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Coupling erythropoietin secretion to mesenchymal stromal cells enhances their regenerative properties

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Aims Mesenchymal stromal cells (MSCs) possess intrinsic features that identify them as useful for treating ischaemic syndromes. Poor *in vivo* survival/engraftment of MSCs, however, limits their overall effectiveness. In this work, we tested whether genetically engineering MSCs to secrete erythropoietin (Epo) could represent a better therapeutic platform than MSCs in their native form.
Methods and results MSCs from C57Bl/6 mice were retrovirally transduced with either an empty vector or one that causes the production of Epo and were then analysed for the alterations in angiogenic and survival potential. Using a mouse model of myocardial infarction (MI), the regenerative potential of null MSCs and Epo-overexpressing MSCs (Epo+MSCs) was assessed using serial echocardiogram and invasive haemodynamic measurements. Infarct size, capillary density and neutrophil influx were assessed using histologic techniques. Using *in vitro* assays coupled with an *in vivo* Matrigel plug assay, we demonstrate that engineering MSCs to express Epo does not alter their immunophenotype or plasticity. However, relative to mock-modified MSCs [wild-type (WT)-MSCs], Epo+MSCs are more resilient to apoptotic stimuli and initiate a more robust host-derived angiogenic response. We also identify and characterize the autocrine loop established on MSCs by having them secrete Epo. Furthermore, in a murine model of MI, animals receiving intracardiac injections of Epo+MSCs exhibited significantly enhanced cardiac function compared with WT-MSCs and saline-injected control animals post-MI, owing to the increased myocardial capillary density and the reduced neutrophilia.
Conclusion Epo overexpression enhances the cellular regenerative properties of MSCs by both autocrine and paracrine pathways.

1. Introduction

Despite improved management and availability of conventional therapies, cardiovascular disease remains the leading cause of death in the Western world. Furthermore, as the incidence of cardiovascular disease increases, the increases in the number of 'no-option' patients who continue to have disabling ischaemia after all conventional revascularization techniques have failed increases. Thus, developing new therapeutic options for these patients is a priority. Under development are strategies involving therapeutic angiogenesis that which attempt to exploit the body's natural ability to develop collateral vessels following ischaemia. Despite promising results in animal models, many of the protein and gene-based strategies used for therapeutic angiogenesis have been clinically disappointing.[1] In particular, the short half-life of recombinant proteins and the inefficient delivery and expression of *in vivo* gene-based strategies continue to be major challenges. In recent years, there has been a growing enthusiasm for the application of cell-based therapies to repair or regenerate ischaemic tissue. In particular, stem/progenitor cells from the bone marrow have demonstrated regenerative and angiogenic properties.[2] Conceptually, cell therapy for cardiovascular disease has evolved from the initial premise that exogenous progenitor or stem cells regenerate injured tissue to a broader hypothesis that cell therapy facilitates complementary aspects of tissue repair.[3] Such complementary aspects might include increased cell survival (limited apoptosis) and tissue oxygenation (angiogenesis) as well as enhanced functional recovery of tissue (positive remodelling).

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In numerous animal models, bone marrow-derived mesenchymal stromal cells (MSCs) have shown promise in the treatment of cardiovascular disease.[4–7] MSCs have intrinsic features that identify them as an ideal cell type for cardiovascular cellular therapy. MSCs possess robust angiogenic and immunomodulatory properties, are the natural component of the host-derived ischaemia response, can be obtained in relatively large numbers through standard clinical procedures and are easily expandable in culture.[8] However, as is the case in all cellular therapies, low MSC persistence post-transplantation limits their overall effectiveness and significantly impacts their clinical usage.[9] Therefore, an obvious enhancement to the use of MSC cellular therapy is to genetically engineer these same cells to synthesize factors they normally do not produce and thereby create a better 'therapeutic bullet'.[10]

With this in mind, appealing cytokines to couple to the MSC cellular platform would be ones that provide an array of effects not only on damaged tissue, but also positively modulate the body's repair response. Erythropoietin (Epo) may be such a candidate. Epo has been shown to protect tissue against ischaemic injuries,[11] stimulate postnatal neovascularization,[12] and block the acute inflammatory response induced by reoxygenation.[13] Furthermore, recent data suggest that MSCs express the Epo receptor (EpoR) and that Epo may directly influence MSC survival.[14,15] Therefore, having MSCs secrete Epo would establish a scenario whereby MSCs could enhance tissue protection through both autocrine (increased survival) and paracrine (increased blood vessel formation) modes of action. Arguably, one can hypothesize that transplantation of Epo-secreting MSCs into ischaemic tissue would maximize Epo's local effects and providing synergistic/additive effects to the natural paracrine regenerative properties of MSCs. In this study, we evaluated whether administration of Epo-secreting MSCs represents a better therapeutic platform than MSCs in their native form. In comparison with the wild-type MSCs (WT-MSCs), we found that Epo-secreting MSCs had markedly enhanced cellular regenerative properties because of both autocrine and paracrine pathways. This was reflected by the increased cell survival, neovascularization and enhanced preservation of heart function following myocardial infarction (MI).

2. Methods

Condensed materials and methods are contained herein. Additional information regarding materials and methods can be found in the online supplement. All animal experiments conducted at the Montreal Heart Institute and Lady Davis Institute for Medical Research (Jewish General Hospital) conform with the Guide for the Care and the Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1 *In vitro* characterization of wild-type and erythropoietin-overexpressing mesenchymal stromal cells

MSC isolation and culture was performed as previously described,[16] then transduced with retroviral supernatant from virus producers containing a murine Epo construct GP+E86-Epo, or with supernatant from virus producers containing an empty vector GP+E86-null (mock transduction). WT and Epo+MSCs were differentiated into osteoblasts and adipocytes using standard induction medium, and flow cytometric analysis was used to characterize the immunophenotype of the MSCs. Epo secretion was evaluated by an ELISA-specific for human Epo (Roche Diagnostics) as previously described.[16] Apoptosis, RT-PCR, western blotting and chemotaxis assays were performed using standard laboratory kits and procedures.[17,18]

2.2 *In vivo* characterization of wild-type and erythropoietin-overexpressing mesenchymal stromal cells

Flow cytometric and histologic immunofluorescence analyses were used to assess angiogenesis, cell survival and differentiation potential *in vivo* utilizing a previously described subcutaneous implantation model of Matrigel-embedded MSCs (Becton–Dickinson, Mississauga, ON, Canada).[19] Blood samples were collected from the saphenous vein of mice with heparinized microhaematocrit tubes.

2.3 PKH26 labelling of mesenchymal stromal cells

Culture expanded MSCs were trypsinized, washed and re-suspended in assay buffer for PKH26 labelling (Sigma–Aldrich, Canada). Cells were then mixed with a 2X stock of PKH26 labelling solution and incubated at room temperature for 5 min. The reaction was stopped and cells washed prior to injection.

2.4 Immunofluorescence

Cells or frozen tissue sections were fixed in ice-cold 4% (v/v) paraformaldehyde, then processed to visualize PKH26 or stained for von Willebrand Factor (vWF), Ki67, Cardiac Troponin I and EpoR as previously described.[20]

2.5 Animal model of myocardial infarction

C57BL/6 female mice 10–12 weeks of age were used. MI was induced with the ligation of left coronary artery as described.[21] For all studies, mice were randomly allocated to three groups: (i) Phosphate Buffered Saline (PBS) vehicle (ii) WT-MSC and (iii) Epo+MSC. Injections were performed 10 min after ligation with a 32 gauge needle (Hamilton, USA) into five different sites bordering the viable myocardium. A total of 1.25×10^6 cells, or the equivalent PBS volume, were injected per mouse.

