Cell fusion contributes to the rescue of apoptotic cardiomyocytes by bone marrow cells

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Abstract

Cardiomyocyte apoptosis is an important contributor to the progressive cardiac dysfunction that culminates in congestive heart failure. Bone marrow cells (BMCs) restore cardiac function following ischaemia, and transplanted BMCs have been reported to fuse with cells of diverse tissues. We previously demonstrated that the myogenic conversion of bone marrow stromal cells increased nearly twofold when the cells were co-cultured with apoptotic (TNF-α treated) cardiomyocytes. We therefore hypothesized that cell fusion may be a major mechanism by which BMCs rescue cardiomyocytes from apoptosis. We induced cellular apoptosis in neonatal rat cardiomyocytes by treatment with hydrogen peroxide (H_2O_2). The TUNEL assay demonstrated an increase in apoptosis from $4.5 \pm 1.3\%$ in non-treated cells to $19.0 \pm 4.4\%$ ($P < 0.05$) in treated cells. We subsequently co-cultured the apoptotic cardiomyocytes with BMCs and assessed cell fusion using flow cytometry. Fusion was rare in the non-treated control cardiomyocytes (0.3%), whereas H_2O_2 treatment led to significantly higher fusion rates than the control group ($P < 0.05$), with the highest rate of $7.9 \pm 0.3\%$ occurring at 25 μM H_2O_2. We found an inverse correlation between cell fusion and completion of cardiomyocyte apoptosis ($R^2 = 0.9863$). An *in vivo* mouse model provided evidence of cell fusion in the infarcted myocardium following the injection of BMCs. The percentage of cells undergoing fusion was significantly higher in mice injected with BMCs following infarction ($8.8 \pm 1.3\%$) compared to mice that did not undergo infarction ($4.6 \pm 0.6\%$, $P < 0.05$). Enhancing cell fusion may be one method to preserve cardiomyocytes following myocardial infarction, and this new approach may provide a novel target for cardiac regenerative therapies.

Keywords: cardiomyocytes ● bone marrow cells ● cell fusion ● apoptosis ● oxidative stress ● hydrogen peroxide

Introduction

Cardiomyocyte apoptosis is an important contributor to the cardiac dysfunction following myocardial ischaemic injury that eventually results in heart failure [1]. Ischaemic necrosis initiates the loss of cardiomyocytes, but progressive apoptosis participates in the cardiac remodelling that results in ventricular thinning and dilatation. Inflammatory signals and oxidative stress caused by reactive oxygen species (ROS) precipitate progressive cardiomyocyte apoptosis. Therefore, regenerative treatments must limit the loss of cardiomyocytes by apoptosis. Cell therapy was initiated to replace the lost myocytes [2, 3], but subsequent studies demonstrated that cell therapy prevented ventricular dysfunction by paracrine influences—inducing angiogenesis, limiting matrix remodelling, recruiting stem cells and reducing apoptosis [4]. However, the optimal cell type to limit myocyte apoptosis has yet to be determined. Bone marrow cell (BMC) therapy is a promising option to restore cardiac function following acute myocardial infarction [5–7]. However, the mechanisms underlying the functional improvements are controversial because of the limited long-term engraftment and differentiation of these cells.

Transplanted BMCs have been shown to fuse with cells of diverse tissues, including epithelial cells, Purkinje neurons, cardiomyocytes and hepatocytes [8–11], although the significance of these

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observations has been questioned because of the extremely low frequency of fusion. However, using a mouse model of tyrosinemia type I (mutated fumarylacetoacetate hydrolase gene), the transplantation of wild-type BMCs was demonstrated to restore liver function by cell fusion and to prevent death [12]. This study illustrates that cell fusion can have significant beneficial effects. In addition, it has been shown that chronic inflammation [9] and heat shock [11] greatly increase the frequency of cell fusion. However, fusion of bone marrow stem cells with cardiomyocytes has not been demonstrated to improve cardiac function following ischaemia, and the effects of cell fusion on the beneficial response to stem cell transplantation have not been demonstrated. We previously reported that the myogenic conversion of bone marrow stromal cells increased nearly twofold when the cells were co-cultured with apoptotic (TNF-α treated) cardiomyocytes [13]. The prominent increase in cell fusion during pathological events (e.g., inflammation and cell death) suggests that this process may be protective and one of several endogenous mechanisms for repair of damaged tissue.

On the basis of previous evidence reported by us and others, we hypothesized that cell fusion may be a mechanism by which BMCs rescue cardiomyocytes from apoptosis. We induced apoptosis in neonatal rat cardiomyocytes by treatment with hydrogen peroxide (H_2O_2) and then co-cultured the apoptotic cardiomyocytes with BMCs. We found evidence of cell fusion and demonstrated an inverse correlation between cell fusion and cardiomyocyte apoptosis. Furthermore, we provide evidence of *in vivo* cell fusion in the infarcted heart of mice following direct injection of bone marrow mesenchymal stromal cells (BMSCs) into the border zone of the infarct.

Materials and methods

Experimental animals

Transgenic Wistar rats expressing GFP (supplied by Dr. Armand Keating, University of Toronto) were used for isolation of BMSCs and haematopoietic stem cells (HSCs). Sprague-Dawley rats (1–2 days old; Charles River Laboratories, Senneville, QC, Canada) were used for isolation of cardiomyocytes. The mice used for the *in vivo* studies were obtained from the Jackson Laboratory, Bar Harbor, ME, USA. The C57BL/6-Tg (ACTb-EGFP) 1Osb/J mouse has a chicken cytoplasmic β-actin promoter with cytomegalovirus enhancer elements driving the expression of an enhanced GFP cDNA, and the Tg (CAG-DsRed*MST) 1Nagy/J mouse has a chicken β-actin promoter with cytomegalovirus immediate early enhancer elements driving the expression of an enhanced dishevelled red (DsRed) cDNA. The study protocol was approved by the Animal Care Committee of the University Health Network, and procedures were performed according to the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, revised 1996).

Cell isolation and culture

Bone marrow mesenchymal stromal cells were harvested from the femora and tibiae of GFP rats. After connective tissue was removed from around the bone, both ends of the bone were cut. Bone marrow plugs were flushed with Iscove's Modified Dulbecco Medium supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin G and 0.1 mg/ml streptomycin. Cells were plated and cultured in the same medium. Three days later, the medium was changed, and non-adherent cells were discarded. The medium was replaced every 3 days, and the cells were subcultured when confluency exceeded 90%. Bone marrow mesenchymal stromal cells from passages two or three were used for the studies. We have previously characterized the BMSCs as positive for CD90.1, CD44 and Sca-1 and negative for CD34, CD45, CD117 and CD31 [13].

Haematopoietic stem cells were harvested from the femora and tibiae of GFP rats as mentioned above. Mononuclear cells were isolated by density gradient centrifugation using histopaque-1077 (Sigma, Oakville, ON, Canada) according to the manufacturer's instructions. Biotin-conjugated CD90.1 (Thy-1.1) antibody (eBioscience, San Diego, CA, USA) and EasySep Biotin Selection Kit (STEMCELL Technologies, Vancouver, BC, Canada) were used to sort HSCs according to the instructions provided by the manufacturer.

