

# Repair of senescent myocardium by mesenchymal stem cells is dependent on the age of donor mice

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## Abstract

Myocardial infarction is one of the leading causes of mortality in aged people. Whether age of donors of mesenchymal stem cells (MSCs) affects its ability to repair the senescent heart tissue is unknown. In the present study, MSCs from young (2 months) and aged (18 months) green fluorescent protein expressing C57BL/6 mice were characterized with p16<sup>INK4a</sup> and  $\beta$ -gal associated senescence. Myocardial infarction was produced in 18-month-old wild-type C57BL/6 mice transplanted with MSCs from young and aged animals in the border of the infarct region. Expression of p16<sup>INK4a</sup> in MSCs from aged animals was significantly higher ( $21.5\% \pm 1.2$ ,  $P < 0.05$ ) as compared to those from young animals ( $9.2\% \pm 2.8$ ). A decline in the tube-forming ability on Matrigel was also observed in aged MSCs as well as down-regulation of insulin-like growth factor-1, fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) compared to young cells. Mice transplanted with young MSCs exhibited significant improvement in their left ventricle (LV) systolic and diastolic function as demonstrated by dp/dt<sub>max</sub>, dp/dt<sub>min</sub>, P<sub>max</sub>. Reduction in the LV fibrotic area was concomitant with neovascularization as demonstrated by CD31 and smooth muscle actin (SMA) expression. Real-time RT-PCR analysis for VEGF, stromal cell derived factor (SDF-1 $\alpha$ ) and GATA binding factor 4 (GATA-4) genes further confirmed the effect of age on MSC differentiation towards cardiac lineages and enhanced angiogenesis. These studies lead to the conclusion that repair potential of MSCs is dependent on the age of donors and the repair of senescent infarcted myocardium requires young healthy MSCs.

**Keywords:** myocardial infarction • mesenchymal stem cells • stem cell aging • p16<sup>INK4a</sup> • senescent heart

## Introduction

Mesenchymal stem cells (MSCs) have been shown to differentiate into cells of many organs [1, 2] including cardiomyocytes [3, 4] and can therefore effectively repair infarcted myocardium resulting in an improved cardiac function [5, 6]. Since the regenerative potential of the body declines with age, stem cells like other cells of the body are also prone to the adverse effects of aging. Nevertheless, there are no clear indications as to what happens within the cellular microenvironment causing the progenitor cells to shift from the quiescent to the senescent state [7]. Cyclin-dependent kinase inhibitors such as p16<sup>INK4a</sup> a marker for cellular senescence have been shown to be involved in haematopoietic stem cell (HSC) regulation [8, 9]. The expression of p16<sup>INK4a</sup> is undetectable in human and rodent tissues and is associated with replicative senescence in cell lines [10, 11]. Down-regulation of

p16<sup>INK4a</sup> mitigates defects in HSC repopulation and apoptosis thus improving stress tolerance and animal survival [12].

Several studies have been carried out to elucidate the effect of aging on stem cell function. Recently, it was demonstrated that a decline in the size of the progenitor pool hampers the ability of stem cells to repair aged muscle [13]. Circulating HSCs and endothelial progenitor cell (EPCs) numbers have also shown to be lower in aged as compared to young individuals [14]. Furthermore, aging can cause a decline in the osteoprogenitors due to decreased proliferative capacity of bone marrow stem cells (BMSCs) [15] and also reduce number of mesenchymal progenitor cells [16].

It is pointed out that incidence of cardiac disorder predominates in old age whereas most studies have focused on resurrecting young mouse heart [6, 17]. These studies have been used as the basis of treatment in human beings; however, source of stem cells for aged patients with myocardial infarction in most clinical trials has been autologous [18, 19] thereby inherently reducing the effectiveness of the therapy. The aim of the present study was to investigate the ability of MSCs obtained from young and aged animals to repair senescent mouse heart. An important finding of the study is the age-induced expression of p16<sup>INK4a</sup> in MSCs and its

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effect on cellular function. The results of the present study indicate a role of p16<sup>INK4a</sup> induced decline in cardiac differentiation potential and functional incapacitation of aged MSCs for the treatment of myocardial infarction.

## Materials and methods

### Laboratory animals

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1985). All animals were treated according to procedures approved by the Institutional Review Board (IRB) at the National Center of Excellence in Molecular Biology, Lahore, Pakistan (protocol FWA00001758).

### Cell isolation and culture

MSCs were isolated according to the procedure described previously [20]. The tibias and femurs were removed from C57BL/6 transgenic green fluorescent protein (GFP) expressing 2 and 18 months old mice. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 20% foetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37°C in humid air and 5% CO<sub>2</sub>. After being seeded for 2 days the MSCs adhered to the bottom of the culture flasks and the HSCs remain suspended in the medium. Medium was changed after every 3 days. Cells were grown for a week till 90% confluent.

### Flow cytometry

For flow cytometry analysis, MSCs from experimental animals were washed with phosphate buffered solution (pH = 7.4) and incubated in the dark for 30 min. at room temperature with CD45FITC (BD Pharmingen, San Diego, CA, USA), CD34PE (BD Pharmingen), CD44PE (BD Pharmingen), CD90FITC (BD Pharmingen) and CD105FITC (BD Pharmingen) antibodies. The specific fluorescence of 10,000 cells was analysed on FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using Cell Quest Pro software.

### Gene profiling of MSCs from young and aged animals

RNA was extracted from MSCs isolated from young and aged animals using trizole reagent (Invitrogen Corporation, Carlsbad, CA, USA) and quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). cDNA synthesis was carried out from 1 µg of RNA sample using M-MLV reverse transcriptase (Invitrogen). RT-PCR analysis for IGF-1, FGF-2, vascular endothelial growth factor (VEGF) and HGF was carried out using a GeneAmp PCR system 9700 (Applied Biosystems, Inc., Foster City, CA, USA) with GAPDH as internal control.

### Analysis of β-galactosidase associated senescence

Senescence associated β-galactosidase staining was performed as described by Dimri *et al.* [21]. Cells were cultured to 80–90% confluence, fixed in 2% formaldehyde and incubated overnight at 37°C with fresh β-gal staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (Sigma-Aldrich, St Louis, MO, USA), 40 mM citric acid, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM MgCl<sub>2</sub>).

### In vitro tube-forming assay

Matrigel was thawed on ice to prevent premature polymerization, aliquots of 50 µl were plated into individual wells of a 48-well tissue culture plate (Corning Incorporated, Corning, NY, USA) and allowed to polymerize at 37°C for at least 30 min. To investigate the tube-forming potential of MSCs from young and aged animals, cells were subjected to hypoxia by treatment with 100 µM H<sub>2</sub>O<sub>2</sub> for 60 min. MSCs were removed with trypsin/ethylenediaminetetraacetic acid after normoxic and hypoxic treatments and plated on Matrigel coated wells in a concentration of 1 × 10<sup>5</sup> cells/well. The formation of tubular structures in the respective wells were examined at 6, 24 and 48 hrs after incubation of cells at 37°C in 5% CO<sub>2</sub> with a phase contrast microscope (IX-51–Olympus, Tokyo, Japan).

### MSCs and neonatal cardiomyocytes co-culture model

Neonatal rat cardiomyocytes were isolated from Sprague-Dawley rat pups (1–2 days old, *n* = 15 per isolation). The cells were separated with neonatal cardiomyocyte isolation system (Worthington Biomedical Corporation, Lakewood, NJ, USA) as described by Xu *et al.* [22]. MSCs from young and aged animals were separately co-cultured with neonatal cardiomyocytes in a ratio of 1:40. Cells were then allowed to grow for a week followed by immunocytochemical analysis.

### Immunocytochemistry

Immunocytochemistry was done as described by Kudo *et al.* [23] on both the cell types after passage 1 (day 4). Primary antibodies used for MSC mouse monoclonal anti-p16<sup>INK4a</sup> (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies. Expression of early cardiac transcription factors was assessed by staining with rabbit polyclonal anti-Myosin enhancing factor-2 (MEF; Santa Cruz) and goat polyclonal anti-GATA binding protein-4 (GATA-4; Santa Cruz) antibodies. Secondary antibodies of donkey anti-rabbit IgG, anti-goat IgG and antimouse IgG (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA) conjugated with rhodamine were applied. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent images were obtained with an Olympus BX-61 microscope equipped with DP-70 digital camera (Olympus).