2.6 Haemodynamic measurements

Cardiac function was assessed by serial echocardiography at baseline (18–24 h after MI), Day 7 and Day 14 after MI. Echocardiography was performed using a S12 Philips Sonos 5500 (Andover, USA) as described.[22] On Day 14 after MI, closed-chest invasive haemodynamics were performed after cannulation of the right carotid artery with a 1.4 F microtip pressure transducer (model SPR 671, Millar Instrument, Houston).

2.7 Histological analysis of myocardium

The MI size was calculated with planimetry using the ratio of circumference method.[23] Capillary endothelial cells and neutrophils were identified using immunohistochemical staining for vWF and MCA771G (Serotec, USA) on paraffin sections.

2.8 Statistical analysis

Statistical analyses were performed using either the two-tailed Student's *t*-test or non-parametric ANOVA, as appropriate. For analysis of cellular treatment on heart function, Dunn's *post hoc* test was performed for three-group comparison. Repeated measures ANOVA was used for serial haematocrit and echocardiogram comparison. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Phenotypic and functional analysis of mesenchymal stromal cells retrovirally engineered to express erythropoietin

We immunophenotyped our WT-MSCs, transduced with an empty vector (mock) and those transduced with our Epo construct (Epo+MSCs) for specific markers of MSCs and endothelial progenitors. The Epo+MSCs were similar to WT-MSCs in that they express constitutively high levels of CD44 and CD105, but did not express significant levels CD45, CD90 or CD31 (*Figure 1A*). We found that when WT-MSCs and Epo+MSCs were exposed to the differentiation media they both could readily differentiate into either adipocytes or osteoblasts, reflecting a preserved mesenchymal plasticity (*Figure 1B*). We measured Epo secretion by MSCs *in vitro*, from 24 h conditioned supernatant, and found that WT-MSCs did not secrete detectable levels of Epo, whereas Epo+MSCs produced $\sim$600 mIU/1 $\times$ 10^6 cells (*Figure 1C*, upper panel). Furthermore, when we fluorescently stained WT-MSCs or Epo+MSCs with an anti-Epo antibody (*Figure 1C*, lower panel) intracellular Epo protein was present only in our Epo+MSCs. Using a subcutaneous Matrigel plug assay system, we tested the host erythropoietic response to Epo+MSCs. Two weeks post-implantation, plugs embedded with Epo+MSCs (range of 0.1–2.0 $\times$ 10^6 cells) produced a significant and dose-dependent increase in haematocrit in tested animals (*Figure 1D*).

3.2 Effect of erythropoietin-overexpression on angiogenic, survival and differentiation potential of mesenchymal stromal cells

We have previously demonstrated that WT-MSCs are capable of producing a robust host-derived angiogenic response *in vivo*[19] and can accurately quantify this neovascularization through flow cytometry.[24] Using the same assay, WT-MSC or Epo+MSC subcutaneous implants were surgically resected at Day 14 post-implantation and analysed for neovascularization and MSC survival. Upon removal, we noticed a marked macroscopic difference between WT-MSC and Epo+MSC implants such that in the Epo+MSC implants vascularization appeared far more prominent (*Figure 2A*, arrows). Histochemical analyses of the implants showed that WT-MSC and Epo+MSC implants were similar in terms of cellular density (*Figure 2B*, upper panel); however, vWF, staining to identify blood vessels demonstrated that Epo+MSC implants had a qualitative increase in blood vessels (*Figure 2B*, lower panel). In a separate experiment, 14-day implants were enzymatically digested to obtain a single cell suspension and the total number of endothelial cells was quantified by flow cytometric analysis. Confirming our immunostaining, we noted a substantial 100% increase in endothelial cells (i.e. $CD31^+/CD45^-$) in the Epo+MSC implants compared with WT-MSC implants (*Figure 2C*). *In vitro*, we tested whether secreted proteins from MSCs had a positive chemotactic effect on endothelial cells. For this purpose, we performed a Boyden chamber transwell

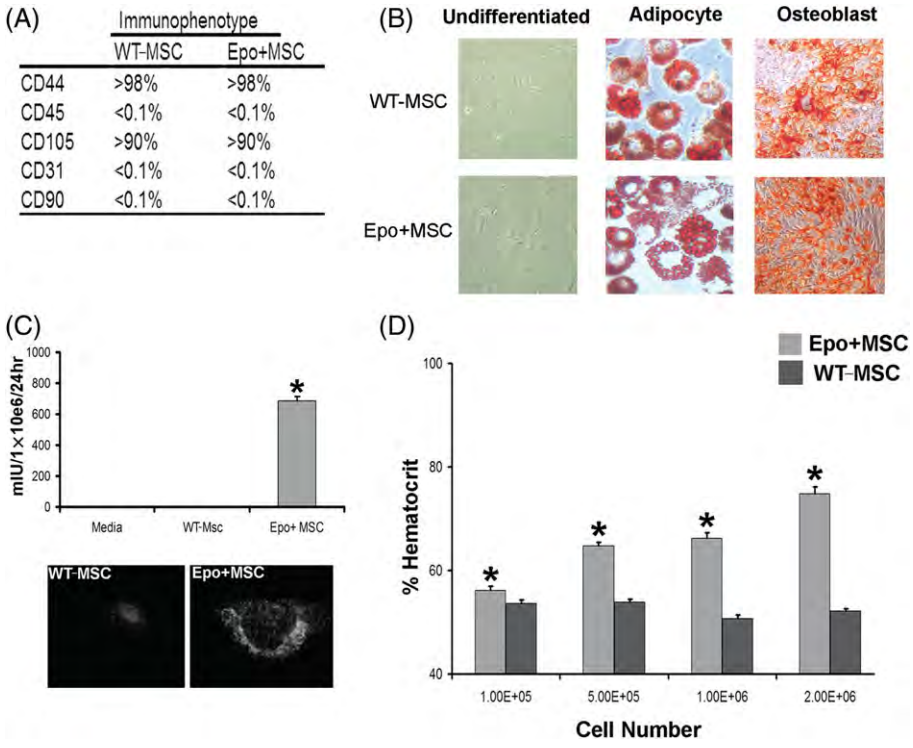

Figure 1 Comparison of murine wild-type mesenchymal stromal cell (WT-MSC) and erythropoietin (Epo)-secreting (Epo+MSC) MSCs. (*A*) Flow cytometry analysis on *in vitro*-cultured WT and Epo+MSCs determined cell surface antigen expression of CD31, CD44, CD45, CD90, CD105, as described in 'Methods' (*B*) Undifferentiated WT-MSCs and Epo+MSCs were cultured in conditions inductive of osteogenic or adipogenic differentiation. Adipogenic differentiation was visualized by Oil Red O staining of lipid droplets, while Alizarin Red staining revealed osteogenic differentiation. (*C*) Secretion of Epo by ELISA, measured on unconditioned culture media or 24 h conditioned media from WT and Epo+MSCs (Upper panel). Immunofluorescence staining for intracellular Epo production on *in vitro*-cultured WT and Epo+MSCs (Lower panel). (*D*) Haematocrit measured on animals 2 weeks after i*n vivo* subcutaneous Matrigel implantation of varying cellular density of WT and Epo+MSCs. (Pictures representative of three separate experiments.) (Epo+MSC vs. WT-MSC implants; $n = 5$ for each cellular density, mean $\pm$ SEM) (*$P < 0.05$ vs. WT-MSC).

