

# Both cultured and freshly isolated adipose tissue-derived stem cells enhance cardiac function after acute myocardial infarction

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## Aims

We assessed whether freshly isolated human adipose tissue-derived cells (fhADCs) or cultured human adipose tissue-derived stem cells (hASCs) have beneficial effects on cardiac function after myocardial infarction (MI), whether the injected cells can survive long term, and whether their effects result from direct differentiation or paracrine mechanisms.

## Methods and results

Myocardial infarction was experimentally induced in severe combined immunodeficient mice, and either fhADCs, cultured hASCs, or phosphate-buffered saline was injected into the peri-infarct region. Myocardial function improved significantly in mice treated with hASCs or fhADCs 4 weeks after MI. Immunofluorescence revealed that grafted hASCs and fhADCs underwent cardiomyogenic differentiation pathway, as indicated by expression of connexin 43 and troponin I in a fusion-independent manner. Some of the injected cells integrated with host cardiomyocytes through connexin 43, and others were incorporated into newly formed vessels. Human adipose tissue-derived stem cells survived in injured hearts up to 4 months, as detected by luciferase-based bioluminescence imaging. Vascular density was significantly increased, and fewer apoptotic cells were present in the peri-infarct region of cell-injected mice.

## Conclusion

This is the first study to systematically compare the effects of fhADCs and hASCs on myocardial regeneration. Both cell types engraft into infarcted myocardium, survive, and improve myocardial function, suggesting that fhADCs, like hASCs, are a promising alternative cell source for myocardial repair after MI.

## Keywords

Myocardial infarction • Angiogenesis • Apoptosis • Stem cells

## Introduction

Myocardial infarction (MI) is characterized by reduced blood supply to the heart and loss of functioning cardiomyocytes. However, the capacity for self-regeneration of adult heart is limited. Stem cell transplantation is being investigated as a novel means to regenerate heart tissue and enhance cardiac function. The potential of adult stem cells to function as cellular therapy for myocardial regeneration is based on their plasticity, clonogenicity, and self-renewal ability. Recent studies have demonstrated

that bone marrow-derived stem cells (BMSCs) have broad differentiation potential and can differentiate into cardiomyocytes. However, the identity of the very small fraction of cells within the bone marrow with the capacity to differentiate into true functional cardiomyocytes is under debate.<sup>1</sup> Furthermore, BMSCs require processing and culturing after bone marrow aspiration, making it difficult to use these cells in an acute clinical setting.

A multipotential stem cell population has been isolated from adipose tissue and was referred to as adipose tissue-derived stem cells (ASCs).<sup>2,3</sup> We and others have demonstrated that

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both freshly isolated and cultured ASCs secrete significant amounts of angiogenic and antiapoptotic factors.<sup>4</sup> Several studies have shown that cultured ASCs improve cardiac function after experimental MI.<sup>5,6</sup> However, less information is available about the therapeutic potential of freshly isolated human adipose tissue-derived cells (fhADCs). A direct comparison of the effects of fhADCs and cultured human ASCs (hASCs) on cardiac function has not been conducted. In addition, it is not known whether the differentiation potential or paracrine factors released by injected ASCs lead to improvements in left ventricular (LV) function. Moreover, the survival and migration of injected ASCs in the ischaemic heart have not yet been addressed.

In this study, using severe combined immunodeficient (SCID) mice as an experimental model of acute MI, we compared the therapeutic effects of intramyocardially injected fhADCs and hASCs on cardiac function. We analysed the cells' differentiation potential [including cardiomyocyte, endothelial cell (EC), and smooth muscle cell (SMC) differentiation pathways] as a possible mechanism for the benefits of stem cell-based therapy, as well as paracrine effects of injected cells. A further aim was to evaluate the long-term local survival of injected hASCs, using luciferase-based bioluminescence imaging (BLI), and migration of the injected cells to other organs.

## Methods

### Cell isolation and expansion

Freshly isolated human adipose tissue-derived cell isolation, hASC culture, flow cytometry, direct immunofluorescence staining, and assays for tube formation, uptake of acetylated low-density lipoprotein, and differentiation *in vitro* are described in Supplementary material online.

### Lentiviral labelling and bioluminescence intensity of human adipose tissue-derived stem cells

Human adipose tissue-derived stem cells at passage 0 were transduced with a lentiviral vector in order to track injected cells in living mice. The lentiviral vector encodes green fluorescent protein (GFP) and luciferase reporter, expressed simultaneously under control of a long-terminal-repeat promoter (Figure 1A). Lentivirus production and cell transduction were performed as reported previously.<sup>7</sup> After transduction, GFP-positive hASCs were sorted by fluorescence-activated cell sorting. The proliferation and differentiation potential of transduced hASCs at passage 3 were analysed as described in Supplementary material online. The correlation between GFP/luciferase-positive hASCs and *in vitro* bioluminescence intensity was determined by preparing various numbers of cells (2000–10 000 per well) in a 96-well plate. Luciferase activity of hASCs was evaluated from bioluminescence images acquired by a Xenogen cooled charge-coupled device optical macroscopic imaging system immediately after adding luciferase substrate (D-luciferin, Gold Bio Technology) at a concentration of 400 µg/mL in medium. The exposure time was 0.15 s. Bioluminescence intensity in different wells was quantified in units of photons per second per centimetre squared per steradian (p/s/cm<sup>2</sup>/sr) by drawing a polygonal region of interest over the signals in images using Living Image 3.0 software (Caliper Life Sciences).

### Mice

All mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas M.D. Anderson Cancer Center. Thirty-three 8- to 12-week-old male SCID mice (Jackson Laboratory), weighing 22–30 g, were subjected to experimental MI (described below) and then randomly assigned to one of three treatment groups: hASCs, fhADCs, and phosphate-buffered saline (PBS) control.

### Myocardial infarction model

Mice were intubated, anaesthetized with a mixture of O<sub>2</sub>/isoflurane, and mechanically ventilated (rate 136 b.p.m., tidal volume 0.15 mL). An anterior thoracotomy was performed, the heart was exposed, and the left anterior descending coronary artery was permanently ligated with an intramural stitch (7-0 prolene suture). Mice were then given an intramyocardial injection of cells (30 µL of 5 × 10<sup>5</sup> hASCs at passage 3 or fhADCs in PBS) or PBS alone (30 µL) into two sites in the peri-infarct zone (15 µL in each site). Successful occlusion was confirmed by myocardial blanching. The thorax was then closed with three layers of sutures.

