Potential risks of bone marrow cell transplantation into infarcted hearts

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Cellular replacement therapy has emerged as a novel strategy for the treatment of heart failure. The aim of our study was to determine the fate of injected mesenchymal stem cells (MSCs) and whole bone marrow (BM) cells in the infarcted heart. MSCs were purified from BM of transgenic mice and characterized using flow cytometry and in vitro differentiation assays. Myocardial infarctions were generated in mice and different cell populations including transgenic MSCs, unfractionated BM cells, or purified hematopoietic progenitors were injected. Encapsulated structures were found in the infarcted areas of a large fraction of hearts after injecting MSCs (22 of 43, 51.2%) and unfractionated BM cells (6 of 46, 13.0%). These formations contained calcifications and/or ossifications. In contrast, no pathological abnormalities were found after injection of purified hematopoietic progenitors (0 of 5, 0.0%), fibroblasts (0 of 5, 0.0%), vehicle only (0 of 30, 0.0%), or cytokine-induced mobilization of BM cells (0 of 35, 0.0%). We conclude that the developmental fate of BM-derived cells is not restricted by the surrounding tissue after myocardial infarction and that the MSC fraction underlies the extended bone formation in the infarcted myocardium. These findings seriously question the biologic basis and clinical safety of using whole BM and in particular MSCs to treat nonhematopoietic disorders. (Blood. 2007;110:1362-1369)

Introduction

Severe heart failure is caused by an irreversible loss of cardiomyocytes and has a poor prognosis regardless of the underlying disease.[1] Since medical treatment is of only limited help, solid organ transplantation was considered until recently the only effective therapy. However, as organ availability decreases, there is an urgent need for alternative treatments. Studies in mice have suggested that myocardial infarctions can be repaired following transplantation of bone marrow (BM)–derived cells into the lesioned myocardium, either through generation of cardiomyocytes or angiogenesis.[2] An underlying assumption of this approach is that the environment will instruct as well as restrict the developmental fate of adult stem cells after their transplantation (for review see Laflamme and Murry[3] or Murry et al[4]). However, the original findings in mice have recently been put into question, since we and others have demonstrated that BM-derived hematopoietic cells do not transdifferentiate into cardiomyocytes in the infarcted myocardium.[5-7]

In this study, we focused on the potential of an enriched population of mesenchymal stem cells (MSCs) that are known to be present in the BM and are multipotent.[8] In contrast to hematopoietic progenitors, MSCs are easy to obtain and to expand in vitro and have therefore emerged as attractive candidates for cellular therapies in heart and other organs.[9,10] However, recent reports have questioned their "transdifferentiation" potential after injection into the myocardium and rather propose benefits via paracrine mechanisms.[11,12] Herein, we investigated and provide novel insights with regard to the fate of enriched populations of BM-derived MSCs as well as whole BM cells comprising both hematopoietic and mesenchymal progenitors after transplantation into the infarcted heart.

Materials and methods

All experiments were approved by the local ethics care committees at Bonn, Cologne, and Lund Universities. Cells for transplantation were isolated from transgenic C57Bl/6 mice expressing enhanced green fluorescent protein (EGFP) under control of the β-actin promoter.[13]

Cell isolation and culture

Fibroblasts were prepared from EGFP$^+$ transgenic mouse embryos (E13.5/E14.5) using standard protocols. Adult BM cells were obtained by flushing femur and tibia of 2- to 3-month-old mice with a 27-gauge needle. Purification of lin$^-$/c-kit$^+$ hematopoietic progenitors and lin$^-$/c-kit$^+$/Sca-1$^+$ hematopoietic stem cells was performed as described earlier.[5] MSCs were generated using standard protocols.[8] Briefly, BM cells were plated on plastic dishes and adherent cultures grown in Dulbecco modified Eagle medium (DMEM) supplemented with 15% fetal calf serum (FCS). Cells were serially passaged before reaching confluence. Cultures of circulating MSCs were established accordingly by plating of peripheral blood cells. In vitro differentiation of cultured MSCs was performed as described earlier[8] with minor modifications. Briefly, adipogenic differentiation was induced with dexamethasone, isobutylmethylxanthine, hydrocortisone, indomethacine, and insulin in DMEM low glucose/10% FCS; chondrogenic differentiation was induced in cell pellets with dexamethasone, ascorbic acid, proline, sodium pyruvate, and transforming growth

Submitted December 18, 2006; accepted April 20, 2007. Prepublished online as *Blood* First Edition paper, May 4, 2007; DOI 10.1182/blood-2006-12-063412.

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factor-β3 in DMEM/F-12/ITS-Supplement; osteogenic differentiation was induced with dexamethasone, ascorbic acid, and β-glycerol phosphate in DMEM low glucose/10% FCS. All chemicals were obtained from Sigma-Aldrich (St Louis, MO), except transforming growth factor-β3 from PeproTech (Rocky Hill, NJ).

Flow cytometry analysis

Flow cytometry analysis was performed on a FACSCalibur using CellQuest (BD Biosciences, Franklin Lakes, NJ). MSCs were trypsinized and stained using PE-labeled antibodies against CD11b, CD44, CD45, CD49e, CD73, Sca-1 (all from BD Pharmingen, Franklin Lakes, NJ), CD105, and CD106 (Santa Cruz Biotechnology, Santa Cruz, CA).

Myocardial infarction, reconstitution, and BM mobilization

Myocardial infarctions were generated in syngeneic 3-month-old C57Bl/6 wild-type mice using a cryolesion or left coronary artery (LCA) ligation (for detail see Nygren et al[5]). Briefly, the mice were anesthetized, their chest opened, and the heart exposed. Cryolesions were generated by pushing a liquid nitrogen–cooled copper probe of 4–mm diameter on the free left ventricular wall 3 times for 20 seconds each. For LCA, a Prolene 8-0 suture was placed around the left coronary artery just distally to the left atrium and tightened, thereafter the myocardium distally to the ligation became immediately pale. Both lesion models led to transmural infarctions and formation of the typical scar tissue a few days after the operation.[5,14] In small rodents, the cryoinjury has distinct advantages compared with LCA, as the lesions are uniform in size and postoperation mortality is much lower. Importantly, in both of these lesion models pathological changes in the form of ossifications/calcifications could be observed after injection of bone marrow–derived MSCs. Since there was no difference in regard to this critical finding, we have combined both lesion types in the statistics.