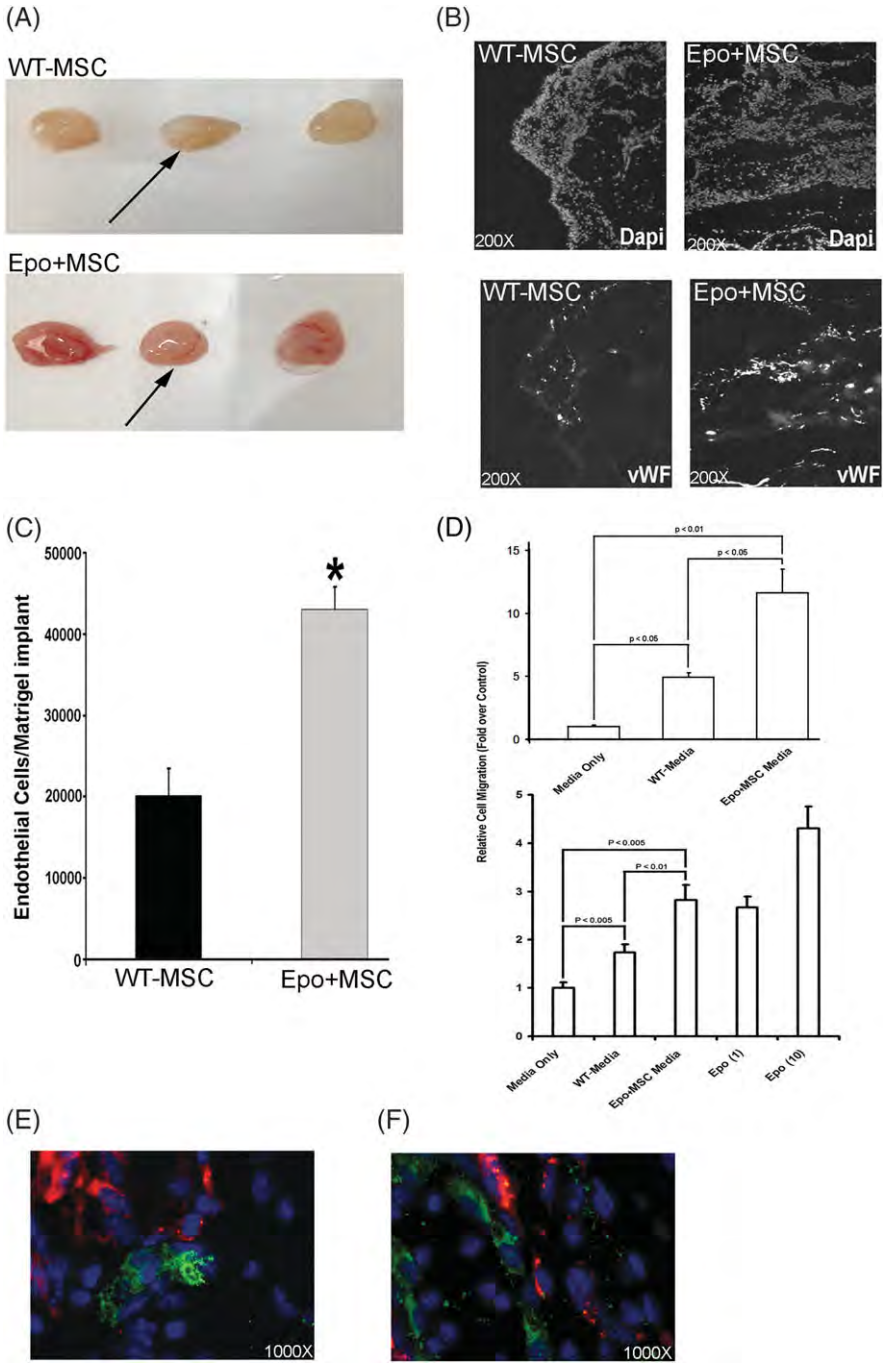

Figure 2 Regenerative potential of wild type-mesenchymal stromal cells (WT-MSCs) and Epo-overexpressing MSCs (Epo+MSCs). (*A*) Macroscopic visual inspection of Matrigel implants from WT-MSCs and Epo+MSCs after 2 weeks for the presence of blood vessels (arrows). (*B*) Morphometry of Matrigel implants from WT and Epo+MSCs after 2 weeks, determined by DAPI staining (upper panel). Blood vessel assessment determined by immunofluorescence staining for vWF (lower panel). (*C*) Flow cytometry analysis on enzymatically digested Matrigel implants from WT and Epo+MSCs 2 weeks post-implantation to quantitative endothelial cells based on cell surface antigen expression of CD31 and CD45. (*D*) Endothelial migration conducted on *in vitro*-cultured Bend3 endothelial cells for 6 (upper panel) and 16 h (lower panel), exposed to 24 h conditioned media from WT and Epo+MSCs and media containing recombinant Epo(1, 10 IU/ml). Immunostaining for blood vessels (vWF-green) to determine endothelial differentiation of PKH26 labelled MSC (red) 2-weeks post-implantation from (*E*) WT and (*F*) Epo+MSC implants. (CD31+/CD45− flow cytometry for Epo+MSC vs. WT-MSC implants; $n = 5$, mean ± SEM) (Bend3 migration Epo+MSC vs. WT-MSC conditioned media; $n = 4$ separate experiments run in duplicate, mean ± SEM) (Pictures representative of minimum three separate experiments, nuclei stained with DAPI-blue).

migration assay using 24 h WT or Epo+MSC conditioned supernatants as bait for an immortalized murine endothelial cell line (Bend3). We found that the secretome derived from Epo+MSCs was significantly better at attracting endothelial cells compared with the secretome of WT-MSCs at both our 6 and 16 h time points (*Figure 2D*). Finally, since a small proportion of MSCs can adopt a endothelial-like phenotype post-transplantation,[24] experiments were conducted to determine whether the increased neovascularization of our Epo+MSC implants might be attributed to the enhanced Epo+MSC endothelial differentiation. By immunostaining for vWF on PKH26-labelled MSC implants, we noted that in both our WT (*Figure 2E*) and Epo+MSC (*Figure 2F*) implants those MSCs in close proximity to blood vessels did not readily

take on an endothelial-like phenotype, but rather acted as accessory cells to the newly developing blood vessels.