### Imaging and histology

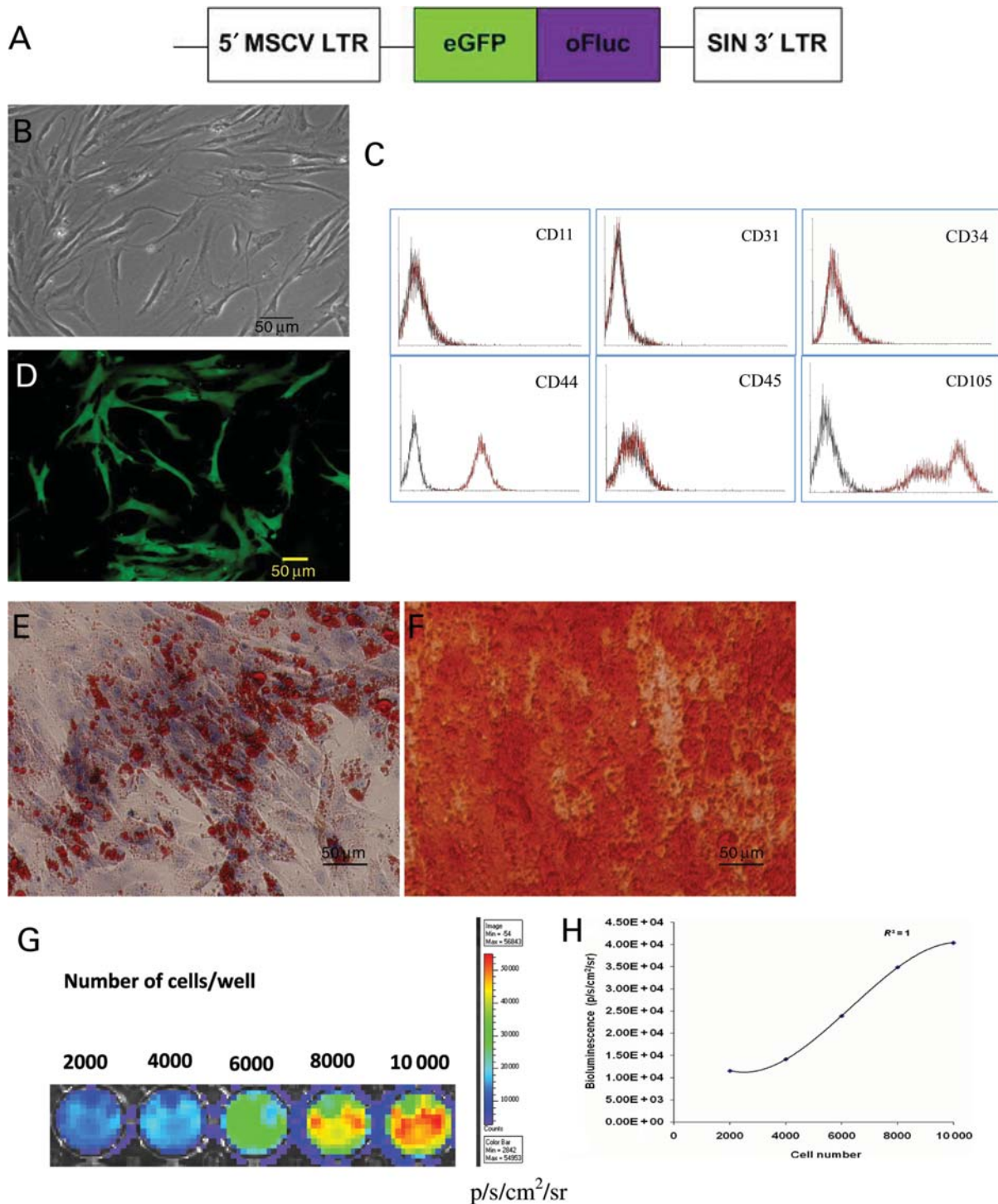
Magnetic resonance imaging (MRI) assessment of cardiac function, histological analysis of vascular density, staining analysis of apoptosis, fluorescence *in situ* hybridization (FISH) analysis of cell fusion, and measurement of infarct size are described in Supplementary material online.

### Confocal images of indirect immunofluorescence staining

Immunofluorescence staining was used to analyse the differentiation, proliferation, and gap junction expression of injected hASCs and fhADCs. Injected human cells were tracked using antibodies against lamin A/C (Novocastra Laboratories), a nuclear membrane marker specific to human cells, as described previously.<sup>8</sup> To confirm the specificity of this antibody, human adipose tissue sections and control mouse heart sections were subjected to immunofluorescence staining with anti-human lamin A/C antibody. Cardiomyocytes were traced by antibody to troponin I (Santa Cruz Biotechnology), ECs by antibody to von Willebrand factor (vWF) (Chemicon), SMCs by antibody to α-smooth muscle actin (SMA) (Sigma), proliferating cells by antibody to Ki67 (Abcam), and gap junction protein by antibody to connexin 43 (Santa Cruz Biotechnology). Tissue sections were fixed with 4% paraformaldehyde for 10 min at room temperature, washed three times with PBS containing 0.3% Triton X-100 (Sigma), and blocked with 10% donkey serum for 30 min at room temperature. Slides were then incubated with combinations of primary antibodies against lamin A/C and either troponin I, vWF, SMA, Ki67, connexin 43, or isotype control for 1 h at 37°C. After three washes with PBS, slides were incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 594-conjugated donkey anti-rabbit or goat IgG (Invitrogen) for 45 min at 37°C. After three more washes with PBS, the slides were stained for nuclei using 4',6-diamidino-2-phenylindole. Images of sections were obtained by a confocal fluorescence microscope.

### Bioluminescence imaging

To determine the minimum number of hASCs detectable by BLI *in vivo*, various numbers of luciferase-positive hASCs (50–3200) were subcutaneously injected into four sites in each of two mice. Each anaesthetized mouse was imaged 20 min (exposure time: 20 s) after intraperitoneal injection of D-luciferin. To trace the survival and



**Figure 1** Characterization of lentivirally transduced, cultured human adipose tissue-derived stem cells expressing green fluorescent protein (eGFP) and luciferase (oFluc) reporter genes. (A) Schematic representation of the lentiviral vector. MSCV LTR, murine stem cell virus long terminal repeat; SIN LTR, self-inactivating viral long terminal repeat. (B) Human adipose tissue-derived stem cells showed fibroblast-like morphology. (C) Flow cytometric analysis shows that human adipose tissue-derived stem cells were negative for CD11, CD31, CD34, and CD45 and positive for CD44 and CD105. Black traces indicate isotype controls; red traces indicate surface antigen expression. (D) Fluorescence image of luciferase-positive human adipose tissue-derived stem cells showing strong expression of green fluorescent protein. (E and F) Human adipose tissue-derived stem cells lentivirally labelled with green fluorescent protein and luciferase maintain the potential to differentiate into adipocytes (E) and osteoblasts (F). (G) Bioluminescence imaging of increasing numbers of human adipose tissue-derived stem cells *in vitro*. Colour scale bar represents range of signals. (H) Bioluminescence imaging is proportional to cell number, with an  $R^2$  value of 1. Scale bar represents 50  $\mu$ m in (B, D–F).

migration of intramyocardially injected hASCs in living animals, five mice were subjected to BLI on Day 2 and Weeks 1–4 after MI (except for two mice that were imaged up to 10 and 16 weeks, respectively). Bioluminescence intensity was quantified by drawing a region of interest over the signals in images in units of p/s/cm<sup>2</sup>/sr. Before *in vitro* imaging of different organs, anaesthetized mice at 10 weeks ( $n = 1$ ) or 16 weeks ( $n = 1$ ) after cell injection were imaged 20 min after intraperitoneal injection of D-luciferin. Hearts, spleens, lungs, kidneys, livers, and brains were then removed from mice and immersed in a six-well plate. Each well contained 3 mL of 400 µg/mL D-luciferin in PBS.

## Statistics

Results are expressed as mean  $\pm$  standard error of the mean. SPSS software version 15 (Chicago, IL, USA) was used to perform one-way analysis of variance (ANOVA) for evaluation of the differences of ejection function and LV volume of mice between different experimental groups (hASCs, fhADCs, and PBS control groups) at each time point (before MI and 1 and 4 weeks after MI) and between the three different time points within each group. The differences of apoptosis, vascular density, and infarct size between the three different experimental groups on Week 4 after MI were also analysed using ANOVA. Pairwise multiple comparisons were to identify the differences of the parameters described above between two experimental groups at each time point or between two different time points within each group using the Tukey test in conjunction with an ANOVA. A  $P$ -value of  $<0.05$  was considered significant. Polynomial regression analysis was performed to evaluate the correlation between cell numbers and bioluminescence intensity of region of interest in the images of *in vitro* using Microsoft Excel.

