

# Spermine Treated-Adipose Tissue-Derived Multi-Lineage Progenitor Cells Improve Left Ventricular Dysfunction in a Swine Model of Chronic Myocardial Infarction

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## Abstract

**Background:** The polyamine spermine enhances differentiation of mouse embryonic stem cells into cardiac lineage. The aim of this study was to determine the effects of spermine on the differentiation of human adipose tissue-derived multi-lineage progenitor cells (hADMPCs) into cardiomyocytes both *in vitro* and *in vivo* and any subsequent functional effect in a swine model of chronic myocardial infarction.

**Methods and results:** Spermine increased the expression of cardiac markers *nkx2.5*, *islet-1*,  $\alpha$ -cardiac actin and cardiac troponin I (to 11.2-, 27.5-, 43.6- and 19.1-fold, relative to baseline, respectively) in hADMPCs. Chronic myocardial infarction model with left ventricular dysfunction was induced by balloon occlusion of the diagonal coronary artery followed by reperfusion, with subsequent similar procedure conducted one week later in the left ascending coronary artery (#6). Four weeks later, the immunosuppressed animals (with CyA 5.0 mg/kg intramuscularly (i.m) body weight/day) were transplanted with spermine-treated hADMPC ( $1 \times 10^5$ ,  $3 \times 10^5$ ,  $1 \times 10^6$  or  $3 \times 10^6$  cells/kg body weight) via the coronary artery (#6). Cardiac function was assessed by echocardiography at 0, 4, 8 and 12 weeks post-transplantation. Transplantation of these cells improved cardiac function and the most effective dose was  $3 \times 10^5$  cells/kg (ejection fraction; 33.4%, 47.0%, 51.5% and 52.9% at 0, 4, 8 and 12 weeks post-transplantation, respectively). At 12-week post-transplantation, spermine-treated hADMPCs differentiated into human-specific troponin I- and  $\alpha$ -cardiac actin-positive cells *in vivo*.

**Conclusion:** Spermine induced differentiation of hADMPCs into cardiomyocytes both *in vitro* and *in vivo* and cellular cardiomyoplasty improved cardiac function. Cellular cardiomyoplasty using hADMPC could be potentially effective cell-based therapy.

**Keywords:** Cellular cardiomyoplasty; hADMPCs; Myocardial infarction; *In situ* reprogramming; Transplantation

## Introduction

Heart failure after myocardial infarction (MI) remains a major clinical challenge despite recent advances in medicinal practice and cardiac transplantation, which are currently the two main treatment options [1,2]. Unfortunately, heart transplantation is limited due to donor shortage and allogenic immuno-rejection, emphasizing the need for novel strategies.

Novel cellular-based therapies have been recently tested in severe cardiac dysfunction [3,4], with some promising results [3-5]. Treatment using mesenchymal stem cells (MSCs) seems particularly promising because these cells have immunoregulatory function, [6] and expand massively *ex vivo* without serious technical issues. MSCs can be obtained from bone marrow but also easily from adipose tissue [7,8]. In a series of publications, our group reported that adipose tissue-derived multi-lineage progenitor cells (ADMPC), which met the criteria of mesenchymal stem cells [9], could be reprogrammed into hepatocytes both *in vitro* and *in vivo* [10,11]. However, human ADMPCs (hADMPCs) *per se* could not survive in cardiac parenchyme longer duration after transplantation and not differentiate into cardiomyocytes *in vivo* [12].

The polyamine spermine was reported recently to induce the differentiation of mouse embryonic stem cells into cardiac lineage [13], and the elucidation of the mechanisms might be underway.

The present study was designed to explore the potential of

hADMPCs in the treatment of MI. For this purpose, we first examined the effects of spermine on the differentiation of hADMPCs into cardiac lineage both *in vitro* and *in vivo*. Second, we monitored left ventricular dysfunction in a swine chronic MI model after transplantation of spermine-treated hADMPCs. The results showed that spermine induced the differentiation of hADMPCs into cardiomyocytes and that cellular cardiomyoplasty using these cells improved left ventricular function in a swine model of chronic MI.