### Myocardial infarction and cell transplantation

Myocardial infarction was produced in female wild-type C57BL/6 aged (18 months old) mice under anaesthesia with sodium pentobarbital (40 mg/kg) as described by Lanza *et al.* [24]. In brief, the heart was exposed by left side

limited thoracotomy and left anterior descending artery (LAD) was ligated with 6–0 silk suture 1 mm from the tip of the normally positioned left auricle. Animals with myocardial infarction were divided into three groups ( $n = 8$ ) each. Group I mice were injected with normal saline and represented the control (sham) group. Groups II and III mice were transplanted with MSCs from aged and young animals, respectively, in the border of the infarct area. MSC concentration used in the experiments was  $1 \times 10^6$ /ml and animals were given intramyocardial injections of MSCs ( $50 \mu\text{l} \sim 50,000$  cells/animal) at the time of LAD ligation.

## Assessment of heart function

Mice were anaesthetized with sodium pentobarbital (40 mg/kg) by intraperitoneal injection. The right carotid artery was cannulated with a micro tip pressure transducer catheter (SPR-839, Millar Instruments, Inc., Houston, TX, USA) connected to MPCU-400 P-V signal conditioning hardware for data acquisition. The inferior vena cava was exposed and inferior vena cava occlusion was performed by external compression. Haemodynamic parameters and PV loops were recorded during steady state. Left ventricle (LV) systolic function was evaluated by  $P_{\text{max}}$ ,  $dp/dt_{\text{max}}$  and arterial elastance. LV diastolic function was evaluated by end-diastolic pressure and  $dp/dt_{\text{min}}$ . Haemodynamic parameter analysis was carried out using Millar's PVAN software (Version 3.3).

## Measurement of infarct size

Fixed hearts were embedded in paraffin and sections were cut from the mid-LV and base. All sections were stained with Masson's Trichrome. Images of the LV area of each slide were taken by an Olympus BX-61 microscope equipped with Digital Camera DP-70 (Olympus). Fibrosis and total LV area of each image were measured using the Image Pro Plus and the percentage of the fibrotic area was calculated as (fibrosis area/LV area)  $\times 100$ .

## Immunohistochemistry

Mice were killed at 2 and 8 weeks after MSC transplantation. Hearts were fixed in 4% paraformaldehyde and then snap frozen in liquid nitrogen. Sections were cut at 5–8  $\mu\text{m}$  in a Cryostat (Microm International GmbH, Walldorf, Germany) at  $-20^\circ\text{C}$ . Sections were stained with mouse monoclonal anti-Ki67 antibody (BD Pharmingen) for cell proliferation, mouse monoclonal anti-p16<sup>INK4a</sup> antibody (Santa Cruz Biotechnology, Inc.) for the identification of senescent myocytes,  $\alpha$ -sarcomeric actin (Sigma-Aldrich) that stains the cardiac muscle and mouse anti-CD31 (Chemicon International, Temecula, CA, USA) and  $\alpha$ -smooth muscle actin (Sigma-Aldrich) antibodies to check evidence for angiogenesis.  $\beta$ -galactosidase associated senescence was also measured in heart sections from all the experimental groups. Secondary antibodies of Donkey anti-rabbit IgG, anti-goat IgG and antimouse IgG (Jackson ImmunoResearch Laboratories, Inc.) conjugated with rhodamine and FITC were used. Capillary density was measured by identifying CD31 and  $\alpha$ -SMA expressing capillaries and small arterioles per five random fields within two separate sections of each group.

## Real-time RT-PCR

RNA was isolated using RNA isolation Midi kit (Qiagen, Inc., Germantown, MD, USA) from the hearts of control and treated groups. Isolated RNA was

used for cDNA synthesis using c-DNA synthesis kit (Fermentas International Inc., Burlington, ON, Canada). Serial dilutions of control mouse c-DNA starting from 10pg were made and used as a standard. Real-time RT-PCR was carried out by using SYBR Green PCR Super Mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 8  $\mu\text{M}$  of each primer, and 100–500 ng/ $\mu\text{l}$  of template DNA on BioRad System iQ5. The relative ratio and standard deviation between the normal and treated samples were calculated using comparative  $C_t$  method ( $\Delta\Delta C_t$  value), as recommended by the BioRad iQ5system.

## TUNEL staining

Terminal dUTP nick end-labelling (TUNEL) assay was performed to determine the level of apoptosis within the MSC transplanted hearts. Heart sections 5  $\mu\text{m}$  thick were stained with MEBSTAIN Apoptosis Kit II (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) as described by Takashi *et al.* [25]. For each heart, the percentage of apoptotic myocyte nuclei in peri-infarcted area was calculated by counting the number of TUNEL<sup>+</sup> myocyte nuclei per total number of nuclei in six sections. Apoptotic myocytes in the infarcted region were excluded from counting.

## Statistical analysis

Quantitative data were obtained from two cover slips each from three separate experiments for MSC characterization. Five random fields per cover slip were analysed and the data were expressed as mean  $\pm$  S.E.M. Haemodynamic parameters were assessed by one-way analysis of variance ANOVA ( $P$ -value of less than 0.05 was considered statistically significant). Analysis of differentiation markers MEF-2 and GATA-4, percentage of fibrosis, comparison of vascular density and measurements of senescent and apoptotic myocytes between groups was performed by Student's unpaired  $t$ -test ( $P$ -value of less than 0.05 was considered statistically significant).

## Results

### *In vitro* studies

#### Characterization of MSCs from young and aged animals

MSCs from experimental animals were confirmed with flow cytometry for the expression of CD44 (93.7%), CD90 (97.9%), CD105 (96.2%), CD34 (1.1%) and CD45 (0.4%) (Fig. 1). Gene profiling of young and aged MSCs showed increased expression of IGF-1, FGF-2, VEGF and HGF compared to aged cells (Fig. 2A). To assess whether MSCs show senescence characteristics with age, expression of p16<sup>INK4a</sup> was observed. MSCs from aged animals showed significantly high expression of p16<sup>INK4a</sup> ( $21.5\% \pm 1.2$   $P < 0.05$ ) compared to MSCs from young animals ( $9.2\% \pm 2.8$ , Fig. 2B, C). Figure 2(D) and (E) demonstrates senescence associated  $\beta$ -galactosidase staining in MSCs from young ( $6.7\% \pm 1.3$ ) and aged ( $16.2\% \pm 1.6$ ) animals.

#### *In vitro* tube-forming studies

*In vitro* formation of tubular structures under normoxic and hypoxic conditions were observed. Tube formation were found

