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Original Article

MiR-182-5p Mediated by Exosomes Derived From Bone Marrow Mesenchymal Stem Cell Attenuates Inflammatory Responses by Targeting TLR4 in a Mouse Model of Myocardial Infraction

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ABSTRACT

Exosomes derived from mesenchymal stem cells (MSCs) could protect against myocardial infarction (MI). TLR4 is reported to play an important role in MI, while microRNA-182-5p (miR-182-5p) negatively regulates TLR4 expression. Therefore, we hypothesize that MSCs-derived exosomes overexpressing miR-182-5p may have beneficial effects on MI. We generated bone marrow mesenchymal stem cells (BM-MSCs) and overexpressed miR-182-5p in these cells for exosome isolation. H_2O_2-stimulated neonatal mouse ventricle myocytes (NMVMs) and MI mouse model were employed, which were subjected to exosome treatment. The expression of inflammatory factors, heart function, and TLR4 signaling pathway activation were monitored. It was found that miR-182-5p decreased TLR4 expression in BM-MSCs and NMVMs. Administration of exosomes overexpressing miR-182-5p to H_2O_2-stimulated NMVMs enhanced cell viability and suppressed the expression of inflammatory cytokines. In addition, they promoted heart function, suppressed inflammatory responses, and de-activated TLR4/NF-κB signaling pathway in MI mice. In conclusion, miR-182-5p transferred by the exosomes derived from BM-MSCs protected against MI-induced impairments by targeting TLR4.

Keywords: Exosome; Inflammatory response; Myocardial infarction; miRNA; TLR4

INTRODUCTION

Myocardial infarction (MI) is with great mortality which causes myocardial damage and finally results in heart failure (1). Although precaution and medical treatment have been developed, there is still obvious amount of death caused by MI each year. Therefore, there is still urgent need for novel effective treatment (2).

TLRs are pattern recognition receptors which play essential role in innate immunity (3). They are expressed both in immune cells and the cells of the cardiovascular system. Increasing

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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

BM-MSC, bone marrow mesenchymal stem cell; LVEDD, left ventricular end-diastolic diameter; LVEDP, left ventricular end diastolic pressure; LVEF, left ventricular ejection fraction; LVESD, left ventricular end-systolic dimension; LVFS, left ventricular fractional shortening; LVSP, left ventricular systolic pressure; MI, myocardial infarction; miR-182-5p, microRNA-182-5p; MSC, mesenchymal stem cell; NMVM, neonatal mouse ventricle myocyte; WT, wild-type.

Author Contributions

Conceptualization: Sun C, Li W, Li Y, Chen J, An H, Zeng G, Wang T; Data curation: Sun C, Li W, Li Y, Chen J, An H, Zeng G, Wang T, Wang C; Formal analysis: Wang T, Guo Y; Funding acquisition: Guo Y, Wang C; Investigation: Li W, Li Y, Chen J, An H, Zeng G, Guo Y, Wang C; Methodology: Sun C, Chen J, An H, Zeng G, Guo Y, Wang C; Resources: Guo Y, Wang C; Supervision: Guo Y, Wang C; Validation: Sun C, Li W, Li Y, Chen J, Zeng G, Wang T, Guo Y, Wang C; Writing - original draft: Sun C, Li W, Li Y, Chen J, An H, Zeng G, Wang T, Guo Y, Wang C; Writing - review & editing: Guo Y, Wang C.

evidences have proved that TLRs contribute to the development of heart diseases (4). One of the TLRs, TLR4 is critical for mediating myocardial inflammation in MI (5). After long term of MI, the TLR4 in cardiomyocytes are up-regulated, which results in amplification of inflammation and exacerbation of heart failure (6). In contrast, inhibition of TLR4 improves heart function in MI (7,8). Therefore, TLR4 is a potential target for MI treatment.

MicroRNA-182-5p (miR-182-5p) has been shown to target TLR4, regulate the inflammation, and attenuate spinal cord injury and ischemia-reperfusion injury (9-12). These activities strongly suggest the potential protective effects of miR-182-5p on MI. Transplantation of mesenchymal stem cells (MSCs) after MI has been shown to ameliorate cardiac dysfunction (13). However, after transplantation, it is a big challenge to preserve MSCs viability and transport them to the damaged sites (14). As membrane-bound extracellular vesicles, exosomes secreted by MSCs contain various active biological elements including RNA, DNA, proteins and lipid (15), which could be suitable for the clinical application. Also, the protection of exosomes against MI have been widely described (16-18). Specifically, miRNAs have been identified in exosomes (19) and exosomes containing specific miRNAs provide enhanced effects on MI (15,20). Taken together, we hypothesized that miR-182-5p-enriched exosome could target TLR4 and display protective function in MI. Here the effects of MSCs exosomes containing miR-182-5p on MI were evaluated.

