Supplementary Materials

Methods

MSCs isolation, culture, and labeling

Allogeneic, male MSCs were isolated from canine bone marrow aspirates as previously described [1]. Briefly, the aspirates were passed through a density gradient to eliminate undesired cell types. The remaining cells were cultured in low glucose DMEM (Gibco, Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin. Hematopoietic cells, fibroblasts, and other non-adherent cells were eliminated during media changes. The remaining purified MSCs population was further expanded (1:3 at each passage) for subsequent experiments.

Passage 2 MSCs were harvested and transduced with lentiviral enhanced green fluorescence protein (EGFP, GENECHEM, Shanghai, China) according to manufacturer's protocol. These cells were qualitatively assessed for EGFP expression within 3 days; 80-85% of cells expressed EGFP determined by two independent observers (XW and LZ). Cells were harvested upon reaching 90% confluence.

Labeled cells were subsequently cryopreserved and stored in a control-rate freezer (-80°C) until implantation. Before *in vivo* infusion, cells were thawed rapidly at 37°C, thoroughly washed, and resuspended in saline (10 mL, 1×10^8 MSCs/mL). At implantation, the cell suspension viability was confirmed to exceed 80% by trypan blue staining.

In vitro migration and differentiation assays

To assess the migration capacity of MSCs, a 24-well transwell dishes (Millipore, Billerica, MA) with 8-μm pore filters was utilized. A total of 1×10^4 MSCs was added to the upper chamber, and 600μL DMEM plus 5% FBS with or without additional growth factors was added to the bottom chamber. After incubation for 12 hours at 37°C, the cells on the upper face were removed by a cotton wool swab. The remaining cells were fixed by 4% paraformaldehyde for 20 minutes. Finally, the membrane was transferred onto glass slides, putting the lower face on the top, and cells were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Saint Louis, MO). The number of migrated cells was counted via fluorescent microscopy (Nikon Eclipse TE2000-S microscope, Nikon, Tokyo, Japan). Each assay was performed in triplicate. The migration ability of MSCs was determined in the presence of 1) 50 ng/mL bFGF, 2) 20 ng/mL vascular endothelial growth factor (VEGF), 3) 2 ng/mL insulin-like growth factor (IGF-1), or 4) medium alone. All growth factors were purchased from Pepro-Tech (Rcky Hill, NJ). All experiments were tested under normoxic and hypoxic conditions. The normoxic chamber was filled with a gas mixture of 5% CO2, 21% O2, and 74% N2, and the hypoxic chamber contained 5% CO2, 1% O2, and 94% N2. Both chambers were regulated at 95% saturated humidity.

To induce cardiomyocyte differentiation, 2×10^4 MSCs were cultured with basal medium in a 24-well plate containing collagen-coated glass coverslips for 24 hours, and incubated with or without 5 ng/mL bFGF for additional 72 hours. The cells were maintained in normal growth medium for 2 weeks. The differentiation capacity was verified by morphology

changes and immunostaining for cardiomyocyte specific troponin I (TnI). Briefly, the cells were fixed by 4% paraformaldehyde for 20 minutes and washed with PBS, followed by incubation with goat antibodies against TnI (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Nuclei were counterstained with DAPI. Cardiomyogenic differentiation was quantified by counting TnI^+ cells. Each assay was performed in triplicate.

MI model

Adult male mongrel dogs weighing 15-20 kg (Weitonglihua Experimental Animal Center, Beijing, China) were subjected to MI via ligation of left anterior descending (LAD) coronary artery and diagonal branches. All animals were anesthetized by intravenous 3% pentobarbital sodium (1 mg/kg) and maintained with isoflurane (1.5-2.0%). The animals were intubated and mechanically ventilated (Newport NMI Wave E200, Covidien, Plainfield, IN). A small left thoracotomy through the fifth intercostal space was performed to expose the heart using a sterile technique. The LAD was isolated distal to its first diagonal branch, and ligated with a 3.0 polypropylene suture, with continuous monitoring of blood pressure and ECG. Lidocaine (1 mg/kg) was injected intravenously when necessary to terminate arrhythmias. Additionally, ligation of the diagonal branch was performed to decrease collateral flow to the infarct area. After hemodynamic stability was achieved, the pericardium and chest were closed, and the animals were allowed to recover.

Retrograde coronary venous infusion

At one week after infarction, all surviving animals underwent retrograde infusion of 10 mL of one of the following: 1) combination bFGF (200 ng/mL) and MSCs (1×10^8 cells), 2) MSCs alone (1×10^8 cells), 3) bFGF alone (200ng/mL), or 4) placebo (phosphate-buffered saline). Arterial access was obtained via the right femoral artery, and the right internal jugular vein was cannulated. Animals were systemically anticoagulated with unfractionated heparin (100 IU/kg). The degree of LAD stenosis and collaterals was determined by coronary angiography. The coronary sinus was cannulated by a 6 Fr multipurpose guide catheter (Abbott Vascular, Redwood, CA). A coronary sinus venogram was performed initially to delineate the anatomy, and provide a roadmap for subsequent cannulations. A 0.014-inch exchange-length extra support guide wire was advanced via the great cardiac vein to the anterior interventricular vein (AIV). Subsequently, an over-the-wire (OTW) balloon catheter (2.5×9 mm or 2.0×9 mm) (Medtronic, Minneapolis, Minnesota) was advanced over the guide wire and positioned in the mid-AIV. From LAD coronary angiography, the position of OTW balloon was consistent with the occlusive site of the LAD. The balloon was inflated, occluding the AIV, and the selected perfusion medium was rapidly (7.6±1.8 sec) injected into the AIV (delivery pressure 105±16 mmHg). The balloon remained inflated for 10 minutes post injection to achieve maximum local delivery.

