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ORIGINAL ARTICLE

Transplantation of Cardiac Mesenchymal Stem Cell-Derived Exosomes Promotes Repair in Ischemic Myocardium

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Abstract

Our previous study demonstrated the beneficial effects of exosomes secreted by cardiac mesenchymal stem cells (C-MSC-Exo) in protecting acute ischemic myocardium from reperfusion injury. Here, we investigated the effect of exosomes from C-MSC on angiogenesis in ischemic myocardium. We intramyocardially injected C-MSC-Exo or PBS into the infarct border zone after induction of acute mouse myocardial infarction (MI). We observed that hearts treated with C-MSC-Exo exhibit improved cardiac function compared to control hearts treated with PBS at one month after MI. Capillary density and Ki67-postive cells were significantly higher following treatment with C-MSC-Exo as compared with PBS. Moreover, C-MSC-Exo treatment increased cardiomyocyte proliferation in infarcted hearts. In conclusion, intramyocardial delivery of C-MSC-Exo after myocardial infarction enhances cardiac angiogenesis, promotes cardiomyocyte proliferation, and preserves heart function. C-MSC-Exo constitute a novel form of cell-free therapy for cardiac repair.

Keywords Cardiac mesenchymal stem cells · Exosomes · Myocardial infarction · Angiogenesis

Introduction

Ischemic heart disease is a leading cause of morbidity and mortality worldwide [1], in part because the adult heart has only very limited capacity to regenerate or repair itself [2]. This results in a cascade of left ventricular remodeling, cardiomyopathy, and eventually chronic heart failure [3]. Stem cell therapy might have the potential to repair muscle with diseases [4–6], and two recent clinical trials (SCIPIO & CADUCEUS) reported that transplantation with cardiac-derived stem cells improves cardiac function [7, 8].

Although the mechanisms underlying stem cell-mediated heart repair are controversial, stem cells appear to function primarily via paracrine effects as opposed to cardiac differentiation and integration [9, 10]. Our previous studies demonstrated that mouse cardiac mesenchymal stem cells (C-MSC) are GATA4-positive mesenchymal stem cells [11, 12]. Numerous studies have demonstrated that mesenchymal stem cells can release angiogenetic cytokines, such as VEGF, bFGF, and SDF-1α, to promote angiogenesis in ischemic myocardium, one potential mechanism of paracrine heart repair [13, 14].

Recent studies have demonstrated that stem cells also secrete exosomes, which play a critical role in their paracrine effects [11, 12, 15–19]. Exosomes are cell-secreted nanovesicles which carry bioactive lipids [20, 21], proteins [22], mRNAs [23, 24], and non-coding RNA including long non-coding RNAs (LncRNAs) [25, 26] and microRNAs (miRNAs) [27–29], which play a key role in cell-cell communication. C-MSC-derived exosomes (C-MSC-Exo) can protect acute ischemic myocardium from reperfusion injury via inhibiting apoptosis [12]. Precise mechanisms whereby MSC-Exo promote cell viability and cardiac repair, however, are not clear yet.

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Recent studies show that bone marrow MSC-derived exosomes could protect infarcted heart from ischemic injury by promoting angiogenesis [30], and miR-21a-5p could be a cardioprotective paracrine factor [31]. Vrijsen et al. [32] reported that exosomes from fetal cardiomyocyte progenitor cells and bone marrow-derived MSCs stimulate angiogenesis in an in vivo Matrigel plug model. However, whether cardiac MSC-derived exosomes contribute to angiogenesis and recovery of cardiac function after myocardial infarction is unclear. The purpose of this study was to determine whether delivery of cardiac MSC-derived exosomes in acute ischemic myocardium provides therapeutic advantages in promoting angiogenesis and improving cardiac function. Our results show that one dose of C-MSC-Exo can activate cell proliferation in the border zone of infarcted heart, enhance angiogenesis, and preserve left ventricular function.

Methods

C-MSC Isolation and Culture

Mouse C-MSCs were isolated from the hearts of 2- to 3-month-old-C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) by a 2-step procedure as previously described with modification [11, 18, 33]. Briefly, in step 1, ventricular heart tissues were minced to a size of 1 mm^3 followed by digestion with 0.1% collagenase IV and 1 U/mL dispase in DMEM/F-12. The digested heart tissue was seeded into a 6-well plate coated with fibronectin/gelatin (0.5 mg fibronectin in 100 mL 0.1% gelatin). Cardiac explant cultures were maintained until small, round, phase-blank cells migrated from adherent explants and proliferated on the fibroblast layer. We then depleted hematopoietic cells using the mouse hematopoietic lineage depletion cocktail kit (Stemcell Technologies) by magnetic activated cell sorting (MACS) followed by enriching Sca-1+cells with Sca-1 magnetic beads (Miltenyi Biotec Inc., Auburn, CA) as instructed by the manufacturer's protocol. The sorted Sca-1 cells were cultured in complete medium (DMEM/F12 containing 10% fetal bovine serum (FBS), 200 mmol/L L-glutamine, 55 nmol/L β-mercaptoethanol, and 1% MEM non-essential amino acid).