Neonatal rat ventricular cardiomyocytes were prepared as described previously [13]. Briefly, atria were removed from the hearts. The ventricles were minced and digested with 0.15% trypsin (Life Technologies, Burlington, ON, Canada) at 37°C, and the isolated cells were pre-plated on a non-coated dish for 30 min to reduce contamination of cardiac fibroblasts. The cells were plated at a density of $1.5 \times 10^5/cm^2$ in 1:1 DMEM and Ham F12 (Life Technologies) containing 10% FBS, 100 U/ml penicillin G and 0.1 mg/ml streptomycin. BrdU (10 mM; Sigma) was added to the cells on the first day to prevent the growth of non-myocytes in the early stages of the experiment. After the first day of culture, BrdU was removed, and the cells were cultured in the same medium containing 8% FBS.

Cardiomyocyte labelling

Neonatal cardiomyocytes were labelled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma) following the manufacturer's instructions. In brief, the single-cell suspension was loaded with a proper volume of Diluent C and PKH26 (10 million cells with 1 ml Diluent C and 2 μl PKH26) and incubated at 25°C for 5 min. The staining reaction was stopped by adding an equal volume of FBS followed by an equal volume of culture medium and centrifugation at $400 \times g$ for 10 min. The labelled cells were washed three times with the culture medium and subsequently plated at a density of $1 \times 10^4/cm^2$.

BMC co-culture with apoptotic cardiomyocytes

Culture medium of the PKH26-labelled cardiomyocytes was replaced with serum-free medium for about 18 hrs on the day after labelling. Following serum starvation, cardiomyocytes were treated with H_2O_2 at various doses in serum-free medium for 18 hrs. H_2O_2-treated cardiomyocytes were co-cultured with BMSCs or HSCs at a ratio of 2:1 in DMEM/F12 with 2% FBS. All analyses of apoptosis following co-culture were performed on total cardiomyocytes.

The dual-chamber system consists of two chambers separated by a semipermeable membrane with a pore size of 4 μm. Cardiomyocytes treated with 25 μM H_2O_2 for 18 hrs were seeded in the lower chamber, and BMSCs were seeded in the upper chamber (the insert) in a 2:1

ratio. After 48 hrs or 72 hrs in culture, the cardiomyocytes were stained for annexin V.

Immunocytochemistry

Immunofluorescent staining was performed to identify the cardiac specific marker α-sarcomeric actinin. In brief, cultured cells were fixed in 2% paraformaldehyde for 10 min, blocked in 5% bovine serum albumin or 5% goat serum and then incubated with monoclonal anti-α-sarcomeric actinin (1:800; Sigma), followed by an Alexa555 goat anti-mouse IgG secondary antibody (1:200; Life Technologies). Nuclei were stained with DAPI (Sigma). The cells were examined using a Nikon Eclipse TE200 fluorescence microscope. Fused cells were identified as GFP/sarcomeric actinin double positive.

Fluorescence-activated cell sorting

PKH26-prelabelled cardiomyocytes co-cultured with either BMSCs or HSCs were harvested using 0.05% trypsin with EDTA (Gibco). For HSC identification, one million cells were removed for antibody staining with FITC-conjugated rat anti-mouse CD 90.1 (eBioscience). Antibody incubation was carried out for 30 min at 4°C in the dark. Isotype-identical IgG served as controls (BD, Mississauga, ON, Canada). Cells were analysed using a Beckman Coulter EPICS XL flow cytometer with EXPO32 ADC software. The fluorescence intensity of 10,000 cells for each sample was quantified.

Annexin V assay

Annexin V-FITC/propidium iodide (PI; BD) staining was used to evaluate apoptosis and necrosis of the cardiomyocytes according to the manufacturer's instructions. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35–36 kDa Ca^{2+}-dependent phospholipid-binding protein that has a high affinity for PS and binds to cells with exposed PS. Thus, it serves as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis. In brief, 5 μl annexin V-FITC and/or 5 μl PI was added to 1 $\times$ 10^5 cells in 100 μl binding buffer. The mixture was gently vortexed and incubated for 15 min at room temperature in the dark, and 400 μl of binding buffer was added to each sample. The samples were analysed within 1 hr using flow cytometry.

TUNEL assay

The *In Situ* Cell Death Detection Kit (Roche, Laval, QC, Canada) was used to label DNA strand breaks by terminal deoxynucleotidyl transferase (TdT), which catalyses polymerization of labelled nucleotides to free 3'-OH DNA ends in a template-independent manner (TUNEL reaction). Adherent cells or cryopreserved heart sections were fixed with a freshly prepared fixation solution (4% paraformaldehyde in PBS, pH 7.4) for 20 min at room temperature. After incubation with freshly prepared permeabilization solution (0.2% Triton X-100, 0.1% sodium citrate) for 15 min, the cells or heart tissue was labelled using the TUNEL reaction mixture in a humidified atmosphere for 60 min at 37°C in the dark. The

cells or heart tissue was subsequently rinsed three times with PBS to stop the reaction. Samples were directly analysed under a Nikon Eclipse TE200 microscope.

In vivo study

Mice were intubated and ventilated with 2% isoflurane. Through a thoracotomy, the pericardium was opened and the left anterior descending (LAD) coronary artery was ligated. Non-ligated (sham-operated) mice underwent thoracotomy only. For cell transplantation, 3 $\times$ 10^5 BMSCs were suspended in 15 μl of serum-free medium and injected immediately following ligation in three injections into the area around the region subtended by the ligated coronary artery (border zone of the infarct) in a predetermined series of manoeuvres that were consistent among animals. The medium control group received 15 μl of serum-free medium without cells. Hearts were collected 3 days after LAD ligation, and the injured and non-injured left ventricular regions were separated prior to digestion with 0.1% collagenase type II (Worthington, Lakewood, NJ, USA) at 37°C for 30 min. After filtration through a 70 μm wire mesh and centrifugation, the cells were washed in PBS supplemented with 2% FBS and 0.1% sodium azide and analysed by flow cytometry. To identify the fused cardiomyocytes *in vivo*, heart tissues were cryopreserved in OCT gel. GFP/RFP double-positive cells were observed using an Olympus Fluoview 2000 laser scanning confocal microscope.

Statistical analyses

Group data are expressed as mean $\pm$ SD. Data were compared between experimental groups using unpaired *t*-tests or one-way ANOVA followed by Tukey's test (if the F ratio of the ANOVA was statistically significant). Differences were considered significant at $P < 0.05$.