MATERIALS AND METHODS

Generation and identification of bone marrow mesenchymal stem cells (BM-MSCs)

BM-MSCs were isolated from male C57BL/6J mice, aging 6 wk and weighing 15–22 g. The BM-MSCs were cultured following the protocol described before (21). Briefly, femur and tibia of mice were excised and bone marrow cells were slowly flushed out from the marrow cavity using DMEM medium (Gibco, Grand Island, NY, USA). EasySep Mouse CD11b Positive Selection Kit (StemCell Technologies, Vancouver, Canada) was used to separate mononuclear granulocytes. Afterwards, the remaining cells were continued to be cultured. After culturing for 3 to 4 days and removing the unattached cells, BM-MSCs were cultured until the confluence reached 75% (22). The surface expression, including CD11b, CD45, CD34, Sca-1, CD105 and CD29 were monitored by flow cytometry as described previously (23).

Segregation of exosomes

The BM-MSCs-derived exosomes were collected and isolated following the protocol reported previously (23). Briefly, the BM-MSCs were cultured with serum-free DMEM. Then, the supernatants were centrifuged at 500 g for 10–12 min and collected. The supernatants were moved to new tubes and subjected to centrifuge at 12,000 g for 20–25 min. Furthermore, the supernatants were collected and subjected to ultra-centrifuge at 100,000 g for 2 h. Then, the supernatants were removed and the exosome pellets were placed in PBS solution. Partial of the exosomes were subjected to transmission electron microscope, Western blot. The size distribution was examined by Nanoparticle Tracking Analysis (Malvern Panalytical, Shanghai, China).

Generation of neonatal mouse ventricle myocytes (NMVMs)

NMVMs were isolated as described before (24,25). Briefly, hearts from C57BL/6 mice (both male and female) with age of 1 day were isolated and minced in PBS. Then heart tissues were

incubated with collagenase II (0.08%) at 37°C for 30 min. The supernatant was transferred to new tubes. DMEM medium with 10% heat-inactivated fetal bovine serum was added to terminate the digestion. After low-speed centrifuge, the cell pellets were re-suspended in DEME culture medium for culture. NMVMs were treated with exosomes for 36 h and then exposed to H_2O_2 (100 μM) for additional 12 h. Then samples were collected for analysis.

Transfection

MiR-182-5p mimic (5'-UUUGGCAAUGGUAGAACUCACACCG-3') and control (5'-UUGUACUACACAAAAGUAGUC-3') were provided by RiboBio (Guangzhou, China). The RNA was transfected into BM-MSCs using Lipofectamine 2000 following the manufacture's protocols.

CCK8 assay

Briefly, 10,000 NMVMs were seeded in the 96-well plates. Next day, the cells were incubated with exosomes for 36 h and then stimulated with 100 μM H_2O_2 for 12 h. CCK-8 (10 μl) was added to each well. The absorbance was measured at 450 nm after incubation for 2 h.

Dual-luciferase reporter assay

Mutant or wild-type (WT) 3'-UTR of TLR4 was cloned into the pmiRGLO vector (Promega, Madison, WI, USA) (26). The HEK-293T cells were co-transfected with miRNA and pmiRGLO plasmid containing MUT or WT 3'-UTR sequences of TLR4 using Lipofectamine 2000. The Renilla luciferase activity was tested according to the published protocol (26).

MI mouse model and mouse treatment

Animal studies were approved by the Ethic Committee of Northwest A&F University (#2020/05/2706). The MI mouse model was established according to the protocol described previously (20,27). C57BL/6J male mice (20 for each group) were injected with 30 mg/kg pentobarbital sodium for anesthetization and then laid down on the operation table. Mice were connected to a respirator and the thoracic surgery was performed. The 3rd and 4th ribs were beveled to expose the hearts, and the coronary artery was ligated using 6-0 nylon thread with 3 knots. Importantly, the left ventricle instantly turned pale upon ligation. 5 μg exosomes were injected intramyocardially at several sites around the infarct region (20). For certain experiments, exosomes were stained with DiR and then 5 μg exosomes were injected intramyocardially at 5 spots around the MI region and normal mouse. After 3 days, exosomes were tracked by Gluc signals (IVIS Lumina Imaging Systems; Xenogen Corporation, Alameda, CA, USA).

ELISA

The level of cytokine in the supernatant was measured using commercial ELISA kits (Abcam, Cambridge, UK) following suggested protocols.

Western blot

The Western blot was conducted following routine procedures, as described previously (20). Briefly, proteins were extracted using radioimmunoprecipitation buffer containing protease inhibitor cocktail (Abcam). Protein concentration was measured by BCA assay kit (Abcam). The 20 μg of protein were loaded on SDS-PAGE gel and then subjected to transfer. The primary antibodies used in the study included: anti-CD63 (1:1,000; Abcam), anti-β actin (1:2,000; Abcam), anti-CD9 (1:1,000; Abcam), anti-Alix (1:1,000; Abcam), anti-TLR4 (1:1,000; Abcam), anti-phospho-p65 (1:1,000; Abcam), anti-p65 (1:1,000; Abcam).