Flow Cytometry

Flow cytometry analyses of cultured C-MSCs were performed with a BD LSRII flow cytometer and BD FACSDiva™ software. Briefly, C-MSCs were blocked with 5% rat serum and stained with a panel of conjugated antibodies, including anti-CD105 (BioLegend, San Diego, CA), anti-CD44 (eBioscience, San Diego, CA), and anti-CD140 (eBioscience, San Diego, CA).

Purification of C-MSC-Derived Exosomes/Microvesicles (C-MSC-Exo)

Exosomes/microvesicles secreted by C-MSCs were purified from conditioned media as previously described with modification [29, 34]. Briefly, culture medium containing 10% exosome-depleted FBS was added to C-MSC. After 48 h, media was collected, and centrifuged at 1000 rpm for 10 min followed by filtration through 0.22-μm filters to remove cell debris. After precipitation of C-MSC-Exo overnight at 4 °C with 5× polyethylene glycol 4000 (PEG 4000, 8.5% final concentration) and 10× NaCl (0.4 mol/L final concentration) followed by centrifugation at 3000 rpm for 30 min, we resuspended the pellets with PBS and stored at − 80 °C until use. We measured the exosome particle size using nanoparticle tracking analysis (NTA) with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) at 23 °C and corresponding software ZetaView 8.02.28 as we described previously [35]. The ZetaView system was calibrated using 100 nm polystyrene particles.

Immuno-Electron Microscopy Imaging

Standard immunoelectron staining with anti-CD63 antibody was performed as previously described [18]. The fixed exosomal preparations were placed on a carbon Formvar-coated 200-mesh nickel grid and incubated for 30 min. The grid was then quenched with 1 M ammonium chloride for 30 min and blocked with 0.4% BSA in PBS for 2 h. The grid was washed with PBS and then incubated with primary rabbit anti-CD63 (1:100 Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h. The grid was then washed with ddH_2O and PBS, and drops of 1.4 nm anti-rabbit nanogold (1:1000, Nanoprobes, Inc.) were applied in blocking buffer for 1 h. After enhancement with HQ Silver (gold enhancement reagent, Nanoprobes, Inc.), the samples were wicked dry and allowed to air dry prior to observation in a transmission electron microscope (JEOL JEM 1230, Peabody, MA). TEM sample preparation and imaging were performed at the Electron Microscopy and Histology Core Laboratory at Augusta University (www.augusta.edu/mcg/cba/emhisto/).

Western Blotting Assay

Exosomes were lysed in RIPA buffer with Triton X-100 (Alfa Aesar, Ward Hill, MA) before brief sonication (30 s, 4 times at 4 °C) using a Bioruptor® sonication device (Diagenode Inc. Denville, NJ). The proteins from C-MSC-Exo were resolved on 10% sodium dodecyl sulfate di-trigel and transferred to nitrocellulose plain film LI-COR Biosicences). For the Odyssey technique, membranes were blocked with Odyssey blocking buffer (LICOR Biosciences, Lincoln, NE), exposed to rabbit anti-Tsg101 (1:1000, Thermo Scientific), rabbit anti-

CD81 (1:1000, Thermo Scientific), and rabbit anti-CD63 (1:250, Santa Cruz Biotechnology, Inc.) overnight at 4 °C. Then membranes were incubated with IRDye 680 goat anti-rabbit IgG (LI-COR Biosciences) at 1:10,000 for 1 h at room temperature. Probed blots were scanned using Odyssey infrared imager.

Tube Formation Assay

Tube formation assay was used to assess the effect of C-MSC-Exo on angiogenesis as described previously with modification [36, 37]. Briefly, growth factor-reduced (GFR) Matrigel (BD Bioscience) was coated on 15-well μ-angiogenesis slides at 10 μl/well (ibidi, Germany). The coated slides were incubated for 1 h at 37 °C, and seeded with human umbilical vein endothelial cell (HUVEC) (10,000 cells/well, Lonza Walkersville Inc. Walkersville, MD) in 50 μl EGM 2-MV medium containing 10 μl PBS or C-MSC-Exo (1 μg/well), and incubated for 20 h at 37 °C to allow tube formation. The wells were then imaged for capillary-like structures using an EVOS microscope (Life Technologies). Quantification of the tubes was performed by taking 4× images of each chamber followed by image analysis using Image J.

