Hypoxia-elicited mesenchymal stem cell-derived exosomes facilitates cardiac repair through miR-125b-mediated prevention of cell death in myocardial infarction

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Detailed Methods

Cell culture

Bone marrow-derived MSCs were isolated from the tibias and femurs of mice and cultured in α-minimal essential medium (α-MEM) added with 10% fetal bovine serum (FBS) [1]. H9C2 rat cardiomyoblasts (ATCC) were cultured in DMEM containing 10% FBS. All media and reagents for cell culture were purchased from Gibco (Carlsbad, CA, USA), and for normal culture, all cells were incubated at 37℃, 5% CO2, 21% O2; for hypoxic culture, MSCs were cultured in exosome isolation medium under 1% oxygen tension for 72 hours, H9C2 cells were cultured in the 37℃, 5% CO2, 1% O2 for 2-24 hrs.

Transmission electron microscopy (TEM), Laser Scattering Microscopy (LSM) nanoparticle tracking analysis (NTA)

The exosomes were fixed with 1% glutaraldehyde, applied onto a carbon-coated copper grid, then stained with 1% phosphotungstic acid. A JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan) was employed to test the samples. LSM images were recorded using PARTICLEMEIRIX system. NTA was performed using a NanoSight NS300 system (Malvern Instruments, Malvern, UK). The exosomes' Brownian motion in PBS was recorded and tracked, generating size distribution data by applying the Stokes-Einstein equation [2].

miRNA Microarray

miRNA profiling was completed at GIMINIX of China using Affymetrix miRNA microarrays, and data analysis was performed by Qiming company (shanghai, China). Hypo-Exos and nor-Exos isolated from the 3 different passages of MSCs were processed and compared for 1100 known mmu-miRNAs. Total RNA from the Hypo-Exos or nor-Exos preparations were extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol (including a DNase step). The expression level signals were scaled in GCOS 1.2 to give a median array intensity of 100. This was done to enable different arrays to be compared.

Validation of Selected miRNAs by real-time Reverse Transcription-PCR (real-time RT-PCR)

The miRNA from each sample was quantified by SYBR Premix Ex Taq qRT-PCR assays (TaKaRa, Japan). Real-time PCR was conducted on an ABI 7500 real-time PCR system, and amplification efficiencies were checked by standard curves. The miRNA expression levels were normalized to U6 expression. [3]

Masson Trichorme Staining and TUNEL Staining

At day 28 post-MI, the infarcted size was measured by Masson trichrome staining. Briefly, frozen tissue sections of heart tissues from different group were fixed in 4% paraformaldehyde, rinsed with PBS, and stained with Masson's trichrome kit. Infarct area and total ventricle area were measured using Image J software and expressed as percentage of infarct size. Mice sacrificed at day 28 post surgery were subject to TUNEL staining (Roche, US) to examine cell apoptosis. [4]

Exosome administration

For the in vivo experiment, exosomes were suspended in 200 μL PBS. MI was induced in mice as described before and randomized to receive immediately locally injection in ischemic heart region of Nor-Exo, Hypo-Exo, negative-control-Hypo-Exo or miR-125b^{KD}-Hypo-Exo with 200 μg each. For targeting study, mouse with MI model were intravenously injected by 200 μg of Cy-5.5-labeled Scr-Exo or IMT-Exo post MI, Cy5.5 or Cy5.5-Exo treatment as the controls. To assess the therapeutic potential of IMT-Exos, 200 μg of Scr-Exo, IMT-Exo, NC-IMT-Exo or miR-125b^{KD}-IMT-Exo per time were injected by tail vein at different time after MI (0 h, 4 hrs, 24 hrs, 48 hrs, 7day).[3]

MicroRNA Treatment

MSCs are grown without antibiotics and transfected with miR-125b-5p inhibitors and negative controls (100nM; Ribobio, Guangzhou) using ribo FECT CP Transfection Kit (Ribobio, Guangzhou) for 48 hrs as per manufacturer's instructions. Exosomes were isolated using the protocol described before.

Analysis of apoptosis by annexin V-FITC staining and proliferation.