The cardiac injury was followed immediately by 2 injections of a total amount of 5 to 6 μL cell suspension or vehicle into the center and the border zone of the infarcted area; the precise location of the injection sites varies due to anatomic differences of the hearts. In some mice, we also performed cryolesions and injected the MSCs in a second operation 4 days later. For mobilization experiments, lethally irradiated mice were reconstituted with $EGFP^+$ transgenic whole BM cells or purified $lin^-/c\text{-}kit^+/Sca\text{-}1^+$ hematopoietic stem cells and engraftment was evaluated by flow cytometry. After 6 weeks, myocardial infarction was induced and the mice were mobilized by 5 daily injections of 5 μg recombinant human Flt-3 ligand and 5 μg recombinant mouse GM-CSF (gifts of Immunex, Seattle, WA) in PBS with 0.02% serum starting one hour after infarction (for detail see Nygren et al[5]).

Histology and immunohistochemistry

Transplanted hearts were harvested and cell engraftment was documented using a fluorescent stereomicroscope. After fixation in 4% paraformaldehyde and cryopreservation in 20% sucrose, hearts were embedded in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA) and cryosectioned (6–10 μm) using a cryostat (Leica CM3050S; Leica Microsystems, Wetzlar, Germany). Alternatively, hearts were fixed in zinc-formalin (Z-fix; Anatech, Battle Creek, MI) and embedded in paraffin, and sections were prepared using a microtome (Leica SM2000R; Leica Microsystems). Semithin sections were generated on an ultramicrotome (Leica Reichert Ultracut R; Leica Microsystems) after fixation of hearts in cacodylate buffer and osmium tetroxide, dehydration to propylene oxide, and embedding in araldite. Histologic stainings (methylene blue, toluidine blue, van Gieson, von Kossa, hematoxylin/eosin, oil red O, Alcian blue) were done using standard protocols. Immunostainings were performed as described before,[14] using antibodies against alpha-actinin, alpha-smooth muscle-actin, desmin (all Sigma-Aldrich), platelet/endothelial cell adhesion molecule (PECAM; BD Pharmingen), osteocalcin (Santa Cruz Biotechnology), and CD45 (Lab Vision, Fremont, CA). Visualization was accomplished with secondary antibodies conjugated to Cy3 or Cy5 (Jackson ImmunoResearch, West Grove, PA) and nuclei were stained with Hoechst 33342 (Sigma-Aldrich).

Image acquisition and preparation

Images for Figures 2B-E, 3E, 4A, 4B inset, 4C inset, and 5F were obtained with a Zeiss Axiovert 200M fluorescent microscope equipped with an Zeiss ApoTome (Carl Zeiss Microimaging, Oberkochen, Germany) using EC Plan-Neofluar 2.5×/0.075 (Figure 2B) or Plan-Neofluar 40×/1.3 Oil (all other images) objectives. Images were photographed with a Zeiss AxioCam MRm camera, acquired by Zeiss AxioVision image acquisition software.

Images for Figures 1D,E, 2A, 2A inset, 3B, 3B inset, 3C-F, 3F inset, 4B, and 5C,D were obtained with a Leica MZ 16F fluorescent stereomicroscope. Images were photographed with a JenOptik ProgRes C10 plus camera (JenOptik AG, Jena, Germany), acquired by JenOptik ProgRes Capture Pro image acquisition software.

Images for Figures 1A,B, 1D inset, 1E inset, 1F, 4A inset, and 5A,E were obtained with a Zeiss Axiovert 40 CFL fluorescent microscope using Zeiss LD A-Plan 20×/0.3 Ph1 (Figure 5E) or Zeiss A-Plan 10×/0.25 Ph1 (all other images) objectives. Images were photographed with a Canon PowerShot G5 digital camera.

Images for Figures 3A and 3A inset were obtained with a Olympus BX50 microscope (Olympus, Center Valley, PA) using a 40 × objective and an Olympus U-POT polarization filter. Images were photographed with a Panasonic XC-003P camera (Panasonic Deutschland, Hamburg, Germany), acquired by Sybex Intervideo WinDVR3 (Sybex, Cologne, Germany) image acquisition software.

Images for Figure 4C were obtained with an Olympus BX51 fluorescent microscope using a 5 × objective. Images were photographed with a Olympus DP70 camera and processed in Adobe Photoshop (Adobe Systems, Munich, Germany) to compose a full image of the whole section.

Image processing was done with Adobe Photoshop or CorelDRAW Graphics Suite (Corel, Unterschleissheim, Germany). Linear adjustments of brightness, contrast, or color balance were applied to the whole image and did not obscure, eliminate, or misrepresent any information present in the original.

Statistics

Only hearts that contained engrafted cells and that were analyzed at least 12 days after surgery were included. Engraftment of $EGFP^+$ cells was proven by fluorescence microscopy up to day 35; at later stages, the EGFP fluorescence often declined and engraftment could not be determined. For all groups of animals, percentages are given and frequency estimates are provided with 95% confidence intervals (CIs). Statistical analysis was performed using the 2-sided Fisher exact test to compare frequencies between groups of animals (Figure 4D).