## Materials and Methods

### Adipose tissue

Adipose tissue samples were resected from 5 human subjects

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during plastic surgery (all females, age, 30-60 years) as excess discards. Ten to 20 grams of subcutaneous adipose tissue were collected from each subject. All subjects provided informed consent. The protocol was approved by the Review Boards for Human Research of Kobe University Graduate School of Medicine, Osaka University Graduate School of Medicine and National Institutes of Biomedical Innovation, Health and Nutrition, Japan.

### Isolation of hADMPCs and spermine-treatment

hADMPCs were prepared as described in detail previously [9-12,14,15]. Briefly, the resected excess adipose tissue was minced and then digested at 37°C for 1 h in Hank's balanced salt solution (HBSS, GIBCO Invitrogen, Grand Island, NY) with Liberase (Roche Diagnostics, Germany). Digests were filtered through a cell strainer (BD Bioscience, San Jose, CA) and centrifuged at  $800 \times g$  for 10 min. Red blood cells were excluded using density gradient centrifugation with Lymphoprep ( $d=1.077$ ; Nycomed, Oslo, Norway), and the remaining cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO Invitrogen) with 10% defined fetal bovine serum (FBS, Biological Industries, Israel) for 24 h at 37°C. Following incubation, the adherent cells were washed extensively and then treated with 0.2 g/l ethylenediaminetetraacetate (EDTA) solution (Nacalai Tesque, Kyoto, Japan). The resulting suspended cells were replated on retronectin (RN) (Takara, Kyoto, Japan)-coated dishes in SteMedia (Cell Science and Technology Institute, Inc., Sendai, Miyagi, Japan),  $1 \times$  insulin-transferring selenium (Cell Science and Technology Institute, Inc.), 1 nM dexamethasone (MSD, Tokyo, Japan), 100  $\mu$ M ascorbic acid 2-phosphate (Sawai Pharmaceuticals Co., Osaka, Japan.), 10 ng/ml epidermal growth factor (EGF, PeproTec, Rocky Hill, NJ), and 5% FBS (Biological Industries). After 6 passages in the same medium, the hADMPCs were cultured in the presence or absence of 100  $\mu$ M spermine (Wako Pure Chemicals, Osaka) for indicated time.

### Co-cultivation of spermine treated-hADMPCs and human cardiomyocytes

Spermine-treated and non-treated hADMPCs ( $2 \times 10^5$  cells/well, each) were seeded onto the lower chamber of a 6-well-type Transwell (Cat. No. 3452. Corning, NY) while human cardiomyocytes ( $2 \times 10^5$  cells/well) (purchased from PromoCell), and pre-cultured for 2 days according to the manufacturer or negative control were seeded onto the upper chamber of the same Transwell in SteMedia, comprising  $1 \times$  insulin-transferring selenium, 1 nM dexamethasone, 100  $\mu$ M ascorbic acid 2-phosphate, 10 ng/ml EGF and 5% FBS. After incubation for 72 hours, the cells in the lower chamber were subjected to real-time-polymerase chain reaction (RT-PCR) to estimate their cardiac commitment.

### RT-PCR

Total RNAs of spermine-pre- and post-treated hADMPCs and co-cultured or non-co-cultured hADMPCs were isolated using an RNeasy kit (Qiagen, Valencia, CA). After treatment with DNase, the cDNA was synthesized using Superscript III RNase H-minus Reverse Transcriptase (Invitrogen). RT-PCR was performed using the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Furthermore, 20 $\times$ Assays-on-Demand™ Gene Expression Assay Mix for human islet-1 (Hs00158126\_m1), GATA-4 (Hs00171403\_m1), Nkx2.5 (Hs00231763\_m1),  $\alpha$ -cardiac actin (Hs01109515\_m1), myosin light chain (MLC) (Hs00166405\_m1), cardiac troponin I (Hs00165957\_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hs99999905\_m1) were obtained from Applied Biosystems.

TaqMan® Universal PCR Master Mix, No AmpErase® UNG (2 $\times$ ), was also purchased from Applied Biosystems. Reactions were performed in quadruplicate for each trial and the mRNA levels were normalized relative to human GAPDH expression.