Using the subcutaneous Matrigel plug system, we also assessed MSC survival post-implantation. Prior to implantation, flow cytometry analysis demonstrated equal PKH26-labelling in both our WT and Epo+MSC populations (*Figure 3A*). This labelling procedure was well tolerated by MSCs and appeared as a punctuate membrane signal (*Figure 3B*). Fourteen days after implantation, the Matrigel plugs were removed and enzymatically digested to obtain a single cell suspension and the cellular content analysed for PKH26-labelled MSCs (*Figure 3C*). We found that a significantly greater fraction of Epo+MSCs persisted when compared with WT-MSCs (*Figure 3D*). Consistent with our flow cytometry data, frozen histologic sections from separate implants showed that qualitatively, compared with WT-MSC implants (*Figure 3E*), PKH26+cells were more abundant in the Epo+MSC implants (*Figure 3F*). As PKH26-labelling can be used to assess cellular proliferation,[25] we conducted immunostaining on an additional series of implants for the

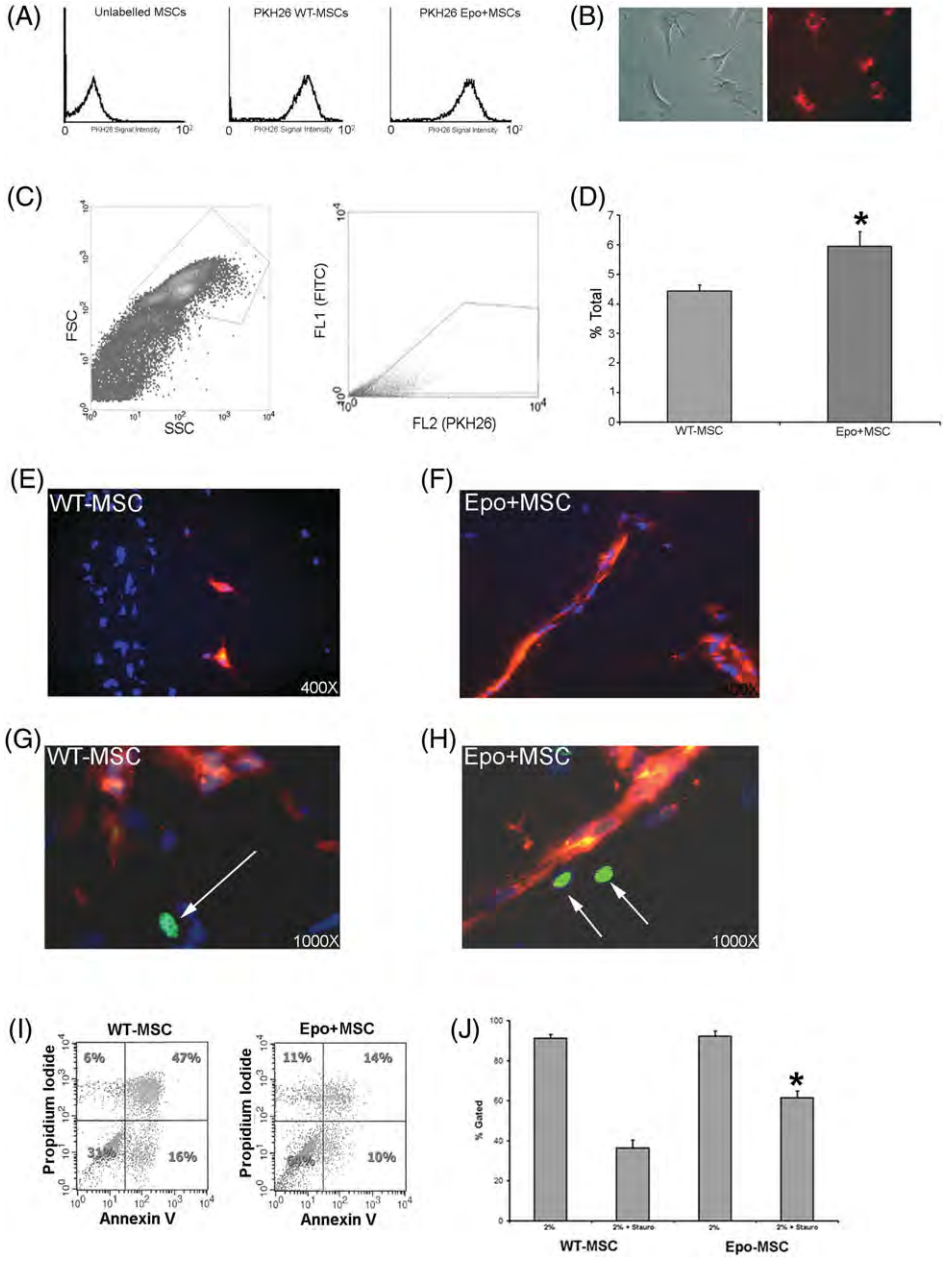

Figure 3 Regenerative potential of wild type-mesenchymal stromal cells (WT-MSCs) and Epo-overexpressing MSCs (Epo+MSCs). (*A*) Flow cytometry to compare PKH26 labelling of WT and Epo+MSCs prior to implantation. (*B*) *In vitro* microscopy confirmation of PKH26 labelling of MSCs. (*C*) Flow cytometry analysis on enzymatically digested Matrigel implants from WT and Epo+MSCs 2 weeks post-implantation to (*D*) quantitative number of surviving MSCs based on PKH26 signal. Matrigel implants from (*E*) WT and (*F*) Epo+MSCs 2 weeks post-implantation to identify surviving MSCs (red). Immunofluorescence for Ki67 (green, arrows) on PKH26-labelled (*G*) WT and (*H*) Epo+MSC Matrigel implants 2 weeks post-implantation to identify proliferating cells. (*I*) Representative Annexin-V/PI flow cytometry analysis plots of 24 h post-staurosporine induced apoptosis on WT and Epo+MSCs. (*J*) Graphical analysis ($n = 4$ separate experiments) of percent healthy (Annexin V, PI negative) WT-MSC and Epo+MSCs following 24 h exposure to staurosporine-induced apoptosis. (PKH26 flow cytometry for Epo+MSC vs. WT-MSC implants; $n = 5$, mean $\pm$ SEM) (Apoptosis, Epo+MSCs vs. WT-MSCs; $n = 4$ separate experiments run in duplicate, mean $\pm$ SEM) (*$P < 0.05$ vs. WT-MSCs). (Pictures representative of minimum three separate experiments, nuclei stained with DAPI-blue).

proliferation marker Ki67 to determine whether MSCs proliferate *in vivo*. At 2 weeks post-implantation, in both our WT (*Figure 3G*) and Epo+MSC (*Figure 3H*) implants, there was evidence of proliferating cells within the implants (arrows); however, neither MSC population appeared to be actively proliferating *in vivo*. Confirming our *in vivo* survival data, when *in vitro* cultured WT-MSCs and Epo+MSCs were exposed to an apoptotic inducer (20 nM staurosporine) for 24 h, Epo+MSCs were better protected against apoptosis, as demonstrated by a 3-fold decrease in the number of cells in mid-apoptosis (*Figure 3I*) and a 50% increase in the number of live cells (i.e. Annexin-V, PI-, *Figure 3J*).