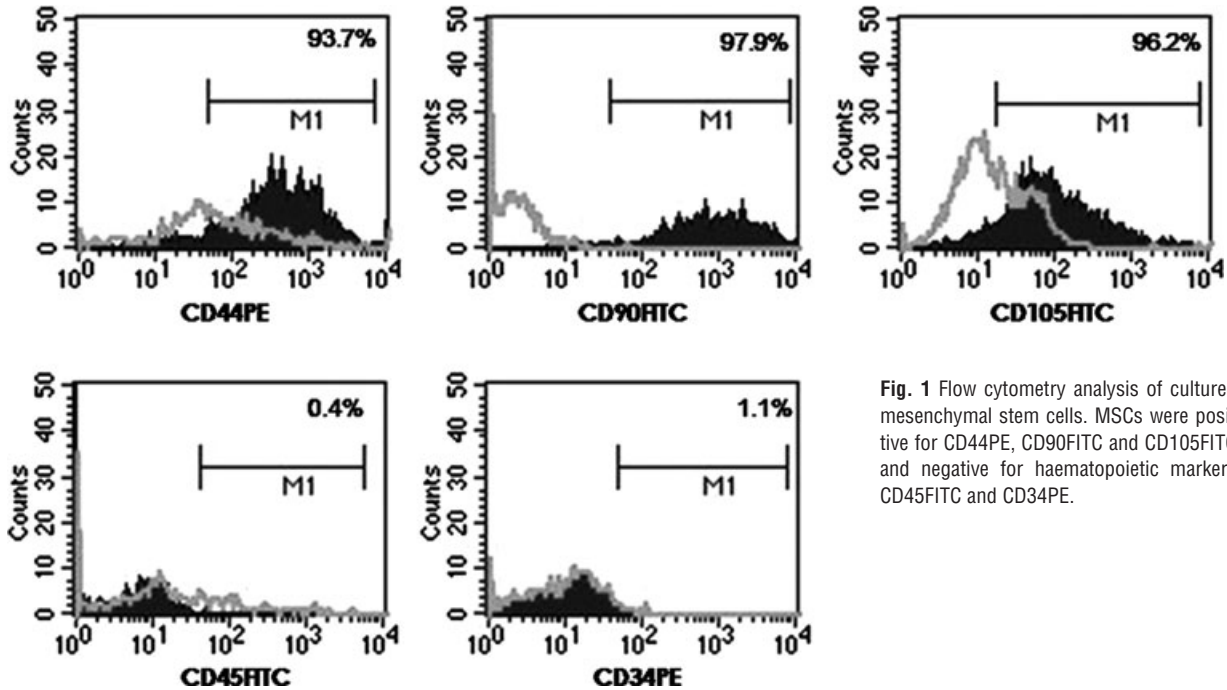


Fig. 1 Flow cytometry analysis of cultured mesenchymal stem cells. MSCs were positive for CD44PE, CD90FITC and CD105FITC and negative for haematopoietic markers CD45FITC and CD34PE.

to be highest in MSCs from young animals (Fig. 2F) compared to MSCs from aged animals after hypoxic treatment (Fig. 2G). Under normoxia, low tube formation activity was observed as compared to hypoxia in young and aged MSCs (data not shown). These results clearly showed the *in vitro* pro-angiogenic ability of young MSCs in response to hypoxic treatment while also highlighting the poor angiogenic response by MSCs from aged animals.

#### Aged MSCs decline in commitment towards cardiac lineages

To determine whether MSCs demonstrate age dependent decline in cardiac commitment, MSCs from young and aged animals were co-cultured with rat neonatal cardiomyocytes and assessed for early cardiac transcription factors MEF-2 and GATA-4. The expression of MEF-2 in MSCs from young animals was  $63.6\% \pm 2.9$  (Fig. 3 A, B) while it was reduced to  $46.8\% \pm 3.4$  in MSCs from aged animals (Fig. 3C, D). Similarly, the expression of GATA-4 in MSCs from young animals was  $55\% \pm 1.8$  (Fig. 3E, F) while it was  $41.6\% \pm 1.0$  in MSCs from aged animals (Fig. 3G, H). The level of senescence in MSCs from young and aged animals after co-culture was analysed by p16<sup>INK4a</sup> expression. The level of p16<sup>INK4a</sup> in MSCs from young animals was  $17.2\% \pm 2.5$  (Fig. 3I, J) while it increased to  $35.8\% \pm 2.0$  in MSCs from aged animals (Fig. 3K, L) showing a significantly high level of senescence. Expression of  $\beta$ -gal in co-culture was found to be  $14.3\% \pm 3.3$  and  $24.9\% \pm 1.3$  in young and aged MSCs, respectively. The expression of different markers of differentiation and senescence are compared in Fig. 3M ( $P < 0.02$ ).

## In vivo studies

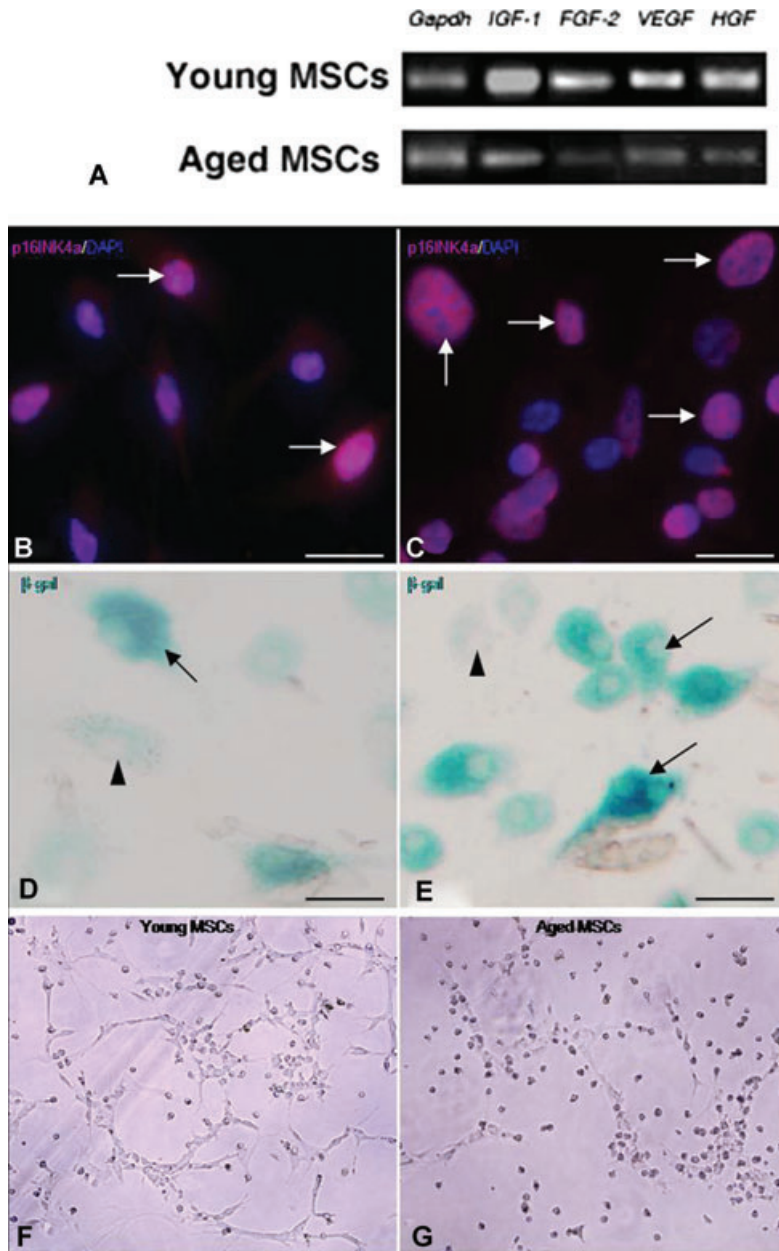
### Homing and proliferation

GFP<sup>+</sup> MSCs after transplantation were able to home to the infarcted area of the myocardium (Fig. 4A, B). In cell transplanted groups, GFP<sup>+</sup> cells observed in the infarcted myocardium were approximately 15–20% of the initially transplanted number. Cell proliferation was observed after 2 weeks in the damaged area of the transplanted hearts by Ki67 which is a nuclear protein expressed by cells in G<sub>1</sub>, S, G<sub>2</sub> and M phases. The number of Ki67<sup>+</sup> nuclei was significantly increased in Group III hearts (Fig. 4E, F) as compared to group II hearts (Fig. 4G, H). The expression of Ki67 within groups is compared in Figure 4J. Enhanced muscle formation was observed in hearts transplanted with Young MSCs as compared to Aged MSCs after 8 weeks of transplantation (Fig. 4C, D). Transplanted cells were able to form connections with the host myocardium as demonstrated by connexin-43 expression (Fig. 4I).

### Haemodynamic parameters

Haemodynamic parameters at 8 weeks in group I mice showed decreased contractility as demonstrated by shifting of the P-V cardiac loops to the right and a decrease in the amplitude of dp/dt<sub>max</sub>. Other parameters also indicated dilated cardiomyopathy as demonstrated by a shift towards right in end-systolic volume (V<sub>es</sub>) and end-diastolic volume (V<sub>ed</sub>).

