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Exosomes derived from circBCRC-3-knockdown mesenchymal stem cells promoted macrophage polarization

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Abstract: Macrophages play an essential role in the myocardial ischemia-reperfusion injury (MIRI), and the macrophage shifting from M1 to M2 phenotypes might be a potential strategy for the treatment of MIRI. It has been reported that miR-182 plays an important role in MSC-Exo-associated macrophage polarization. As circBCRC-3 is a newly discovered circle RNA that worked as a sponge of miR-182, this research aimed to find if circBCRC-3 plays a role in MSC-Exo-associated macrophage polarization. Firstly, circBCRC-3 was identified by divergent primers in mesenchymal stem cells (MSCs). Secondly, the exosome of MSCs was isolated and identified by transmission electron microscopy (TEM), nanoparticle-tracking analysis, and western blotting analysis. The expression level of circBCRC-3 in MSCexos was detected by RT-PCR. Finally, the polarization of the RAW264.7 cell phenotype was analyzed by flow cytometry. Moreover, we first identified circBCRC-3 in MSCs. The results further confirmed that MSCexo could effectively shift the macrophage polarization state from M1 towards the M2 phenotype, which indicated its role in MIRI cure.

Introduction

Authors Acute myocardial infarction (MI) has been one of the leading causes of death in the world. The reperfusion therapy, which is a common process to MI patients, could cause myocardial ischemia-reperfusion injury (MIRI) that triggers an inflammatory cascade reaction in the myocardial cells (Hausenloy and Yellon, 2013). The macrophage-associated immune response plays an important role in MIRI. After reperfusion, macrophages in M1 status create a pro-inflammatory environment and clear away dead cells. Later, macrophages in M2 status through anti-inflammatory cytokines, secrete growth factors, processing scar formation. Thus, the two macrophage phenotypes and the regulations of changing two statuses are important for infarct healing (Ong *et al.*, 2018). By promoting earlier and more M2 macrophage infiltration, shifting the balance between M1 and M2 macrophages might be a potential way of treating MIRI.

Exosomes are membrane nanovesicles that exist in almost all biological fluids (Elahi *et al.*, 2020; Kourembanas, 2015; Raposo and Stoorvogel, 2013). They were reported as the important mediator of paracrine mechanisms and potential clinic applications in lung injury, cardiovascular disease, regenerative medicine, and therapy of inflammatory diseases (Elahi *et al.*, 2020; Liu *et al.*, 2020; Ha *et al.*, 2020; Harrell *et al.*, 2019; Wang *et al.*, 2020; Chen *et al.*, 2020). Recent studies have shown that exosomes were associated with many pathological and physiological conditions (Chen *et al.*, 2020; Elahi *et al.*, 2020; Ha *et al.*, 2020; Harrell *et al.*, 2019; Liu *et al.*, 2020; Wang *et al.*, 2020). Emerging evidence suggests that exosome derived from a mesenchymal stem cell (MSC) (MSCexo) could exert beneficial effects on some diseases, including MI (Liu *et al.*, 2017; Ma *et al.*, 2018; Zhang *et al.*, 2016; Zhao *et al.*, 2015), hepatic fibrosis (Li *et al.*, 2013; Jiang *et al.*, 2018; Qu *et al.*, 2017), and cancers (Phinney and Pittenger, 2017; Ono *et al.*, 2014; Kim *et al.*, 2018; Lee *et al.*, 2013; Qi *et al.*, 2017; Reza *et al.*, 2016).

CircRNA is a new member of non-coding RNAs, produced by a back-splicing event from pre-mRNA. CircRNA could tolerate the digestion of exonuclease for lacking 5' cap and 3' poly (A), which suggests that it is much more stable than linear RNA. Moreover, circRNA

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mainly locates in the cytoplasm and exerts its function by acting as a sponge of miRNAs (Hansen *et al.*, 2013; Jeck *et al.*, 2013). It only has been revealed that circDLPAG4 (Chen *et al.*, 2020; Wang *et al.*, 2019), circNCX1 (Li *et al.*, 2018), and circACR (Zhou *et al.*, 2019) could play roles in MIRI. Recently, it was reported that miR-182 in MSCexo played a role in the macrophage polarization of MIRI (Zhao *et al.*, 2019), and circBCRC-3 could bind to miR-182 (Xie *et al.*, 2018). Therefore, we speculate that circBCRC-3 may play a regulatory role in the macrophage polarization of MIRI. In this study, we showed that an exosome derived from a circBCRC-3 knockdown mesenchymal stem cell could promote macrophage polarization indicating its potential in MIRI cure.