3.3 Erythropoietin receptor expression on mouse mesenchymal stromal cells is permissive for an autocrine loop on erythropoietin-overexpressing mesenchymal stromal cells

The presence of the EpoR has been demonstrated in several non-haematopoietic cells including neuronal, cardiomyocyte, endothelial, vascular smooth muscle cells (VSMCs)[26] and recently MSCs.[14,15] We also demonstrate that the EpoR is expressed by murine MSCs. *Figure 4* illustrates the detection of the EpoR in murine MSCs on both a transcriptional and protein level. RT-PCR analysis revealed that both C57Bl/6 (*Figure 4A* left) and Balb/C (data not shown) MSC

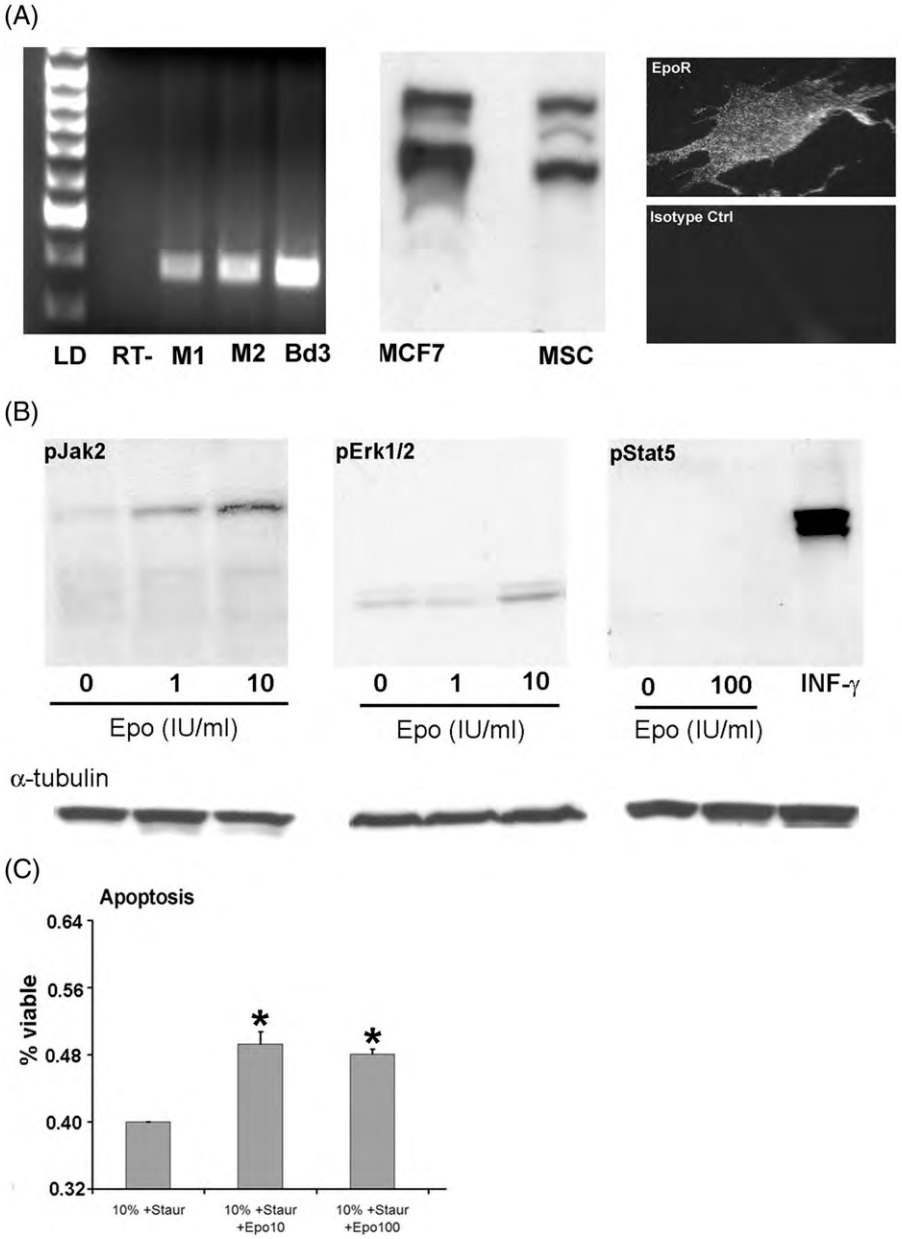

Figure 4 Erythropoietin receptor (EpoR) on murine mesenchymal stromal cells (MSCs). (*A*) EpoR primers used for RT-PCR analysis of mouse MSCs (M1 and M2) and Bend3 (Bd3) cells (Left panel). Western blot of EpoR protein on whole cell lysates from human breast cancer cell line MCF-7 and murine MSCs (Middle panel). Immunofluorescence of murine MSCs stained for EpoR and isotype control (Right panel). (*B*) Recombinant Epo signalling on MSCs. western blot(s) of signalling proteins on murine MSC whole cell lysates following 15 min stimulation with recombinant Epo or INF-γ (Upper panel). Left Jak2 phosphorylation, middle Erk1/2 phosphorylation, right Stat5 phosphorylation. α-Tubulin loading controls (Lower panel). (*C*) Apoptosis (right panel) on murine MSCs following recombinant Epo stimulation at different doses, using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay. (MTS-apoptosis, Epo+MSCs vs. WT-MSCs; $n = 4$ separate experiments run in triplicate, mean ± SEM) (*$P < 0.05$ vs. 10%+Staur).

populations express EpoR mRNA, as do human MSCs (see Supplementary material online, *Figure S1*). Western blot analysis demonstrated that like MCF-7 cells, mouse MSCs have EpoR immunoreactive band at 64 and 72 kDa (*Figure 4A*, middle) which correspond to the unprocessed cytosolic form and the membrane form, respectively.[27,28] Additional experiments using immunofluorescence under non-permeablizing conditions confirmed that EpoR is found on the membranes of MSCs as evidenced by a punctuate membrane staining pattern, which was not apparent using an isotype control antibody (*Figure 4A*, right).

Several studies have suggested that despite detectable levels of EpoR on cancer cell lines, Epo does not necessarily evoke a signalling cascade.[29] Thus, in addition to describing the presence of EpoR on mouse MSCs, we undertook studies to establish whether EpoR exerts a function on MSCs. Generally, Epo, upon binding to EpoR, initiates signalling via autophosphorylation of Jak2.[26] Fifteen-minute stimulation of mouse MSCs with the increasing levels of Epo demonstrated a dose-dependent increase in Jak2 activation using phosphorylation-specific Jak2 antibody (*Figure 4B*, left). The same stimulation with Epo was also capable of dose dependently increasing the phosphorylation of Erk1/2 (*Figure 4B*, middle). In contrast to haematopoietic cells,[30] but similar to VSMCs, exposure to recombinant Epo did not activate Stat5 signalling in our MSC populations (*Figure 4B*, right), although our MSC populations were capable of activating Stat5 signalling following INF-γ treatment (*Figure 4B*, right).

Using the MTS assay as a measure of mitochondrial activity, we assessed whether Epo could protect mouse MSCs from apoptosis. Staurosporine-treated MSCs showed a significant reduction in mitochondrial activity ($\sim$60%) compared with untreated controls, consistent with increased cell death. However, pre-treatment of MSCs with Epo afforded a significant level of protection such that at Epo concentrations of 10 and 100 IU/ml MTS conversion was increased by $\sim$20% compared with non-Epo-exposed staurosporine-treated MSCs (*Figure 4C*). We performed Annexin V-PI flow cytometry analysis of MSCs pre-exposed to 100 U/ml Epo and subjected to staurosporine-induced apoptosis. We found that compared with non-Epo-exposed staurosporine-treated MSCs, Epo treatment caused a 17% decrease in overall apoptosis (data not shown).