Results

Murine MSC lines were established from whole BM of β-actin EGFP mice (Figure 1A,B) using standard protocols.[8,9] After 3 passages, the cultured BM cells were devoid of cells of the hematopoietic lineage and highly enriched for MSCs as shown by lack of the hematopoietic markers CD11b and CD45 and expression of Sca-1, CD44, CD49e, CD73, CD105, and CD106 using flow cytometry (Figure 1C). The multipotent nature and functional integrity of the MSCs was further confirmed by their in vitro differentiation capacity into the osteogenic (Figure 1D), adipogenic (Figure 1E), as well as chondrogenic (Figure 1F) lineage. Then, MSCs (3rd-11th passage) were injected ($1\text{-}2 \times 10^5$ cells) into the lesioned area of left coronary artery (LCA) occluded or cryoinfarcted mouse hearts.[5] Prominent engraftment of MSCs into and beyond the injured areas was seen in 94.0% (47 of 50; CI, 0.84 of 0.98) of hearts 7 to 132 days after transplantation (Figure 2A,B). However, neither $EGFP^+$ cardiomyocytes (Figure 2C,D) nor $EGFP^+$ endothelial or smooth muscle cells of vessels (Figure 2E) were detected, ruling out a transdifferentiation of MSCs into cardiac and vascular lineages. In order to further determine the fate of the MSCs, we used araldite embedding and semithin sections. Surprisingly, in a large fraction of the hearts we detected extended pathological abnormalities in form of encapsulated structures in the myocardial lesions (Figure 3A). These formations consisted of

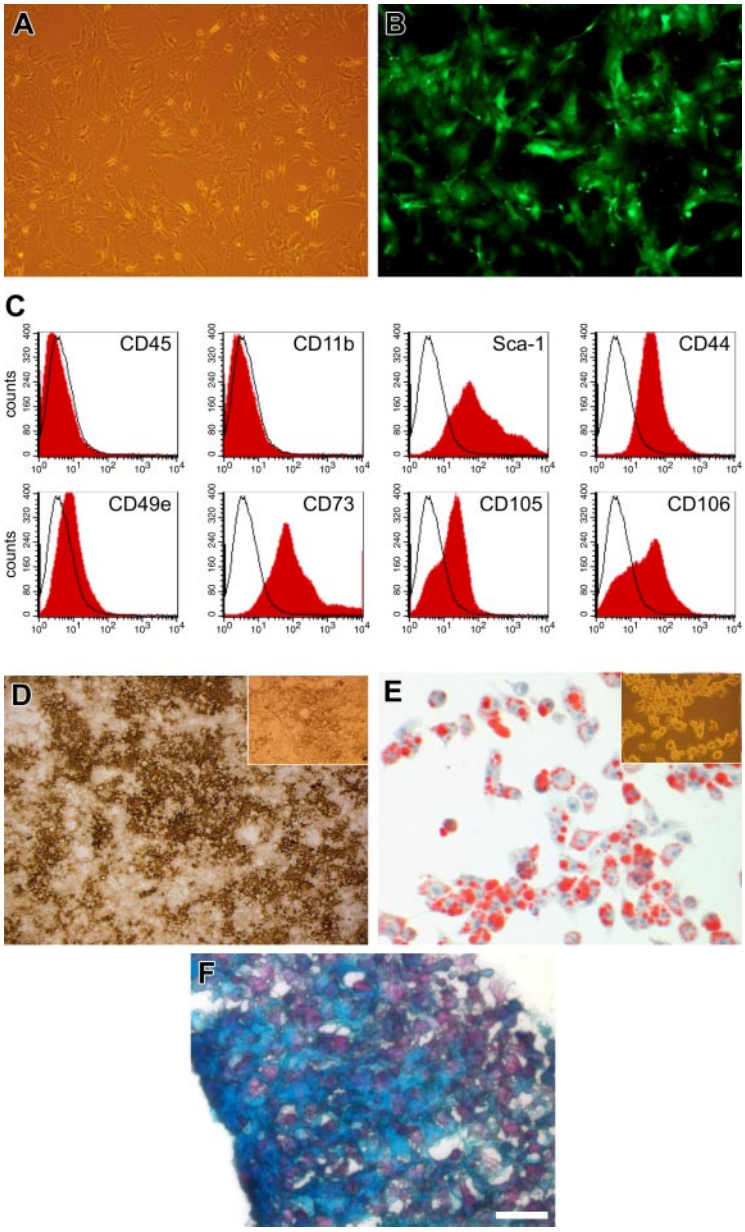

Figure 1. Characterization of MSCs. (A) MSCs (passage 4) displayed a fibroblast-like morphology in culture and were (B) EGFP$^+$ as shown by fluorescence microscopy. (C) Flow cytometry analysis of enriched MSCs (passage 5) proved typical expression of surface markers; note the lack of the hematopoietic cell markers CD45 and CD11b. (D) Osteogenic differentiation of MSCs (passage 10) in vitro led to formation of aggregates and trabecular structures (inset, 17 days); Ca^{2+} deposition was demonstrated by von Kossa staining. (E) In vitro differentiation of MSCs (passage 7) into adipocytes. The accumulation of lipid droplets in vacuoles (inset, 2 weeks in culture) was confirmed by staining with oil red O. (F) Chondrogenic differentiation of MSCs (passage 10) in vitro was determined using combined Alcian blue/nuclear fast red staining. Bar represents 180 μm (panels A,B), 860 μm (panel D), 600 μm (panel D inset), 100 μm (panel E), 300 μm (panel E inset), and 550 μm (panel F). See "Materials and Methods; Image acquisition and preparation" for microscopy details.

injected EGFP$^+$ cells within amorphous material and often showed almost transmural distribution in the scar. Polarization microscopy revealed the presence of calcifications within these structures (Figure 3A inset), and this finding was confirmed by von Kossa staining (Figure 3B). Further analysis with combined von Kossa and van Gieson staining proved that the calcifications were restricted to the scar (Figure 3C), and subsequent sections demonstrated that these areas contained high numbers of the injected EGFP$^+$ MSCs (Figure 3D). In fact, von Kossa–positive areas were found as early as 14 days after transplantation in 51.2% (22/43; CI, 0.37–0.65; median = 28 days) of LCA occluded (4/4) as well as cryoinfarcted (18/39) hearts. Because of these findings and our earlier results that the fate and long-term engraftment of embryonic cardiomyocytes and BM-derived cells are identical in the cryoinjury and LCA model,[5,15] both lesion models are combined in the statistics (Figure 4D). To better imitate earlier studies in rodents[16,17] and also currently used protocols in patients, we have infarcted mice and injected the MSCs in a second operation 4 days later. In 5 of 7 mice, EGFP$^+$ MSCs were found after 4 weeks and in all of these 5 hearts von Kossa–positive areas were detected (data not shown). Thus, the observed results are independent of both lesion type and time point of cell injection.