Heart function in groups II and III mice after MSC administration demonstrated an increase in contractility demonstrated by higher values of V<sub>es</sub> and V<sub>ed</sub>. Arterial elastance (E<sub>a</sub>) of groups II and III was

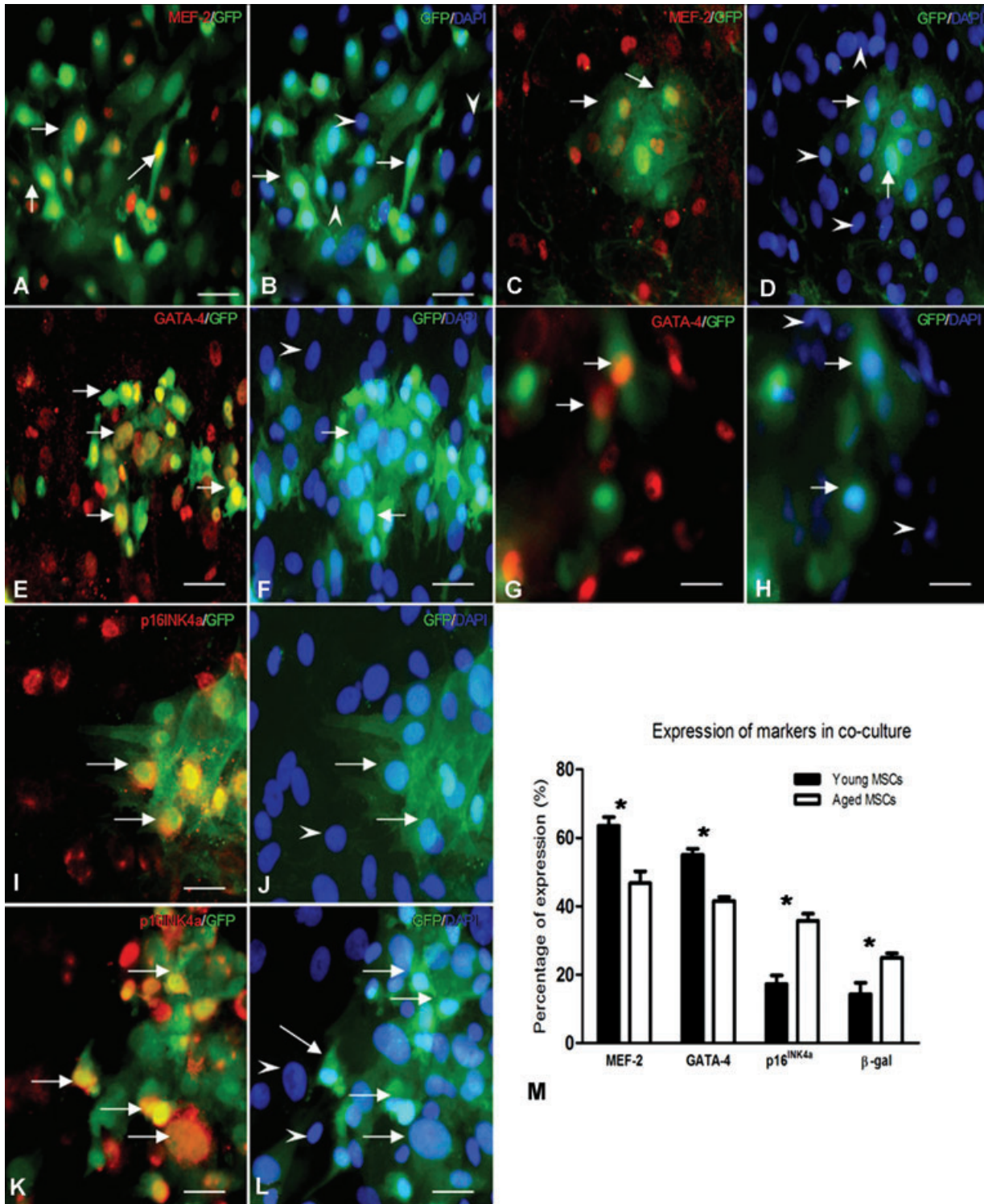


**Fig. 2** Characterization of MSCs from young and aged animals. **(A)** Expression of various growth factors in MSCs from young and aged animals. **(B)** p16<sup>INK4a</sup> expression in young animals. **(C)** The expression of p16<sup>INK4a</sup> was significantly higher in MSCs from aged animals. **(D)** Expression of β-gal in MSCs from young animals (arrow indicates β-gal<sup>+</sup> cells, arrowhead indicates β-gal<sup>-</sup> cells). **(E)** High expression of β-gal in MSCs from aged animals (400×, Scale bar ~ 20 μm). **(F)** Young MSCs show highest activity and formation of tubular structures after hypoxic treatment. **(G)** Low tube formation activity after hypoxic treatment in aged MSCs.

also higher as compared to group I demonstrating a high efficiency of LV work in MSC treated senescent hearts. However, improvement in the heart function was more pronounced in group III mice treated with MSCs from young animals as compared to group II mice transplanted with MSCs from aged animals. Load-dependent parameters such as ejection fraction (EF) revealed marked increases in both MSC administration groups. LV-diastolic function as indicated by end-diastolic pressure ( $P_{ed}$ ),  $\tau$ -(Glantz) and  $dp/dt_{min}$  was significantly improved ( $P < 0.05$ ) after MSC treatment as compared to control mice (Table 1).

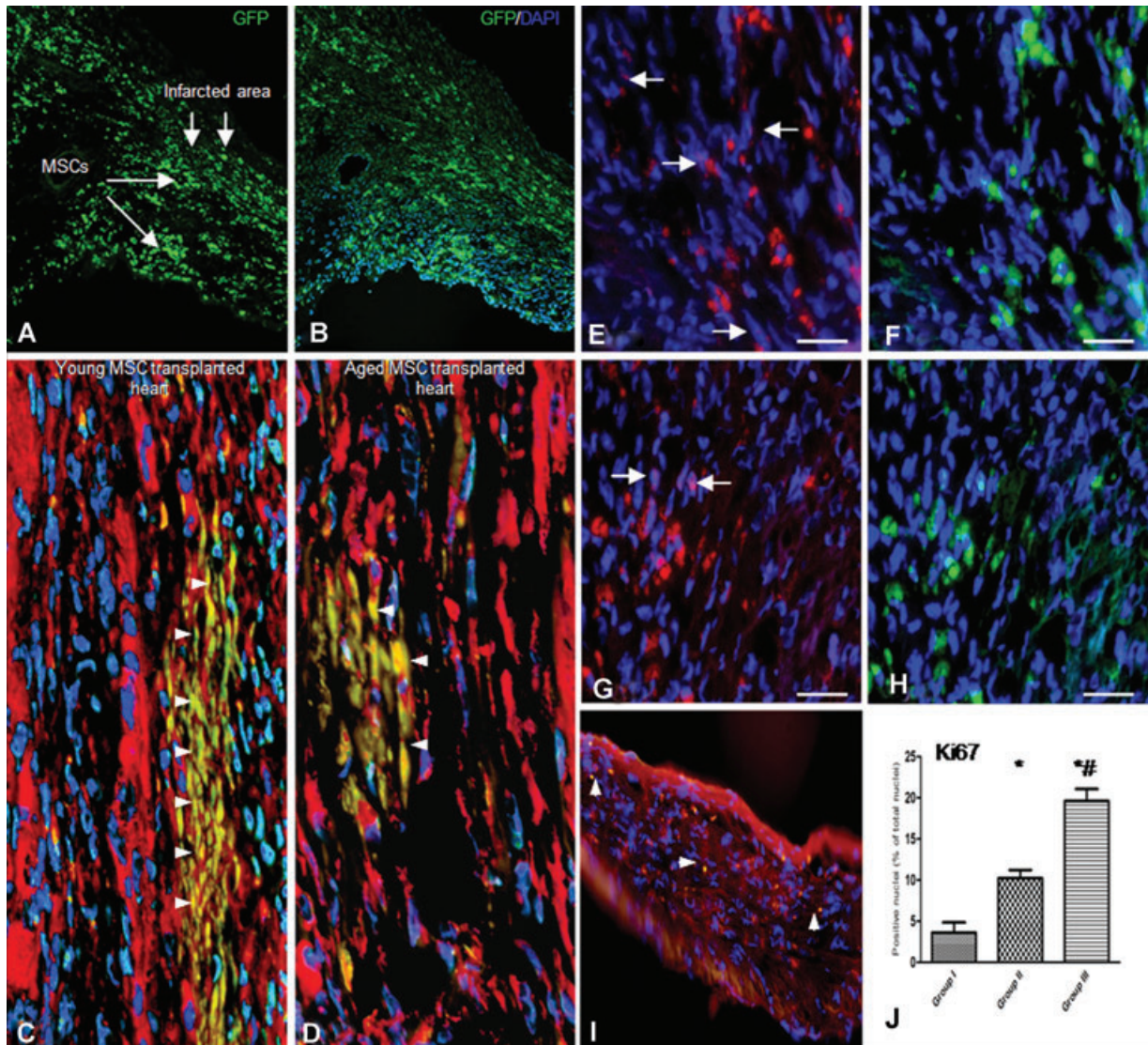
#### Reduction of fibrosis in the senescent heart