3.4 The effect of erythropoietin-overexpressing mesenchymal stromal cells on ventricular function following acute myocardial infarction

3.4.1 Tissue persistence

Using a clinically relevant murine model of MI, we tested the therapeutic potential of Epo+MSCs. We subjected mice to myocardial ischaemia by the ligation of the left coronary artery followed by the injection of either PBS, WT-MSCs or Epo+MSCs at five different points (250 000 cells per injection in 5 μl) in the borderzone of the infarct 10 min after ligation. The peri-operative mortality was not statistically different between treatment groups (data not shown, $P = 0.37$). In one set of experiments, we pre-labelled MSCs with PKH26 to assess cell survival and persistence at 7 and 14 days post-MI. Because of high levels of autofluoresence in the ischaemic heart, in both the FL2 (PKH26 channel) and FL1 (FITC channel) fluorescent spectra, we qualitatively determined surviving implanted MSCs based on a FL2 signal that was non-overlapping with an autofluorescent FL1 signal (*Figure 5A*, upper panel). Thus, only those cells that were PKH26+/FITC− were considered to be surviving MSCs, whereas those cells that appeared to be both PKH26+ and FITC+ were considered necrotic or dying cells (*Figure 5A* lower panel, arrows). At 7 days post-implantation, we identified WT-MSCs and Epo+MSCs within the myocardium and noted that the majority of the PKH26+/FITC− MSCs were clustered closely together and had not migrated into the infarcted myocardium (*Figure 5B* and *C*). At 14 days post-implantation, we again could not definitively identify WT or Epo+MSCs within the infarcted heart, but did find surviving MSCs bordering the infarcted area. When we performed immunostaining for cardiac troponin I on these hearts we found that regardless of whether surviving cells were from our WT or Epo+MSCs or whether the MSCs were found near the surface of the heart (*Figure 5D*) or deep within the myocardium (*Figure 5E*), these cells remained uncommitted to a myocardial phenotype. Furthermore, we also noted that like our Matrigel plug data, these same MSCs (both WT and Epo+) did not readily differentiate into endothelial cells when injected into the heart, but again acted like accessory cells for the blood vessels (*Figure 5F* and *G*). The distribution of the surviving cells at 7 and 14 days post-implantation and their lack of differentiation towards either a myocardial or endothelial lineage suggests that any beneficial cardiac effects we observe from the WT and Epo+MSC groups will likely be the result of the paracrine actions of MSCs and not their transdifferentiation potential.

3.4.2 Effect on left ventricular remodelling post-myocardial infarction

In a second set of experiments, we followed animals for 14 days after left coronary artery ligation and intracardiac injections of PBS, WT-MSCs or Epo+MSCs. Cardioprotective effects of MSCs after MI were evaluated using serial echocardiograms (see Supplementary material online, *Figure S2A*). LV dysfunction, assessed by fractional shortening (FS), was evident in the three groups at baseline when compared with non-operated animals (data not shown), but were similar between the three experimental groups (Epo+MSC; $24.2 \pm 11.1\%$, WT-MSC; $28.3 \pm 8.2\%$ and PBS; $24.9 \pm 7.0\%$, $P = 0.54$). FS declined progressively throughout the course of the study for the PBS and WT-MSC-injected animals; however, this decline was not apparent in the Epo+MSC-injected animals (*Figure 6A*). At Day 14, fractional shortening was significantly higher in the Epo+MSC group compared with the WT-MSC and PBS groups ($29.7 \pm 8.5\%$ vs. $19.9 \pm 6.6\%$ and $19.1 \pm 7.4\%$, respectively, $P = 0.02$) (*Figure 6B*). LV invasive haemodynamic measurements were consistent with the echocardiograms and confirmed an enhanced preservation of the contractility and relaxation in the Epo+MSC and WT-MSC groups compared with the PBS control group ($+dP/dT$ of 3839 ± 653, 3269 ± 742 and 2725 ± 803 mmHg, respectively, $P < 0.05$) (*Figure 6C*). Despite these improvements, no variation in MI size was observed between the treatment groups (*Figure 6D*). A modest (13%) increase in haematocrit was observed in the Epo+MSC group at Day 7, but was no longer apparent by Day 14 (see Supplementary material online, *Figure S2B*).