Real time PCR

The RT-PCR was conducted following standard protocol, as described previously (20). Briefly, total RNA was extracted by Trizol (Thermo Fisher, Waltham, MA, USA) and then reverse transcribed to cDNA using Hi-Fi cDNA Synthesis Kit (Abcam). The real time PCR was conducted using SYBR™ Green PCR Master Mix in QuantStudio 5 system (Thermo Fisher). The sequences of the primers were provided in **Supplementary Table 1**. U6 or GAPDH was employed as internal control. $2^{-\Delta\Delta Ct}$ method was used for the mRNA expression analysis.

Detection of cardiac function

The left ventricular end-diastolic diameter (LVEDD), left ventricular ejection fraction (LVEF), left ventricular end-systolic dimension (LVESD), and left ventricular fractional shortening (LVFS) were examined using an ultrasound instrument (Philip Technologies, Cambridge, MA, USA). The left ventricular end diastolic pressure (LVEDP), left ventricular systolic pressure (LVSP), left indoor pressure dropping rate (−dp/dt), and left indoor pressure rising rate (+dp/dt) were measured as described previously (20).

Statistics analysis

Data were illustrated as mean ± SD. Student's *t*-test or 1/2-way ANOVA followed by Dunn's multiple comparisons test was employed for statistical comparison. The difference was regarded as significant when $p<0.05$. The experiments have been biologically repeated for at least 3 times to confirm the results.

RESULTS

Identification of BM-MSCs and BM-MSC-derived exosomes

The morphology of exosomes was analyzed by TEM (**Fig. 1A**), which indicated that exosomes with the diameter of 30–150 nm were round and had complete lipid bilayer membrane. The Nanoparticle Tracking Analysis demonstrated that the major exosomes had the diameter around 100–120 nm (**Fig. 1B**). We further analyzed the levels of exosome markers. We found the exosomes were enriched with CD9, CD63 and Alix but not β actin (**Fig. 1C**).

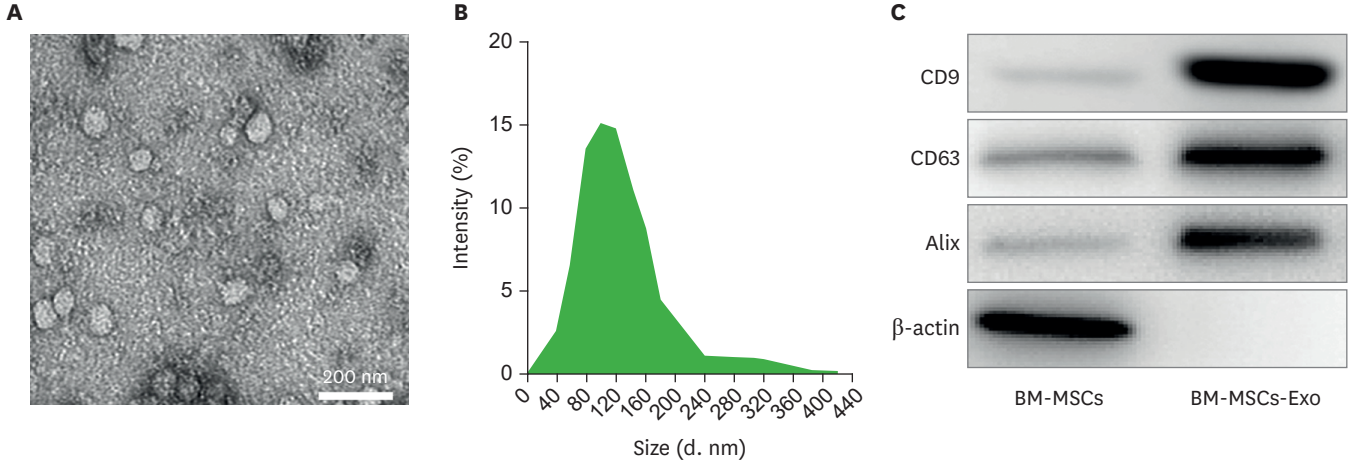

Figure 1. Isolation and characterization of BM-MSC-derived exosomes. (A) Identification of exosomes by TEM. (B) Nanoparticle Tracking Analysis of exosomes. (C) Detection of CD9, CD63 and Alix expression by Western blot.