Heart sections from all the experimental mice groups were stained with Masson's trichrome to measure the level of fibrosis as mentioned earlier. There was extensive fibrosis of the LV ( $33.9\% \pm 2.8$ ) with thinning of the LV wall in group I. Administration of MSCs from aged and young animals significantly reduced the percentage of fibrosis from  $21.6\% \pm 1.0$  in group II to  $11.5\% \pm 3.2$  in group III, respectively, thus showing marked reduction as compared to group I (Fig. 5A–C). However when compared between groups II and III, the percentage of





**Fig. 3** Expression of transcription factors in co-culture. (A) GFP<sup>+</sup> Young MSCs (arrows) show high expression of MEF-2 (red). (B) GFP<sup>+</sup> Young MSCs (arrows) with GFP<sup>-</sup> cardiomyocytes (arrowheads) and nuclei stained with DAPI. (C) Low MEF-2 (red) expression in GFP<sup>+</sup> Aged MSCs (arrows). (D) GFP<sup>+</sup> Aged MSCs (arrows) with GFP<sup>-</sup> cardiomyocytes (arrowheads) and nuclei stained with DAPI. (E) High GATA-4 (red) expression in GFP<sup>+</sup> MSCs from young animals was observed. (F) Corresponding picture showing GFP<sup>+</sup> Young MSCs (arrows) with GFP<sup>-</sup> cardiomyocytes (arrowheads) with nuclei stained with DAPI. (G) Low GATA-4 (red) expression in GFP<sup>+</sup> Aged MSCs. (H) GFP<sup>+</sup> Aged MSCs (arrows) with GFP<sup>-</sup> cardiomyocytes (arrowheads) and nuclei stained with DAPI. (I) GFP<sup>+</sup> Young MSCs (arrows) show low expression of p16<sup>INK4a</sup> (red). (J) GFP<sup>+</sup> Young MSCs (arrows) with GFP<sup>-</sup> cardiomyocytes (arrowheads) and nuclei stained with DAPI. (K) High p16<sup>INK4a</sup> (red) expression in GFP<sup>+</sup> Aged MSCs (arrows). (L) GFP<sup>+</sup> Aged MSCs (arrows) with GFP<sup>-</sup> cardiomyocytes (arrowheads) and nuclei stained with DAPI. (200×, Scale bar ~ 20μm). (M) Differentiation markers after co-culture of MSC with Cardiomyocytes. The values are expressed in means ± S.E.M. \**P* less than 0.02 was considered as significant.



**Fig. 4** MSC homing, proliferation and formation of new muscle in the infarcted area. (A) GFP<sup>+</sup> MSCs home to the infarcted area. (B) Same as (A), nuclei stained with DAPI. (C and D) Young and Aged MSC transplanted heart show new muscle formation (arrowheads) after colocalization of GFP and  $\alpha$ -sarcomeric actin (red). (E) Group III heart with high Ki67 expression. (F) Same as (A) showing Young GFP<sup>+</sup> MSCs and nuclei stained with DAPI (G) Group II heart with low Ki67 expression. (H) Same as (C) showing Aged GFP<sup>+</sup> MSCs and nuclei stained with DAPI. (I) Connexin-43 expression between transplanted cells and host myocardium. (J) Bar graph shows Ki67<sup>+</sup> nuclei in the border zone. All values expressed as mean ± S.E.M. \**P*-value of <0.05 versus group (I); #*P*-value of <0.05 versus group II was considered significant.

**Table 1** Haemodynamic parameters

Haemodynamic parameters	Group I	Group II	Group III
Body weight (BW g)	25.5 ± 0.5	27.4 ± 0.7	30.1 ± 0.5
Ves. (μl)	20.86 ± 0.84	18.68 ± 0.13*	7.61 ± 0.19* <sup>#</sup>
Ved (μl)	22.62 ± 0.25	16.75 ± 0.25*	13.61 ± 0.18* <sup>#</sup>
P <sub>max</sub> (mmHg)	71.49 ± 0.25	81.17 ± 0.13*	85.41 ± 0.29* <sup>#</sup>
P <sub>ed</sub> (mmHg)	12.25 ± 0.19	10.44 ± 0.13	7.61 ± 0.91
EF (%)	10.58 ± 0.2	19.53 ± 0.16*	24.41 ± 0.25* <sup>#</sup>
Ea (mmHg/μl)	17.19 ± 0.16	22.84 ± 0.16*	28.57 ± 0.19* <sup>#</sup>
dp/dt max (mmHg/sec.)	3799 ± 69.39	4577 ± 123.02*	6053 ± 102.62* <sup>#</sup>
dp/dt min (mmHg/sec.)	-3337.66 ± 24.39	-3936.66 ± 24.66*	-5885.66 ± 86.16* <sup>#</sup>

Haemodynamic parameters: V<sub>es</sub>: end-systolic volume, V<sub>ed</sub>: end-diastolic volume, P<sub>max</sub>: maximum pressure, P<sub>ed</sub>: end-diastolic pressure, EF: ejection fraction, E<sub>a</sub>: arterial elastance, τ (Glantz): τ-Glantz method.

\**P* < 0.05 versus group I, <sup>#</sup>*P* < 0.05 versus group II. All data are expressed as mean ± S.E.M., *n* = 8 for each group.

fibrosis was significantly (*P* < 0.05) reduced in group III mice treated with MSCs from young animals. Comparison of fibrosis within groups is shown in Figure 5D.

### Angiogenesis in the senescent heart

The angiogenic response in senescent hearts treated with MSCs from young and aged animals was observed with CD31 and α-SMA expression primarily in the capillary endothelial cells and vascular smooth muscle cells of the infarct region. The expression of CD31 (Fig. 6A–C) and α-SMA (Fig. 6D–F) was higher in group III (43.8 ± 1.5 and 24 ± 1.5) and II (29.8 ± 2.1, 14.2 ± 3.5) as compared to group I (11 ± 2.4, 4.8 ± 1.05). However, the capillary density in group III was significantly higher (*P* < 0.01) than in group II (Fig. 6G, H).

### Expression studies in the senescent heart

Real-time RT-PCR analysis for the VEGF, SDF-1α and GATA-4 gene in senescent hearts was done for all the experimental groups. The expression of VEGF, SDF-1α and GATA-4 gene was observed to be significantly increased in group III mice treated with MSCs from young animals as compared to group II and group I mice (Fig. 6I). GAPDH primers were used as the loading control.

### Senescent myocytes

Senescent myocytes in the non-infarcted area were identified by immunohistochemistry with p16<sup>INK4a</sup>. The number of p16<sup>INK4a</sup> myocytes was reduced to 7.2 ± 1.9 in group III from 16.3 ± 1.6 in group II and 28.8 ± 2.1 in group I (Fig. 7A–C). However the expression was significantly (*P* < 0.05) reduced in group III as compared to groups I and II (Fig. 7D). Level of β-gal associated senescence was observed to be significantly lower (*P* < 0.05) in hearts treated with young MSCs compared to remaining groups and is compared in Figure 7D.