Murine Myocardial Infarction Model and Intramyocardial PBS/C-MSC-Exo Delivery

To evaluate the effect of C-MSC-Exo on cardiac repair after induction of myocardial infarction, mice were subjected to acute MI as previously described [38, 39]. Briefly, C57BL/6 mice were anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. The mice were orally intubated with a 24-gauge tube and ventilated with room air using a Harvard Rodent Ventilator (Model 55-7058, Holliston, MA). The thorax was opened by a lateral thoracotomy and the heart was exposed by a pericardial incision. An 8-0 nylon suture (Ethicon, Somerville, NJ) was placed under the left anterior inferior artery (LAD) for permanent ligation. Immediately after coronary occlusion, mice were injected intramuscularly with 30 μl PBS or C-MSC-Exo (50 μg, 30 μl) in the infarct border zone. The chest was closed by layers, and the mice were allowed to recover. Animals were sacrificed 1 month after LAD ligation for tissue harvesting and histological assay. Animals were treated according to approved protocols and animal welfare regulations of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Histology

For cell staining, C-MSCs were plated on 8-well chamber slides (Millipore, Billerica, MA) and fixed with 4% paraformaldehyde. After blocking with 5% goat serum, cells were incubated with rabbit anti-GATA4 antibody (1:100; Aviva System Biology, San Diego, CA) at 4 °C overnight. Primary antibodies were resolved via secondary staining with goat anti-rabbit Alexa Fluor 555-conjugated (1:400, Life Technologies, Carlsbad, CA). Slides were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA).

To quantify proliferation of endothelial cells and cardiomyocytes in ischemic myocardium, we performed double immunostaining of Ki-67/CD31 and Ki-67/cTnI in mouse hearts. Briefly, mouse hearts were fixed with 10% formalin followed by 30% sucrose, frozen in OCT and processed for sectioning. We performed heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) followed by 5% goat serum blocking and streptavidin/biotin blocking (Vector Laboratories, Inc. Burlingame, CA). Heart sections were stained overnight at 4 °C with biotinylated anti-mouse/rat Ki67 (1:100, eBioscience), rabbit anti-CD31 (1:100, Cell Signaling Technology), and rabbit anti-cTnI (1:50, Santa Cruz Biotechnology). Slides were incubated with anti-rabbit secondary antibody conjugated to Alexa 488 and streptavidin Alexa Fluor 555 conjugate (1:400, Life Technologies, Carlsbad, CA). Slides were mounted using VECTASHIELD HardSet mount media with DAPI. Staining was analyzed by a Zeiss 780 laser scanning microscope (Carl Zeiss, Thornwood, NY). Calculate the number of capillaries and vessels manually, and express them as capillary density per 10,000 μm^2. Proliferative cardiomyocytes were classified as cTnI positive cells with Ki67 positive nuclei in each field.

Masson trichrome staining was performed using a commercial kit according to previous protocol [40]. The thickness of infarct wall was assayed by Image J.

Echocardiography

Echocardiographic studies were performed using a Vevo 2100 imaging system (VisualSonics Inc.) as previously described [29]. Briefly, echocardiography was obtained at baseline, 1 day and 1 month after MI. The mice were anesthetized with 2% isoflurane inhalation and the heart rate was maintained at 400 to 500 beats per minute. The M-mode image at the level of the mid-papillary muscle image was used to measure the left ventricular end-systolic volume (LVESV) and end-diastolic volume (LVEDV). Left ventricular ejection fraction (EF) was calculated as [LVEDV − LVESV] / LVEDV, and left ventricular fractional shortening (FS) was calculated as [LVIDd − LVIDs] / LVIDd. Digital images were analyzed offline by blind observers using the Vevo 2100 Workstation 1.7.1.

Statistical Analysis

Results are presented as the mean ± standard error of the mean (SEM). Comparisons between two groups were made by two-tailed Student's *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Characterization of C-MSC

As previously mentioned, we isolated C-MSC from the adult mouse heart using a two-step method [41]. Immunofluorescent staining showed that C-MSCs express the early cardiac transcription factor GATA4 (Fig. 1A). Flow cytometry showed that C-MSCs express high levels of MSC-specific cell surface markers CD105, CD44, and CD140 (Fig. 1B). Taken together, these data indicate that C-MSCs represent a subpopulation of cardiac-derived mesenchymal stem cells.

Characterization of C-MSC-Derived Exosomes

Morphological analysis of C-MSC-Exo using electron micrography demonstrated the typical appearance of microvesicles (Fig. 2A). Western blot analysis confirmed the presence of exosome markers, including TSG101, CD81, and CD63 (Fig. 2B). ZetaView®, a nanoparticle tracking analyzer that uses Brownian motion, was employed to measure the size of the microvesicles. The particles exhibited an average diameter of 120 nm, consistent with the characteristic size range of exosomes (Fig. 2C).