### Animal model of MI and cell transplantation

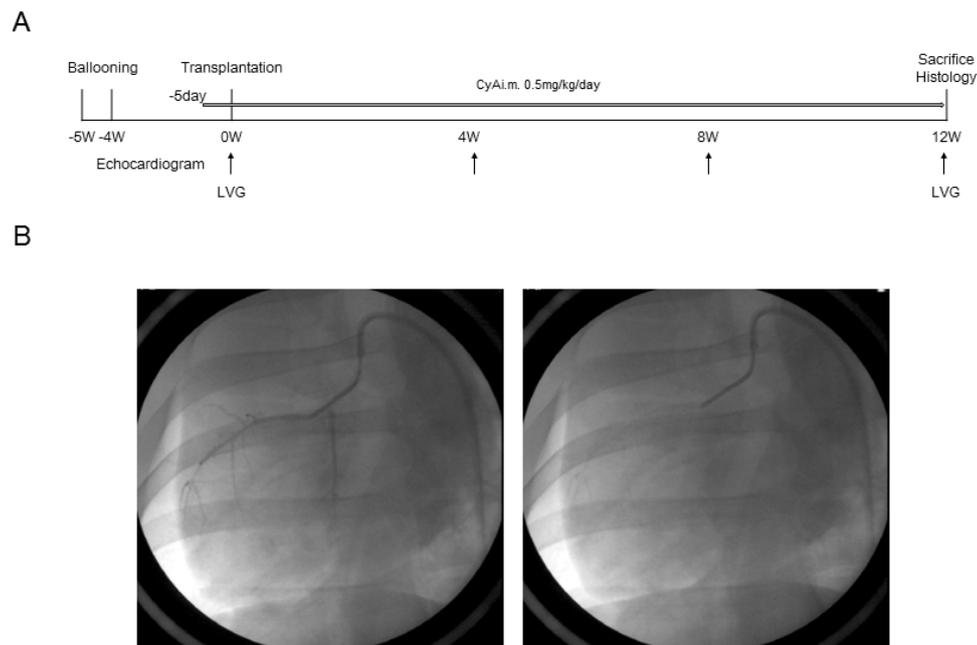
A chronic MI swine model was prepared as described in detail previously [12]. The protocol was shown in Figure 1A. Briefly, 5 weeks before transplantation, the first diagonal branch (D1; #9) of the coronary arteries of pigs was balloon-occluded for 60 min followed by reperfusion. One week later, the left ascending coronary artery of the same animal was 90-min balloon-occluded just proximal to the divergence of the first septal branch (#6), followed by reperfusion (Figure 1B). To ensure better baseline survival and to obtain a swine model of severe old MI, two separate reperfusion infarcts one week apart were performed. From day 5 before cell transplantation to the end of the protocol, the animal received 5.0 mg/kg/day CyA intramuscularly to produce immunosuppression. The CyA-immunosuppressed chronic MI animals (ejection fraction <40% by echocardiography, n=22) were randomly assigned to receive intracoronary transplantation of spermine-treated hADMPCs [ $1 \times 10^5$  (n=4),  $3 \times 10^5$  (n=4),  $1 \times 10^6$  (n=4), and  $1 \times 10^7$  cells/kg (n=5), at density of  $1 \times 10^6$  cells/mL), or placebo lactic Ringer's solution with heparin (n=5), at 4 weeks after the second occlusion/reperfusion. The transplantation procedure was performed as follows: a hockey stick type catheter was placed in the left coronary artery, and then the cell-suspension or placebo control solution was instilled via a straight type 5Fr catheter that was introduced by a guide wire into the left anterior descending artery (#6). The Osaka University Graduate School of Medicine Standing Committee on Animals approved the experimental protocol.

### Assessment of swine cardiac function

Left ventriculography (LVG) was performed just before and 12 weeks after transplantation. The right anterior oblique (RAO) views were used for analysis. Cardiac ultrasonography was performed before and at 4, 8 and 12 weeks after cell transplantation using a VIVID 7 system (GE Healthcare Biosciences, Uppsala, Sweden). The studies were reviewed using the M-mode with short axis view observed from the left 5th intercostal space, and cardiac function was assessed by calculating the ejection fraction using Teichholz equation.