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miR-182-5p targeted TLR4 and negatively regulated TLR4 expression

MiR-182-5p could target TLR4 (9). We identified the paring sequence of miR-182-5p and TLR4 (**Fig. 2A**). We further made mutations in binding site sequence of TLR4 mRNA and evaluated the binding of miR-182-5p and TLR4 mRNA via luciferase assay. Using the luciferase assay, we detected significantly decreased luciferase activities in cells transfected with miR-182-5p and wild type 3' UTR of TLR4, confirming that miR-182-5p targeted TLR4 (**Fig. 2B**). Transfection of miR-182-5p in BM-MSCs resulted in significantly increased miR-182-5p level (**Fig. 2C**). Correspondingly, significantly decreased mRNA level (**Fig. 2D**) and protein level (**Fig. 2E and F**) of TLR4 were detected in these transfected cells. Interestingly, overexpression of miR-182-5p did not affect the expression of TLR1, TLR2 and TLR5 (**Supplementary Fig. 1**), suggesting miR-182-5p specifically targeted TLR4.

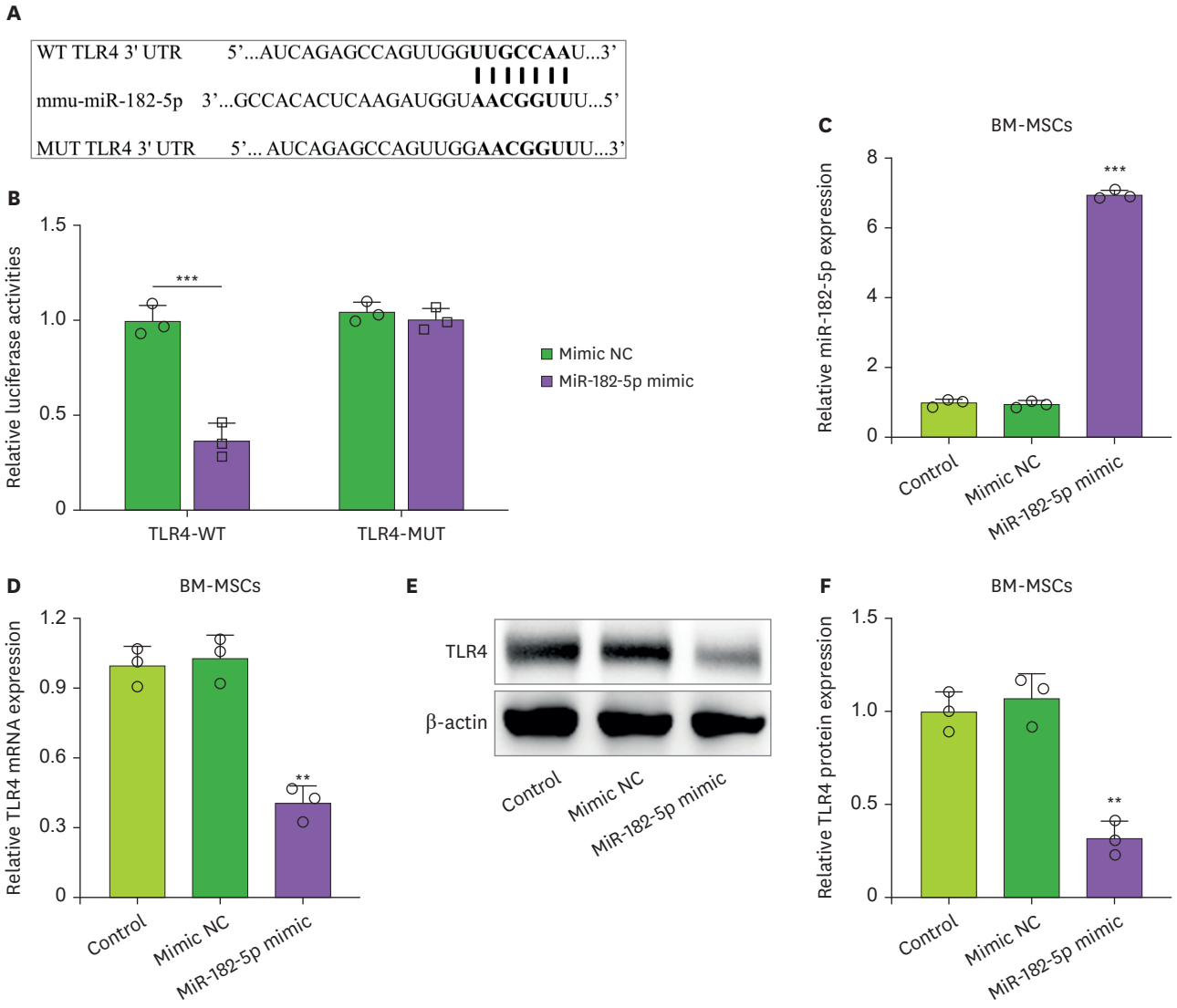

Figure 2. MiR-182-5p targets TLR4. (A) The predicted binding site of miR-182-5p and 3'-UTR of TLR4 mRNA is shown, as well as mutated 3'-UTR of TLR4. (B) HEK-293T cells were co-transfected with luciferase reporters. Relative luciferase activity was measured at 48 h post transfection. Two-way ANOVA followed Turkey's multiple comparisons test. BM-MSCs were transfected with miR-182-5p mimics or NC for 24 h. qRT-PCR was used to measure the (C) miR-182-5p expression and (D) TLR4 expression in BM-MSCs. (E) Western blot was used to analyze the protein expressions of TLR4. (F) β-actin was used as a loading control and the expressions were normalized to control (n=3 for each group). One-way ANOVA followed Dunn's multiple comparisons test.
NC, negative control.
$^{**}p<0.01$, $^{***}p<0.001$ compared to control.

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MiR-182-5p mediated by BM-MSCs-derived exosomes attenuated H_2O_2 induced inflammatory responses in NMVMs

We isolated the exosomes from miR-182-5p-overexpressed BM-MSCs. The overexpression of miR-182-5p did not affect the expression of BM-MSCs markers (**Table 1**). Compared to exosomes isolated from normal BM-MSCs and BM-MSCs transfected with control miRNA, the exosomes isolated from BM-MSCs transfected with miR-182-5p were highly enriched with miR-182-5p (**Fig. 3A**). Then we established the H_2O_2-treated NMVMs cell model and treated NMVMs with exosomes. As shown in **Fig. 3B**, H_2O_2 treatment significantly decreased the cell viability. In contrast, NMVMs pre-treated with exosomes from normal BM-MSCs (we termed normal exosome) had significantly increased cell viability after H_2O_2 treatment. NMVMs pre-treated exosomes from miR-182-5p overexpressed BM-MSCs (we term exosome-miR-182-5p) had the highest cell viability when compared to NMVMs or NMVMs pretreated with normal exosomes, suggesting