H9C2 cells were cultured overnight at a seeding density of 1×10^5 cells/ 6-well tissue culture plates and treated with miR-125b^{KD}-Exos or Negative-control-Exos as described before H9C2 subjected to hypoxia. Annexin V/PI assay was carried out according to the manufacturer's instructions. Briefly, the cells were harvested using 0.25% trypsin in PBS (Invitrogen, Carlsbad, CA), washed once with cold PBS and twice with 1X binding buffer. The final cell pellet was resuspended with 100 μl of freshly prepared PI (Sigma, St. Louis, MO) and annexin V-FITC solution (BD Biosciences, US) and incubated at room temperature in the dark for 15 min. Additionally 400 μl of binding buffer was added to the sample and analyzed within 30 min using flow cytometer (FACS Aria III, BD Biosciences, San Jose, CA). The percentage of late apoptotic cells was analyzed using BD FACS DIVA software. Cell proliferation was determined using the CCK-8 Kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol[5]. Briefly, the Exos treated cells were seeded (5×10^3 cells/well) on 96-well plates and cultured for 24 hrs. At the indicated time, 10 μl of the CCK-8 solution was added to each well and incubated for 2 h. The absorbance of the plate was then measured at 450 nm with a microplate reader (Molecular Devices, Menlo Park, CA). The experiment was repeated three times.

Immunofluorescence staining and confocal imaging

For immunofluorescence assay, Scr-Exo or IMT-Exo were labeled by the membrane intercalating dye PKH67. According to the previous report, 1.5 μL PKH67 was diluted in 100 μL diluent C, and IMT-Exo resuspended in 2 mL PBS. Subsequently, transfer the exosomes mixed with diluent C to the tube containing PKH67 in diluent C. After incubation for 3 min at room temperature, the reaction was quenched by adding 2 mL PBS containing 10% exosome-deficient FBS. Then, the exosomes were centrifuged at 164,000 g for 90 min to remove uncombined dye. After washing with PBS for 1-2 times, the exosomes were resuspended in pbs and stored at -80 ℃ for further usage. To investigate the cellular localization of the exosomes in ischemic heart, 200 μg PKH67-labeled Scr-Exo, or IMT-Exo was intravenously administrated when left ascending artery ligation and 4 hrs post MI. 24 hrs later, the mice were

perfused with 25 mL PBS and then with 25 mL 4% paraformaldehyde. The hearts were isolated and cryosectioned in 10-μm thickness. The sections were blocked by 0.3% Triton-100 for 30 min and by 3% BSA or 5% goat serum for 2 h, and then immunostained with anti-SMA (Sigma) or anti-cTnI (abcam) antibody over night at 4 ℃. After washing 3 times by PBST (PBS containing 0.1% Triton-100), the samples were incubated with Alexa 594-conjugated secondary antibodies (Invitrogen, Grand Island, NY, USA) for 1 h at room temperature (RT). After washing 3 times by PBST, staining by DAPI (5 μg/mL), the tissue slides were imaged by an FV-1200 confocal microscope (Olympus, Tokyo, Japan). Images were processed and analyzed by ImageJ software (NIH). The relative fluorescence intensities or positive area were calculated.

Western blot analysis

Cells were lysed for 30 min at 4 °C in a lysis buffer. Total cell protein concentration was determined using BCA protein assay kit. Total protein (50 μg) was resolved by SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis. The primary antibodies for bak1 (Cell signaling Technology) or p53 (Cell signaling Technology) and horseradish peroxidase-conjugated secondary antibody (Santa cruz) were used. The bands were visualized using enhanced chemiluminescence reagents and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst version 1.1).

RNA Quantification by real-time RT-PCR

Total RNA was isolated using a TRIzol-based (Invitrogen, USA) RNA isolation protocol. RNA concentration was quantitated by applying the Nano Drop ND-100 Spectrophotometer (Nano Drop Technologies, Wilmington, DE). For detection of bak1, *p53* and GAPDH, cDNAs were synthesized by using PrimeScriptTM RT reagent kit (TaKaRa, Japan) according to the manufacturer's instructions. Real-time PCRs were performed by using SYBR Premix Ex Taq qRT–PCR assays (TaKaRa,Japan) with *bak1* and *p53*. GAPDH specific primers (Sangon Biotech, Shanghai) under ABI 7500 real-time PCR system (Life Technologies, USA). GAPDH was used as internal standard to normalize the miRNAs and mRNA expression level using 2-ΔΔCt method. The experiments were repeated for three times.