### Histological analysis

For histological analysis, the swine hearts were dissected out at the end of the experiment and immediately fixed overnight in 4% paraformaldehyde, washed in 70% alcohol, dehydrated through a graded ethanol series, cleared with xylene, and finally processed for embedding in paraffin wax. Paraffin sections were cut at 3- $\mu$ m thickness, delineated on the microscope slide, deparaffinized in xylene, and then rehydrated through a graded ethanol series in distilled water. The sections were then immersed in Target Retrieval Solution (Dako, Glostrup, Denmark) and boiled, followed by cooling at room temperature for 20 min. Sections were then washed twice with Tris-buffered saline (TBS), pH 7.4, followed by 1% polyoxyethylene sorbitan monolaurate (Tween20) in TBS (TBS-T), and then incubated overnight with 10% blocking solution (Nacalai tesque) in TBS-T. Sections were then incubated in a humidity chamber for 16 h at 4°C with mouse monoclonal antibodies to human  $\alpha$ -cardiac actin (American Research Products, Belmont, MA), human actinin (Abcam, Cambridge, UK) diluted in blocking solution, followed by Alexa Fluor 488-labeled donkey anti-mouse IgG (Molecular Probes, Eugene, OR) with counter DAPI-staining. Hematoxylin and eosin staining, Masson trichrome



**Figure 1:** Commitment of hADMPCs into cardiac-lineage. (A) The mRNA expression levels of islet-1, GATA-4, nkx2.5,  $\alpha$ -cardiac actin ( $\alpha$ -CA), myosin light chain (MLC), cardiac troponin I and myosin heavy chain (MHC) analyzed by quantitative-polymerase chain reaction with TaqMan Probe<sup>®</sup>. After incubation with spermine for the indicated time, the cells showed upregulation of expression of the indicated cardiac lineage markers. The expression levels are relative to those at day 0. (B) Analysis of cardiac lineage commitment of spermine-treated (+) and untreated (-) cells after co-culture with human isolated cardiomyocytes, as examined for nkx2.5 and  $\alpha$ -CA expression by QT-PCR. The expression levels are relative to that of GAPDH.

staining and Sirius red staining were also performed. The stained slides were viewed on a BioZero laser scanning microscope (Keyence, Osaka).

## Results

### Spermine-treatment commit hADMPCs into cardiac lineage *in vitro*

We evaluated first whether spermine enhances the commitment of hADMPCs into cardiomyocytes *in vitro*, which was tested by analyzing the mRNA expression of several cardiac differentiation markers by RT-PCR before and after treatment with spermine, including islet-1 (a cardiac stem cell marker); Nkx2.5 and GATA-4 (transcription factors required for subsequent cardiac differentiation); and  $\alpha$ -cardiac actin (CA), myosin light chain (MLC), cardiac troponin I, and myosin heavy chain (MHC) (markers of cardiac differentiation) (Figure 2A). For each of the above markers, the expression level after induction of hADMPC was expressed relative to that of the baseline (before induction). At day 1 post-induction, the expression levels of islet-1, Nkx2.5, GATA-4,  $\alpha$ -CA, MLC, cardiac troponin I, and MHC in hADMPC were 10.1, 660, 21, 95, 11, 13, 5.1 times higher than the baseline, respectively. These results indicated that the appropriate treatment-duration with spermine steered hADMPCs for commitment into cardiac lineage *in vitro* was 1 day.

### Augmentation of cardiac commitment of spermine treated-hADMPCs after co-culture with cardiac myocytes

To analyze the commitment of spermine-treated hADMPCs for differentiation into cardiac lineage *in situ*, the spermine-treated and untreated hADMPCs were cultured with or without human cardiomyocytes, and evaluated for NK $\times$ 2.5,  $\alpha$ -CA, MLC and cTnI mRNA expression levels (representative cardiac lineage markers),