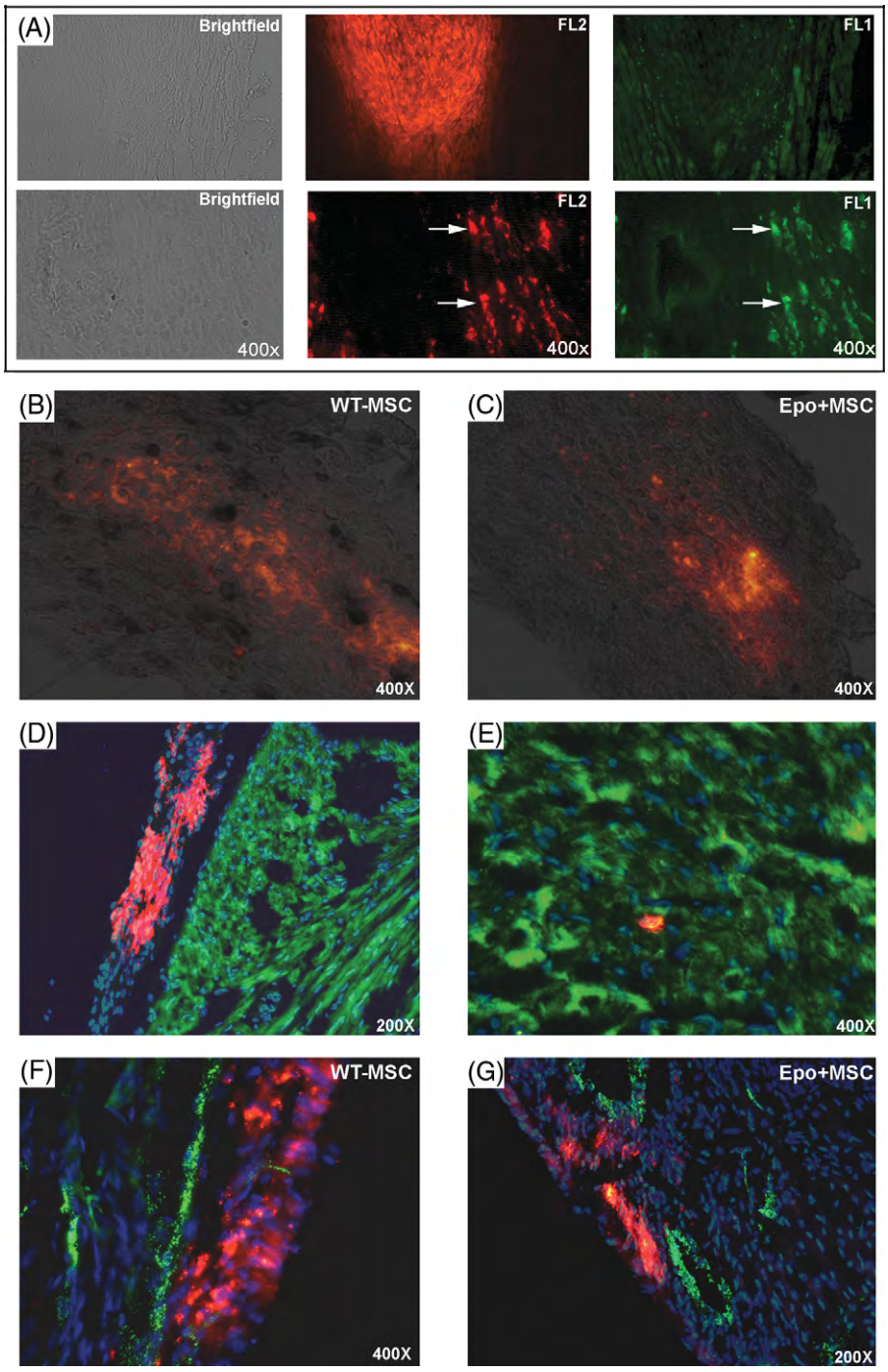

Figure 5 Persistence, and differentiation potential of intra-cardiac mesenchymal stromal cell (MSC) injections following myocardial infarction (MI). (*A*: upper panel) Seven day post-MI assessment of PKH26− labelled MSC persistence determined by non-overlapping signal in FL2 (PKH26 channel; upper middle panel) and FL1 (FITC channel; upper left panel). (*A*: lower panel) Autofluorescence determined by equal signal in both FL2 (PKH26 channel; lower middle panel, arrows) and FL1 (FITC channel; lower left panel, arrows). Seven day post-MI engrafted PKH26-labelled MSCs were found primarily as clusters of cells located in the borderzone of the infarct for both (*B*) wild-type and (*C*) Epo-overexpressing MSCs (Epo+MSCs). Immunostaining for cardiac troponin I (green) for differentiation of 14 day post-MI surviving MSCs found at the surface (*D*) and interior (*E*) of the myocardium. Immunostaining for blood vessels (vWF-green) to determine endothelial differentiation of 14 day post-MI surviving PKH26-labelled (red) (*F*) WT and (*G*) Epo+MSCs. (Pictures representative of minimum three separate experiments, nuclei stained with DAPI-blue).

To further understand the mechanism underlying the preservation of LV function conferred by Epo+MSCs, we used immunohistochemical techniques to assess the degree of myocardial vascularization and neutrophilic infiltration on these animals at 14 days post-MI. Immunohistochemical staining for vWF and quantitative analysis of blood vessel density revealed that vascular density of the MI borderzone in Epo+MSC-treated mice was significantly higher than that of PBS and WT-MSC treated animals (*Figure 6E*). Interestingly, in addition to having the greatest number of blood vessels, Epo+MSC-treated animals also exhibited the lowest degree of myocardium neutrophilic infiltration (*Figure 6F*). Supporting this observation, we also observed that neutrophil express the EpoR and demonstrate and that high local levels of Epo

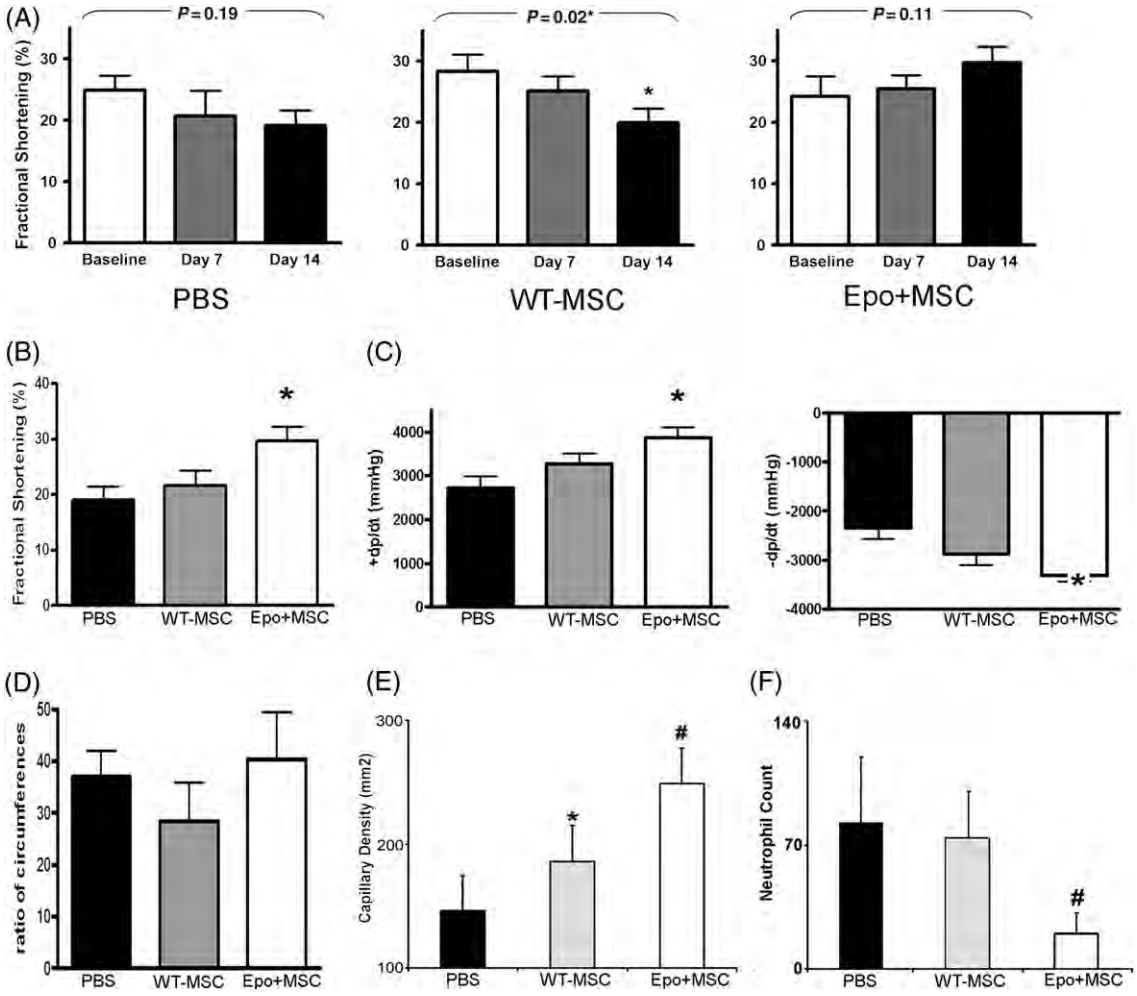

Figure 6 Haemodynamic and morphometric impact of intra-cardiac mesenchymal stromal cell (MSC) injections following myocardial infarction (MI). (*A*) Left ventricular (LV) function over time by treatment group. A progressive LV dysfunction, as illustrated by decline in fractional shortening is present in both PBS and wild-type MSC (WT-MSC) animals. Epo-overexpressing MSCs (Epo+MSCs) treated animals did not exhibit this LV fractional shortening decline over time. (*B*) At Day 14, Epo+MSC treated animals exhibited a significantly higher fractional shortening. (*C*) Closed-chest invasive haemodynamic measurements performed at Day 14 determined that LV contractility ($+dP/dT$, middle pannel), and relaxation ($-dP/dT$, right pannel) was better preserved in Epo+MSC treated animals compared with PBS controls. (*D*) At 14 days, the infarct size assessed by planimetry was similar between the PBS, WT-MSC and Epo+MSC injected groups. (*D*) Immunohistochemical quantification of blood vessels density in the borderzone of mice injected with PBS, WT or Epo+MSCs, 14 days post-MI. (*E*) Immunohistochemical quantification of neutrophils in the borderzone and infarct of mice injected with PBS, WT-MSCs or Epo+MSCs, 14 days post-MI. (haemodynamic and immunohistochemical $n = 8$–10 per group, mean ± SEM) ($^{*}P < 0.05$ vs. PBS; $^{\#}P < 0.05$ vs. all groups).

can mitigate neutrophil migration towards a chemotactic stimulus (see Supplementary material online, *Figure S3*).