In order to determine the precise nature of the amorphous material and the potential source of calcifications, we next used the bone-specific marker osteocalcin. The encapsulated structures stained positive for osteocalcin, proving ossification. The injected MSCs were cemented within the bone structures (Figure 3E), suggesting that these cells generated the amorphous material. Besides osteogeneic differentiation, we still identified EGFP$^+$ cells that were located at the periphery beyond the calcifications/ossifications. These had a fibroblast-like morphology and adopted neither cardiomyocyte, endothelial, nor smooth muscle fate (data not shown). We also performed stainings using oil red O and Alcian blue as well as histologic analysis of hearts after injection of MSCs. We could identify neither fat cells in and around the infarcted areas nor chondrocytes. Although slightly positive Alcian blue staining was found in the scar tissue, it was clearly weaker than control stainings of cartilage. Moreover, histologically no evidence of cartilage differentiation was found within and around the infarcted areas.

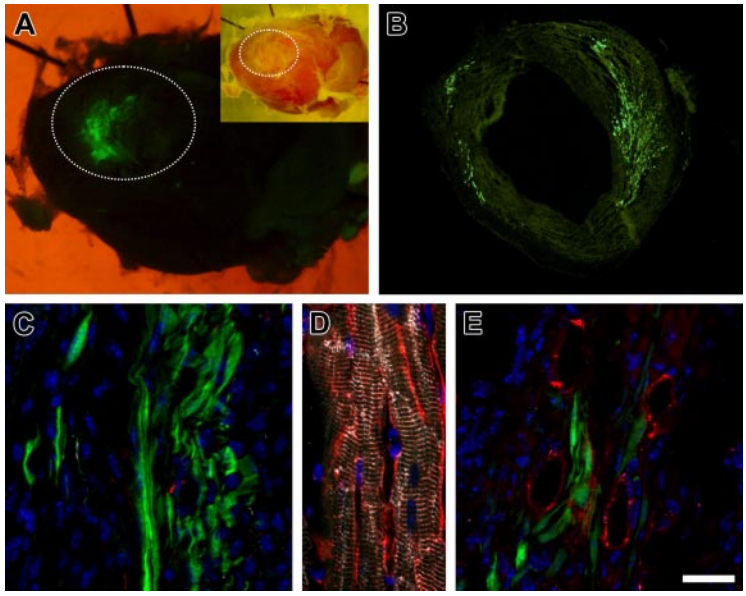

Figure 2. Engraftment of MSCs into the infarcted murine heart; lack of transdifferentiation of MSCs. (A) Massive engraftment of EGFP$^+$ MSCs (green) in a cryoinfarcted heart 29 days after injection of 2×10^6 cells (passage 3); inset shows a transmission light picture of the heart; the infarcted area is marked by a white dotted line. (B) Cryosection of border zone of the infarction demonstrating prominent engraftment of EGFP$^+$ MSCs (green) 7 days after injecting 1×10^5 cells (passage 4). (C) Engrafted EGFP$^+$ MSCs (green) in the infarcted area 24 days after transplantation of 1×10^5 cells (passage 3); these displayed round or elongated shape but were negative for alpha-actinin (red) and desmin (white). (D) Cardiomyocytes in the intact heart (same section as shown in panel C) displayed typical shape and distinct cross-striation. (E) PECAM (red, endothelial marker)– and alpha-smooth muscle-actin (magenta)–positive small vessels were identified in vicinity to engrafted MSCs (green) within and around the lesioned area. None of the vessels showed EGFP$^+$ endothelial or smooth muscle cells. Some of the vessels were missing the smooth muscle layer, indicating vessel neoformation. Nuclei were stained with Hoechst dye (blue). Bar represents 1000 μm (panel A), 2500 μm (panel A inset), 470 μm (panel B), 18 μm (panels C,E), and 8 μm (panel D). See "Materials and Methods; Image acquisition and preparation" for microscopy details.

Although MSCs are known to differentiate into mesenchymal tissue types and preferentially into osteoblasts,[10] we established 3 MSC lines to exclude atypical behavior of a single preparation. All the highly enriched MSC lines were able to produce bone in vitro and found to generate ossifications in vivo. Importantly, in untreated MSC cultures, no signs of osteogenic differentiation were found using von Kossa and osteocalcin staining, excluding that the ossifications in the infarcted hearts did result from transplantation of predifferentiated osteoblasts. To rule out a dose-escalation effect as underlying mechanism, we also injected lower amounts of MSCs (1×10^4) into myocardial infarctions and detected again calcifications/ossifications in the majority of those hearts (4 of 6, 66.7%; CI, 0.30-0.90; median = 29 days), although of smaller size