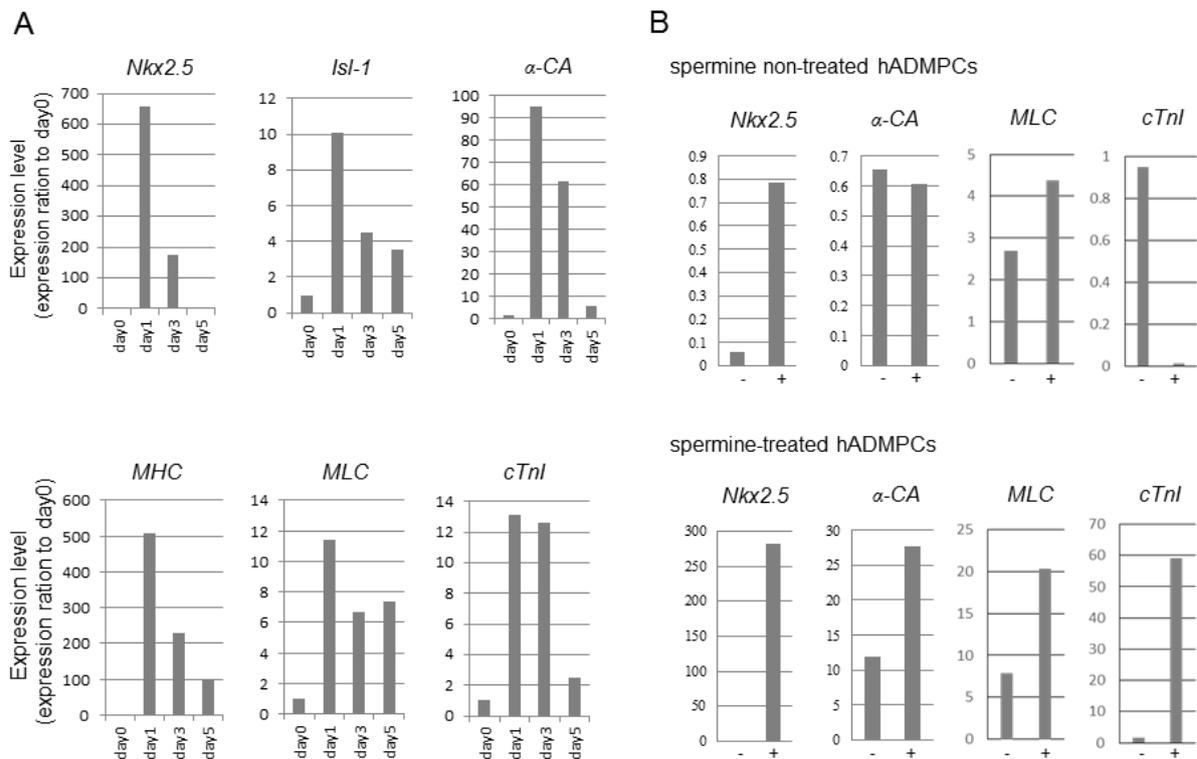
by RT-PCR (Figure 2B). Co-culture with human cardiomyocytes increased NK $\times$ 2.5 and MLC expression in both treated and untreated cells, compared with increased  $\alpha$ -CA and cTnI in only spermine-treated hADMPCs. These results suggest that the spermine-treated hADMPCs could be induced into cardiac lineage.