## Results

### Human adipose tissue-derived stem cell population is homogeneous

Human adipose tissue-derived stem cells cultured in medium containing 20% foetal bovine serum showed a fibroblast-like morphology (Figure 1B). Flow cytometric analysis indicated that hASCs from three donors were positive for stem cell markers CD44 ( $93.56 \pm 7.17\%$ ) and CD105 ( $94.57 \pm 3.73\%$ ) but negative for haematopoietic lineage markers CD11b ( $0.05 \pm 0.03\%$ ), CD45 ( $0.16 \pm 0.14\%$ ), and CD34 (also expressed in ECs;  $0.29 \pm 0.39\%$ ; Figure 1C). These results revealed that cultured hASCs were homogeneous and did not contain ECs or haematopoietic lineages, consistent with previous reports.<sup>9</sup>

To track injected cells in living mice, we transduced hASCs with a lentiviral vector expressing luciferase and GFP reporter (Figure 1D). Lentiviral transduction and luciferase–GFP expression did not change the proliferation or differentiation potential of hASCs (Figure 1E and F). Additionally, there was a positive correlation between cell number and bioluminescence intensity ( $R^2 = 1$ ; Figure 1G and H).

### Freshly isolated human adipose tissue-derived cell population is heterogeneous

In contrast to cultured hASCs, fhADCs from three samples contained not only a significant number of stem cells, as identified

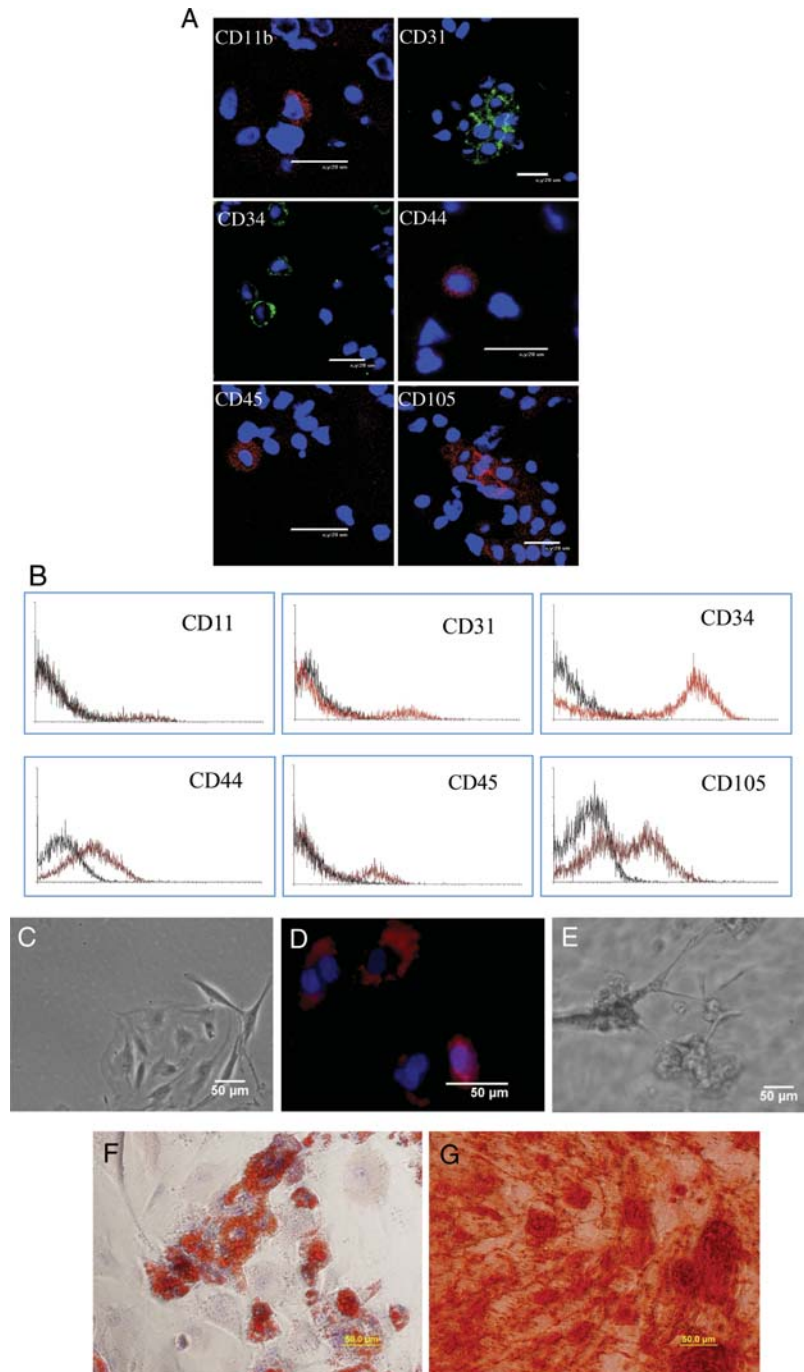
by expression of CD44 ( $35.13 \pm 8.16\%$ ) and CD105 ( $40.32 \pm 7.15\%$ ) (Figure 2A and B), but also a certain percentage of haematopoietic lineages, identified by the expression of CD11b ( $4.72 \pm 0.33\%$ ), CD34 ( $46.50 \pm 5.53\%$ ), and CD45 ( $6.21 \pm 2.05\%$ ); ECs, identified by CD31 ( $9.96 \pm 1.43\%$ ); and SMCs, identified by SMA ( $3.67 \pm 0.51\%$ ). To confirm the presence of EC and stem cell fractions among fhADCs, we cultured fhADCs in EC medium EGM-2 (ECs died in regular stem cell growth medium; data not shown). After 4 days of culture, we observed EC-like (cobblestone) and fibroblast-like (spindle) cells (Figure 2C). The EC-like cells took up acetylated low-density lipoprotein, as indicated by red fluorescence signals, and formed capillary-like tubes when cultured in Matrigel (Figure 2D and E). Freshly isolated human adipose tissue-derived cells differentiated into adipocytes (Figure 2F) and osteoblasts (Figure 2G) when cultured in induction medium for 3 weeks. These results indicated that fhASCs constitute a heterogeneous population, including functional ECs and stem cells.

### Human adipose tissue-derived stem cells and freshly isolated human adipose tissue-derived cells improve cardiac function after myocardial infarction