Results

Oxidative stress induces cardiomyocyte apoptosis

We used H_2O_2, as a potent generator of ROS, to induce apoptosis in neonatal rat cardiomyocytes. To assess apoptosis, we first examined the morphological changes in the cardiomyocytes following H_2O_2 treatment (Fig. 1A). Before H_2O_2 treatment, most of the cardiomyocytes beat spontaneously at a frequency of about 40 beats per min. After H_2O_2 treatment, the cardiomyocytes remained adherent but stopped beating. Some cells lost cell-cell contact as their cytoplasm retracted and demonstrated pycnosis followed by karyorrhexis. Second, we identified apoptotic cardiomyocytes using the TUNEL assay. Cardiomyocytes were stained both for sarcomeric actinin and TUNEL (Fig. 1A). The proportion of double-positive cells in the H_2O_2-treated group was 19.0 $\pm$ 4.4%, which was significantly higher than that of the control group (4.5 $\pm$ 1.3%, $P < 0.05$, Fig. 1A). We then used annexin V flow cytometry to examine the effect of varying doses of H_2O_2 treatment on cardiomyocyte apoptosis and varying incubation
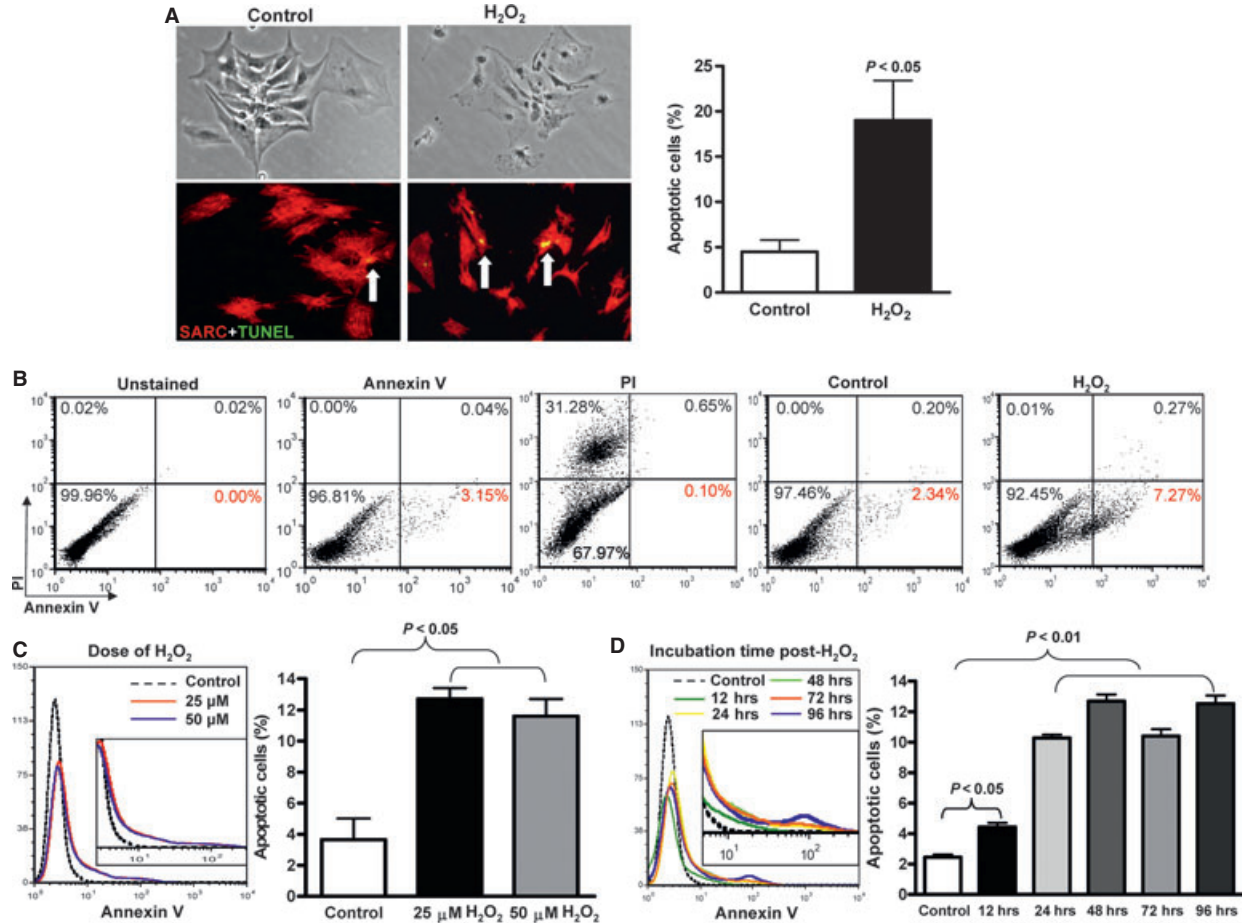
Fig. 1 Reactive oxidative stress causes cardiomyocyte apoptosis (A) Representative micrographs (200×) show the morphology of the cultured cardiomyocytes (left panels) and H_2O_2-treated apoptotic cardiomyocytes (right panels). Cardiomyocytes were stained for sarcomeric actinin (SARC; red) and TUNEL (green) before and after H_2O_2 treatment (25 μM, 18 hrs). Arrows indicate double-positive cells. Quantification of the TUNEL[+] cardiomyocytes showed that the proportion of apoptotic cardiomyocytes was significantly increased after H_2O_2 treatment ($n = 6$/group). (B) Representative dual-coloured flow cytometry of control and H_2O_2-treated cardiomyocytes for annexin V (apoptotic cardiomyocytes) and propidium iodide (PI; necrotic cardiomyocytes). For PI only staining, cardiomyocytes were treated with H_2O_2 (400 μM for 1 hr). (C) Quantification of the dose response showed a significant increase in cardiomyocyte apoptosis after treatment with both 25 μM and 50 μM H_2O_2 ($n = 3$/group) compared to control, but no significant difference between doses. Inset=enlarged tail of plot. (D) The percentage of apoptotic cardiomyocytes at different time-points after H_2O_2 treatment (25 μM) was significantly different than the non-treated control group, but was further increased at 24 hrs post H_2O_2 treatment. Cellular apoptosis plateaued at 48 hrs after H_2O_2 treatment ($n = 3$ to 5/group). Inset=enlarged tail of plot.

times following H_2O_2 treatment (Fig. 1B–D). As shown in Figure 1C, the percentage of apoptotic cardiomyocytes treated with either 25 μM or 50 μM H_2O_2 was approximately three times higher than that of the non-treated cardiomyocytes ($P < 0.05$), but there was no difference between doses. Furthermore, the percentage of apoptotic cardiomyocytes at 12 hrs after H_2O_2 treatment was significantly higher than that of non-treated cardiomyocytes ($P < 0.05$, Fig. 1D), and it further increased at 24 hrs ($P < 0.01$). Apoptosis reached its peak at 48 hrs after H_2O_2 treatment. The results from both the TUNEL and annexin V assays demonstrate that oxidative stress from H_2O_2 treatment induces apoptosis in neonatal rat cardiomyocytes.

BMSCs fuse with apoptotic cardiomyocytes

In an attempt to induce cell fusion, rat BMSCs expressing GFP were co-cultured with H_2O_2-treated cardiomyocytes at a ratio of 1:2 for 72 hrs. To distinguish the two cell types, cardiomyocytes were pre-stained with PKH26. PKH/GFP double-positive cells (Fig. 2A) observed with fluorescent microscopy suggested that cell fusion had occurred.