Effect of C-MSC-Exo on Tube Formation In Vitro

To investigate the effect of C-MSC-Exo on HUVEC-mediated angiogenesis, in vitro Matrigel tube formation assay was performed. As illustrated in Fig. 3A, B, treatment with C-MSC-Exo, in comparison with PBS, had a significant effect on capillary tube formation, suggesting a pro-angiogenetic effect of C-MSC-Exo in vitro.

Effect of C-MSC-Exo on Left Ventricular Function by Echocardiography

To assess whether delivery of C-MSC-Exo at the time of induction of ischemia has functional benefit, we performed echocardiographic measurement at baseline, 1 day and 1 month after MI. There was no significant difference in left ventricular EF and FS at baseline or 1 day after MI in mice treated with PBS and C-MSC-Exo (Fig. 4A–C). At 1 month, the PBS-treated mice showed progressive deterioration of LV function, with the mean EF decreasing about 8% (from 43% at 24 h to 35% at 4 weeks). However, mean EF decreased only about 4% (from 52% at 24 h to 48% at 4 weeks) in C-MSC-

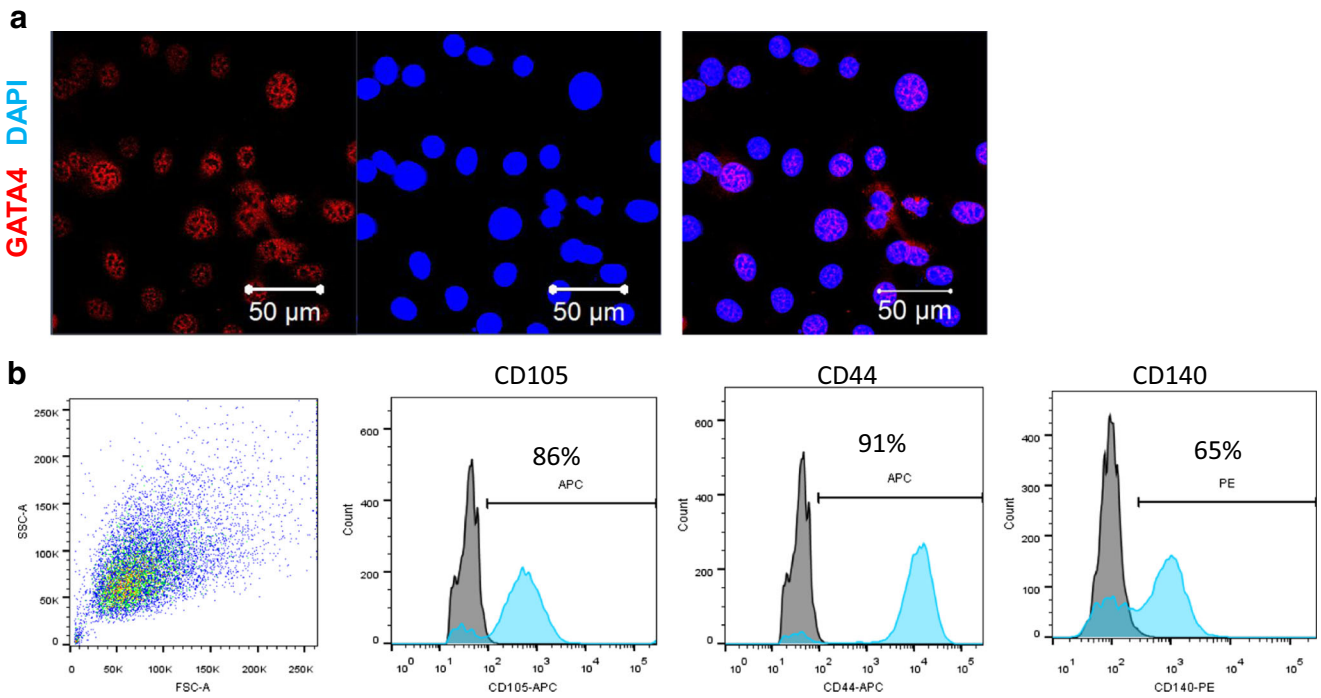

Fig. 1 Phenotypic characterization of C-MSC cells. **A** Immunofluorescent staining of C-MSC cells for expression of the cardiac transcription factors GATA4 (red); cell nuclei were counterstained with DAPI (blue). **B** Flow cytometric analyses of C-MSC cells for expression of the mesenchymal cell surface markers CD105, CD44, and CD140