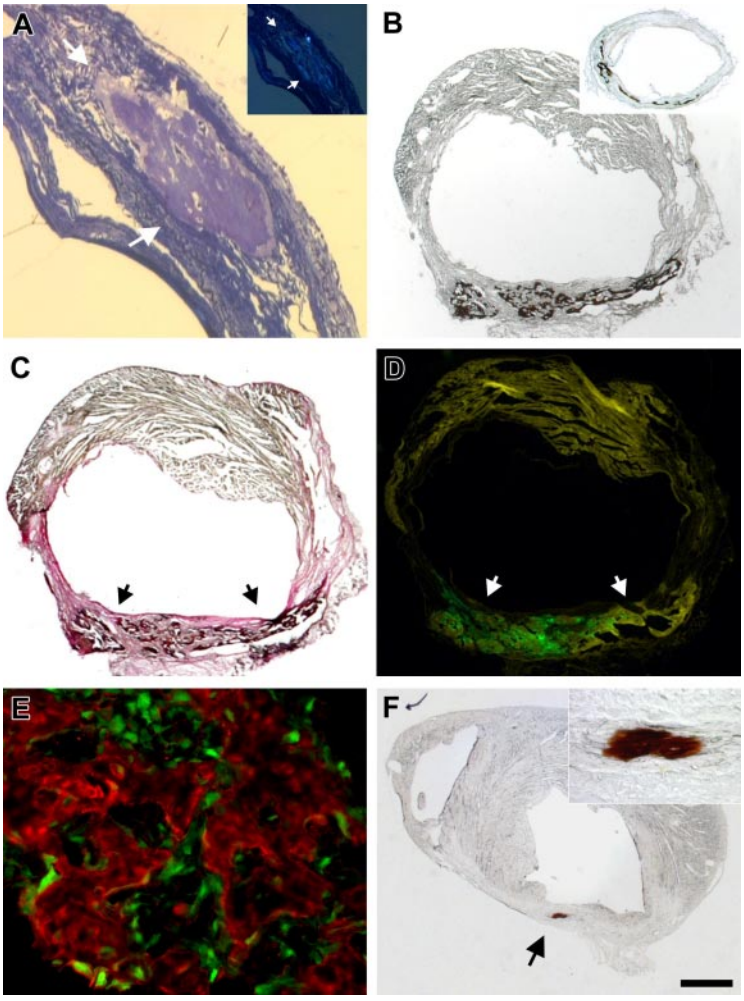

Figure 3. Calcifications and bone formation in the lesioned heart after injection of enriched MSCs. (A) Methylene blue–stained semithin section through the infarcted area of a heart 29 days after injecting 2×10^5 enriched MSCs (passage 3). Transmission light microscopy revealed an encapsulated formation (arrows) filling out almost the entire width of the scar. Polarization microscopy showed fluorescent areas within the amorphous material indicating calcifications (panel A inset). (B) Von Kossa staining evidenced massive calcifications (black deposits) 29 days after injecting 1×10^5 MSCs (passage 6) into the cryoinjured mouse heart. (Panel B inset) Similar calcifications were seen using von Kossa staining in an LCA mouse heart 28 days after injecting 1×10^5 MSCs (passage 5). (C) Combined van Gieson/von Kossa staining (same heart as shown in panel B) demonstrated that the calcifications (black, marked by arrows) were restricted to the infarcted area (red) and (D) contained high numbers of the injected EGFP$^+$ MSCs (green, subsequent section). (E) Immunostaining with osteocalcin (Cy3, red) demonstrated that the injected EGFP$^+$ MSCs (green) were cemented within trabecular-like bone structures (sample obtained from same heart as shown in panel B). (F) Similar calcifications but of smaller size were revealed by von Kossa staining (arrow) 21 days after injecting only 1×10^4 enriched MSCs (passage 6). The infarcted area of the heart is characterized by the thinned-out ventricular wall. Inset shows the area of calcification at higher magnification. Bar represents 180 μm (panel A), 550 μm (panel A inset), 650 μm (panel B), 1300 μm (panel B inset), 600 μm (panels C,D,F), 50 μm (panel E), 100 μm (panel F inset). See "Materials and Methods; Image acquisition and preparation" for microscopy details.

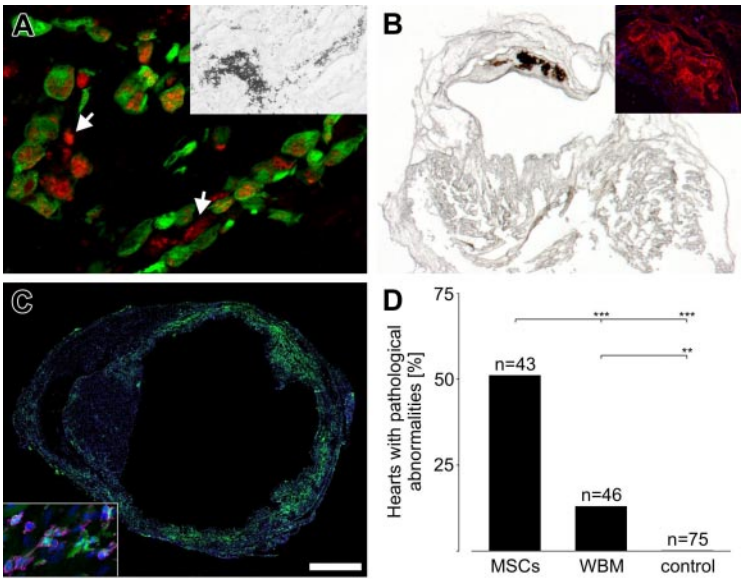

Figure 4. Bone formation origins from the MSC fraction of BM. (A) Cytosolic and extracellular (some marked by arrows) osteocalcin staining (Cy3, red) proved bone formation in a cryoinfarcted heart 13 days after injecting 5×10^6 EGFP$^+$ whole BM-derived cells (green). The osteocalcin accumulation in the injected (EGFP$^+$) cells revealed that these produce the bone tissue. Von Kossa staining in the same also heart showed calcifications (panel A inset). (B) Distinct calcifications (von Kossa staining) inside the lesioned, thinned-out ventricular wall of a cryoinfarcted heart harvested 369 days after injection of 1×10^6 EGFP$^+$ whole BM cells. Osteocalcin staining (Cy3, red) of a subsequent section of the same heart revealed distinct bone formation without enclosed cells (panel B inset). Nuclei were stained with Hoechst dye (blue). (C) Prominent engraftment of EGFP$^+$ cells (green) into the lesioned area 28 days after LCA and consecutive cytokine-induced mobilization of BM cells. Nuclei were stained with Hoechst dye (blue). CD45 staining (magenta) proved the hematopoietic origin of the engrafted cells (panel C inset). (D) Statistics of hearts with pathological abnormalities after infarction and injection of enriched MSCs (4 LCAs), whole BM cells (WBM, 2 LCAs), and controls (injection of vehicle, fibroblasts, hematopoietic progenitor cells, and mobilization of BM cells in reconstituted mice, 32 LCAs). $P < .001$ for MSCs vs WBM; $P < .001$ for MSCs vs control; and $P = .002$ for WBM vs control (2-sided Fisher exact test). Bar represents 24 μm (panel A), 50 μm (panel A inset), 750 μm (panel B), 380 μm (panel B inset), 940 μm (panel C), 40 μm (panel C inset). See "Materials and Methods; Image acquisition and preparation" for microscopy details.

(Figure 3F). The encapsulated structures containing the calcifications/ossifications were not induced by the surgical procedure and/or the lesion since none was observed in infarcted control hearts injected with vehicle (0 of 30; CI, 0.00-0.11; median = 28.5 days). Moreover, application of other cell types into the lesion did also not cause these alterations as proven by the injection of syngeneic fibroblasts (0 of 5; CI, 0.00-0.43; median = 56 days) into infarctions. Thus, preselected MSCs engraft into injured myocardium, do not transdifferentiate into cardiomyocytes, endothelial, or smooth muscle cells, and can generate large calcified bonelike structures due to apparent lack of tissue-restricted differentiation.