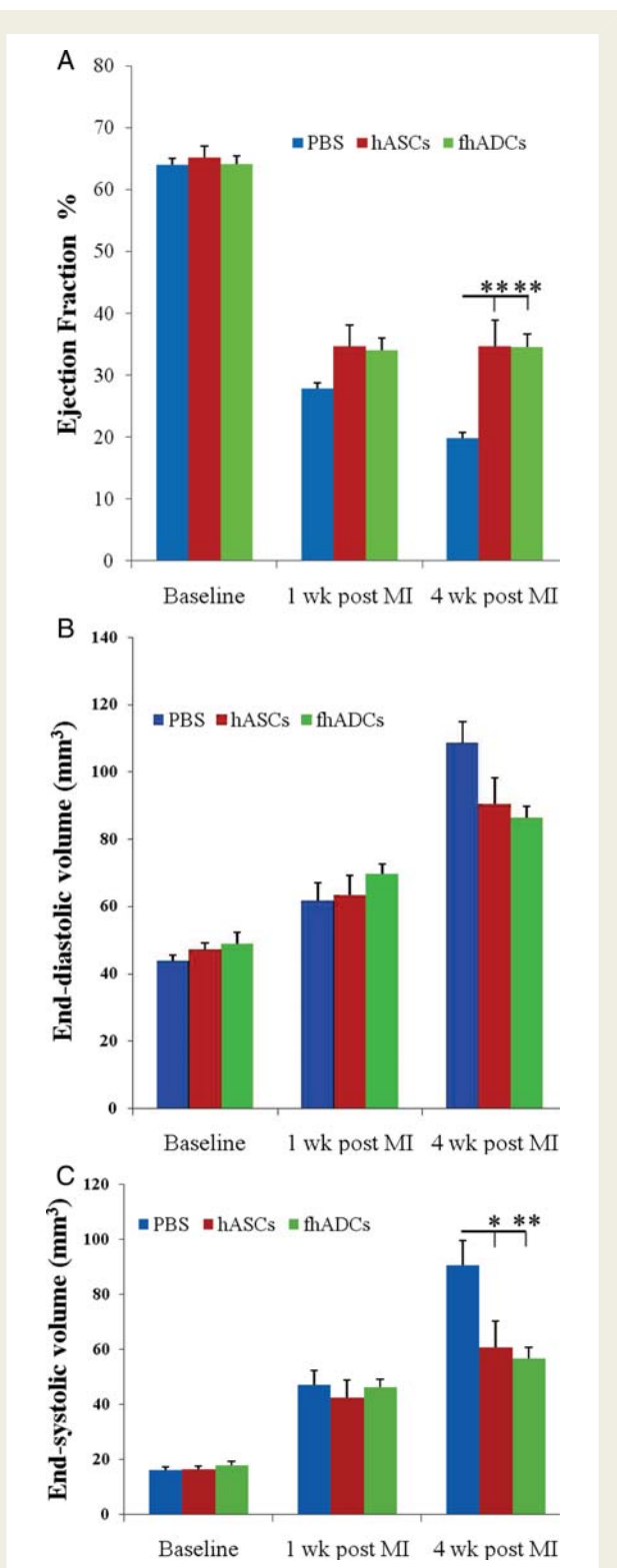
Left ventricular function of mice ( $n = 7$  mice per group) was evaluated by MRI at baseline before MI and 1 and 4 weeks after cell transplantation or PBS injection. Seven days after MI, ejection fraction in all groups ( $34.62 \pm 3.48\%$  for hASCs,  $34.02 \pm 2.00$  for fhADCs, and  $27.82 \pm 3.98\%$  for PBS; Figure 3A) had significantly declined compared with baseline, indicating that global myocardial function in all mice was severely impaired. Further significant functional loss continued up to 28 days after MI in the PBS-injected hearts. In contrast, hearts that received cell injections did not deteriorate further at 4 weeks [ $34.68 \pm 4.25\%$  for hASCs and  $34.51 \pm 2.71\%$  for fhADCs vs.  $19.78 \pm 3.82\%$  for PBS;  $P = 0.008$  (hASCs vs. PBS) and  $P = 0.009$  (fhADCs vs. PBS)]. No significant difference was observed in LV end-diastolic volume between the two cell-injected groups and the PBS group (Figure 3B). End-systolic volume was lower in both the hASC ( $60.82 \pm 9.52 \text{ mm}^3$ ) and fhADC ( $56.85 \pm 3.93 \text{ mm}^3$ ) groups compared with the PBS group ( $90.63 \pm 8.91 \text{ mm}^3$ ;  $P = 0.016$  and  $0.008$ , respectively; Figure 3C). Supplementary material online, Figure S1 shows representative examples of end-systolic and end-diastolic short-axis images. Supplementary material online, Videos S1–S6 show MRI short-axis cine views of representative hearts from the three groups before MI and 4 weeks after MI.

### Transplanted human adipose tissue-derived stem cells and freshly isolated human adipose tissue-derived cells differentiate but do not fuse with host cells

Injected human cells were tracked using antibody to human lamin A/C, which specifically binds to human, but not mouse, nuclei (Figure 4A). Both hASCs and fhADCs were detected in all eight hearts from cell-treated groups ( $n = 4$  per group) 28 days after cell implantation, suggesting successful engraftment and survival of the injected cells.



**Figure 2** Characterization of freshly isolated human adipose tissue-derived cells. (A) Cell surface markers of freshly isolated human adipose tissue-derived cells were analysed by immunofluorescence staining. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue) and surface markers were stained with antibodies (red or green). (B) Cell surface marker expression in freshly isolated human adipose tissue-derived cells was confirmed by flow cytometry and demonstrated that freshly isolated human adipose tissue-derived cells constitute a heterogeneous cell population. (C) Endothelial cell-like (cobblestone) or fibroblast-like (spindle) shapes were observed for freshly isolated human adipose tissue-derived cells cultured in endothelial cell medium for 4 days. (D and E) Presence of endothelial cells within freshly isolated human adipose tissue-derived cell population was confirmed by their capacity to take up acetylated low-density lipoprotein (D, red signals) and to form capillary-like structures on Matrigel (E). (F and G) Stem cells within freshly isolated human adipose tissue-derived cell population differentiated into adipocytes (F) and osteocytes (G). Scale bars represent 20 μm in (A) and 50 μm in (C–G).



**Figure 3** Magnetic resonance imaging analysis of cardiac function. Mice were intramyocardially injected with phosphate-buffered saline alone, human adipose tissue-derived stem cells, or freshly isolated human adipose tissue-derived cells. Magnetic resonance imaging was performed before myocardial infarction and at 1 and 4 weeks after myocardial infarction. (A) Left ventricular ejection fraction was significantly higher in human adipose tissue-derived stem cell- and freshly isolated human adipose

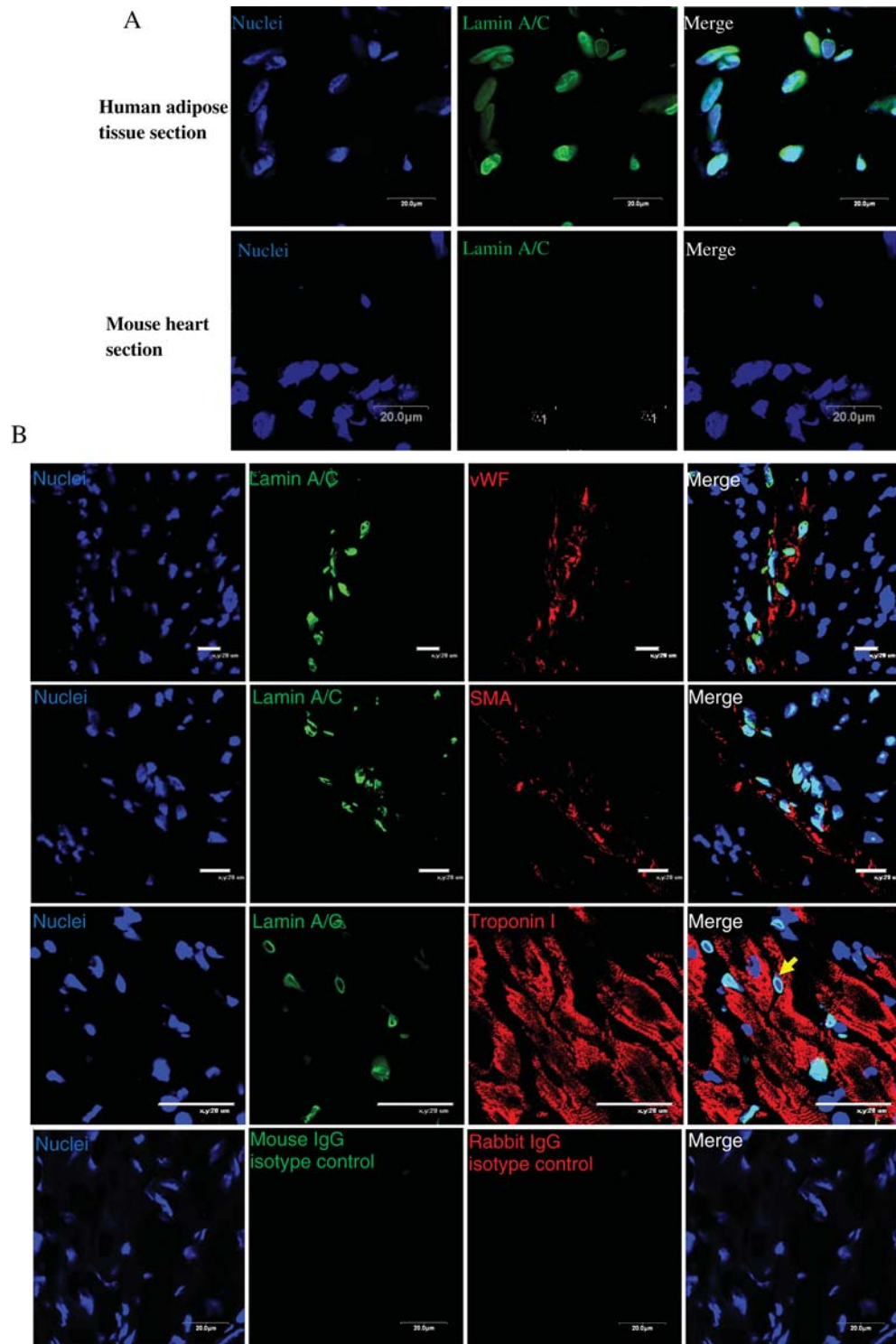
We observed clusters of injected cells in both the infarct region and the border zone, but not within viable myocardium among the infarcted area. Both engrafted hASCs and fhADCs expressed vWF or SMA, indicating that some injected cells differentiated into vascular cells. Because fhADCs contain ECs and SMCs, these cells might directly incorporate into blood vessels without further differentiation. Importantly, both hASCs and fhADCs indicate a cardiomyogenic differentiation pathway at the border zone. We did not observe any fluorescent signals on tissue sections incubated with isotype-control antibodies alone (Figure 4B and C). Connexin 43 was expressed in a punctate pattern between injected cells and host cardiomyocytes (Figure 5A). Some injected cells remained in a proliferative cell cycle, as evidenced by the expression of proliferating cell marker Ki67 (Figure 5B). Extensive FISH analysis did not reveal fusion between injected human cells and host cells of mouse heart (Figure 5C). These results suggest that the injected cells most likely proliferate and undergo a cardiomyocyte, EC, or SMC pathway in a fusion-independent way.