To validate our finding, the percentage of fused cells (cardiomyocytes and BMSCs) was quantified using flow cytometry (Fig. 2B). PKH26 pre-labelled, H_2O_2-treated cardiomyocytes were co-cultured
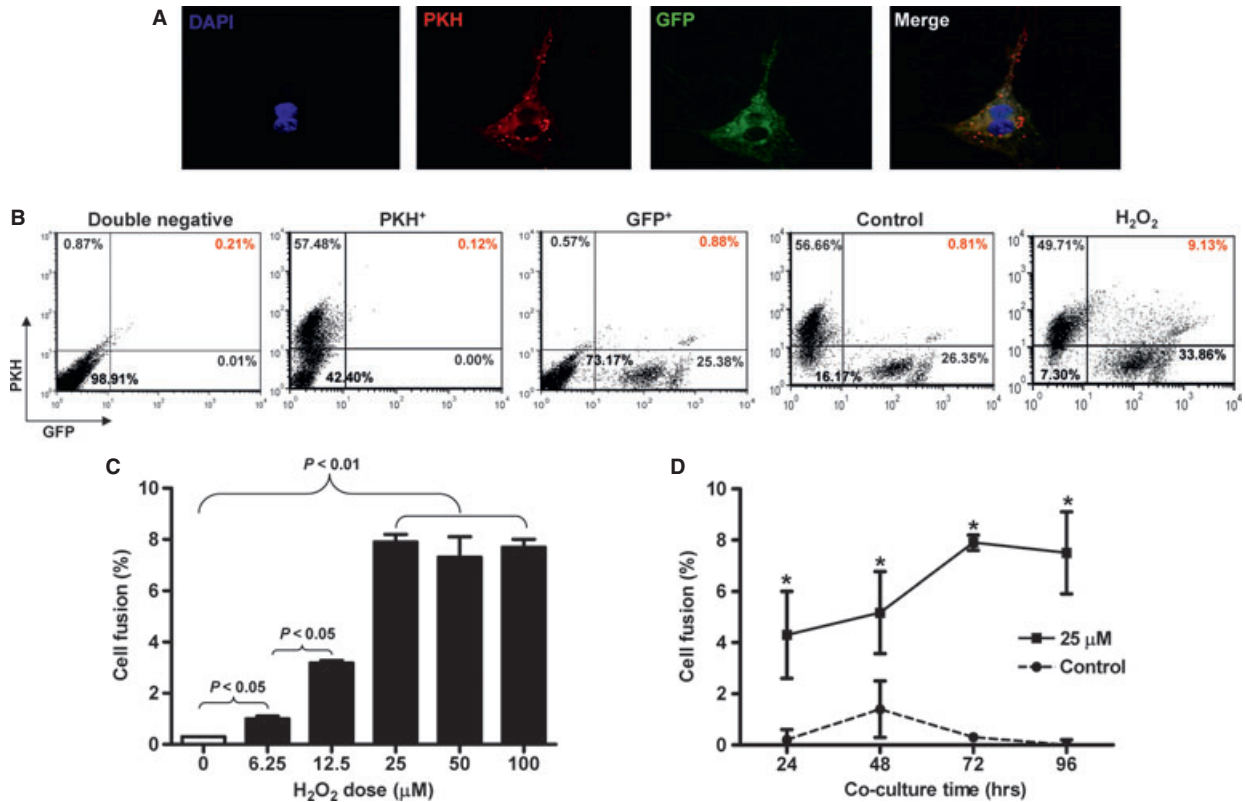
Fig. 2 BMSCs fuse with H_2O_2-treated cardiomyocytes (A) Representative confocal images ($600\times$) illustrate fusion of H_2O_2-treated cardiomyocytes with BMSCs. Cardiomyocytes were pre-stained with PKH26 membrane fluorescent dye (red), treated with H_2O_2 and co-cultured with BMSCs expressing GFP (green) for 72 hrs. Cell nuclei were stained with DAPI (blue). Merged image shows a triple-positive cell. (B) Flow cytometry provided the number of BMSCs fused with cardiomyocytes (GFP on the x axis, PKH26 on the y axis). Double negative: Cardiomyocytes mixed with BMSCs in 2:1 ratio. PKH+: PKH26 pre-labelled cardiomyocytes mixed with BMSCs. GFP+: Cardiomyocytes mixed with BMSCs expressing GFP. Control: Pre-labelled cardiomyocytes co-cultured with GFP+ BMSCs in a 2:1 ratio. H_2O_2: PKH26 pre-labelled, H_2O_2-treated cardiomyocytes co-cultured with GFP+ BMSCs. Fused cells were PKH/GFP double positive. (C) Quantification of flow cytometry results indicated that cell fusion between BMSCs and cardiomyocytes was H_2O_2 dose dependent up to 25 μM ($n = 4$/group). (D) Quantification of flow cytometry also showed that the per cent fusion between BMSCs and cardiomyocytes was greatest at 72 hrs of co-culture. The per cent fusion of the H_2O_2-treated cardiomyocytes was significantly greater than that of the control cardiomyocytes at all co-culture time-points (*$P < 0.05$, $n = 3$ to 6/group).

with BMSCs, and the fused cells were identified as PKH/GFP double positive. The cardiomyocytes were treated with increasing doses of H_2O_2 (Fig. 2C). Fusion rarely took place in the non-treated control cardiomyocytes (0.3%), whereas all H_2O_2 treatment groups showed significantly higher fusion rates than the control group ($P < 0.05$). We observed a H_2O_2 dose-dependent response from 6.25 μM to 25 μM; however, cell fusion was saturated at 25 μM as higher doses showed no further increase in fused cells. At this dose, the rate of cell fusion was 7.9 ± 0.3%, 26.3 times greater than that in the non-treated control.

We then evaluated the time course of fusion between the H_2O_2-treated cardiomyocytes and BMSCs. Cardiomyocytes treated with 25 μM H_2O_2 were co-cultured with BMSCs from 24 hrs to 96 hrs. Cell fusion increased from 24 hrs to 72 hrs and then reached a plateau (Fig. 2D). The percentage of fused cells in the H_2O_2 treatment

group was significantly higher than that of the non-treated control group at all co-culture time-points ($P < 0.05$). From these studies, we concluded that 25 μM was the optimal H_2O_2 dose and 72 hrs was the optimal co-culture duration to induce cell fusion; therefore, these parameters were used in the subsequent studies.

Fusion with BMSCs is associated with reduced cardiomyocyte apoptosis

To evaluate whether cell fusion could ameliorate the H_2O_2-induced apoptosis of cardiomyocytes, the cardiomyocytes were pre-stained with PKH26 membrane fluorescent dye (red), treated with H_2O_2 and co-cultured with BMSCs in a 2:1 ratio from 48 hrs to 96 hrs. PKH/annexin V double-positive cells were identified as apoptotic

cardiomyocytes (Fig. 3A). Control group cells were treated with H_2O_2, but without BMSC co-culture. The annexin V assay showed a stepwise decrease in cardiomyocyte apoptosis as the co-culture time increased (Fig. 3B). The percentage of apoptotic cells at each time-point was significantly different from that of the control group ($P < 0.05$) and from each other ($P < 0.05$).