Since the MSC lines we developed might be transformed during culture expansion, we next investigated the incidence of pathological abnormalities after injection of 1 to 5×10^6 unfractionated BM cells into the infarcted myocardium. By using this cell population, we could analyze the differentiation fate of both hematopoietic progenitors and unmanipulated MSCs. This approach appeared also important since most patients in ongoing clinical trials receive whole BM rather than preselected cells. Strikingly, we detected encapsulated structures in as many as 6 hearts, representing 13.0% of mice that underwent transplantation (6 of 46; CI, 0.06-0.26; median = 18 days; Figure 4D); 3 of those displayed von Kossa– and osteocalcin-positive areas (Figure 4A,B). We found evidence of an early stage of ossification 13 days after transplantation with osteocalcin staining not only in the extracellular space, but also within transplanted EGFP$^+$ cells (Figure 4A). This clearly demonstrated that the injected cells were the source of bone formation and that the osseous material was subsequently released by those cells. At later stages, the von Kossa/osteocalcin-positive areas appeared larger and devoid of EGFP$^+$ MSCs and other cells (Figure 4B). The delayed onset and minor extent of bone formation in these hearts compared with those transplanted with the enriched MSC population suggested that the appearance of bone tissue was dependent on the quantity of MSCs in the unfractionated cell suspension from BM. This was also supported by the observation of smaller pathological abnormalities after injecting 1×10^4 MSCs. Importantly, the massive bone formation at later stages clearly shows that the ossification is an ongoing time-dependent process.

Although our experiments using cultured MSCs support the involvement of this cell fraction in the generation of the pathological abnormalities observed after injection of whole BM cells, we performed additional experiments to distinguish between the role of hematopoietic cells and MSCs. We injected 1 to 2×10^5 lin$^-$/ckit$^+$ hematopoietic progenitors[5] directly into the infarction after LCA ligation, and in none of these hearts (0 of 5; CI, 0.00-0.43; median = 28 days) were pathological abnormalities detected (data not shown). In recent clinical trials, cytokine treatment rather than direct injection of BM cells was tested in myocardial infarction patients,[18,19] and thus we used a similar experimental approach. In mice reconstituted with EGFP$^+$ whole BM (n = 29) or purified lin$^-$/c-kit$^+$/Sca-1$^+$ hematopoietic stem cells (n = 6), infarctions were induced by LCA ligation (n = 27) or cryoinjury (n = 8) and followed by cytokine treatment. This led, in accordance with our earlier findings,[5] to prominent mobilization of cells and progenitors of hematopoietic lineages from the BM as shown by strong infiltration of the infarction area exclusively with CD45$^+$ cells (Figure 4C inset). However, despite the massive engraftment of EGFP$^+$ cells (Figure 4C) no pathological abnormalities (0 of 35; CI, 0.00-0.11; median = 28 days) were found, suggesting that the related osteogenic cell population was not directed to the heart. Since it is still unclear whether cytokine treatment induces mobilization of MSCs from the BM, we harvested and cultured peripheral blood from mice with (n = 6) and without (n = 4) cytokine application. Similar numbers of colonies were obtained from both groups of mice and the cells displayed typical morphology and markers of MSCs (Figure 5A,B). Their nature was further corroborated by successful in vitro differentiation (data not shown). Thus, standard cytokine treatment does not mobilize additional MSCs from the BM into peripheral blood, and this result correlates with the lack of encapsulated structures in the hearts of cytokine-treated mice.

As only direct injection of cultured BM-derived MSCs or unfractionated BM cells into the infarction induced calcifications/ossifications—whereas the MSCs present in peripheral blood appeared to not give rise to pathological abnormalities—we next tried to reconcile this apparent discrepancy by injecting cultured MSCs intravenously (10^4-10^6) into mice after cryoinjury. The mice died (n = 5, most likely of right heart failure) when injecting the cells into the jugular or femoralis vein but survived when slowly injecting the cells into the tail vein (n = 5). No EGFP$^+$ cells were found in the hearts of these 2 groups directly or up to 42 days after application, while most MSCs were trapped in the lung and in the spleen (Figure 5C,D). This suggests that MSCs do not migrate into the injured heart muscle following myocardial infarction and that