### Apoptosis

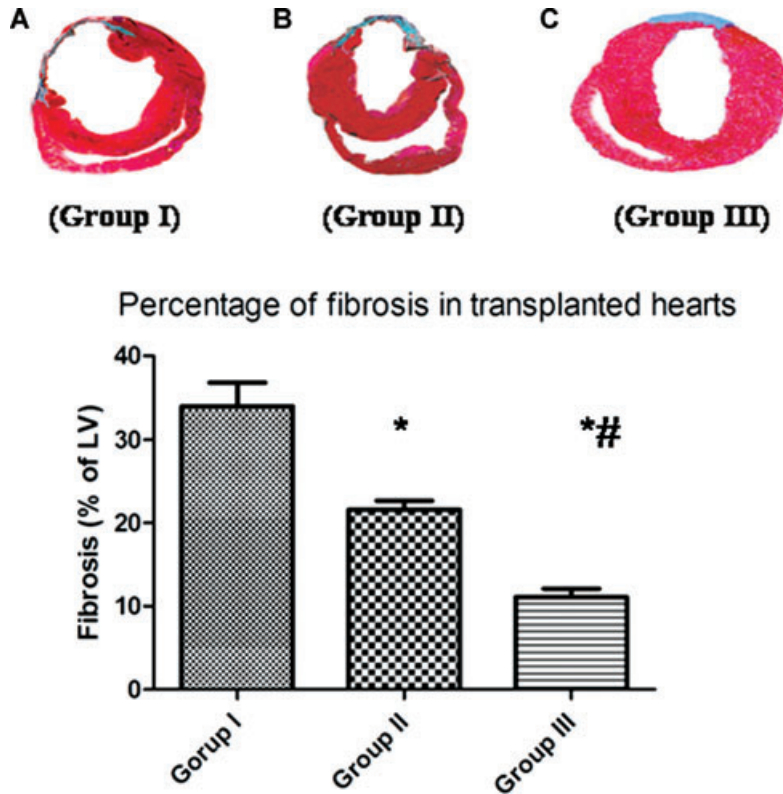
Level of apoptosis was analysed at 2 weeks by TUNEL assay. A reduction in the TUNEL<sup>+</sup> nuclei (8.6% ± 1.0, Fig. 8A) was observed in group III mice. When compared with group II mice, a reduction in the TUNEL<sup>+</sup> nuclei (19.6% ± 2.0, Fig. 8B) was observed as compared to group I mice (34.7% ± 1.7). The number of apoptotic nuclei was significantly less in group III (*P* < 0.03) as compared to group II and group I mice (Fig. 8C).

## Discussion

The aim of the study was to determine the effect of aging on MSCs and for this purpose cells from young and aged animals were characterized with p16<sup>INK4a</sup>, a marker for cellular senescence [26]. The results show a significantly high expression of p16<sup>INK4a</sup> in MSCs from aged animals and provide evidence for a possible role for p16<sup>INK4a</sup> induced aging in MSCs. There have been reports relating p16<sup>INK4a</sup> activation with age and a consequent decline in forebrain progenitors [27] and islet regenerative potential [28]. Recent data also suggests that expression of p16<sup>INK4a</sup> induces an age-dependent decrease in the proliferative capacity of certain tissue-specific stem cells and unipotent progenitors [29]. We also observed a significantly high expression of β-galactosidase in MSCs from aged animals after 7 days of culture and this has been shown as an effective way for the identification of senescent cells in culture [30]. This finding is in concordance with previous reports [16] and further validates senescent characteristics of the p16<sup>INK4a</sup> expressing MSCs from aged animals.

MSCs from young and aged animals were co-cultured with rat neonatal cardiomyocytes to assess the expression of MEF-2 and GATA-4. MSCs were easily distinguished by the expression of GFP and the presence of multiple nucleoli. MEF-2 transcription factor is involved in cardiac morphogenesis [31] and its expression level was found to be significantly higher in MSCs from young animals. GATA-4 has been shown to control cardiac structural and regulatory gene





**Fig. 5** Masson's trichrome staining of the heart sections after 8 weeks shows infarcted region of the LV wall with fibrosis. (A) Group I heart. (B) Group II heart. (C) Group III heart. (D) Bar graph indicates the % of Infarct size in the whole LV area. \*P-value of <0.05 versus group I; #P-value of <0.05 versus group II.

expression [32]. Our studies showed that MSCs from young animals were able to express high level of GATA-4 suggesting the superior ability of these cells to form cardiac phenotypes. MSCs from aged animals were positive for GATA-4 but their number was significantly low. Our results demonstrate a decline in commitment of MSCs from aged animals towards cardiac lineages and can be compared to previous reports that show a loss in the ability of MSCs to differentiate into osteogenic lineages with age [33, 34].

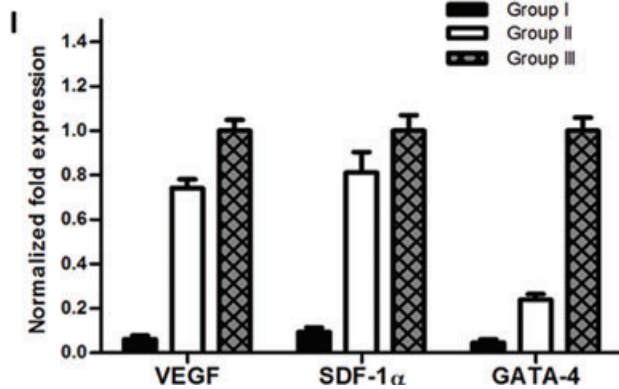
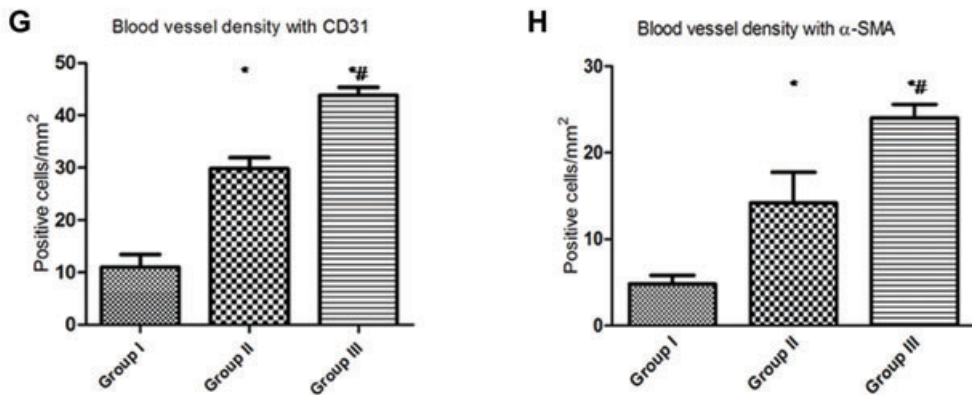
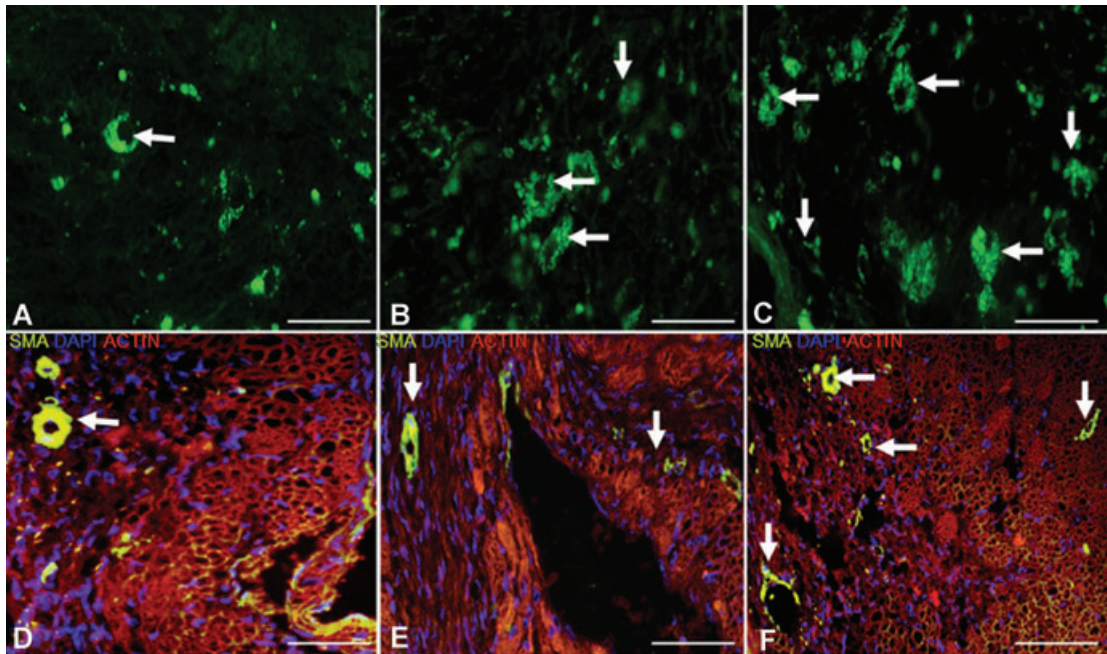
One of the important aspects of the study was to determine whether aging limits the ability of MSCs for the repair of senescent infarcted heart. Our results demonstrated that MSCs from young animals were able to improve function of the senescent heart significantly as compared to MSCs from aged animals. Our results show a possible association between improvement in senescent heart function and enhanced expression of paracrine mediators IGF-1, FGF-2, VEGF and HGF in young MSCs. Our finding concurs with previous report showing decline in expression of various paracrine factors [17] and also points towards susceptibility of paracrine capability of MSCs to advanced age. There have been reports documenting a diminished role for aged MSCs in the repair of infarcted myocardium [35]. Nevertheless, there is no report documenting the impact of aging on MSC mediated repair of the senescent heart. Myocardial repair potential of aged bone marrow stromal cells, skeletal muscle cells and smooth muscle cells has been compared previously [36]. However, this work does not document differences in the ability of young and aged MSCs