4. Discussion

The present study tested whether coupling Epo production to MSCs could enhance MSC-based treatment of cardiovascular disease. We demonstrate that MSCs do not naturally produce Epo, but show a direct signalling response of MSCs to Epo and correlate that to changes in Epo+MSC behaviour both *in vitro* and *in vivo*. We found that MSCs can be retrovirally engineered to secrete high levels of Epo without altering their mesenchymal phenotype or plasticity. Epo+MSCs could secrete ~600 mIU Epo/1 × 10^6 cells in 24 h, which physiologically speaking is substantial as normal Epo levels in the blood are between 5 and 25 mIU/L.[26] Therefore, an implant containing as little as 100 000 cells can lead to continuous and substantial *in situ* Epo production. We have previously shown that MSCs can promote a robust, VEGF-dependent, host-derived angiogenic response[19], and herein, we demonstrate that Epo+MSCs are clearly more potent in this regard. Furthermore, our Matrigel plug assay confirmed that Epo+MSCs survive better *in vivo* compared with WT-MSCs. In the final portion of this study, we conducted a proof-of-principle experiment in a clinically relevant model of acute MI and demonstrated that Epo+MSCs were more potent than WT-MSCs in preserving myocardial contractility, promoting neovascularization and reducing neutrophilic cellular infiltration.

This study ultimately defines two important justifications for continued development of Epo+MSCs for the treatment of cardiovascular disease. First, our data support the recent assertion by Zhang *et al.*[14] that peripheral Epo infusion can augment the therapeutic potential of MSCs; however, within their study, repetitive high-dose peripheral infusions of Epo were required for the observed effect. In fact, the experimental dose used in that study would translate into multiple injections of ~400 000 U in an 80 kg patient, a dose that is normally administered over a 10 week period for the treatment of patients with severe anaemia. At present, such a

high dose has not been proven to be safe in humans and may theoretically lead to thrombotic events.[31] The necessity for high peripheral Epo doses is likely because of the bone marrow acting as a biological sink as well as low bioavailability of Epo to the compromised myocardium and transplanted MSCs because of reduced local perfusion. Together these factors raise concerns in regards to clinically implementing a combinational therapy using peripheral Epo and local MSC injection. In contrast, transplantation of MSCs secreting Epo can lead to the continuous local Epo delivery at the site of injury, thereby maximizing Epo's local effects and providing synergistic/additive effects to the natural paracrine regenerative properties of MSCs.[32] Our data support this notion as the haematocrit was only slightly elevated in our MI animals injected with Epo+MSCs, since MSCs stay clustered close to their site of injection, do not actively proliferate *in vivo* and do not readily differentiate into cardiac or endothelial cells. Furthermore, since MI scar size was not altered in any of our treatments, we can infer that the enhanced preservation of cardiac function we observed in our Epo+MSCs-treated animals was the direct result of Epo+MSCs creating a more amenable local environment that can enhance the influx of endothelial cells and/or block recruitment of inflammatory cells. As such we can postulate that, by using Epo+MSCs for cardiovascular therapy, fewer cells would be required to observe a beneficial effect. Since Epo production would be local, the risk of thrombotic events would be minimal.

The second advantage is that by having Epo continuously secreted by MSCs, an autocrine loop is established, and this bestows these MSCs with enhanced survival potential. Originally, the EpoR was considered to be restricted to those cells involved in erythropoiesis.[26] However, in recent years, EpoR has been recognized on an increasing number of non-erythropoietic cells including endothelial cells, cardiomyoctes and VSMCs.[26] In this work, we confirm and extend the findings of Zhang[14] and Zwezdaryk[15] that the EpoR is found on MSCs. Specifically, we have demonstrated that mouse MSCs possess the EpoR and that Epo stimulation influences MSC survival. In comparison with the Zwezdaryk *et al*. study,[15] which used hypoxia as an indirect means of establishing functional EpoR signalling, we directly demonstrate that Epo can initiate signalling in MSCs through phosphorylation of Jak2 and Erk1/2. Together these data provide a mechanistic explanation for why our Epo+MSCs have enhanced survival both *in vitro* and *in vivo*. Our data provide additional support to the studies of Mangi[5] and Wang[4] for coupling gene engineering with MSC cellular therapy for the treatment of cardiovascular disease. However, unlike Mangi's study, which used overexpression of Akt, a pro-survival gene, in MSCs to enhance persistence in heart,[31] or the Wang study, which used overexpression of VEGF, a protein already known to be produced by MSCs,[4] we over-expressed Epo, a protein not normally expressed by MSCs. As such, we not only take advantage of Epo's natural paracrine ability to accelerate functional tissue recovery[33] via reduced apoptosis[34] and increased neovascularisation,[35] but also exploit Epo's abilities to enhance MSC survival via an autocrine loop. Together, coupling Epo production to MSCs establishes powerful paracrine and autocrine actions making the Epo+MSC platform a promising tool.

It is important to note that this study did not attempt to determine whether engineering MSCs to secrete Epo should be performed in lieu of Epo administration post-MI, but rather set out to determined whether coupling Epo secretion to MSCs can enhance the potential benefit of MSC therapy when cellular therapy is warranted. Our data clearly suggest that Epo+MSCs are more potent than WT-MSCs. Aside from MI, we believe that this platform could be used to treat a variety of ischaemic disorders including stroke, peripheral vascular disease and idiopathic pulmonary hypertension. Indeed, administration of Epo-secreting MSCs could open a window of therapeutic opportunity where a single dose after an ischaemic episode offers enhanced immediate and long-lasting benefits over unmodified MSCs.

With the growing enthusiasm for the use of MSCs in the treatment of ischaemic disorders and the potential enhancement *ex vivo* gene modification of these cells may afford, several biosafety issues must be addressed. In particular, concerns that MSCs may be prone to malignant transformation during *in vitro* expansion and that this may be exacerbated by insertional mutagenesis during gene modification must be resolved.[36] Recently, two studies have suggested murine MSCs can undergo malignant transformation;[37,38] however, this appears to be a species-specific phenomenon as human bone marrow-derived MSCs do not undergo transformation after long-term *in vitro* culture.[39] We are unaware of any studies describing oncogenic transformation of MSCs because of retroviral genetic manipulation, and based on our proliferation studies we can conclude that the vast majority of our WT or Epo+MSCs did not proliferate *in vivo* and thus were unlikely to have been transformed. Despite this, prior to clinical implementation, the biosafety of *ex vivo* gene engineered MSCs should be confirmed by molecular karyotyping.[39] Additional levels of biosafety may also be achieved by pre-screening engineered MSCs to select clones with transgene insertion at desirable sites within the chromosome and by implementing insulators into vector design to minimize positional effects. Ultimately, if retroviruses are still a concern, the use of non-intregrating gene transfer platforms including naked DNA plasmids, adenovirus and adeno-associated virus could be employed and would likely maintain the therapeutic potential of this platform since MSCs do not readily proliferate *in vivo*.

In conclusion, Epo-secreting MSCs combine the desirable cardioprotective, proangiogenic and anti-inflammatory features of Epo to yield a cellular vehicle with similar properties. We believe the clinical translation of such a strategy is feasible and represents a novel second generation platform for the development of cell-based therapeutics.

Supplementary material

Supplementary Material is available at *Cardiovascular Research* Online.

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