### Human adipose tissue-derived stem cells and freshly isolated human adipose tissue-derived cells promote angiogenesis and inhibit apoptosis, and injected human adipose tissue-derived stem cells remain and survive in infarcted hearts

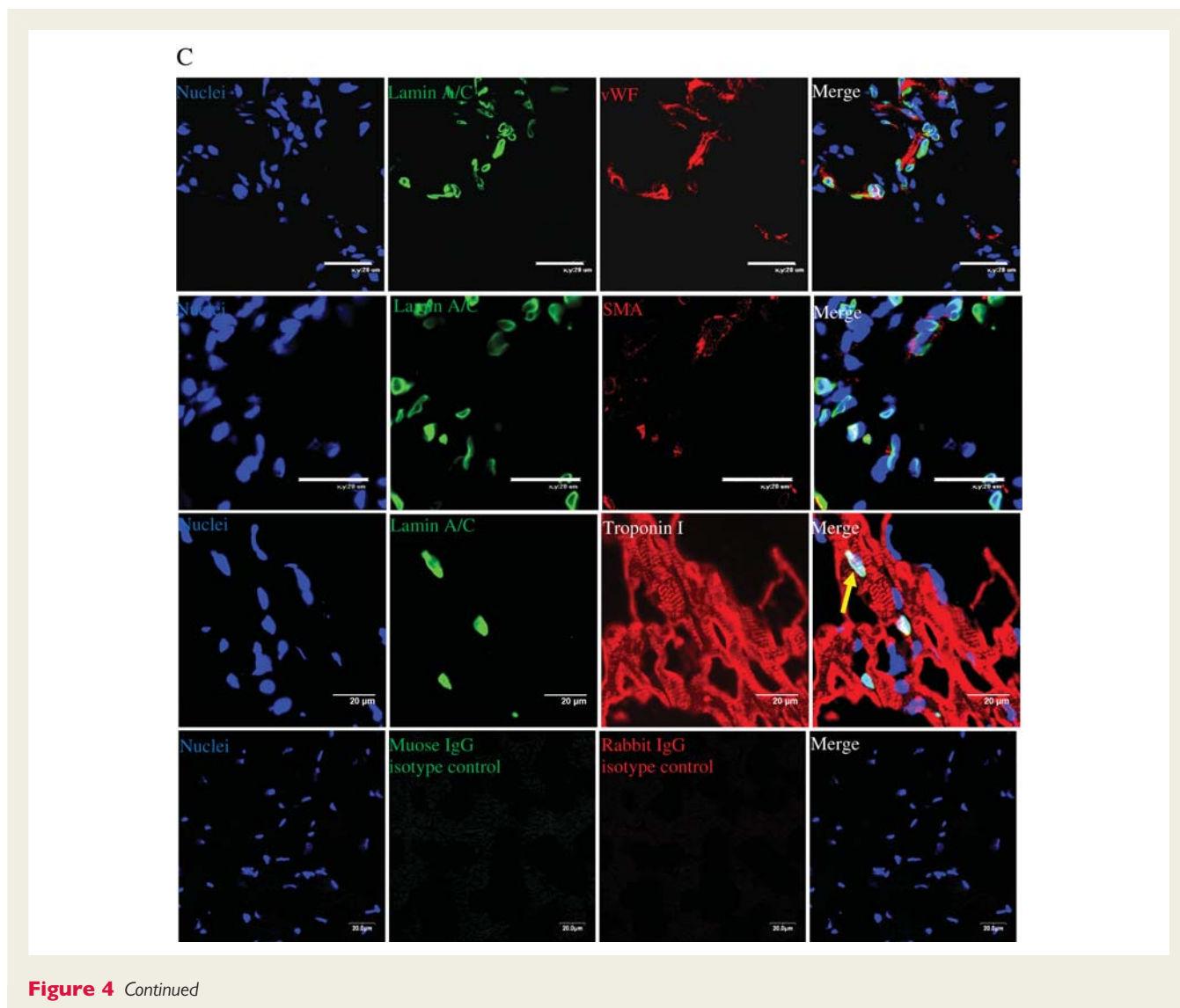
Both hASCs and fhADCs significantly promoted angiogenesis (Figure 6) and inhibited apoptosis ( $n = 6$  mice per group; Figure 7) (described in Supplementary material online).

*In vivo* bioluminescence calibration studies demonstrated that 800 hASCs were sufficient to produce a detectable signal after subcutaneous injection. The signal intensity was in linear proportional to the number of transplanted cells (see Supplementary material online, Figure S2). Representative images taken in five mice after cell injection showed that bioluminescent signals within the same mouse were detectable in cardiac areas over the 28 days of the experiment (Figure 8A). During the initial 2–14 days after cell injection, bioluminescent signals decreased from  $(15.9 \pm 5.4) \times 10^5$  to  $(5.98 \pm 2.24) \times 10^5$  p/s/cm<sup>2</sup>/sr. After that, the bioluminescent signals gradually increased to  $(9.13 \pm 3.02) \times 10^5$  ( $n = 5$ ) on Day 28 after cell injection (Figure 8B). We did not observe bioluminescent signals in other organs. The *in vivo* finding was confirmed by *ex vivo* imaging of explanted whole hearts. No signals were detected in lungs, brains, spleens, kidneys, or livers for up to 16 weeks after initial cell transplantation (Figure 8C).

tissue-derived cell-treated hearts than in phosphate-buffered saline-treated hearts. (B and C) End-diastolic (B) and end-systolic (C) volumes increased in the three groups at 1 and 4 weeks after myocardial infarction compared with baseline. End-systolic volume in human adipose tissue-derived stem cell- and freshly isolated human adipose tissue-derived cell-treated hearts was lower at 4 weeks after myocardial infarction compared with phosphate-buffered saline.  $^{***}P < 0.01$  and  $^{*}P < 0.05$ ;  $n = 7$  in each group.



**Figure 4** Immunofluorescence analysis of differentiation 4 weeks after myocardial infarction. (A) Immunofluorescence staining for human-specific lamin A/C in the nuclear membranes of human cells, but not of murine cells. Upper panels show human adipose tissue sections; bottom panels show heart sections from mice that did not receive human cell injections. (B and C) Sections of hearts intramyocardially injected with human adipose tissue-derived stem cells (B) or freshly isolated human adipose tissue-derived cells (C) were triple-stained with 4',6-diamidino-2-phenylindole (nuclei), antibodies to lamin A/C (nuclear membrane), and von Willebrand factor (endothelial cell marker), smooth muscle actin (smooth muscle cell marker), or troponin I (cardiomyocyte marker) as indicated. Scale bars represent 20  $\mu\text{m}$ .