for the repair of senescent infarcted heart. We also observed that the cardiac function declined in the senescent myocardium with age and this is in unison with previous reports [37, 38].

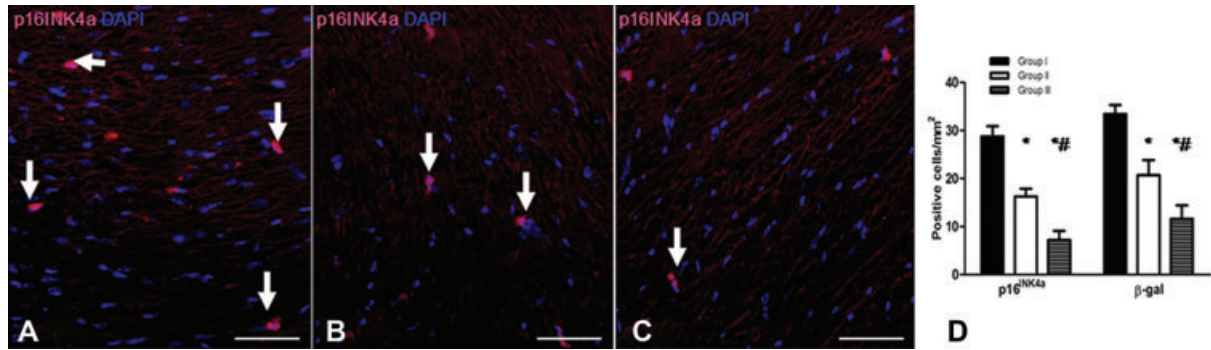
The ability of cells to reduce apoptotic levels is an important factor in the MSC mediated improvement of the senescent heart function and this was demonstrated by TUNEL assay and immunohistochemistry with p16<sup>INK4a</sup>. Our decision to employ p16<sup>INK4a</sup> as a marker for the identification of senescent myocytes is in concordance with recent reports correlating p16<sup>INK4a</sup> and myocyte aging [39, 40]. Myocyte apoptosis in animals and human beings is restricted to p16<sup>INK4a+</sup> cells which do not express telomerase and typically show severe telomere shortening [41].

It is known that the senescent heart is prone to cardiac failure mainly due to weakened angiogenesis [42]. Angiogenic response within the heart involves different cytokines and growth factors. Among them, VEGF has been shown to be essential for the improvement of angiogenesis in the senescent heart [43]. Our data provided a direct comparison between MSCs from young and aged animals highlighting the poor angiogenic response of MSCs from aged animals. These findings are in concordance with previous reports demonstrating the attenuation of the angiogenic ability of stem cells with age [35].

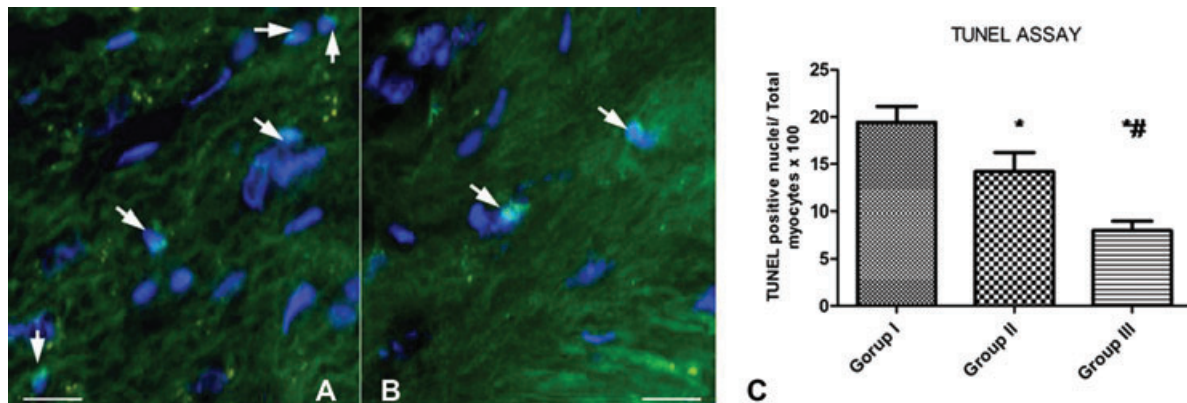
The ability of MSCs from aged animals to repair the senescent infarcted heart as demonstrated by our results is severely hampered with age and is in concordance with studies demonstrating declined stem cell function with advanced age [15, 16, 44]. Our mouse model for heart repair mimics the clinical pathology and



**Fig. 6** Evidence for angiogenesis in the senescent heart demonstrated with CD31 and  $\alpha$ -SMA expression. (A and D) Group I reduced angiogenesis. (B and E) Group II hearts with new blood vessel formation. (C and F) Enhanced neovascularization in group III, cells stained with CD31 and  $\alpha$ -SMA (green), cardiac muscle stained with sarcomeric actin (red) and nuclei positive for DAPI (400 $\times$ , Scale bar~ 10  $\mu$ m). (G, H) Blood vessel density analysis by fluorescent immunostaining for the expression of CD31 and  $\alpha$ -SMA. \**P*-value of <0.05 versus group I; #*P*-value of <0.01 versus group II. (I) Real-time RT-PCR comparative analysis of VEGF, SDF-1 $\alpha$  and GATA-4 genes. Group III showed significantly higher neovascularization and commitment towards cardiac lineages as compared to groups I and II.



**Fig. 7** Expression of p16<sup>INK4a</sup> in myocytes of the peri-infarct region in Control and MSC treated hearts. (A) Group I hearts, (B) Group II hearts, (C) Group III hearts (400×, Scale bar~ 10 μm). (D) Expression of p16<sup>INK4a</sup> and β-gal associated senescence in hearts treated with young and aged MSCs. \*P-value of <0.05 versus group I; #P-value of <0.05 versus group II.