To confirm our observations from the annexin V assay, the TUNEL assay was used to detect the percentage of apoptotic cardiomyocytes after co-culture with BMSCs. Similar results were observed with the TUNEL assay (Fig. 3C). Apoptotic cardiomyocytes were identified as sarcomeric actinin/TUNEL double positive. The percentage of apoptotic cardiomyocytes in the co-culture group was $8.4 \pm 3.4\%$, which was a 55.8% decrease from that in the control group ($19.0 \pm 4.4\%$, $P < 0.05$). Plotting fusion rates (Fig. 2D) against cardiomyocyte apoptosis (Fig. 3A) from 48 hrs to 72 hrs of co-culture, we found an inverse correlation between these two variables: as cell fusion increased, cardiomyocyte apoptosis decreased ($R^2 = 0.9863$, Fig. 3D).

We used a dual-chamber system separated by a semipermeable membrane to evaluate the potential of paracrine factors released from the BMSCs to prevent cardiomyocyte apoptosis. This system allows the diffusion of secreted factors, but prevents physical contact between the cell populations. Cardiomyocytes treated with 25 μM H_2O_2 were seeded in the lower chamber, and BMSCs were seeded in the upper chamber. After 48 hrs or 72 hrs in culture, the cardiomyocytes were stained for annexin V to assess apoptosis. We used our usual cell co-culture system to evaluate the synergistic effect of cell fusion and paracrine factors. We showed that at 72 hrs, apoptosis was reduced by culturing cardiomyocytes and BMSCs in separate chambers ($P < 0.05$, Fig. 3E), suggesting that apoptosis was reduced by paracrine factors alone. However, the percentage of apoptotic cells at both 48 hrs and 72 hrs was reduced further by the co-culture of cardiomyocytes and BMSCs ($P < 0.05$, Fig. 3E), indicating that cell fusion further reduced cardiomyocyte apoptosis.

Correlation of cell fusion and reduced cardiomyocyte apoptosis also occurs with hematopoietic stem cells

Mouse HSCs were isolated by magnetic cell sorting, as shown in Figure 4A. Haematopoietic stem cells expressing GFP were co-cultured for 72 hrs with H_2O_2-treated cardiomyocytes immediately after sorting. Cardiomyocytes were stained for sarcomeric actinin, and fused cells were positive for both GFP and sarcomeric actinin (Fig. 4B). Fused cells had yellow cytoplasm, and the nuclei appeared to be merging.

We used the same co-culture method described above for BMSCs to evaluate the fusion of HSCs with apoptotic cardiomyocytes. PKH26 pre-labelled, H_2O_2-treated cardiomyocytes were co-cultured with HSCs for 72 hrs, and the percentage of fused cells was determined using flow cytometry (Fig. 4C). The per cent fusion of H_2O_2-treated cardiomyocytes with HSCs was significantly greater than that of non-treated cardiomyocytes ($P < 0.05$). There was no significant difference in the percentage of fused cells for H_2O_2-treated cardiomyocytes co-cultured with HSCs or BMSCs. Thus, HSCs fused with apoptotic cardiomyocytes at the same frequency as BMSCs. The annexin V assay was used to detect the percentage of apoptotic cardiomyocytes after co-culturing with HSCs (Fig. 4D). PKH26 pre-labelled, H_2O_2-treated cardiomyocytes were co-cultured with HSCs for 72 hrs and the percentage of PKH26/annexin V double-positive cells was quantified. Cardiomyocyte apoptosis in the group treated with H_2O_2 and co-cultured with HSCs was $6.5 \pm 1.0\%$, which was not statistically different from the group co-cultured with BMSCs ($7.6 \pm 0.4\%$); however, it was a 37.5% decrease compared to treated cardiomyocytes without co-culturing ($10.4 \pm 0.8\%$, $P < 0.05$). Therefore, co-culture with HSCs was also associated with reduced apoptosis of H_2O_2-treated cardiomyocytes, to a level comparable to that of BMSCs.