## Discussion

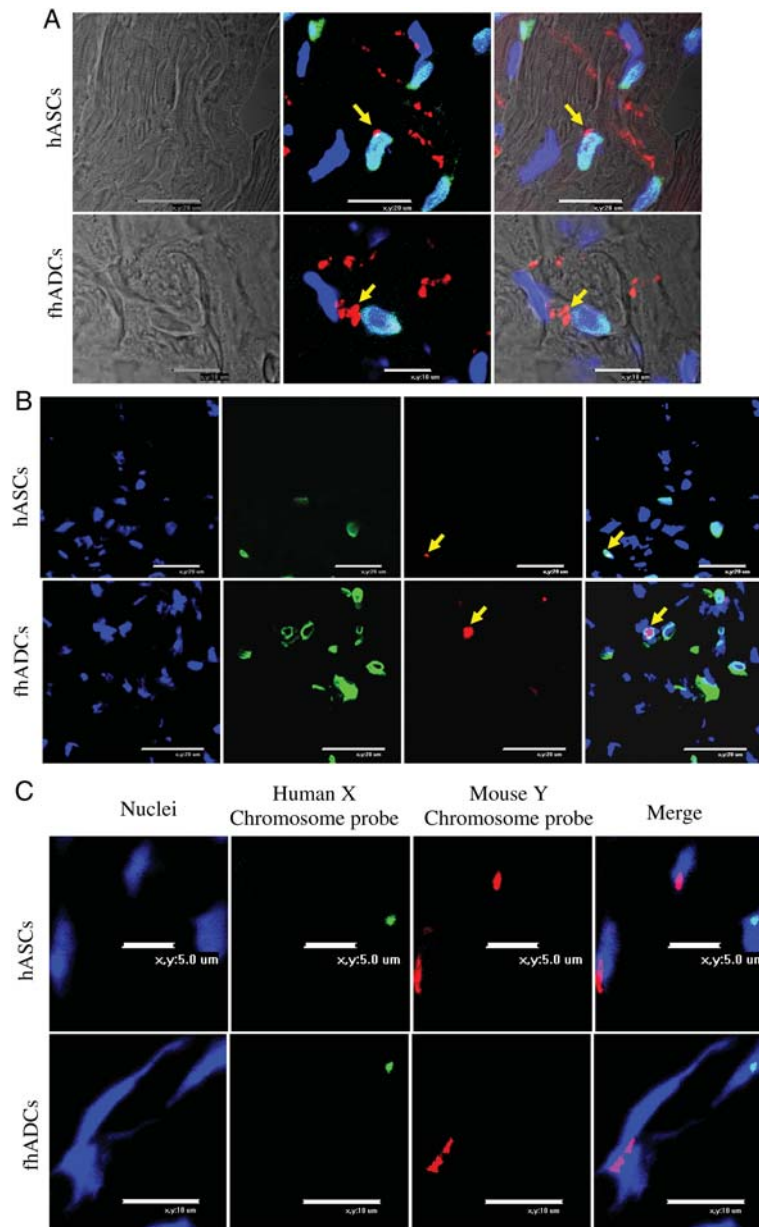
This is the first study to demonstrate that freshly isolated human ASCs, like cultured hASCs, have the ability to engraft and thereby improve cardiac function when transplanted directly into the hearts of mice subjected to an acute MI. In addition, this study provides insight towards a better understanding of the mechanisms underlying the beneficial effect of ASCs on cardiac function. First, the injected hASCs survived in injured hearts up to 4 months after MI and did not migrate in a significant number into other organs. Secondly, engrafted ASCs indicate a cardiomyogenic and vascular cell differentiation pathway without evidence of cell fusion. In addition, the injected cells promote angiogenesis and reduce the apoptosis rate in cardiomyocytes at the border zone.

Adipose tissue is an attractive cell source for stem cell-based treatment of injured myocardium because it is relatively easy to harvest, available in sufficient quantities, and yields a significantly higher number of uncommitted stem cells compared with other

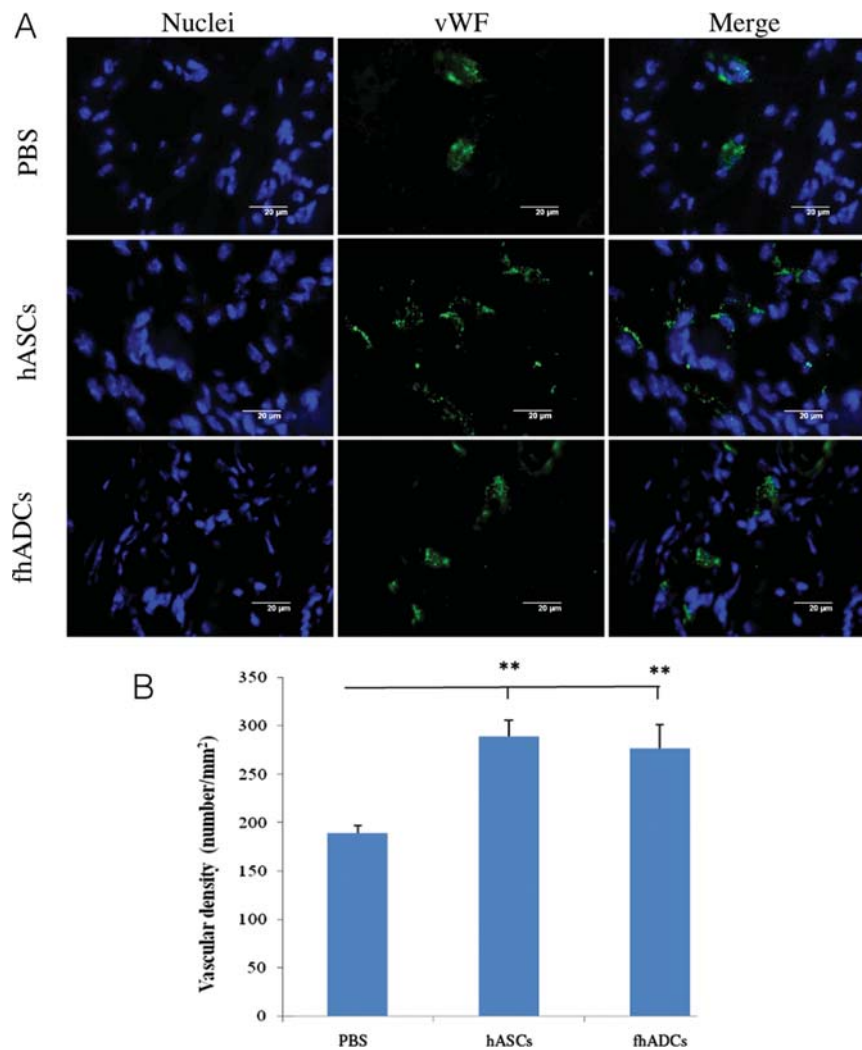
sources such as bone marrow. The advantage of using adipose tissue as the source of uncultured stem cells (fhADCs) obviates the need for prior cell expansion *in vitro*, thus potentially allowing for immediate autologous cell transplantation in the case of an acute infarction.