miR-182-5p prevented H_2O_2-induced cell death. Correspondingly, exosome-miR-182-5p treatment suppressed the expression of pro-apoptotic protein Bax while promoted the expression of anti-apoptotic protein Bcl2 (**Supplementary Fig. 2**). H_2O_2 induced the expression of IL-6 (**Fig. 3C**), MCP-1 (**Fig. 3D**), and TNF-α (**Fig. 3E**) in supernatant of NMVMs. Pre-treatment of normal exosomes significantly decreased the H_2O_2-induced expression of these cytokines in NMVMs. NMVMs pre-treated with exosomes-miR-182-5p had significantly decreased expression of these cytokines when compared to normal NMVMs or NMVMs pre-treated with normal exosomes. H_2O_2 induced the mRNA expression of Cox2 in NMVMs while pretreatment of normal exosomes or exososme-miR-182-5p significantly decreased H_2O_2-induced mRNA expression of Cox2 (**Fig. 3F**). We detected significantly increased mRNA (**Fig. 3G**) and protein (**Fig. 3H and I**) level of TLR4 in H_2O_2-stimulated NMVMs. Pretreatment of normal exosomes did not affect the mRNA or protein level of TLR4 after H_2O_2 stimulation. In contrast, pretreatment of exosome-miR-182-5p resulted in significantly decreased mRNA (**Fig. 3G**) and protein (**Fig. 3H and I**) level of TLR4.

Exosomes derived from BM-MSCs overexpressing miR-182-5p promoted cardiac function in MI mice

The effects of exosomes on echocardiographic indicators of MI mice were further evaluated. Three days post intra-myocardial injection, the exosomes can be still detected myocardial area (**Supplementary Fig. 3**). Compared to mice in sham group, MI mice displayed obviously increased LVEDD (**Fig. 4A**), LVESD (**Fig. 4B**), while they had significantly decreased LVEF (**Fig. 4C**) and LVFS (**Fig. 4D**). In contrast, when treated with normal exosomes, the MI mice could have significantly decreased LVEDD (**Fig. 4A**), LVESD (**Fig. 4B**), while they had significantly increased LVEF (**Fig. 4C**) and LVFS (**Fig. 4D**) when compared to non-treated MI

Table 1. Cell surface marker expression analysis in BM-MSCs and transfected with miR-182-5p mimics by flow cytometry (%)

Variables	Control	Mimic NC	MiR-182-5p mimic	p-value
CD29	95.87±2.31	96.72±1.87	94.57±2.41	0.164
CD105	97.35±1.48	95.38±1.92	96.09±2.35	0.275
Sca-1	94.98±3.37	95.16±4.07	95.90±3.11	0.096
CD34	3.44±1.16	2.86±1.08	1.69±0.83	0.265
CD45	2.35±0.92	1.47±0.83	1.95±1.07	0.164
CD11b	5.09±1.22	3.68±1.07	4.31±0.96	0.351

Values are presented as mean ± SD. Data were collected from 3 independent experiments. The p-values were acquired from 1-way ANOVA test. BM-MSCs among different groups were analyzed for CD29, CD105, Sca-1, CD34, CD45, and CD11b expressions using by Fluorescence Activated Cell Sorting with flow cytometry at passage 3. The negative expressions of CD45, CD34, CD11b and positive expressions of CD105, CD29, Sca-1 indicated a mesenchymal stem cell lineage of BM-MSCs and transfected with miR-182-5p mimics.
NC, negative control.