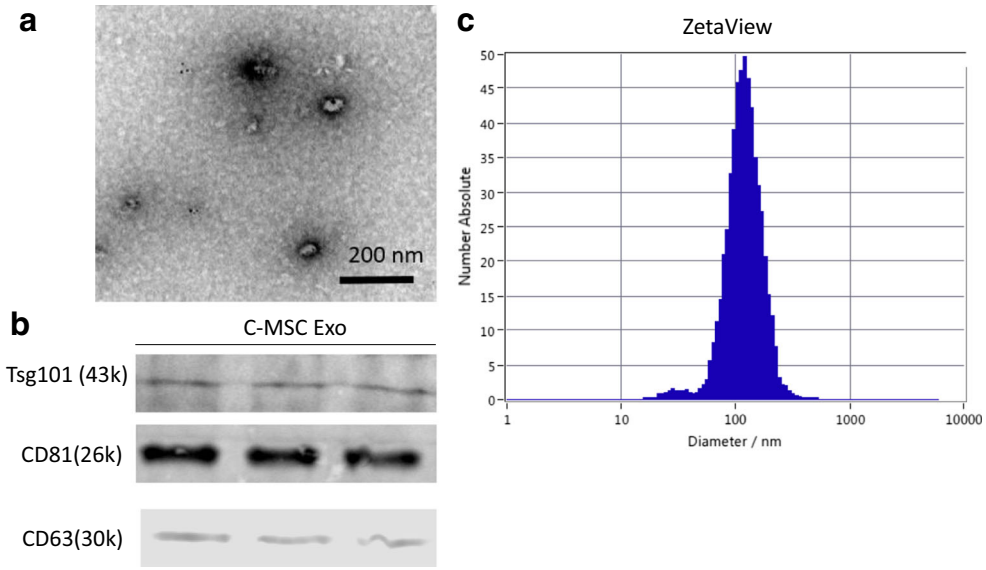

Fig. 2 Characterization of C-MSC derived exosomes. **A** Transmission electron micrograph image of C-MSC-derived exosomes after immunoelectron labeling with anti-CD63 antibody. Scale bar = 200 nm. **B** Western blot results demonstrate the expression of Tsg101, CD81, and CD63 in exosomes derived from C-MSC. **C** Particle size distribution in purified pellets consistent with size range of exosomes (average size 120 nm), measured by ZetaView® Particle Tracking Analyzer

Exo-treated mice, indicating that C-MSC-Exo treatment has functional benefit (Fig. 4B).

Effect of C-MSC-Exo on Angiogenesis in the Ischemic Myocardium

One month post-MI, Mason trichrome staining shows that the scar of the C-MSC-Exo-treated hearts is much thicker and contains more live cardiomyocytes in comparison with PBS-treated hearts, suggesting beneficial effect of C-MSC-Exo in preserving ischemic cardiomyocytes after MI (Fig. 5A, B). Next, we want to determine the mechanisms of the increased scar thickness, previous studies have shown that stem cell therapy can improve the healing of infarcted myocardium by promoting angiogenesis or activating cardiomyocyte proliferation via paracrine effects [42]. To determine whether C-MSC-Exo have similar effects, we first measured the capillary density in hearts of mice treated with PBS or C-MSC-Exo (Fig. 6A). Immunofluorescent staining for CD31 showed a significantly higher density of capillaries in the infarcted hearts treated with C-MSC-Exo than PBS (Fig. 6B). Furthermore, we observed more proliferative cells in C-MSC-Exo-treated hearts than PBS-treated control hearts by immunostaining for Ki67 (Fig. 6C), indicating that C-MSC-Exo promote angiogenesis and cell proliferation in infarcted hearts.

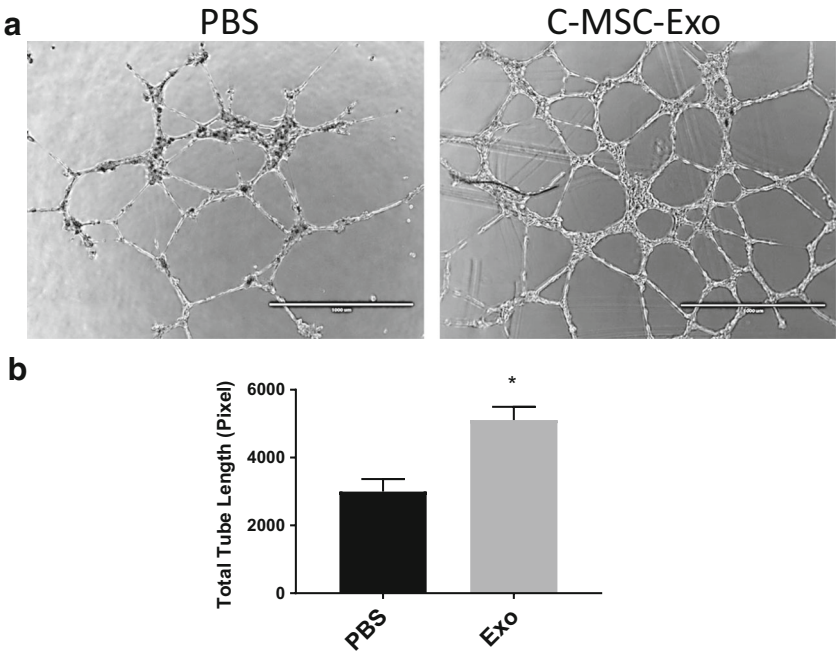

Fig. 3 Effects of C-MSC-Exo on capillary tube formation by HUVEC. **A** Culturing HUVEC were seeded on Matrigel-coated wells in medium containing PBS or C-MSC-Exo; **B** The total tube length per field of view was quantified after 20 h. Values are expressed as mean ± SEM, * $P < 0.05$, $n = 6$