It has been postulated that adult peripheral blood CD34-positive cells injected into SCID mice transform into cardiomyocytes after experimentally induced MI and that most of the newly formed cardiomyocytes result from cell fusion.<sup>10</sup> Fusion has also been proposed as a major mechanism for the generation of hepatocytes<sup>11</sup> and Purkinje neurons.<sup>12</sup> However, fusion may be more difficult and unlikely in constantly beating myocardium. Considering low frequency of the fusion events found by others, it is questionable whether generation of new cardiomyocytes by cell fusion could significantly improve cardiac function after MI. Injected BMSCs have been observed to fuse with host cells, but the frequency of cellular fusion was too low to be considered as the mechanism of stem cell-mediated cardiac recovery.<sup>13</sup> We





**Figure 5** Immunofluorescence and fluorescence *in situ* hybridization analysis of injected cells 4 weeks after myocardial infarction. (A) Immunofluorescence analysis of gap junction marker connexin 43. Human adipose tissue-derived stem cell- or freshly isolated human adipose tissue-derived cell-injected heart sections were triple-stained with 4',6-diamidino-2-phenylindole (blue) and antibodies to lamin A/C (green) and connexin 43 (red). Left column shows phase-contrast images; middle column shows fluorescence images; and right column shows merged images with connexin 43 expressed in a punctate pattern (pointed by arrows) between injected human cells and mouse host cells. Scale bars represent 20 μm in upper panels and 10 μm in lower panels. (B) Immunofluorescence analysis of proliferation marker Ki67. Heart sections were triple-stained with 4',6-diamidino-2-phenylindole (blue), and antibodies to lamin A/C (green) and Ki67 (red). Merged images in right column show that both injected human adipose tissue-derived stem cells and freshly isolated human adipose tissue-derived cells were positive for Ki67 (pointed by arrows). Scale bars represent 20 μm. (C) Fluorescence *in situ* hybridization analysis of cell fusion. Heart sections from male mice receiving human adipose tissue-derived stem cells or freshly isolated human adipose tissue-derived cells (from male patients) were hybridized with probes specific for human X chromosome (green) and mouse Y chromosome (red). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue). Merged images did not indicate injected human adipose tissue-derived stem cells or freshly isolated human adipose tissue-derived cells to be fused with host cells. Scale bars represent 5 μm in upper panels and 10 μm in bottom panels.



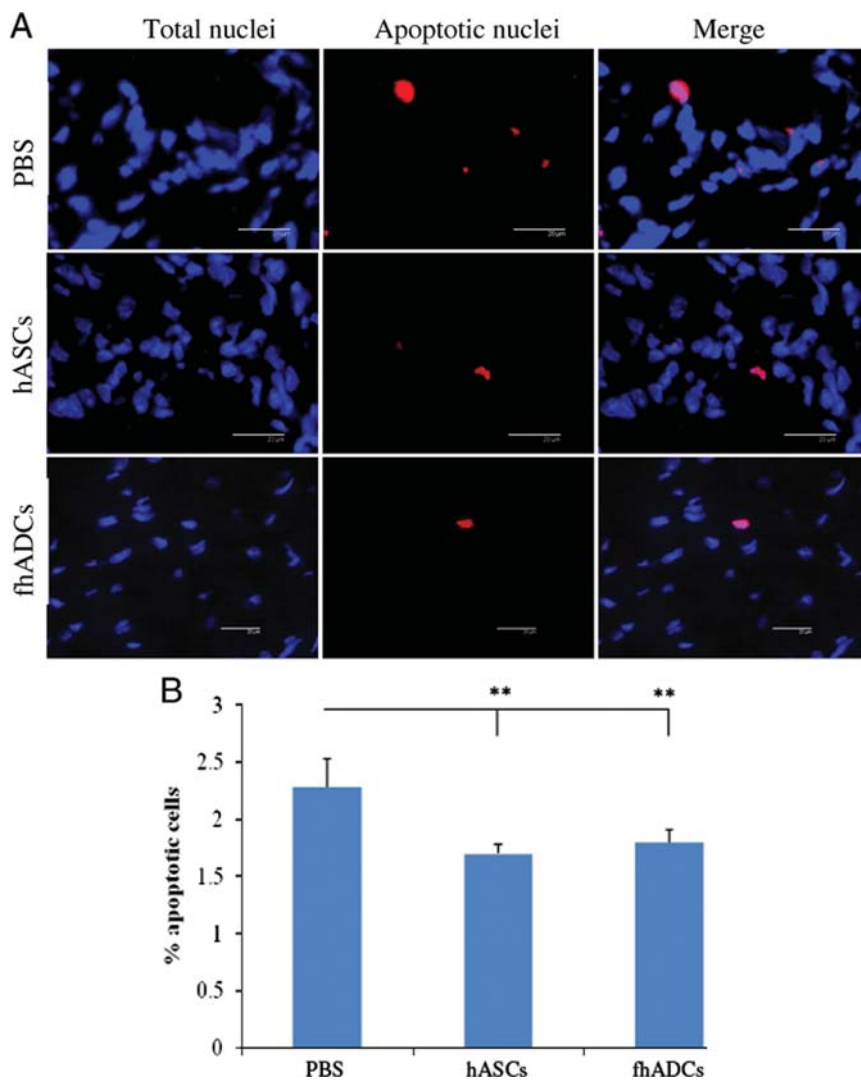
**Figure 6** Immunofluorescence analysis of vascular density in the peri-infarct zone 4 weeks after myocardial infarction. (A) Phosphate-buffered saline-, human adipose tissue-derived stem cell-, or freshly isolated human adipose tissue-derived cell-treated hearts were stained with 4',6-diamidino-2-phenylindole (blue) or antibody to endothelial cell marker von Willebrand factor (green). Scale bars represent 20 μm. (B) Quantification of vessel density in the three groups. Vessel density was significantly higher in the cell-injected groups than in the phosphate-buffered saline control group.  $n = 6$  per group.  $^{***}P < 0.01$ .

performed an extensive FISH analysis to assess whether the differentiation of injected cells could be the result of fusion between the injected cells and cardiac tissue-resident cells. Despite a careful and extensive analysis, we were unable to detect any fused cells, suggesting that the cardiomyocyte, EC, and SMC phenotypes of donor hASCs and fhADCs observed *in vivo* did not result from cell fusion.

We previously showed that hASCs express various functional ion channels.<sup>14</sup> In the present study, we detected clusters of injected hASCs and fhADCs in the border zone and infarct area of the hearts. Some of the cells developed a cardiac phenotype, incorporated into healthy myocardium surrounding the infarct area, and formed connexin 43-positive gap junctions between themselves and host cardiomyocytes. This is important for transplanted cells to exert an antiarrhythmic effect.<sup>15</sup> Our previous

studies have showed that transplantation of pig-derived ASCs increased the electrical stability of the heart.<sup>16</sup> However, whether the injected cells differentiated into functional cardiomyocytes remains to be confirmed by electrophysiological studies.

Tracking the survival and migration of injected ASCs *in vivo* is of major importance to elucidate the mechanisms underlying their favourable therapeutic effects. Previous studies reported poor survival of injected stem cells in injured hearts; in one study, no injected mouse ASCs were detected in hearts 30 days after transplantation.<sup>17</sup> Bone marrow-derived stem cells also survive poorly in infarcted injured hearts.<sup>18</sup> Several non-invasive tools, including iron particle and radiotracer, have been used to track cell survival, proliferation, and distribution *in vivo*. However, MRI signals do not reflect cell viability and proliferation because the iron particles might persist within dead cells, transfer to neighbouring spaces,