Materials and Methods

Isolation of exosome of mesenchymal stem cell (MSCexo)
Two 6–8 weeks-old c57bl/6 mice were sacrificed. The femurs and tibias of the mice were obtained, then they were soaked and washed in cold PBS. The epiphysis of the femur and tibia was cut off, exposing the medullary cavity. PBS was used to blow and suspend marrow cells. Mesenchymal stem cells in marrow tissue were collected through centrifugation (1000 rpm, 10 min). MSCs were resuspended with 5 mL PBS and washed twice. Then, MSCs were resuspended in DMEM and maintained cell culture as described. The ExoQuick™ Plasma Prep and Exosome Precipitation Kit (SBI System Biosciences, USA) were used to isolate MSCexo from the MSC cell supernatants according to the manufacturer's instructions.

Cell culture
RAW264.7 cells (from ATCC) were cultured in DMEM medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) at a cell culture incubator (Thermo Scientific HeraCell 240i) at 37°C. For M1 macrophage induction, 500 ng/mL LPS was used.

Reverse Transcription polymerase chain reaction (PCR) assay
Total RNA was extracted from cells using TRIzol Reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. Using a Primescipt RT reagent kit with gDNA Eraser, the cDNA was then synthesized with reverse transcriptase (RTase) following the manufacturer-provided protocols (with random primers). Polymerase chain reactions were run using PrimeSTAR® Max DNA Polymerase (TaKaRa), following manufacturer's instructions. Real-time-polymerase chain reactions (RT-PCR) were run using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa), following manufacturer's instructions.

CircRNA BCRC-3 siRNA and transfection
A siRNA against the reverse splicing site of Circular RNA BCRC-3 was designed with the online prediction tool (Sidirect2), with the target sequence CTGTTTCCATCG AGTCACTGAATAGAATGAACCC. MSC cell transfection was performed according to the manufacturer's instruction. 5 μL Lipofactamine 2000 (Invitrogen, USA) was added to 50 μL optiMEM (Invitrogen, USA), and 2 μg siRNA was added into 50 μL optiMEM; two solutions were mixed and stayed for 15 min. The siRNA mixture was then added into the serum-free cell culture; after incubating for 6 h, the cell culture was changed to the complete medium. After 48 h, the cell culture was collected to isolate exosomes as described.

TABLE 1

Primers used for PCR

Name of Primer	Primer's Sequence
divergent–H-BCRC3-F	GTCAGGAGGGCAGCAGTAGA
divergent–H-BCRC3-R	AACTCAATAGCCATTTCACCAC
convergent–H-BCRC3-F	CTTTAGTAATGTATGGGAGGAT
convergent–H-BCRC3-R	GTTTGCTATAACGGTTGC
divergent–H-GAPDH-F	GAAGGTGAAGGTCGAGTC
divergent–H-GAPDH-R	GAAGATGGTGATGGGATTTC
convergent–H-GAPDH-F	CAATGACCCCTTCATTGACC
convergent–H-GAPDH-R	TTGATTTTGGAGGGATCTCG

Western blotting
Western blotting was performed using the ECL Western Blotting Substrate Kit (Abnova) and antibodies, Calnexin (Abcam, ab22595), CD63 (Abcam, ab216230), TSG101 (Abcam, ab30871).