Fig. 3 Fusion with BMSCs is associated with reduced cardiomyocyte apoptosis. Cardiomyocytes were pre-stained with PKH26 membrane fluorescent dye (red), treated with H_2O_2 and co-cultured with BMSCs in a 2:1 ratio from 48 hrs to 96 hrs. PKH/annexin V double-positive cells were identified as apoptotic cardiomyocytes. (A) Representative dual-coloured flow cytometry of control H_2O_2-treated cardiomyocytes or H_2O_2-treated cardiomyocytes co-cultured with BMSCs. Annexin V on the x axis and PKH-labelled cardiomyocytes on the y axis. PKH-labelled cardiomyocytes without annexin V staining were used to set up the gate. (B) Annexin V assay demonstrated that the percentage of apoptotic cells in the co-culture groups at 48, 72 and 96 hrs was significantly different from each other and from the control group (H_2O_2-treated cardiomyocytes without BMSC co-culture, at 48 hrs; $n = 3$/group). Apoptosis decreased as the co-culture time increased. Inset=enlarged tail of plot. (C) Representative micrographs (200×) of cardiomyocytes stained for sarcomeric actinin (SARC; red) and TUNEL (green) following H_2O_2 treatment, with and without BMSC co-culture, at 48 hrs. Arrows indicate double-positive cells. The percentage of apoptotic cells in the control group (no co-culture, $n = 6$) was significantly higher that that of the co-culture group ($n = 8$). (D) The fusion of cardiomyocytes with BMSCs from 48 hrs to 72 hrs of co-culture was inversely correlated with cardiomyocyte apoptosis. (E) H_2O_2-treated (25 μM, 18 hrs) cardiomyocytes were seeded in the lower chamber of a two-chamber system separated by a semipermeable membrane, and BMSCs were seeded in the upper chamber (the insert) in a 2:1 ratio. After 48 hrs or 72 hrs in culture, cardiomyocytes were stained for annexin V. For the usual co-culture system, cardiomyocytes were treated with H_2O_2 (25 μM, 18 h) and co-cultured with BMSCs in a 2:1 ratio. Representative histogram plot (left panel) shows the control H_2O_2-treated cardiomyocytes (at 48 hrs), H_2O_2-treated cardiomyocytes co-cultured with BMSCs (solid line) and H_2O_2-treated cardiomyocytes in the dual-chamber system with the BMSC insert (dashed line) stained for annexin V. The percentage of apoptotic cells (right panel) in the co-culture groups at 48 hrs and 72 hrs was significantly lower than that of both the control group and the respective insert group (*$P < 0.05$ vs. control, #$P < 0.05$ *versus* respective insert group; $n = 3$/group).
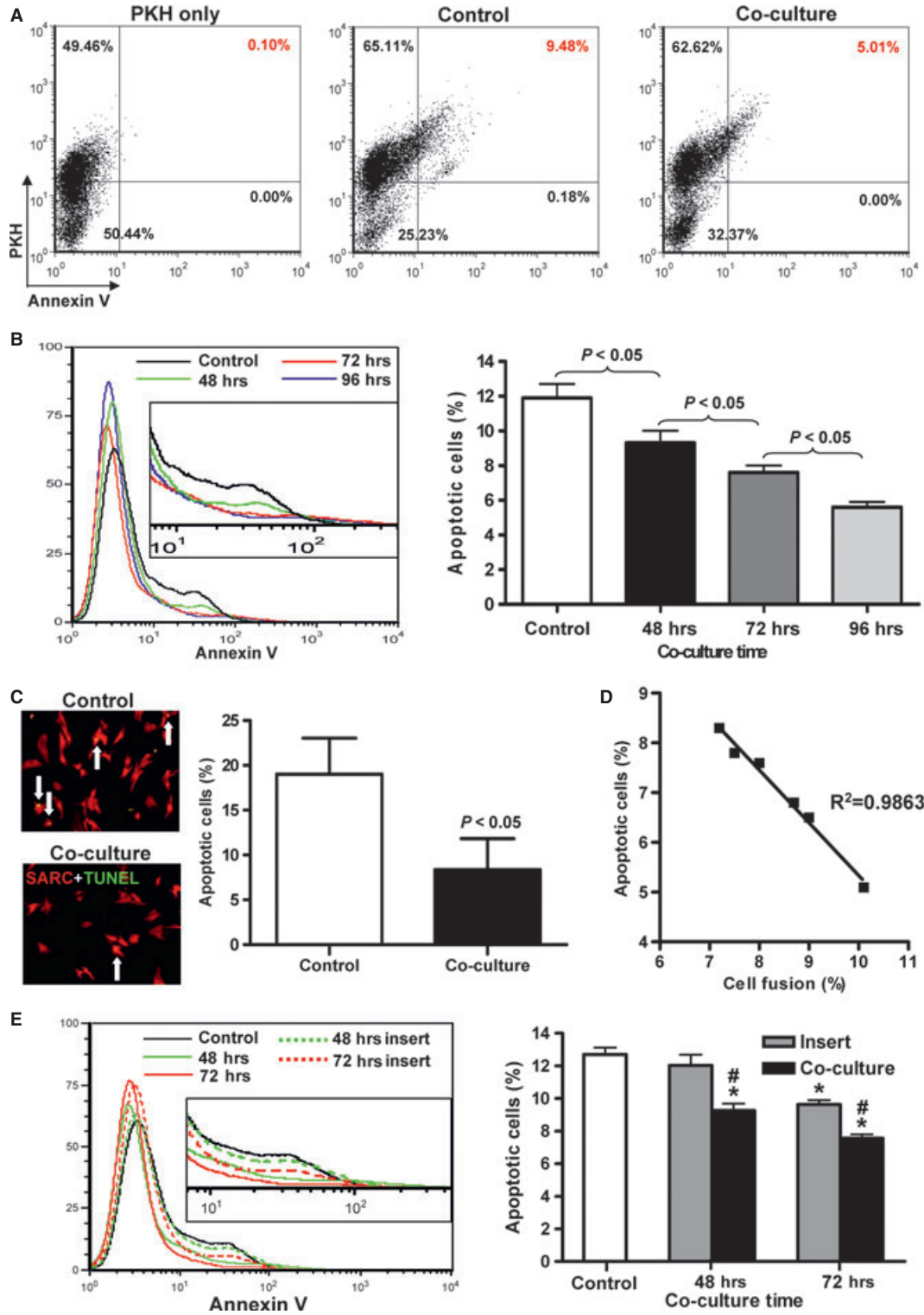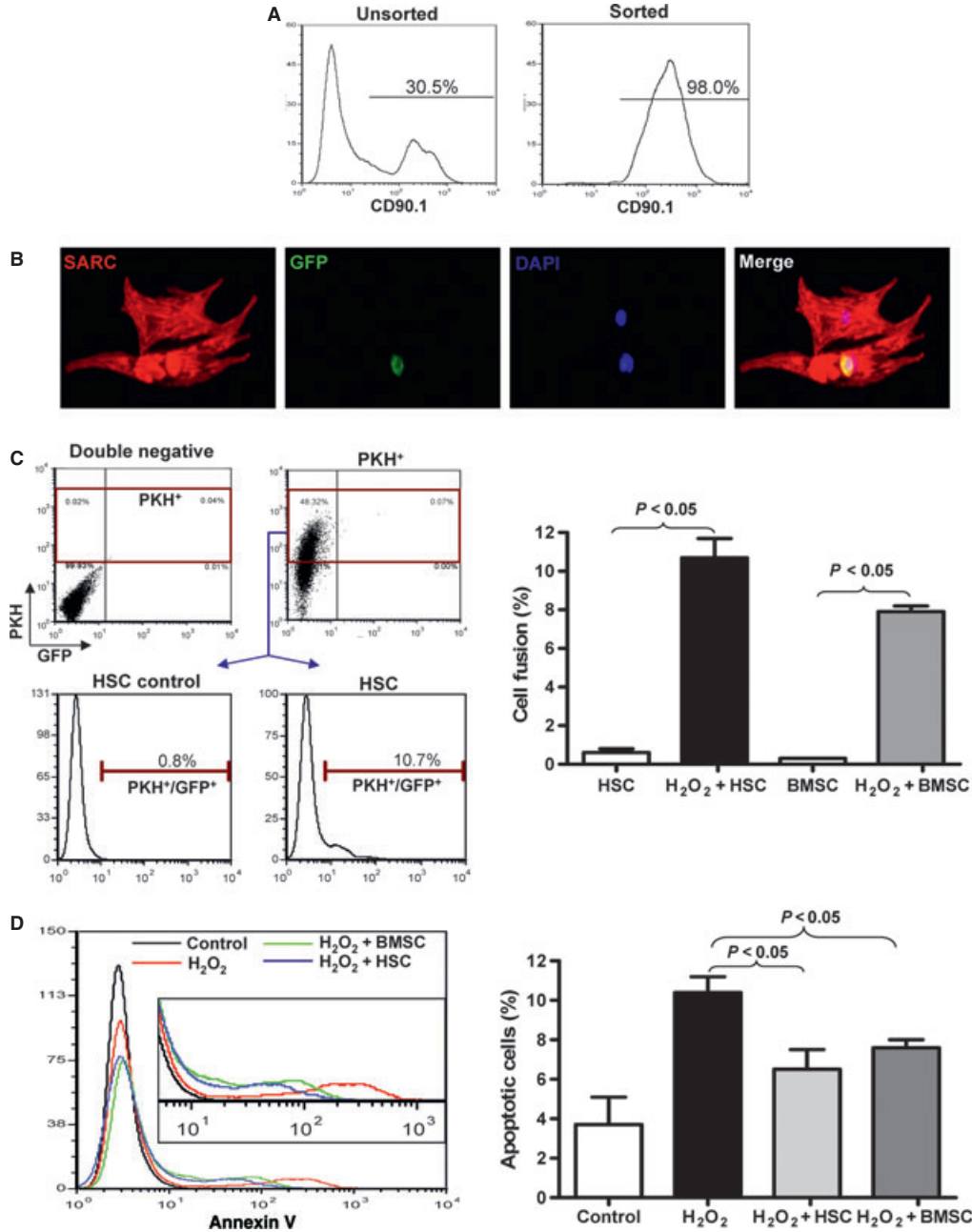