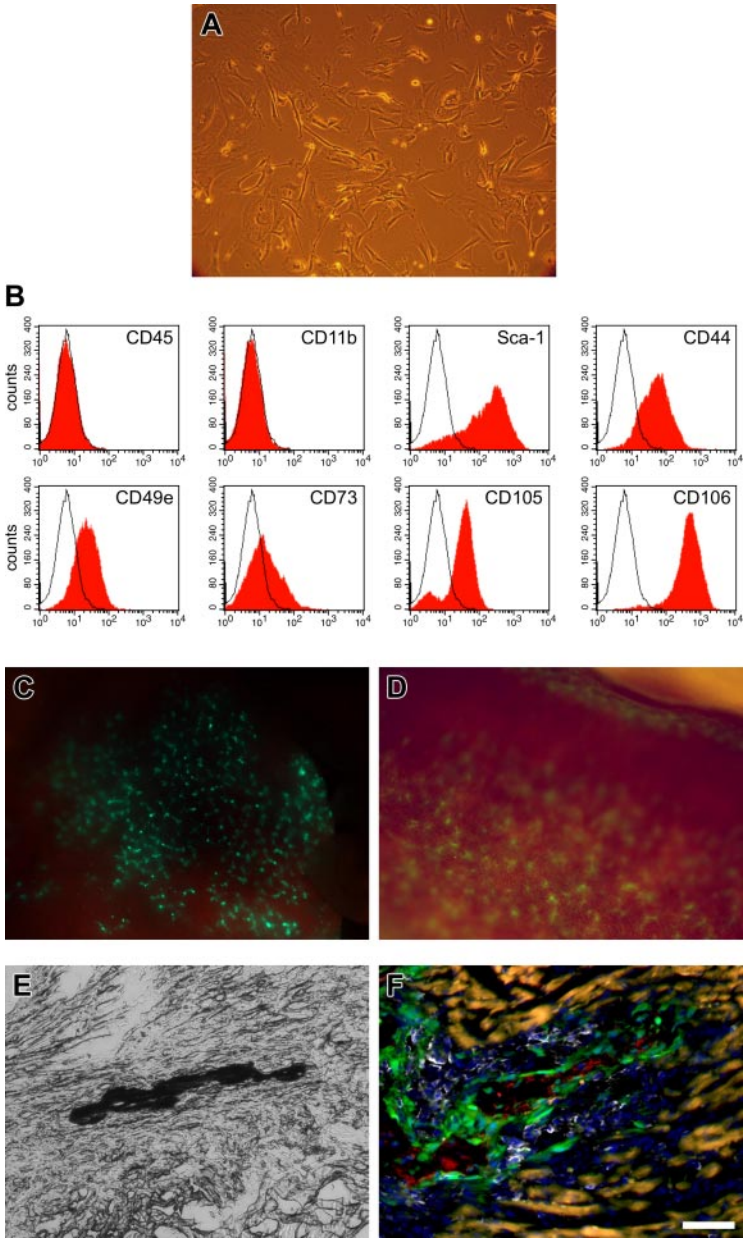

Figure 5. MSCs from peripheral blood; cell administration and tissue injury govern the fate of MSCs. (A) Adherent cell culture established from the peripheral blood of mobilized mice (passage 2); the cells displayed a fibroblast-like morphology as BM-derived MSCs. (B) Peripheral blood–derived MSCs (passage 6) expressed typical MSC markers as shown by flow cytometry analysis. (C) $EGFP^+$ cells (green) were found in the lung directly after systemic injection of 1×10^6 MSCs (passage 8) into the femoralis vein; the mouse died immediately, likely because of acute right heart failure due to obstruction of lung capillaries by MSCs. (D) $EGFP^+$ cells (green) were found in the spleen 5 days after slow injection of 1.5×10^6 MSCs (passage 8) into the tail vein. (E) Transplantation of 1.2×10^5 enriched MSCs (passage 6, 21 days) into the intact, uninfarcted heart resulted in calcifications (black, von Kossa staining) that were clearly restricted to the injection channel. (F) Tissue damage due to the injection needle was accompanied by inflammation documented by strong invasion of hematopoietic cells (CD45 staining, white). Engraftment of MSCs (green) was limited to the lesion (injection channel); osteocalcin staining (red) proved ossification. Autofluorescence of intact cardiomyocytes appeared yellowish. Bar represents180 μm (panel A), 700 μm (panel C), 350 μm (panel D), 100 μm (panel E), 60 μm (panel F). See "Materials and Methods; Image acquisition and preparation" for microscopy details.

direct administration to the infarcted myocardium is required to induce the observed adverse pathology. The importance of the injury was further investigated by injection of cultured MSCs (1.2×10^5, passage 6) into intact myocardium of mice (n = 7). Notably, in the majority (6 of 7, 85.7%; CI, 0.49-0.97; median = 27 days) of these hearts, we observed calcifications/ossifications, but they were exclusively restricted to the injection channel (Figure 5E) where damage to cardiomyocytes and inflammation was detected (Figure 5F). These data demonstrate that tissue injury and direct access of MSCs to the injury site are the two necessary requirements to induce the pathological abnormalities.

Discussion

Our data do not support the common assumption that the damaged tissue will direct and restrict the cellular fate of transplanted adult multipotent stem cells.[3,4] In contrast, we show that these cells can adapt with high frequency fates with potentially deleterious effects in the engrafted tissue. This finding is well established for embryonic stem cells that are pluripotent and known to develop teratomas upon transplantation into adult recipients,[20] while it is a rather unexpected and disturbing finding that the injection of whole BM cells into infarcted myocardium carries a considerable risk for bone formation.

The pathological abnormalities were seen only after direct injection of MSCs alone or unfractioned BM cells, but not vehicle, fibroblasts, or hematopoietic progenitors. This excluded tissue-derived heterotopic calcifications as underlying cause that were found after acute myocardial infarction in a few human patients[21,22] and more frequently in rats.[23] Direct involvement of the transplanted cells in the generation of calcifications/ossifications rather than an unspecific tissue response was clearly supported by the observation that transplanted cells generated osteocalcin at an early stage, whereas at later stages the massive bone formation led to decellularized central areas. Calcifications after injection of BM cells into the infarcted rat myocardium were reported earlier.[24] However, this study had little impact on ongoing clinical trials because of the lack of information in respect to origin and mechanisms underlying the observed calcifications due to the fact that they could

determine neither the cellular origin (recipient or donor-derived) nor the responsible cell type. We here addressed these important questions by using (1) genetically labeled cells, (2) whole BM and different fractions of BM-derived cell populations, (3) careful characterization of the cell biologic properties of the MSCs and their in vitro differentiation potential, (4) different types of myocardial infarction models, (5) a large number of different control conditions, and (6) direct injection of cells as well as BM mobilization. Our present study in mice and the earlier in rats[24] could not identify, as reported recently for rat hearts,[23] a high incidence of calcifications after myocardial infarction independent of cell transplantation.

The involvement of the MSC fraction of BM was clearly demonstrated by injection of purified hematopoietic progenitor cells and by the cytokine treatment where the lack of MSC mobilization was accompanied by lack of pathological abnormalities in infarcted hearts. Similarly, intravenous injections of cultured MSCs did not reach the myocardium because of trapping of the cells in the lung and spleen. Since in healthy individuals no calcifications are observed our experimental evidence postulates that a special microenvironment in combination with MSC enrichment is needed to give rise to these pathological abnormalities. This is clearly supported by the experiments where MSCs were injected into the intact myocardium, proving that tissue damage and inflammation apparently represent the required conditions for calcifications/ossifications. We also identified peripheral MSCs, which in regard to marker expression and in vitro differentiation potential showed similar properties as the BM-derived MSCs. Future work is required to determine their provenience, multipotency, and long-term fate in vivo. Thus, the most important and novel findings of our study are the demonstration that the calcifications/ossifications originate from the injected cells and that the responsible cell fractions of BM are the MSCs and that tissue injury and/or inflammation are required to induce the calcifications/ossifications.