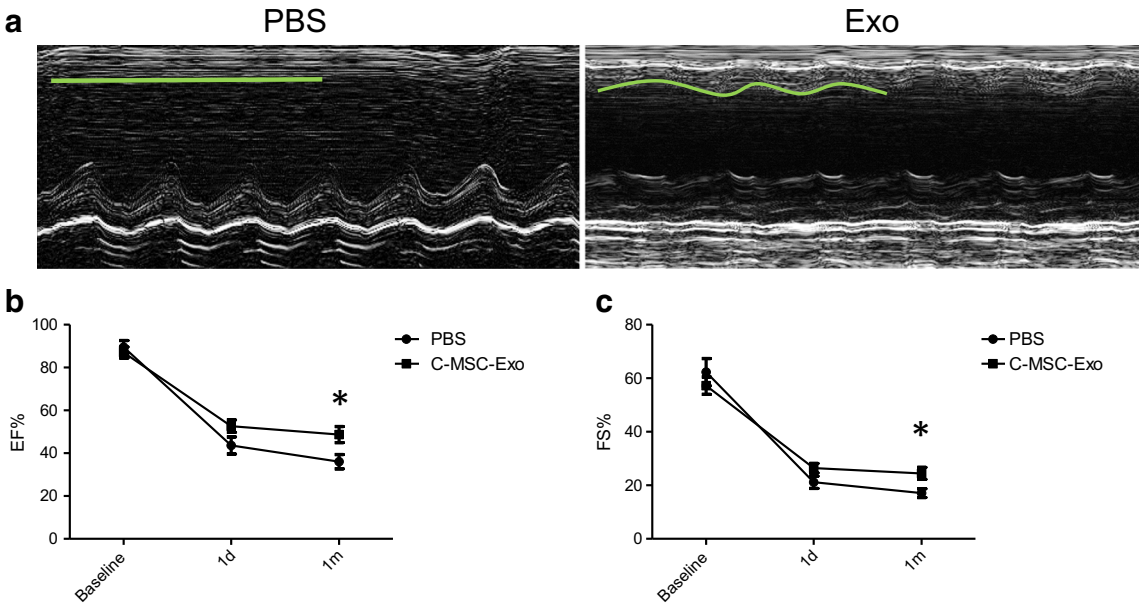

Fig. 4 Echocardiographic measurements of cardiac function at baseline, 1 day and 1 month after PBS or C-MSC-Exo treatment. **A** M-mode images of mice treated with PBS or C-MSC-Exo at 1 month post MI; **B–C** Average LVEF and FS at baseline, 1 day and 1 month after PBS and C-MSC-Exo treatment in infarcted hearts (*, $P < 0.05$, $n = 7$ for PBS group, $n = 8$ for Exo group)

Next, to study whether C-MSC-Exo activate the proliferation of resident cardiomyocytes, we performed dual immunofluorescent staining for Ki67 and cTnI. We detected a significantly higher number of Ki67+ proliferative cTnI+ cardiomyocytes in infarcted hearts treated with C-MSC-Exo than in PBS-treated hearts, suggesting that C-MSC-Exo treatment can activate proliferation of resident cardiomyocytes (Fig. 7A, B).

Discussion

In this study, we found that injection of C-MSC-Exo into ischemic myocardium promotes angiogenesis, stimulates the proliferation of cardiomyocytes, and preserves heart function post-MI. Our findings support the growing body of evidence suggesting that MSC transplantation promotes heart repair through diverse paracrine effects [43].

We have reported that cardiac-derived mesenchymal stem cells are unique, and distinct from bone marrow-derived mesenchymal stem cells in GATA4 expression (11, 12, 18); GATA4 is an early cardiac-specific transcription factor, which plays a critical role in the late embryonic heart development [44]. We have previously demonstrated that administration of C-MSC-Exo inhibits apoptosis in cardiomyocytes in a mouse model of acute myocardial ischemia/reperfusion [12]. Yu B et al. [45] reported that therapeutic potential of bone marrow