Fig. 4 Correlation of cell fusion and reduced cardiomyocyte apoptosis also occurs with HSCs (A) Representative histogram plots show mouse CD90.1 (Thy-1.1) HSCs before and after magnetic cell sorting. (B) Representative micrographs (400×) illustrate fusion between HSCs expressing GFP (green) and H_2O_2-treated cardiomyocytes. Cardiomyocytes were co-cultured with HSCs for 72 hrs and stained for sarcomeric actinin (SARC; red). Cell nuclei were stained with DAPI (blue). (C) Flow cytometry demonstrated the fusion of HSCs with cardiomyocytes (GFP on the x axis, PKH26 on the y axis). Double negative: Cardiomyocytes mixed with HSCs in 1:1 ratio. PKH+: PKH26 pre-labelled cardiomyocytes mixed with BMSCs. HSC control: Pre-labelled cardiomyocytes mixed with BMSCs. Cells gated on PKH+ were also plotted for GFP. HSC: PKH26 pre-labelled, H_2O_2-treated cardiomyocytes co-cultured with BMSCs for 72 hrs. Fused cells were PKH/GFP double positive. Quantification of flow cytometry results showed that fusion of H_2O_2-treated cardiomyocytes with HSCs was significantly greater than that of non-treated cardiomyocytes (n = 4/group). There was no difference in the per cent of fused cells between HSCs and BMSCs. (D) Flow cytometry of annexin V assay showed that the percentage of apoptotic cells in the H_2O_2-treated cardiomyocytes without co-culture was significantly higher than that of treated cardiomyocytes co-cultured for 72 hrs with either HSCs or BMSCs (n = 3 to 4/group). Inset=enlarged tail of plot.
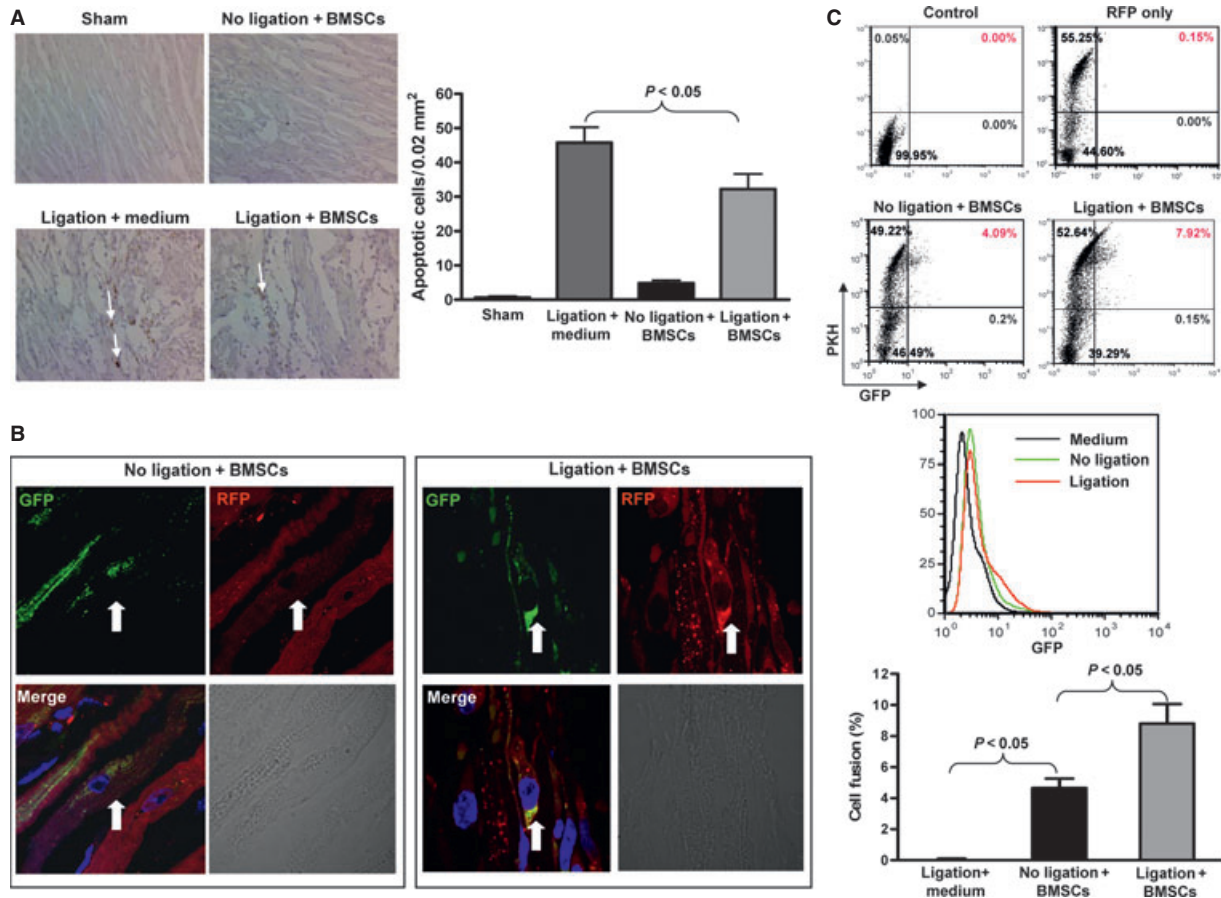
Fig. 5 Fusion between BMSCs and apoptotic cardiomyocytes occurs *in vivo* (A) Representative micrographs (200×) show cellular apoptosis in the sham, ligation+medium, no ligation+BMSCs and ligation+BMSCs groups. Arrows indicate apoptotic cells. Cellular apoptosis was significantly lower in the ligation+BMSCs group compared with the ligation+medium group (n = 5/group). (B) Confocal images (600×) show the *in vivo* cell fusion of GFP⁺ BMSCs with the RFP⁺ cardiomyocytes in both the no ligation+BMSCs and ligation+BMSCs groups. Arrows indicate cells that underwent fusion. Nuclei are stained with DAPI (blue). (C) Representative dual-coloured flow cytometry of single-cell suspensions collected 3 days after coronary artery ligation and BMSC transplantation. RFP/GFP double-positive cells indicate cell fusion. The percentage of fused cells was significantly higher in the no ligation+BMSCs group than in the ligation+medium group. However, the per cent of fused cells was significantly higher in the ligation +BMSCs group than the no ligation+BMSCs group (n = 4 to 5/group).