Flow cytometry of detection of the polarization of macrophages
Cells were resuspended and adjusted to a concentration of 1×10^6 cells/mL in staining buffer. After 48 h treatment, cells were washed three times with PBS and collected. Then cells were stained for the antibody iNOS-FITC (FabGennix, P35228) CD206-PE (Biolegend, 141708) and PI (50 μg/mL) and kept in the dark. All samples were then run on a BD Accuri™ C6 (BD Bioscience) with a four-color (FITC, PE, PerCP Cy5.5, and APC) fluorescence flow cytometry analysis.

TEM and size analyze for MSCexo identification
The MSCexo were dropped on the gilder grids and dyed with uranyl acetate. Then the morphology was observed and photographed under a JEM-2100F transmission electron microscope was used to identify the exosomes isolated from MSC. The size of exosomes was measured by Malvan Mastersizer™.

Statistical analysis
All data analyses were completed using R Statistical Software (v 2.15.0, http://www.r-project.org/). Analysis of variance (ANOVA) was used to determine the differences in circBCRC-3 expression levels between groups. The *p*-values which are smaller than 0.05 were regarded as statistically significant.

Results

Identification of circBCRC-3 in MSCs
CircBCRC-3 (circBase ID: hsa_circ_0001110) is located at proteasome 26S subunit, non-ATPase 1 (PSMD1) gene locus, and its post-splicing sequence length is 1002 bp (Fig. 1A). Agarose gel electrophoresis was used to determine the specificity of the PCR product of circBCRC-3. Divergent

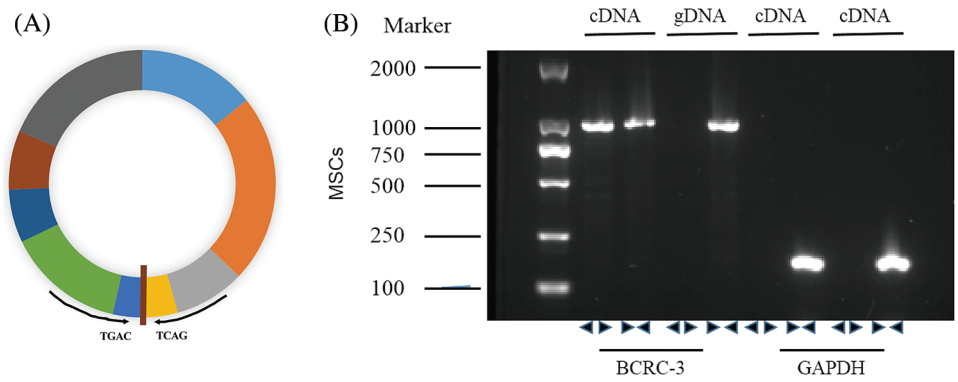

FIGURE 1. Identification of circBCRC-3 in MSCs.
A. The genomic structure indicates that circular RNA BCRC-3 consists of nine exons (1,002 bp) from the PSMD1 gene. B. Agarose gel electrophoresis analysis of PCR product with divergent and convergent primers of circBCRC-3 in cDNA and gDNA. GAPDH was used as the negative control.