In vivo imaging

Cy5.5-labeled exosomes were used for in vivo imaging. For the targeting study, 24 hrs after intravenous administration, the mice were anesthetized and sacrificed. The heart, lungs, liver, spleen, and kidneys were dissected and fixed in 4% paraformaldehyde. An FMT 4000 in vivo imaging system (PerkinElmer, Waltham, MA, USA) was employed to capture images. Relative fluorescence intensity was measured using Image J software.

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Figure S1

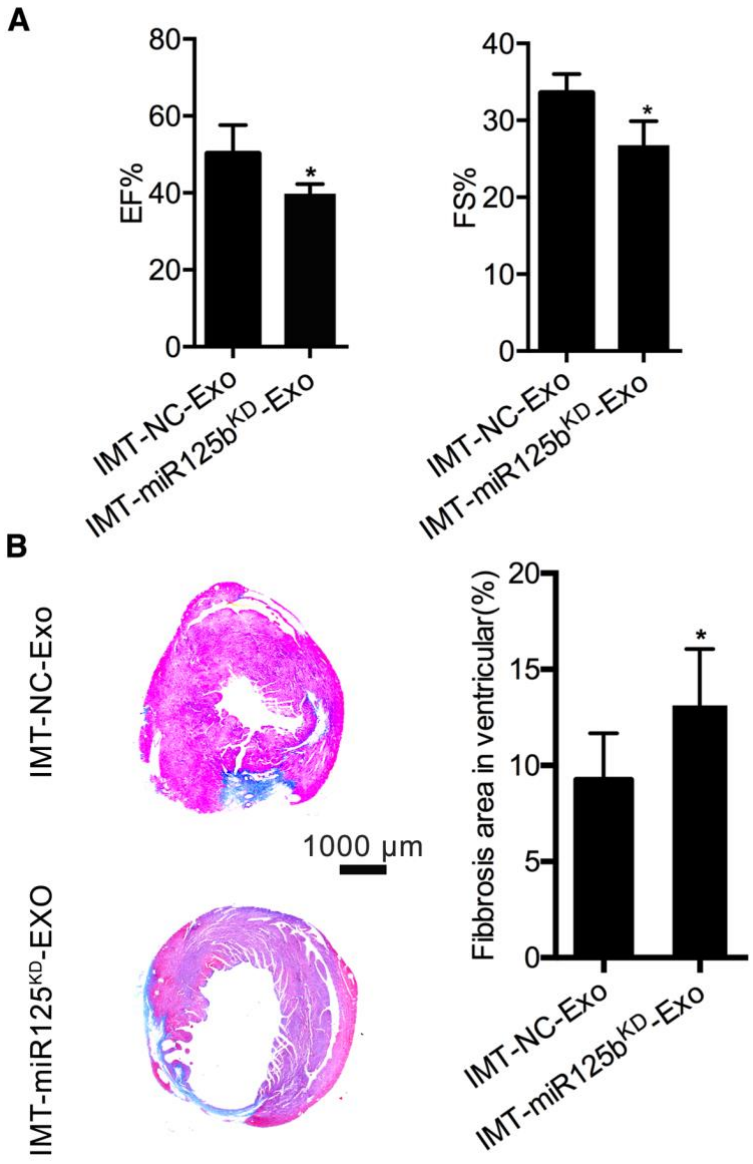

Figure S. Loss of miR125b in Hypo-Exo with IMT modification results in loss of its cardiac protection in vivo. (A) Quantification of EF% and FS% measured by echocardiography between IMT-NC-Exo and IMT-miR125b^{KD}-Exo groups in the mouse with MI model. **(B)** Representative Masson trichrome stained myocardial sections of hearts from mice in the IMT-NC-Exo and IMT-miR125b^{KD}-Exo groups at 28 d after MI. Scale bar, 1mm. *P<0.05 for IMT-miR125b^{KD}-Exo vs. IMT-NC-Exo.