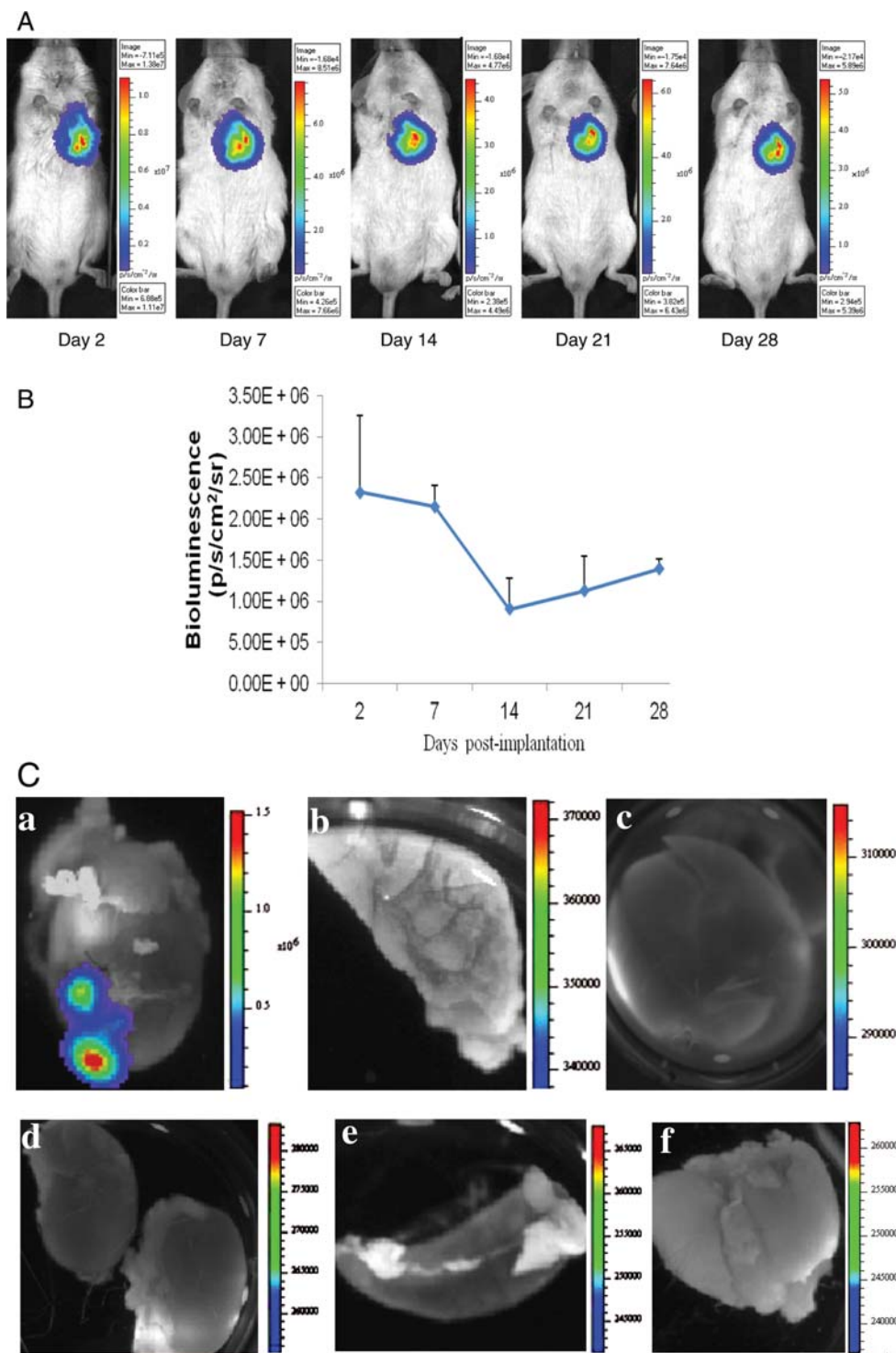


**Figure 7** Effect of transplanted human adipose tissue-derived stem cells and freshly isolated human adipose tissue-derived cells on cardiomyocyte apoptosis in ischaemic myocardium 4 weeks after myocardial infarction. (A) Cardiac sections from phosphate-buffered saline-, human adipose tissue-derived stem cell-, or freshly isolated human adipose tissue-derived cell-treated mice were stained with 4',6-diamidino-2-phenylindole for nuclei (blue) and with TUNEL reagent for apoptosis (red). Scale bars represent 20  $\mu\text{m}$ . (B) Quantitation of TUNEL-positive apoptotic cells in cardiac sections. There were significantly fewer apoptotic cells in the cell-treated groups compared with the phosphate-buffered saline control group.  $n = 6$  per group.  $**P < 0.01$ .

or be engulfed by resident macrophages after the transplanted cells die. Nuclear imaging based on radiotracers is also limited by concerns such as relatively short half-lives of radiotracers hampering long-term imaging of the cells, and adverse effects of the radiotracer at high dosage on stem cell viability and differentiation capacity.<sup>19,20</sup> In this study, hASCs were transduced with a lentiviral vector for stable genetic integration of the luciferase gene into the donor hASCs, resulting in production of bioluminescence signals by injected living cells and their progeny. In our calibration study, we showed that the intensity of bioluminescence was positively correlated with the number of hASCs *in vitro* and *in vivo*. Thus, in the present study, luciferase-based BLI was used as an indicator of living cells to trace the survival, migration, and proliferation of

the injected cells in the infarct zone in living mice. *In vivo* bioluminescence signals from injected hASCs decreased within first 2 weeks after MI, suggesting that some donor cells died as a result of ischaemic and inflammatory environment. This pattern concurs with other reports about BMSCs.<sup>18</sup> However, gradually increasing signal intensity was observed between Days 14 and 28, and strong signals were observed in the heart regions of the two mice we followed up to 10 and 16 weeks, indicating that BLI can track long-term cell behaviour *in vivo*, and hASCs can effectively survive and proliferate in that environment.

The prolonged survival of stem cells can be explained by several mechanisms. In the present study, ASCs demonstrate cardiomyogenic and vascular cell differentiation pathways. In addition, ASCs



**Figure 8** Bioluminescence imaging tracing of injected human adipose tissue-derived stem cells *in vitro* and *in vivo*. (A) Images of a representative mouse that received intramyocardial transplantation of  $5 \times 10^5$  human adipose tissue-derived stem cells expressing luciferase reporter gene over 4 weeks after myocardial infarction. Strong bioluminescence signals in the area overlying the heart were visualized throughout the 28 days, indicating survival of injected human adipose tissue-derived stem cells in the ischaemic heart. (B) Bioluminescence imaging of injected human adipose tissue-derived stem cells over time ( $n = 5$ ). Bioluminescence imaging decreased 2–14 days after myocardial infarction but then gradually increased again. (C) Bioluminescence imaging of various organs collected 16 weeks after human adipose tissue-derived stem cell transplantation. Bioluminescence was observed in the heart (a) but not in other organs, including the lung (b), liver (c), kidney (d), spleen (e), and brain (f). Coloured scale bar is in units of p/s/cm<sup>2</sup>/sr.

promoted angiogenesis via secretion of angiogenic factors, as shown previously. Further mechanisms include antiapoptotic effects afforded by IGF-1 and other paracrine factors secreted by stem cells.<sup>21</sup> We previously demonstrated that tumour necrosis factor- $\alpha$  (a pro-inflammatory cytokine) protects hASCs from H<sub>2</sub>O<sub>2</sub>-induced apoptosis through NF- $\kappa$ B,<sup>22</sup> which might contribute to the long-term survival of hASCs in injured hearts.

Various routes of cell delivery, including intravenous, intramyocardial, intracoronary, and direct LV cavity injection, have been used in animal experiments and clinical trials.<sup>20</sup> Cells delivered by systemic routes may end up at unwanted locations. In the present study, we used direct intramyocardial injection. The injected hASCs were retained in the heart and did not migrate in a significant number (the minimum number of cells able to be detected by BLI *in vivo* is 800) into other organs or tissues up to 4 months after transplantation. Post-mortem histological analysis of the engraftment and survival of injected fhADCs confirmed that these cells were also retained in the heart, and no fhADCs were found in other organs. Expression of Ki67 in fhADCs confirmed their viability.

In conclusion, fhADCs, like cultured hASCs, improve cardiac function after MI. The improvement in cardiac function is associated with a fusion-independent differentiation pathway, enhancement in neovascularization, and reduction in apoptosis, suggesting that injected cells functionally engraft, proliferate, differentiate, and produce paracrine growth factors in the infarcted hearts.

## Supplementary material

Supplementary material is available at *European Heart Journal* online.

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