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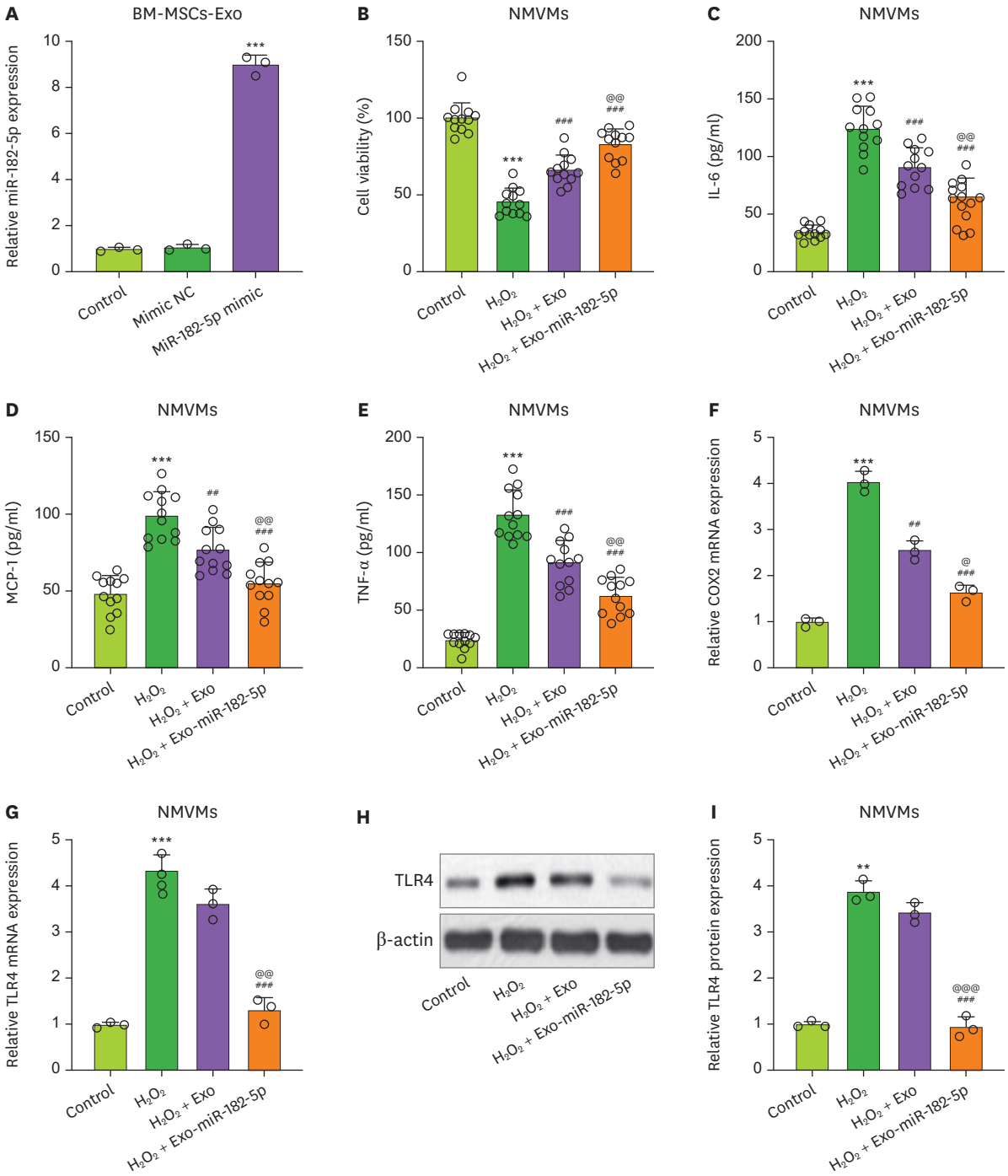

Figure 3. BM-MSC derived exosome-mediated transfer of miR-182-5p attenuated H_2O_2 induced inflammatory responses in NMVMs. (A) qRT-PCR was used to measure miR-182-5p expression in exosomes derived from BM-MSCs. (B) Cell viability was analyzed in NMVMs. The levels of IL-6 (C), MCP-1 (D), TNF-α (E) in cell supernatants were measured by corresponding ELISA assays. (F) The mRNA expression of COX2 in NMVMs was measured by RT-PCR. (G-I) qRT-PCR and Western blot were used to analyzed the mRNA and protein expressions of TLR4 in NMVMs. Data were shown as mean ± SD (n=3 in each group for qRT-PCR and Western blot, n=12 in each group for CCK-8 and ELISA). One-way ANOVA followed Dunn's multiple comparisons test.
qRT-PCR, quantitative RT-PCR; NC, negative control.
$^{**}p<0.01$, $^{***}p<0.001$ compared to control; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to H_2O_2 treated group; $^{@}p<0.05$, $^{@@}p<0.01$, $^{@@@}p<0.001$ compared to H_2O_2 and exosome treated group.