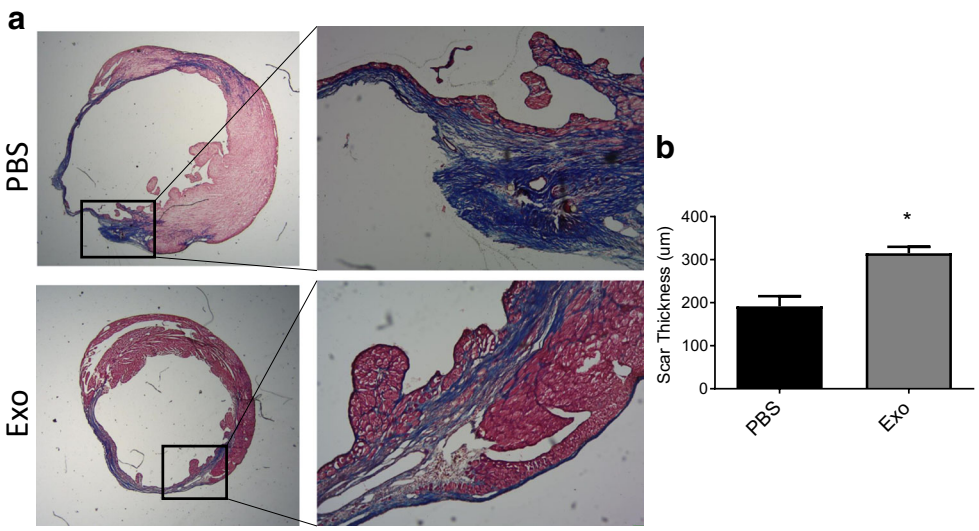

Fig. 5 C-MSC-Exo treatment preserves wall thickness in myocardium post-MI. **A** Representative Masson's trichrome images of LV sections show a thicker scar with increased numbers of viable myocardium (red cells) in the infarcted hearts treated with C-MSC-Exo in comparison with PBS. **B** Scar thickness measurement in both PBS and C-MSC-Exo treated MI mice revealed significantly thicker scar in 1 month post-MI C-MSC-Exo treated mice's hearts (*, $P < 0.05$, $n = 4$ for PBS and C-MSC-Exo treated mice)

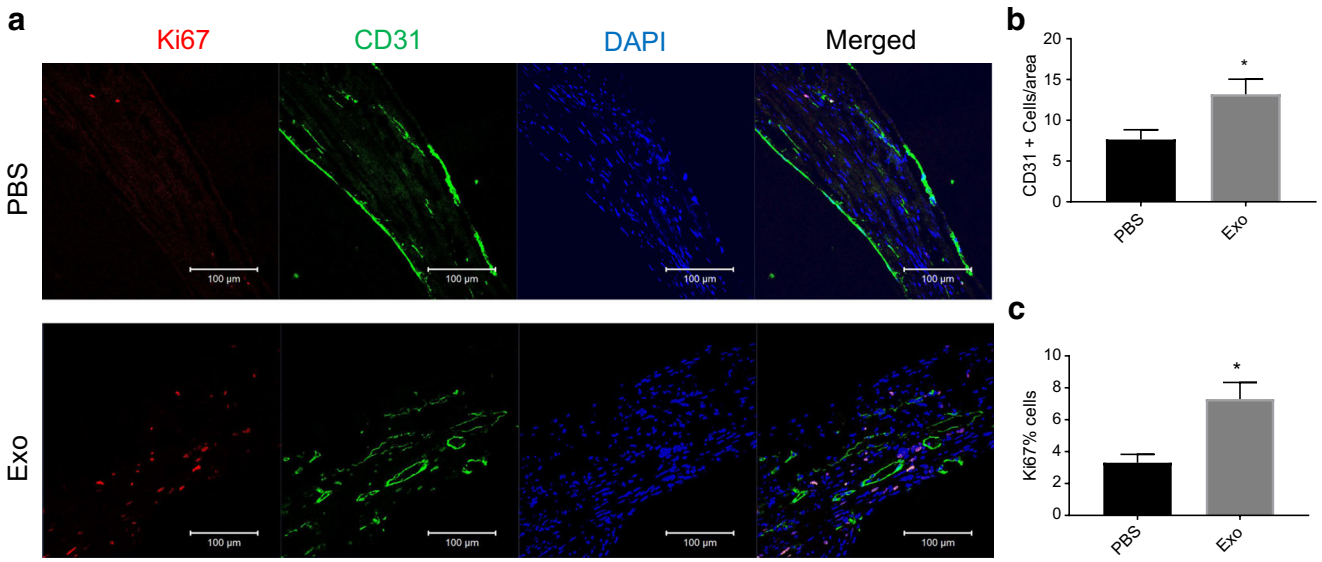

Fig. 6 Stimulation of cardiac angiogenesis in C-MSC-Exo-treated hearts. **A** Immunofluorescent staining of CD31 and Ki67 was performed to detect vessel density and cell proliferation in infarcted hearts 1 month post-MI. **B** The comparison of CD31+ cells per area (10,000 μm^2) between PBS and C-MSC-Exo treated infarcted hearts (*, $P < 0.05$, n = 6). **C** The comparison of percentage of Ki67-positive cells between PBS- and C-MSC-Exo-treated infarcted hearts (*, $P < 0.05$, n = 6)

MSC-Exo transplantation could be further improved by genetically modifying MSC with GATA4, which enhanced cardiomyocyte survival. These findings suggest a role for GATA4 expression in paracrine-mediated mechanisms whereby MSC and their Exo regulate apoptosis.