Echocardiography

Transthoracic echocardiography was performed at baseline, 1 week after MI (before infusion), and 4 weeks after infusion (just prior to euthanasia) via Aplio Artida cardiac ultrasound

system (TOSHIBA, Tokyo, Japan) with 5.0-MHz transducer. Parasternal long-axis views were obtained with both M-mode and 2D echo images. Left ventricular (LV) end-diastolic diameter (LVEDD) and end-systolic diameter (LVESD) were measured perpendicular to the long axis of the ventricle. The LV end-diastolic volume (LVEDV) and end-systolic volume (LVESV) were calculated from the Teichholz formula [2]. LV ejection fraction (LVEF) and fractional shortening (FS) were calculated as follows: LVEF= [(LVEDV-LVESV)]/LVEDV×100; FS=[(LVEDD-LVESD)]/LVEDD×100. Treatment outcome effect was calculated by final parameter minus pre-infusion parameter. Each variable was acquired from 3 consecutive cardiac cycles and averaged. An experienced cardiologist (QWS) interpreted all echocardiograms in a blinded fashion.

Morphology and histology

After final echocardiography study, each animal was euthanized with intravenous potassium chloride. Cardiectomy was performed, and specimens were fixed in 10% buffered formalin. Thereafter, the LV myocardium was transversely sliced into 5 rings of 1.0 to 1.5-cm thickness along the apical-basal axis. For morphologic analysis, infarct wall thickness was measured at the level of the papillary muscle, and calculated as a percentage of septal wall thickness.

Each ring sliced was separated into 8 portions, including the anterior, anterolateral, lateral, posterolateral, and posterior LV free wall; anterior, mid, and posterior interventricular septum. Representative heart samples from the infarct, border, and remote zones were obtained, fixed in 10% formalin, embedded in paraffin, and sectioned (5 μm). Hematoxylin and eosin, and Masson trichrome staining were performed as described [3]. Fibrotic area was

calculated by the proportion of collagen-stained areas to total tissue area. Five randomly selected areas from each section were acquired and averaged. Images were analyzed in a blinded manner via Image Pro Plus 5.0 (Nikon, Tokyo, Japan).

Blood vessel density was determined at the border zone of the myocardium by immunohistochemistry. In brief, paraffin-embedded sections were deparaffinized, rehydrated, and exposed to boiling citrate buffer (pH=6) for antigen retrieval, followed by overnight incubation with primary antibodies against factor VIII-related antigen (FVIII, Santa Cruz Biotechnology, Santa Cruz, CA) and α-smooth muscle actin (α-SMA, Sigma-Aldrich, Saint Louis, MO) at 4°C, and then incubated with secondary antibodies at 4°C for 1 hour and detected with 3,3'-diami-nobenzidine. Vascular structures positive for FVIII and α-SMA expression were counted in 5 fields per slide, 5 slides per animal, and expressed as number per mm^2.

Apoptosis was detected with an in situ cell death detection kit (Roche Applied Science, Germany) per manufacturer protocol. Cryosections from the LV myocardial infarct border region were subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Cell nuclei were counterstained with DAPI. $TUNEL^+$ cells were visualized as green nuclei, and counted in 5 fields per slide, 5 slides per animal, and expressed as a percentage of the total cells.

Immunofluorescence

The engraftment and differentiation potentials of MSCs were evaluated in the MSCs (n=3) and bFGF+MSCs (n=3) groups. Frozen sections (7 μm) were fixed in 4% paraformaldehyde

for 20 minutes and washed with PBS, followed by overnight incubation with antibodies against FVIII, α-SMA, TnI, and cardiac myosin heavy chain (MHC) (Abcam, Cambridge, MA) at 4°C, and then incubated with tetramethylrhodamine isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). After counterstaining with DAPI, sections were mounted and observed by fluorescent microscopy.

The survival of engrafted cells was examined in the infarct zone, and quantified by manually counting EGFP-positive ($EGFP^+$) cells. The differentiation of implanted MSCs was evaluated based upon colocalization of EGFP with the endothelial cell marker FVIII, smooth muscle cell marker α-SMA, and cardiomyocyte marker TnI. Images were taken from 5 fields per slide, 5 slides per block, and acquired with a Nikon Eclipse TE2000-S microscope (Nikon, Tokyo, Japan) and examined by two blinded investigators (XW and HTM).

References:

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