Overall, our findings are not that surprising in light of the fact that MSCs are known to have the potential to differentiate into mesenchymal tissue types including bone.[8] Moreover, cultured MSCs have been reported to bear karyotype alterations and to develop osteosarcomas in the lung upon in vivo injection.[25] In accordance with this study on mice[25] and previous studies on human MSCs,[26] we also identified karyotype changes in our MSC lines with longer culture times (M.B., unpublished observations, April 2006), further suggesting that clinical trials using MSCs could be affected not only by the herewith reported lack of fate restriction but also by genetic instability. The karyotype alterations at higher passage numbers do not explain, however, the observed calcifications/ossifications as these were also detected after injection of unmanipulated, uncultured BM cells into the infarcted myocardium. The demonstration of the inability of injured myocardium to restrict the fate of MSCs makes it likely that unwanted differentiation fates of MSCs could occur also upon transplantation into other tissues and makes the findings of considerable relevance for the planned use of MSCs for cell replacement therapy in a wide variety of different diseases.[10]

Although in this study the pathological abnormalities were found consistently in infarcted hearts, it is surprising that similar findings were not reported by other studies. We could not identify major differences in the isolation and the cell biologic characteristics of the MSCs and their transplantation into infarcted hearts. However, epigenetic modulation of MSCs with 5-azacytidine[27-29] could influence the fate of the cells. After application of cell fractions that were enriched for different types of progenitors (eg, AC133,[30] c-kit,[2] side-population[31]), the lack of calcifications/ossifications can be explained in accordance with our data with the lack of MSCs in those transplanted cell populations. The most obvious difference was that in the other studies a rather short follow-up of the fate of injected cells was used. Therefore, the potential abnormalities would be most likely small in size and difficult to identify. In fact, we detected these abnormalities accidentally by analyzing semithin sections and only after becoming aware of the alterations we found these on a regular basis also in cryosections and paraffin-embedded material.

This study, as well as reported clinical[32] and experimental[33] incidents, reveals potential risks of the clinical trials using BM transplantation in patients with myocardial infarction[34-38] and in particular with regard to ongoing or planned trials with purified MSCs.[3,39] Although a direct comparison of mouse data with the human situation is not possible, in the first clinical study using MSCs for the treatment of heart infarction published so far, very high numbers of cells ($8\text{-}10 \times 10^9$) were injected into the afflicted coronary artery.[39] This high dosage could potentially increase the risk of early onset and extent of the calcifications/ossifications, as not only direct intramyocardial application but also intracoronary injection leads to enrichment of BM-derived stem cells in the infarcted myocardium.[40] The fact that in most of the other clinical trials unfractionated BM cells that contain a very low percentage of MSCs (approximately 1 in 100 000) are injected may reduce the number of engrafting MSCs into the lesion and delay but not prevent the potential formation of calcifications/ossifications as demonstrated by our dose-dependency and whole BM transplantation experiments. In any case, it is reasonable to advise long-term follow-up for patients enrolled in the ongoing clinical trials using unfractionated BM for possible calcifications/ossifications that may negatively affect the electrical and mechanical stability of the heart. On a positive note, our findings suggest that patients receiving cytokine treatment appear not at increased risk of calcifications/ossifications in the heart. Since we identified the MSC population being responsible for bone formation, also enrichment of MSC-free BM cells or the use of other progenitor populations in BM[2,31,41] may lower the risk of uncontrolled differentiation. Conversely, enrichment into cells of the hematopoietic lineage may reduce the therapeutic potential, as the MSCs appear to be the biologically most attractive subpopulation because of their multipotency. In fact, our study does not exclude that MSCs may prove a helpful cell source for the treatment of heart infarction and other disorders.[11] Also, pluripotent embryonic stem cells are known for their lack of fate restriction after transplantation that leads to tumor formation. Therefore novel in vitro differentiation protocols as well as lineage selection techniques are being developed.[15] Similar approaches ought to be pursued for the further clinical use of multipotent BM-derived cells.

Overall, our data further demonstrate that the mechanisms involved in wound healing and stem cell differentiation are complex and that the developmental fate of adult multipotent BM cells after transplantation in nonhematopoietic organs requires further experimental studies.

Acknowledgments

This study was supported by a grant from the Deutsche Forschungsgemeinschaft to B.K.F. and W.R. (FL-276/4–2/3), from the Juvenile Diabetes Research Foundation and Swedish Research Council to S.E.W.J., from the scientific exchange program North Rhine Westphalia-Sweden to B.K.F., and in part by the EC-FP6-project DiMI, LSHB-CT-2005–512146. The Lund Stem Cell Center is supported by a center of excellence grant from the Swedish Foundation for strategic research.

We thank A. Koester and M. Ghilav for technical help, Drs A. and A. Zimmer (University of Bonn, Germany) for support with karyotyping, L. Mürtz for cell culture work, and Dr I. P. Hall (University of Nottingham, United Kingdom) for helpful comments on an earlier version of the paper.

Authorship

Contribution: M.B. undertook generation, cultivation, and characterization of MSC clones, harvesting and fixation of mouse hearts, stainings, analysis, and paper preparation; T.B. and W.R. did mouse operations, harvesting and fixation of hearts, preparation of cryosections, stainings, and analysis; Y.X. contributed to mouse operations and harvesting and fixation of hearts; O.D. generated and analyzed paraffin sections; J.M.N. performed mouse operations and harvesting and fixation of hearts; K.T. provided functional measurements of hearts; J.W.U.F. did pathological analysis of hearts and stainings; H.B. accomplished characterization of MSCs with flow cytometry; S.E.W.J. and J.H. supervised stem cell work and were involved in design of experimental protocols, analysis of data, and writing the paper; A.W. supervised the microsurgery; W.B. performed ultrastructural analysis and histologic analysis of the semithin and paraffin sections, stainings, and preparation of figures. B.K.F. was initiator and supervisor of the project (PI), and was responsible for the experimental and analytical proceedings and writing the paper. M.B., T.B., and W.R. contributed equally to the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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