Fusion between BMSCs and apoptotic cardiomyocytes occurs *in vivo*

To investigate whether or not cell fusion would occur with apoptotic cardiomyocytes *in vivo*, we transplanted BMSCs into transgenic mice expressing red fluorescent protein (RFP) following myocardial ischaemia. The LAD coronary artery was ligated to produce ischaemia and to induce cardiomyocyte apoptosis; sham-operated animals did not undergo ligation. Bone marrow mesenchymal stromal cells expressing GFP were injected into the myocardium immediately following ligation; medium alone was used as a control.

Cellular apoptosis was significantly lower in the ligated mice that received BMSCs compared with the ligated mice that received medium only (P < 0.05, Fig. 5A). Confocal microscopy showed the *in vivo* cell fusion of BMSCs and cardiomyocytes in both ligated and non-ligated mice that received BMSCs (Fig. 5B).

Single-cell suspensions were obtained 3 days following injection to assess cell fusion. Fused cells were RFP/GFP double positive (Fig. 5C). The percentage of cells undergoing fusion was significantly higher in non-ligated mice injected with BMSCs (4.6 ± 0.6%) compared to ligated mice injected with medium only (0.03 ± 0.07%, P < 0.05, Fig. 5C). Ligation and BMSCs produced the highest percentage of cell fusion (8.8 ± 1.3%). Our *in vivo* data suggest that cell fusion between transplanted BMSCs

and cardiomyocytes was promoted by cardiomyocyte exposure to ischaemia.

Discussion

The accepted paradigm of BMC therapy is that these cells home to the site of injury and then either differentiate into the cell types of the damaged tissue [7, 14, 15] or promote repair by creating a microenvironment that is conducive to regeneration by endogenous cells [16–18], such as the secretion of soluble factors that act through paracrine signalling [4]. However, some studies suggest that stem cells can adopt the phenotype of other cells *via* cell fusion and have called into question the true plasticity of the stem cells [19, 20]. On the basis of the results of our study, we propose that cell fusion may be one mechanism by which injured cardiomyocytes may be repaired.

Our *in vitro* studies demonstrated that co-culturing healthy cardiomyocytes with BMCs resulted in an extremely low (0.3%) frequency of spontaneous cell fusion. However, when BMCs were co-cultured with cardiomyocytes that had suffered oxidative stress, in the form of H_2O_2 treatment, the frequency of cell fusion increased more than 20-fold. We found a significant correlation between increasing rate of cell fusion and decreasing rate of cardiomyocyte apoptosis following H_2O_2 treatment, indicating that BMCs can rescue cardiomyocytes from apoptosis through cell fusion. On the basis of these results, we suggest that cardiomyocyte damage may be a prerequisite to stimulate cell fusion.

The cardiomyocytes used in our *in vitro* studies suffered oxidative stress by treatment with H_2O_2, which generates free radicals that are harmful to the biomembranes and filaments of the cardiomyocytes and results in FAS-mediated apoptosis. It has been shown that H_2O_2-induced oxidative stress causes several cardiomyocyte abnormalities, such as membrane phospholipid peroxidation, thiol oxidation and ATP loss [21, 22]. There is also an increase in the turnover of cardiomyocyte membrane constituents and lethal disruption of the cardiomyocyte sarcolemma [21, 22]. The H_2O_2-treated cardiomyocytes in our *in vitro* studies developed both morphological and functional changes. They underwent pycnosis and karyorrhexis, lost cell-cell contact as their cytoplasm retracted and stopped spontaneously beating.

We found that both BMSCs and HSCs could fuse with apoptotic cardiomyocytes, at a frequency of $7.9 \pm 0.3\%$ and $10.7 \pm 1.0\%$, respectively. Although our ability to observe the cell fusion process was limited by lack of an immunofluorescent microscope with time-lapse capability, we intermittently observed the co-cultured cells under an immunofluorescent microscope. The BMSCs clung to the apoptotic cardiomyocytes. Membrane contact occurred first, then the cell plasma began to merge, suggesting that direct cell-cell contact was a requirement for cell fusion. The fused cells assumed the characteristic morphology of cardiomyocytes, and some of them underwent nuclear fusion with the BMSCs. Although high-definition microscopy was not employed, the cell fusion did not appear to be overlap of the cells. First, the cell densities were low ($1 \times 10^4/cm^2$ for cardiomyocytes, $0.5 \times 10^4/cm^2$ for BMCs) so that most cells were in a monolayer; second, the cardiomyocytes and BMCs were both adherent, growing cells; and, third, we also used flow cytometry to estimate the extent of fusion, and the results of this assessment are independent of overlapping cells.

The results of our *in vivo* mouse model support our *in vitro* data. We demonstrated fusion of endogenous cardiomyocytes and transplanted BMSCs following ischaemic injury. We believe that the oxidative stress (*in vitro*) and the inflammatory microenvironment following myocardial ischaemia (*in vivo*) induced cardiomyocyte apoptosis, which subsequently promoted cell fusion. Interestingly, the mice that received BMSCs but did not undergo coronary ligation also had evidence of cell fusion, although at a lower rate. The trauma of the thoracotomy and intramyocardial injection of cells may have activated local and systemic inflammatory responses, resulting in the release of cytokines that may have subsequently promoted cardiomyocyte apoptosis and stimulated cell fusion even in the absence of ischaemic injury.

Other researchers have demonstrated that inflammation is a potent stimulus for the induction of cell fusion. Fusion of BMCs with Purkinje neurons, a cell type that cannot be regenerated, was significantly increased in the presence of chronic inflammation during experimental autoimmune encephalitis (a mouse model of multiple sclerosis) [9]. Heat shock has also been shown to increase the rate of cell fusion between BMCs and small airway epithelial cells in co-culture [11].

We found that cell fusion between injured cardiomyocytes and BMCs reduced the incidence of, and may have rescued the cardiomyocytes from, apoptosis. We propose that cell fusion may be one way that cells repair themselves and that cell fusion may contribute to cardiac regeneration. Cell fusion may be one mechanism by which cell therapy improves cardiac function, regardless of whether the cells engraft or undergo myogenic differentiation. Paracrine signalling with the release of soluble factors may also contribute to cardiomyocyte regeneration. Therefore, the secretion of soluble factors by BMCs may be another potential mechanism for the decrease in the number of apoptotic cardiomyocytes that we observed. Using a dual-chamber culture system to prevent contact between cell populations, we demonstrated that paracrine factors from the BMSCs can reduce cardiomyocyte apoptosis after injury; however, apoptosis was significantly further reduced by cell fusion. The process of cell fusion should be further elucidated [23], but on the basis of our study, we believe that cell fusion may be a protective response to cell injury. Future studies will be required to characterize the fused cells with respect to their biochemistry, electrophysiology and gene expression.

Maintaining the number of functioning cardiomyocytes is the ultimate goal of therapy following myocardial infarction. Rather than trying to regenerate cardiomyocytes, a feat that has proven difficult to achieve, a novel alternative approach is to prevent cardiomyocyte apoptosis. Our results demonstrate that the potential of BMSC transplantation as cell therapy may be related to the fusion of BMSCs and injured cardiomyocytes, opening up new therapeutic targets to enhance fusion and increase cardiomyocyte survival following ischaemic injury. Future studies may increase cell fusion to achieve more extensive preservation of the ischaemic cardiomyocytes.

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Conflicts of interest

The authors confirm that there are no conflicts of interest.

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