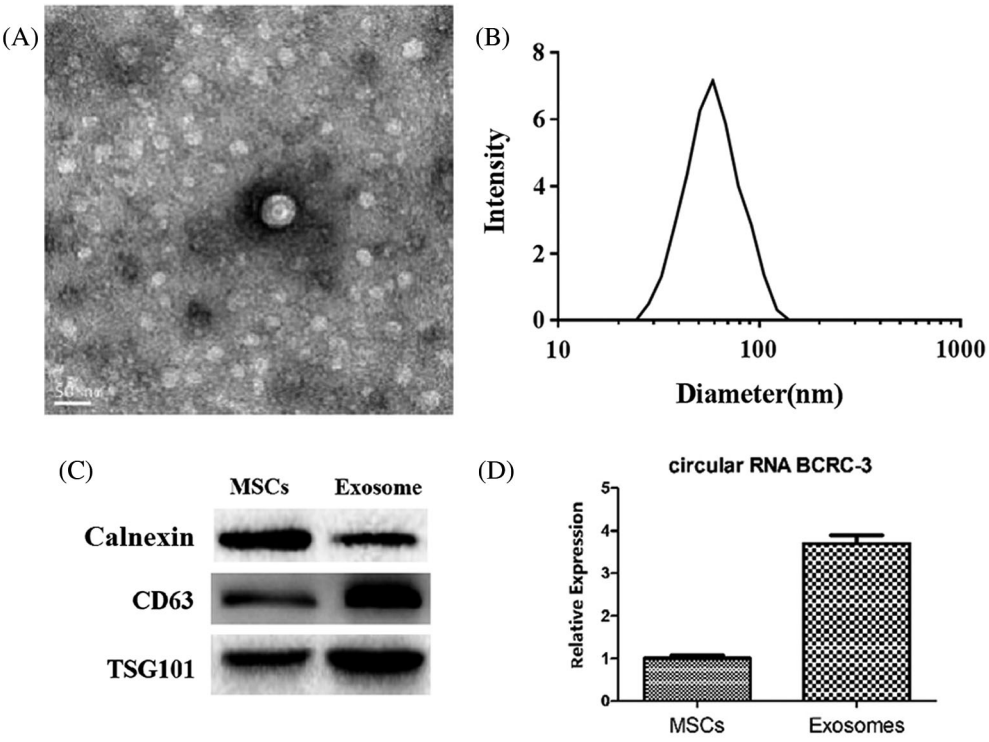

FIGURE 2. A. The morphology of exosomes under a transmission electron microscope (scale bar, 100 nm).
B. With Malvern Mastersizer, the size of exosomes was measured. The x-axis is the diameter distribution of exosomes (nm); the y-axis is the intensity of exosomes. C. The protein expression of exosome and MSC cells. Calnexin: ER marker, CD63, and TSG101: exosome markers. D. The circBCRC-3 expression in MSCs and MSC-derived exosomes detected by RT-PCR.

primers (Tab. 1) detected circular RNA BCRC-3 in cDNA but not genomic DNA (gDNA) (Fig. 1B), which further characterize its circular form.

Characterization of exosome of MSC

Multiple approaches were employed to characterize the morphology features and molecular markers of the isolated extracellular vesicles of MSC in order to identify exosomes. The MSCexo were studied under transmission electron microscopy (TEM). The morphological features of exosomes could be clearly observed: a round or elliptical shape with a diameter range of 30–100 nm (Fig. 2A). Then, the size distribution of extracellular vesicles was assessed with the nanoparticle-tracking analysis, which showed that the mean size of extracellular vesicles was 132.5 ± 37.4 nm; and most of the extracellular vesicles were distributed within the range of the exosome diameter (30–150 nm) (Fig. 2B). Moreover, the expressions level of CD63 and TSG101 (molecular markers of exosomes) were determined using western blotting analysis. High levels of CD63 and TSG101 were detected in the isolated exosomes, whereas little calnexin (a molecular marker of the endoplasmic reticulum) could be found (Fig. 2C). Meanwhile, the expressions of circBCRC-3 in MSCexo and MSCs were compared. The RT-PCR results indicated that the expression of circBCRC-3 was much higher in purified exosomes than in donor MSCs (Fig. 2D). The difference was significant ($p < 0.05$*).

CircBCRC-3 involvement in MSCexo mediated macrophage polarization in vitro

To study the effects of circBCRC-3 on macrophage polarization, flow cytometry analysis was performed to detect the levels of M1 ($iNOS^+CD206^-$) and M2 ($iNOS^-CD206^+$) markers. After the lipopolysaccharide (LPS) treatment, most raw264.7 cells transformed into M1 macrophage ($iNOS^+CD206^-$). With siRNA treatment, the percentage of M2 macrophage ($iNOS^-CD206^+$, the second quadrant) was elevated (Fig. 3A). The results of Fig. 3A demonstrated that the polarization of macrophages from M1 to M2 under the inflammatory environment was facilitated by treating with circBCRC-3 siRNA, and it suggested that circBCRC-3 might be the key regulatory factor determining the macrophage polarization.