### Transplantation of spermine-treated hADMPCs improves cardiac function

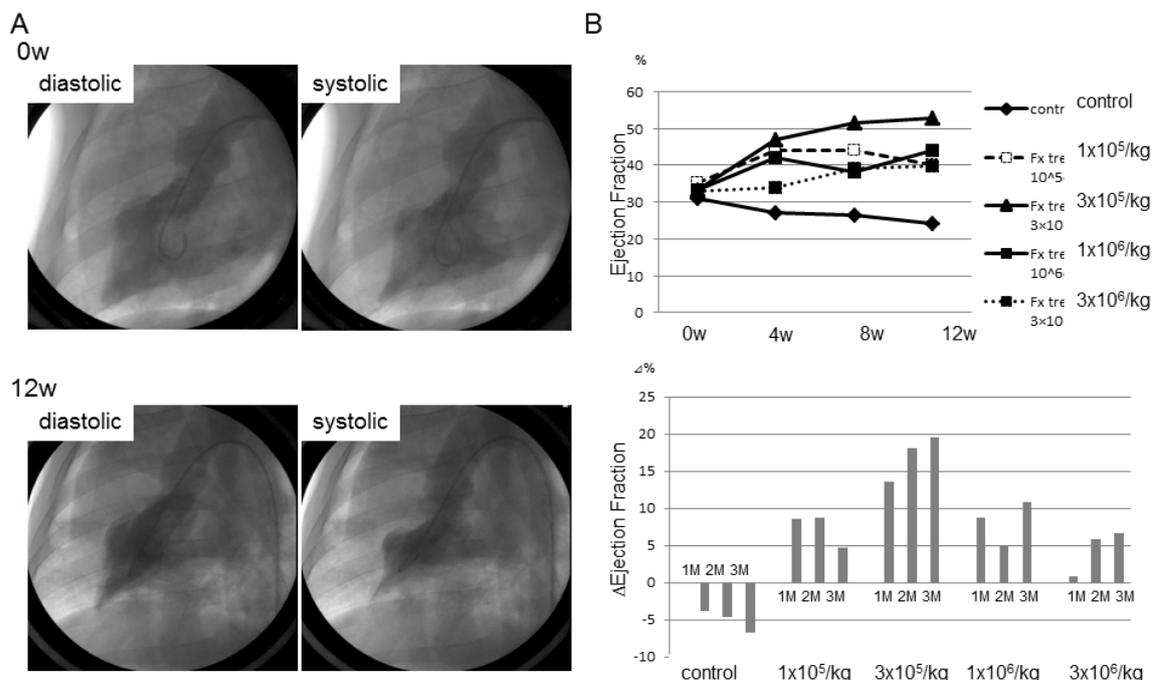
The left ventricle anterior wall motion was poor at baseline (before hADMPCs transplantation) (Figure 3A, top 2 panels), but improved at 12 weeks post-transplantation (Figure 3A, bottom 2 panels). Quantitative analysis of cardiac wall motion by echocardiography showed improvement of left ventricular ejection fraction (EF) and  $\Delta$ EF in the implanted group but not in the control swine, and that the most effective dose was  $3 \times 10^5$  cells/kg in the dose-escalation study (Figure 3B). Improvement of cardiac function at the latter dose was observed at 4-week post-transplantation and maintained in the later phase of the experiment. These results indicate that intracoronary transplantation of spermine-treated hADMPCs improves cardiac function.

### Effects of spermine-treated hADMPCs transplantation on cardiac structure

Twelve weeks after transplantation, the treated swine were sacrificed and cardiac tissues prepared for histological examination for further analysis of cardiac structure and delineate the difference between spermine-treated hADMPCs transplanted animals and controls (Figure 3). Hematoxylin/eosin (HE), Masson's trichrome (MT) and Sirius red (SR) staining showed the presence of a thin layer of cardiac muscles and massive fibrosis in the scarred anterior left ventricular wall of the control (Figure 4A). In contrast, the same staining techniques in spermine-treated hADMPCs-transplanted swine showed layers of cardiomyocytes on the anterior ventricular wall (Figure 4A).



**Figure 2:** Study protocol and administration of spermine-treated hADMPs. (A) From day 5 before cell transplantation to the end of the experiment, the animal received CyA for immunosuppression. At day 0, the animals with chronic severe MI were applied for the experiment. (B) At day 0, pigs with experimentally-induced chronic myocardial infarction were transplanted with spermine-treated hADMPs via the left anterior descending coronary artery (LAD #6).



**Figure 3:** Effects of transplantation of spermine-treated hADMPs on cardiac function. (A) RAO view of left ventriculography (LVG) performed at day 0 (top 2 panels) and 12 weeks after transplantation (bottom 2 panels) in a representative animal. (B) Cardiac function was assessed by echocardiography before and every 4 weeks after transplantation. The left ventricular ejection fraction (EF) and  $\Delta$ EF improved in the transplantation group, but not in the control group. The most effective dose was  $3 \times 10^5$  cells/kg body weight.