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mice, suggesting exosomes ameliorated cardiac dysfunction in those MI mice. Notably, when treated with exosomes-miR-182-5p, the MI mice had significantly less LVEDD and LVESD than MI mice treated with normal exosomes. MI mice treated with exosomes-miR-182-5p had significantly higher LVEF and LVFS than MI mice treated with normal exosomes, indicating exosomes overexpressing miR-182-5p had better effects compared to normal exosomes. Echocardiography image also confirmed the improvement of heart function in MI mice treated with exosomes-miR-182-5p (**Supplementary Fig. 4**). Similarly, exosomes-miR-182-5p significantly enhanced the LVSP (**Fig. 4E**) and decreased LVEDP (**Fig. 4F**). Exosomes-miR-182-5p also rescued the value of pressure rising rate (**Fig. 4G**), as well as pressure dropping

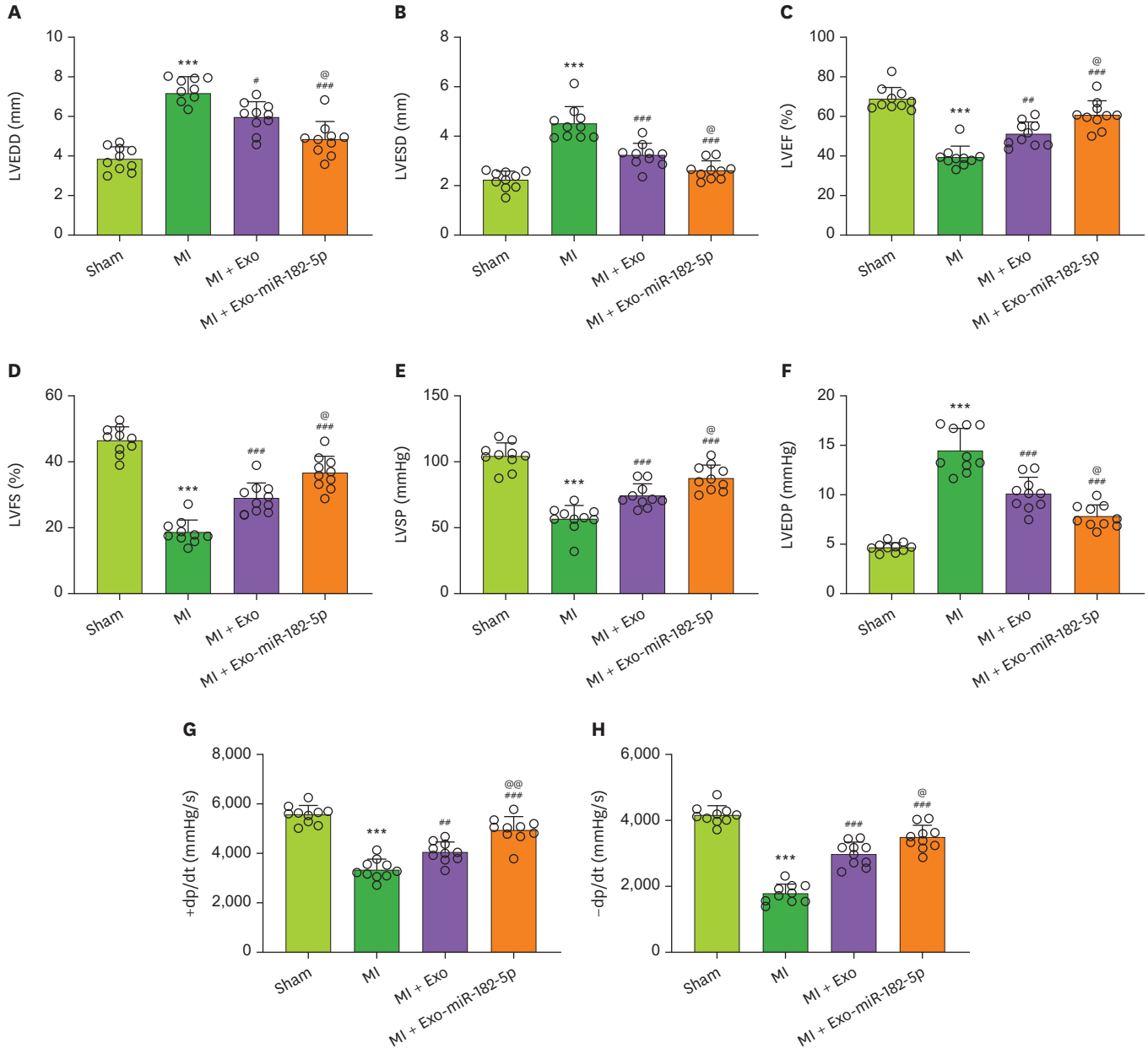

Figure 4. Effects of BM-MSC derived exosome-mediated transfer of miR-182-5p on LVEDD (A), LVESD (B), LVEF (C), and LVFS (D) from echocardiography. Effects of BM-MSC derived exosome-mediated transfer of miR-182-5p on LVSP (E), LVEDP (F), +dp/dt (G), and −dp/dt (H) from left ventricle hemodynamic measurements (n=10 [10 animals] in each group). One-way ANOVA followed Dunn's multiple comparisons test.
***p<0.001 compared to sham; *p<0.05, **p<0.01, ***p<0.001 compared to MI; @p<0.05, @@p<0.01 compared to MI + exosome group.

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rate (**Fig. 4H**). Collectively, our results demonstrated that BM-MSCs-derived exosomes overexpressing miR-182-5p promoted cardiac function in MI mice.

Exosomes derived from BM-MSCs overexpressing miR-182-5p suppressed inflammation in MI mice

Then, we measured the expression of pro-inflammatory cytokines in heart tissues from MI mice with different treatments. We found that MI mice had remarkably upregulated mRNA level of IL-6 (**Fig. 5A**), IL-1β (**Fig. 5B**), TNF-α (**Fig. 5C**), MCP-1 (**Fig. 5D**), Cox-2 (**Fig. 5E**) as well as iNOS (**Supplementary Fig. 5**) while normal exosome treatment and exosome-miR-182-5p treatment significantly suppressed mRNA level of these inflammatory mediators. Correspondingly, the protein level of MCP-1 (**Fig. 5F**), IL-6 (**Fig. 5G**), IL-1β (**Fig. 5H**) and TNF-α (**Fig. 5I**) in MI mice treated with exosome-miR-182-5p were significantly decreased. Taken together, these results showed BM-MSCs-derived exosomes-mediated transfer miR-182-5p inhibited inflammation in MI mice.