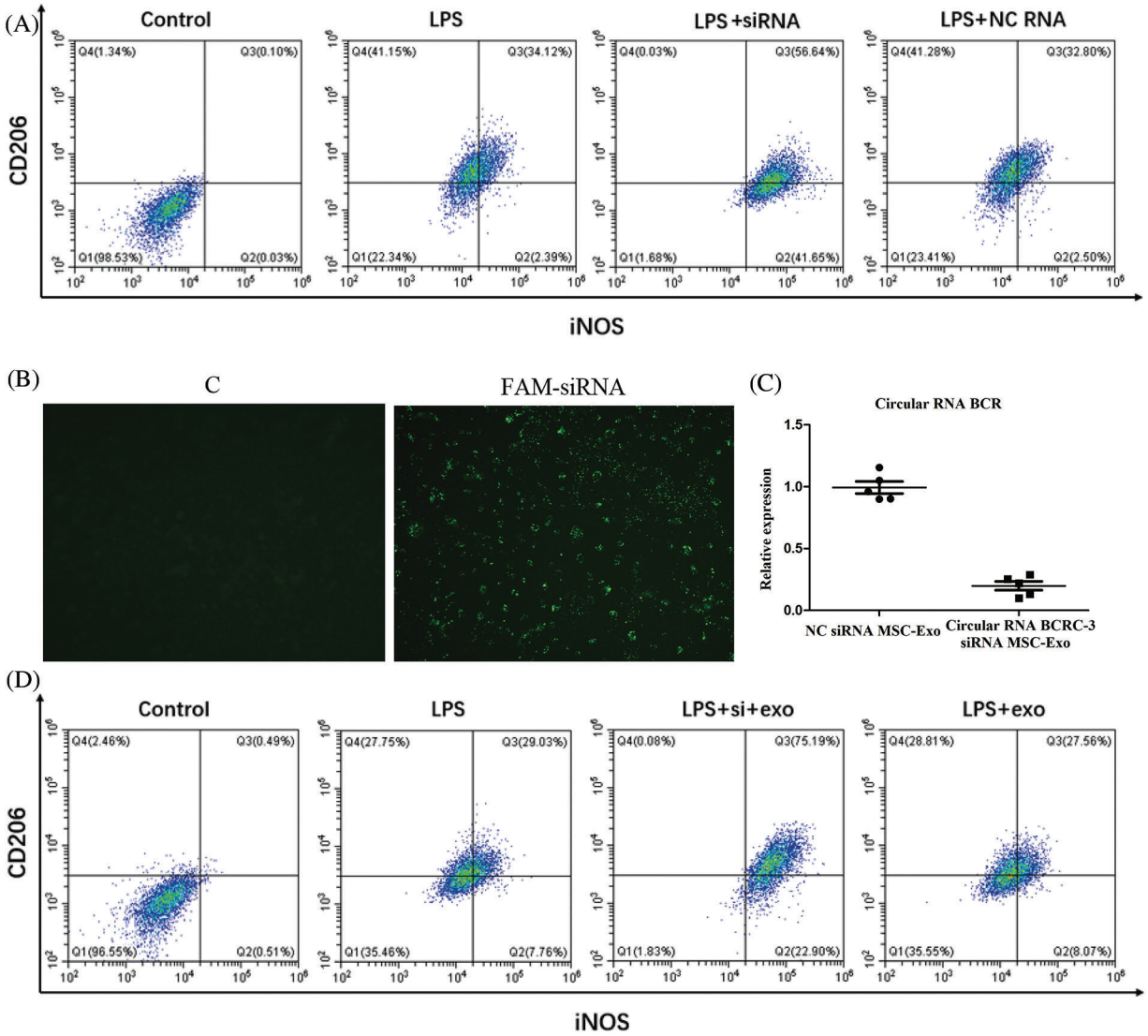

FIGURE 3. CircBCRC-3 is involved in MSC-Exo mediated macrophage polarization *in vitro*.
A. Representative flow cytometry plots showing the percentages of M1 ($iNOS^+CD206^-$) and M2 ($iNOS^-CD206^+$) phenotype in LPS-stimulated RAW264.7 cells after transfection with circBCRC-3 siRNA or NC siRNA for 48 h. B. Representative images of the uptake of circBCRC-3 transfected MSCs. C. Real-time PCR analysis of circBCRC-3 levels in exosomes derived from NC siRNA MSC-Exo and circBCRC-3 siRNA MSC-Exo (N = 5). D. Representative flow cytometry plots showing the percentages of M1 ($iNOS^+CD206^-$) and M2 ($iNOS^-CD206^+$) phenotype in LPS-stimulated RAW264.7 cells treated with circular circBCRC-3 siRNA MSC-Exo or NC siRNA MSC-Exo for 48 h.

To confirm the role of circBCRC-3 in MSCexo, MSCs were transfected with circBCRC-3 siRNA, and the exosomes were subsequently isolated from the culture supernatants. As circBCRC-3 siRNA was fluorescein amidites (FAM)-labeled, it was seen under a fluorescence microscope (Fig. 3B). RT–PCR analysis revealed that the expression level of circBCRC-3 was significantly decreased in circBCRC-3 siRNA transfected MSCexo compared to negative control (NC) siRNA transfected MSCexo (Fig. 3C). LPS-stimulated macrophages were then treated with NC siRNA MSCexo or circBCRC-3 siRNA MSCexo for 48 h, and then the cells were collected for flow cytometry analysis. Compared to the LPS treatment group, myocardial macrophages treated with the circBCRC-3 siRNA transfected exosomes showed more percentage of M2 macrophage ($iNOS^+CD206^-$, 22.9% to 7.76%) (Fig. 3D). The result showed that the polarization of macrophages from M1 to M2 was significantly elevated by circBCRC-3 siRNA MSCexo (Fig. 3D), suggesting that MSCexo from circBCRC-3 knocked down MSC could be a key factor that affected macrophage polarization.

Discussion