## Integration of spermine treated-hADMPCs with the swine cardiac milieu

The *in situ* differentiation capacity of the implanted spermine-treated hADMPCs into cardiomyocytes after grafting onto the scarred myocardium was assessed by immunohistochemical staining for human specific  $\alpha$ -CA and human specific  $\alpha$  actinin. Human  $\alpha$ -CA- and  $\alpha$  actinin-positive cells were identified in the swine myocardium (Figures 4A and 4B, respectively). Intracellular localization studies showed  $\alpha$ -CA and  $\alpha$  actinin immunoreactivities in the sarcomeres (Figures 4C and 4D, respectively), indicating that at least a proportion of spermine-treated hADMPCs integrated *in situ* as cardiomyocytes with the cardiac milieu.

## Discussion

The main findings of the present study were: 1) spermine upregulated the expression of cardiac progenitors' nuclear transcriptional factors and cardiac structural molecules on hADMPCs, 2) engraftment of spermine-treated hADMPCs into the cardiac milieu following instillation into coronary artery and their differentiation into cardiomyocyte-like cells *in situ*, and 3) integration of these cardiomyocyte-like cells with the cardiac milieu improved cardiac function in the chronic MI swine model.

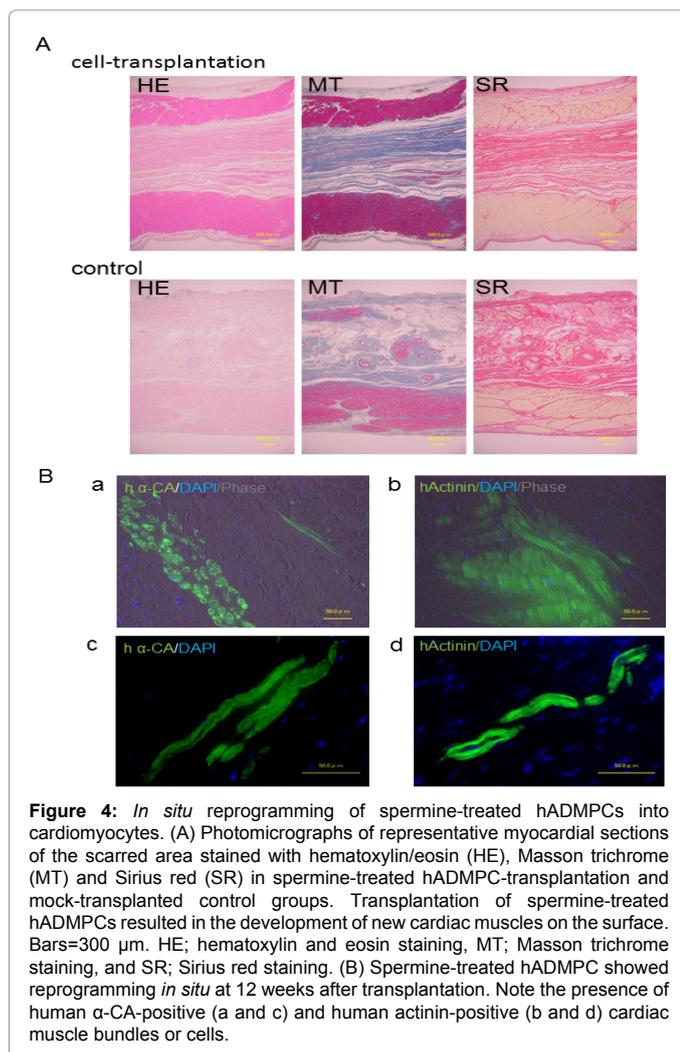
Intracoronary transplantation of spermine-treated hADMPCs in MI-related chronic heart failure offers several advantages. First, adipose tissue, the source of hADMPCs, is easily and safely obtainable without critical ethical problems. Second, the spermine-treated hADMPCs easily and rapidly differentiated into cardiac lineages *in situ* after intracoronary transplantation. Finally, the route of administration of these cells is friendly to cardiologists and includes the use of commonly available catheters.