**Fig. 8** Apoptosis measured by TUNEL assay. (A) Group II heart shows high number of apoptotic cells. (B) Reduction in the apoptotic cells in group III hearts. Nuclei are stained with DAPI (400×, Scale bar ~ 10 μm). (C) Level of apoptotic nuclei within hearts of group I, II and III and \*P-value of <0.05 versus group I; #P-value of <0.03 versus group II was considered significant.

points towards the incapacity of autologous MSCs isolated from aged patients. Therefore our results strongly support the use of MSCs from young individuals for the treatment of infarcted myocardium in aged patients. Moreover, there may be a pivotal role for p16<sup>INK4a</sup> in the functional impairment of MSCs with age and requires further investigations to validate this assumption. Therefore, it would be beneficial to transplant cells from young healthy donors and at the same time identify various factors involved in the impaired stem cell function with age.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

### Table S1 Primer sequences 5'-3' end

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

## References

- Krause DS. Plasticity of marrow-derived stem cells. *Gene Therapy*. 2002; 9: 754–8.
- Sharif S, Nakagawa T, Ohno T, *et al*. The potential use of bone marrow stromal cells for cochlear cell therapy. *Neuroreport*. 2007; 18: 351–4.
- Kuramochi Y, Fukazawa R, Migita M, *et al*. Cardiomyocyte regeneration from circulating Bone marrow cells in mice. *Pediatr Res*. 2003; 54: 319–25.
- Makino S, Fukuda K, Miyoshi S, *et al*. Cardiomyocytes can be generated from marrow stromal cells *in vitro*. *J Clin Invest*. 1999; 103: 697–705.
- Amado LC, Saliaris AP, Schuleri KH, *et al*. Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci USA*. 2005; 102: 11474–9.
- Orlic D, Kajstura J, Chimenti S, *et al*. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001; 410: 701–5.
- Sussman MA, Anversa P. Myocardial aging and senescence: where have the stem cells gone? *Ann Rev Physiol*. 2004; 66: 29–48.
- Cheng T, Rodriguez N, Shen H, *et al*. Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science*. 2000; 287: 1804–8.
- Walkey CR, Fero ML, Chien WM, *et al*. Negative cell-cycle regulators cooperatively control self-renewal and differentiation of hematopoietic stem cells. *Nature Cell Biol*. 2005; 7: 172–8.
- Krishnamurthy J, Torrice C, Ramsey MR, *et al*. *Ink4a/Arf* expression is a biomarker of aging. *J Clin Invest*. 2004; 114: 1299–307.
- Zindy F, Quelle DE, Roussel MF, *et al*. Expression of the p16<sup>INK4a</sup> tumour suppressor *versus* other INK4 family members during mouse development and aging. *Oncogene*. 1997; 15: 203–11.
- Janzen V, Forkert R, Fleming HE, *et al*. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16<sup>INK4a</sup>. *Nature*. 2006; 443: 421–6.
- Carlson ME, Conboy IM. Loss of stem cell regenerative capacity within aged niches. *Aging Cell*. 2007; 6: 371–82.
- Thijssen DH, Vos JB, Verseyden C, *et al*. Hematopoietic stem cells and endothelial progenitor cells in healthy men: effect of aging and training. *Aging Cell*. 2006; 5: 495–503.
- Stenderup K, Justesen J, Clausen C, *et al*. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone*. 2003; 33: 919–26.
- Stolzing A, Scutt A. Age-related impairment of mesenchymal progenitor cell function. *Aging Cell*. 2006; 5: 213–24.
- Jiang S, Haider KH, Ahmed RPH, *et al*. Transcriptional profiling of young and old mesenchymal stem cells in response to oxygen deprivation and reparability of the infarcted myocardium. *J Mol Cell Cardiol*. 2007; 44: 582–96.
- Schachinger V, Erbs S, Elsasser A, *et al*. Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. *N Engl J Med*. 2006; 355: 1210–21.
- Wollert KC, Meyer GP, Lotz J, *et al*. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: BOOST randomized controlled clinical trial. *Lancet*. 2004; 364: 141–8.
- Khan M, Manzoor S, Mohsin S, *et al*. IGF-1 and G-CSF complement each other in BMSCs migration towards infarcted myocardium in a novel *in vitro* model. *Cell Biol Int*. 2009; 33: 650–7.
- Dimri GP, Lee X, Basile G, *et al*. A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci USA*. 1995; 92: 9363–7.
- Xu M, Wani M, Dai YS, *et al*. Differentiation of bone marrow stromal cells into the cardiac phenotype requires intercellular communication with myocytes. *Circulation*. 2004; 110: 2658–65.
- Kudo M, Wang Y, Wani MA, *et al*. Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. *J Mol Cell Cardiol*. 2003; 35: 1113–9.
- Lanza R, Moore MA, Wakayama T, *et al*. Regeneration of the infarcted heart with stem cells derived by nuclear transplantation. *Circ Res*. 2004; 94: 820–7.
- Takashi E, Wang Y, Ashraf M. Activation of mitochondrial K(ATP) channel elicits late preconditioning against myocardial infarction *via* protein kinase C signaling pathway. *Circ Res*. 1999; 85: 1146–53.
- Anversa P, Rota M, Urbanek K, *et al*. Myocardial aging: a stem cell problem. *Basic Res Cardiol*. 2005; 100: 1–12.
- Molofsky AV, Slutsky SG, Joseph NM, *et al*. Increasing p16<sup>INK4a</sup> expression decreases forebrain progenitors and neurogenesis during ageing. *Nature*. 2006; 443: 448–52.
- Krishnamurthy J, Ramsey MR, Ligon KL, *et al*. p16<sup>INK4a</sup> induces an age-dependent decline in islet regenerative potential. *Nature*. 2006; 443: 453–7.
- Kim WY, Sharpless NE. The regulation of INK4/ARF in cancer and aging. *Cell*. 2006; 127: 265–75.
- Lee BY, Han JA, Im JS, *et al*. Senescence-associated  $\beta$ -galactosidase is lysosomal  $\beta$ -galactosidase. *Aging Cell*. 2006; 5: 187–95.
- Black BL, Olson EN. Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF-2) proteins. *Ann Rev Cell Dev Biol*. 1998; 14: 167–96.
- Molkentin JD. The zinc finger-containing transcription factors GATA-4, -5 and -6 ubiquitously expressed regulators of tissue-specific gene expression. *J Biol Chem*. 2000; 275: 38949–52.
- Fehrer C, Lepperdinger G. Mesenchymal stem cell aging. *Exp Gerontol*. 2005; 40: 926–30.
- Ross SE, Hemanti N, Longo KA, *et al*. Inhibition of adipogenesis by Wnt signaling. *Science*. 2000; 289: 950–3.
- Edelberg JM, Tang L, Hattori K, *et al*. Young adult bone marrow-derived endothelial precursor cells restore Aging-impaired cardiac angiogenic function. *Circ Res*. 2002; 90: e89–93.
- Zhang H, Fazel S, Tian H, *et al*. Increasing donor age adversely impacts the beneficial effects of bone marrow but not smooth muscle myocardial cell therapy. *Am J Physiol Heart Circ Physiol*. 2005; 289: H2089–96.
- Yang B, Larson DF, Watson R. Age-related left ventricular function in the mouse: analysis based on *in vivo* pressure-volume relationships. *Am J Physiol Heart Circ Physiol*. 1999; 46: H1906–13.
- Pacher P, Mabley JG, Liaudet L, *et al*. Left ventricular pressure-volume relationship in a rat model of advanced aging-associated heart failure. *Am J Physiol Heart Circ Physiol*. 2004; 287: 2132–7.

39. **Rota M, Hosoda T, De Angelis A, et al.** The young mouse heart is composed of myocytes heterogeneous in age and function. *Circ Res.* 2007; 101: 387–99.
40. **Beusejour CM, Campisi J.** Ageing: balancing regeneration and cancer. *Nature.* 2006; 443: 404–5.
41. **Torella D, Rota M, Nurzynska D, et al.** Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. *Circ Res.* 2004; 94: 514–24.
42. **Rivard A, Fabre JE, Silver M, et al.** Age-dependent impairment of angiogenesis. *Circulation.* 1999; 99: 111–20.
43. **Iemitsu M, Maeda S, Jesmin S, et al.** Exercise training improves aging-induced downregulation of VEGF angiogenic signaling cascade in hearts. *Am J Physiol Heart Circ Physiol.* 2006; 291: H1290–8.
44. **Fehrer C, Laschober G, Lepperdinger G.** Aging of murine mesenchymal stem cells. *Ann NY Acad Sci.* 2006; 1067: 235–42.