Macrophages are central inflammatory mediators of the heart tissue, involving in both the initiation and resolution of the inflammatory process. Multiple reports have highlighted the significance of macrophages in MIRI models (de Couto *et al.*, 2017; de Couto *et al.*, 2015). Increasing evidence suggested that MSC could trigger the macrophage to switch to the anti-inflammatory M2 phenotype (Kudlik *et al.*, 2016; Ben-Mordechai *et al.*, 2013). Our work further confirmed that MSCexo could effectively shift the macrophage polarization state from M1 towards the M2 phenotype. Stem cells have a strong ability of proliferation, and multi-directional differentiation and could secrete chemokines, growth factors, microbubbles, cytokines, and exosomes to

the injured site, which promotes the differentiation, proliferation, and chemotaxis of the injured site cells. Among these secretions, exosomes play an important role in signal transduction, intercellular transportation, and tissue regeneration (Zhang *et al.*, 2015; Hu *et al.*, 2015).

Mammalian macrophages are induced to a variety of phenotypes in response to different external stimuli. Some researchers have noted that the change of a subset of miRNA expression was repeatedly found to be involved in the macrophage polarization (Chen *et al.*, 2009; Cheng *et al.*, 2012; Forrest *et al.*, 2010; Cai *et al.*, 2012; Zhang *et al.*, 2013; Rückerl *et al.*, 2012; Chaudhuri *et al.*, 2011). CircRNAs, always as miRNA sponges, are stable transcripts expressed from different genomic locations and have been recently recognized as important regulators for cellular miRNA abundance and thus are major players in the miRNA-mediated post-transcriptional regulatory network. With the interactions between circRNAs and miRNAs, circRNAs are potentially involved in many disease processes, cell processes, and gene expressions (Memczak *et al.*, 2013; Ghosal *et al.*, 2013).

As a circBCRC-3 is a sponge of miR-182, we testified that circBCRC-3 knockdown MSCexo could promote macrophage changed from M1 to M2, which indicated its role in MIRI therapy. Although our data provided circBCRC-3 as a target for MIRI treatment, its clinical application needs further exploration.

Availability of Data and Materials: All data generated or analyzed during this study are included in this manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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