Selection of appropriate cell origin is the first critical issue for successful cellular therapy. The adipose tissue is easily obtainable and can be safely resected without serious ethical issues, and therefore can be applied for not only autologous but also allogenic cellular therapy, and the cells can be obtained in large quantities by liposuction, yielding from 100 ml to >3L of lipoaspirated tissues [16]. Bjornorp et al. [17] reported a simple method for isolation of cells from the rat foot pad adipose tissue. This procedure was then modified for the isolation of cells from human specimens [18-20]. In this context, Zuk et al. [7] reported the presence of cells with properties similar to those of mesenchymal stem cells (MSCs) in adipose tissue stroma and labelled them adipose tissue-derived stromal/stem cells (ADSCs/ASCs). While the *in vitro* differentiation of ADSCs/ASCs has been reported, only a few studies reported their *in vivo* differentiation into cardiomyocytes [21,22]. Previous studies demonstrated improvement of cardiac structure and function after placement of ADSC-sheet transplantation onto scarred myocardium in both laboratory rats and pigs [23,24]. In these studies, histological analysis demonstrated the formation of new blood vessels but no engraftment of ADSCs into the scarred myocardium. In this context, Gimble et al. [25] suggested that hADMPCs might secrete angiogenic factors and/or antiapoptotic factors.

In a series of studies, we have characterized hADMPCs as a novel and minor cell population in human adipose tissues and reported that these cells have stem cell properties including their ability to be reprogrammed *in situ* into cardiomyocytes in rats with MI and into hepatocyte-like cells in the rabbit hepatic parenchyma [9-12]. Based on their advantages, hADMPCs are promising source of cells for cell therapy.

In this study, we demonstrated the differentiation of spermine-treated hADMPCs into cardiac lineages *in situ* after intracoronary transplantation, and that such differentiation had a positive impact on functional outcome. Several groups have demonstrated the differentiation of induced pluripotent stem cells-derived cardiac progenitor cells into cardiomyocytes *in situ* and subsequent improvement of cardiac function of diseased heart [26,27]. The mode of action is probably similar to that of spermine-treated hADMPCs. While it is hoped that iPSCs-derived cardiac progenitor cells can be used therapeutically, evidence suggests that the risks of teratoma-, tumor- and/or carcinoma-formation could prevent their clinical use on a wide scale [28].

With regard to the route of administration, we selected coronary artery transplantation in this study and determined the optimal dose of cells to be  $3.0 \times 10^5$  cells/kg body weight. Various methods of transplantation of cellular products have been described previously, such as cell-patch placement, intramyocardial injection and intracoronary instillation [27]. Cell-patch placement can deliver cells with high viability, but the patch could be placed only on the anteriolateral wall of the heart and the procedure requires highly invasive open heart surgery. On the other hand, intramyocardial injection is used for direct application of the cells onto the diseased cardiac muscle, but the procedure carries the risk of cardiac perforation



**Figure 4:** *In situ* reprogramming of spermine-treated hADMPCs into cardiomyocytes. (A) Photomicrographs of representative myocardial sections of the scarred area stained with hematoxylin/eosin (HE), Masson trichrome (MT) and Sirius red (SR) in spermine-treated hADMPC-transplantation and mock-transplanted control groups. Transplantation of spermine-treated hADMPCs resulted in the development of new cardiac muscles on the surface. Bars=300  $\mu$ m. HE; hematoxylin and eosin staining, MT; Masson trichrome staining, and SR; Sirius red staining. (B) Spermine-treated hADMPC showed reprogramming *in situ* at 12 weeks after transplantation. Note the presence of human  $\alpha$ -CA-positive (a and c) and human actinin-positive (b and d) cardiac muscle bundles or cells.

[29]. For intracoronary administration, which was selected in the present study, the cells are delivered through percutaneous coronary intervention technique friendly to the cardiologists using catheters available in daily clinical practice.

Despite the positive outcome, our technique need to overcome certain issues. First, the risk-benefit based approach should be applied in the infarcted or affected tissues after transplantation. Second, the functional benefits, if any, of transplantation of spermine treated-hADMPCs should be examined by the Investigational New Drug approval before stepping into clinical trials and clinical practice.

## Conclusion

We demonstrated in the present study the successful engraftment of spermine-treated hADMPCs into the scarred myocardium with *in situ* reprogramming and that transplantation of these cells via the coronary artery resulted in recovery of cardiac function. These results suggest that intracoronary transplantation of spermine-treated hADMPCs could be a potentially viable therapeutic approach for patients with heart diseases.

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