We and other groups have reported that transplanted MSC secreted angiogenic factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and stem cell homing factor to promote angiogenesis in ischemic myocardium [13, 14, 46]. In this study, we used a permanent myocardial ischemia model and observed that a single injection of C-MSC-Exo significantly stimulated angiogenesis in the infarct hearts. The mechanism underlying this observation is unclear. Endothelial cells can locally proliferate during angiogenesis; alternatively, stem cells or bone marrow-derived circulating endothelial cells can participate in angiogenesis. C-MSC-Exo might stimulate ischemic myocardium to release chemokines, such as SDF or VEGF, to recruit these cells to the infarcted region. Further experiments are necessary to elucidate the source of CD31+ cells in the infarcted hearts.

The mechanism of C-MSC-Exo-mediated cardiomyocyte proliferation is likewise unclear. Shao et al. reported that bone marrow-derived MSC-Exo stimulate the proliferation of H9C2 cells, prevent H_2O_2-induced apoptosis, and inhibit the transformation of TGF-beta-induced fibroblasts into myofibroblasts [47]. It is possible that C-MSC-Exo transport microRNAs into ischemic myocardium which are capable of

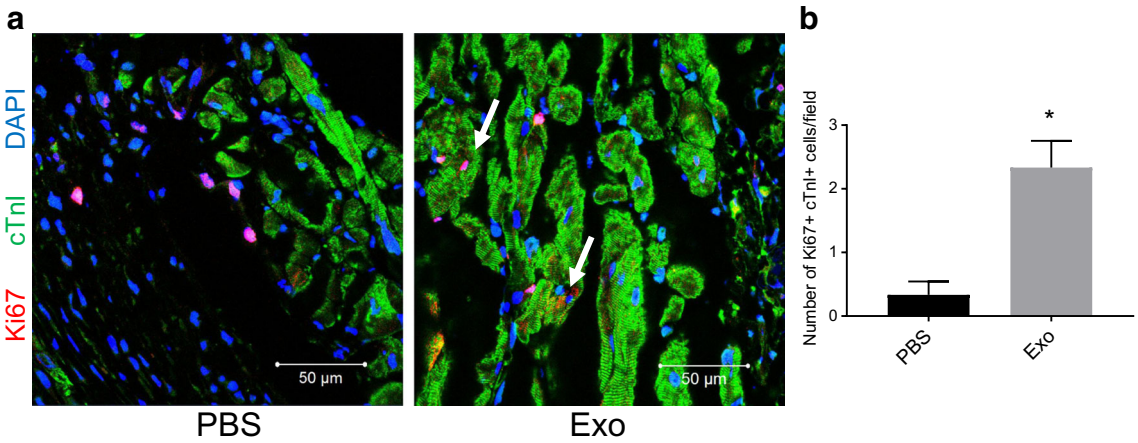

Fig. 7 C-MSC-Exo treatment activates proliferation of cardiomyocytes in infarcted hearts. **A** Immunofluorescent staining of Ki67 and cTnI was performed to detect proliferation of cardiomyocytes one month after treatment. **B** The comparison of Ki67+ cTnI+ cells per field between PBS- and C-MSC-Exo-treated infarcted hearts (*, $P < 0.05$, n = 6)

activating the cell cycle program in recipient cells [48]. Li P et al. [49] reported that plasma exosomes had the protective effects against cardiomyocyte apoptosis by the activation of ERK1/2 signaling pathway. Our recent study shows that exosomes from Suxiao Jiuxin Pill (SJP)-preconditioned C-MSC can increase HL-1 cell proliferation via downregulation of H3K27 demethylase UTX expression (11). Therefore, C-MSC-Exo might promote proliferation of cardiomyocytes via multiple signaling pathways. In the future, we need to optimize stem cell therapy by promoting cardioprotective exosome release via drug stimulation or electrical stimulation [18, 50].

In conclusion, our results suggest that a single administration of C-MSC-Exo can enhance cardiac angiogenesis, increase cardiomyocyte proliferation in ischemic myocardium, and thus preserve cardiac function in a mouse model of myocardial infarction. This finding supports the development of cardiac mesenchymal stem cell-derived exosomes as a cell-free therapy for ischemic cardiac disease.

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Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

Ethical Approval All applicable institutional guidelines for the care and use of animals were followed.

This article does not contain any studies with human participants performed by any of the authors.

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