Exosomes derived from BM-MSCs overexpressing miR-182-5p suppressed TLR4/NF-κB signaling pathway in MI mice

Finally, the effects of exosomes on TLR4 and NF-κB signaling pathway were examined. We noticed notably upregulated mRNA level (**Fig. 6A**) and protein level (**Fig. 6B and C**) of TLR4 in heart tissues of MI mice when compared to those in sham mice. Interestingly, mRNA and protein levels of TLR4 could be significantly reduced by exosome treatment. In addition, treatment of exosome-miR-182-5p promoted the downregulation of TLR4, indicating miR-182-5p reduced TLR4 expression. We also found that exosomes treatment decreased the protein level of phosphorylated-p65 but not affect the protein level of p65 (**Fig. 6B and D**). Exosome-miR-182-5p promoted the downregulation of phosphorylated-p65 (**Fig. 6B and D**). Collectively, our data showed that exsome-miR-182-5p suppressed TLR4/NF-κB activation in MI mice. In addition, we further identified that miR-182-5p functioned through TLR4 as knocking down TLR4 impaired the protective effects of miR-182-5p (**Supplementary Fig. 6**).

DISCUSSION

Here we generated the BM-MSCs exosomes enriched in miR-182-5p and evaluated the effects of exosomes on MI mice. We found that exosomes-miR-182-5p prevented H_2O_2-induced cell death and inflammation in NMVMs. When administrated the exosomes-miR-182-5p to MI mice, these exosomes improved the cardiac function and suppressed inflammatory response in heart tissues. We further determined that exosomes-miR-182-5p down-regulated TLR4 and inhibited TLR4/NF-κB signaling pathway. Our results strongly demonstrate that exosomes-miR-182-5p could be a useful approach for MI treatment.

During MI, the dead myocardial cells and the fibrosis of infarcted heart lead to heart failure. Although there are surgical techniques and drug available, these treatments cannot rescue the function of infarcted myocardial cells (28,29). Because of the distinct characteristics including their ability to differentiate to cardiomyocytes, anti-fibrosis, MSCs are supposed to be critical in MI treatment (29). However, due to the restricted environment condition such as lacking of oxygen and nutrients, MSCs undergo necrosis and apoptosis quickly after transplantation, resulting in clinical application limitation (30). Increasing evidences have demonstrated that it is the paracrine activity of the transplanted MSCs, but not their differentiation to myocardium, that contribute to their protective effects on MI (31). Studies

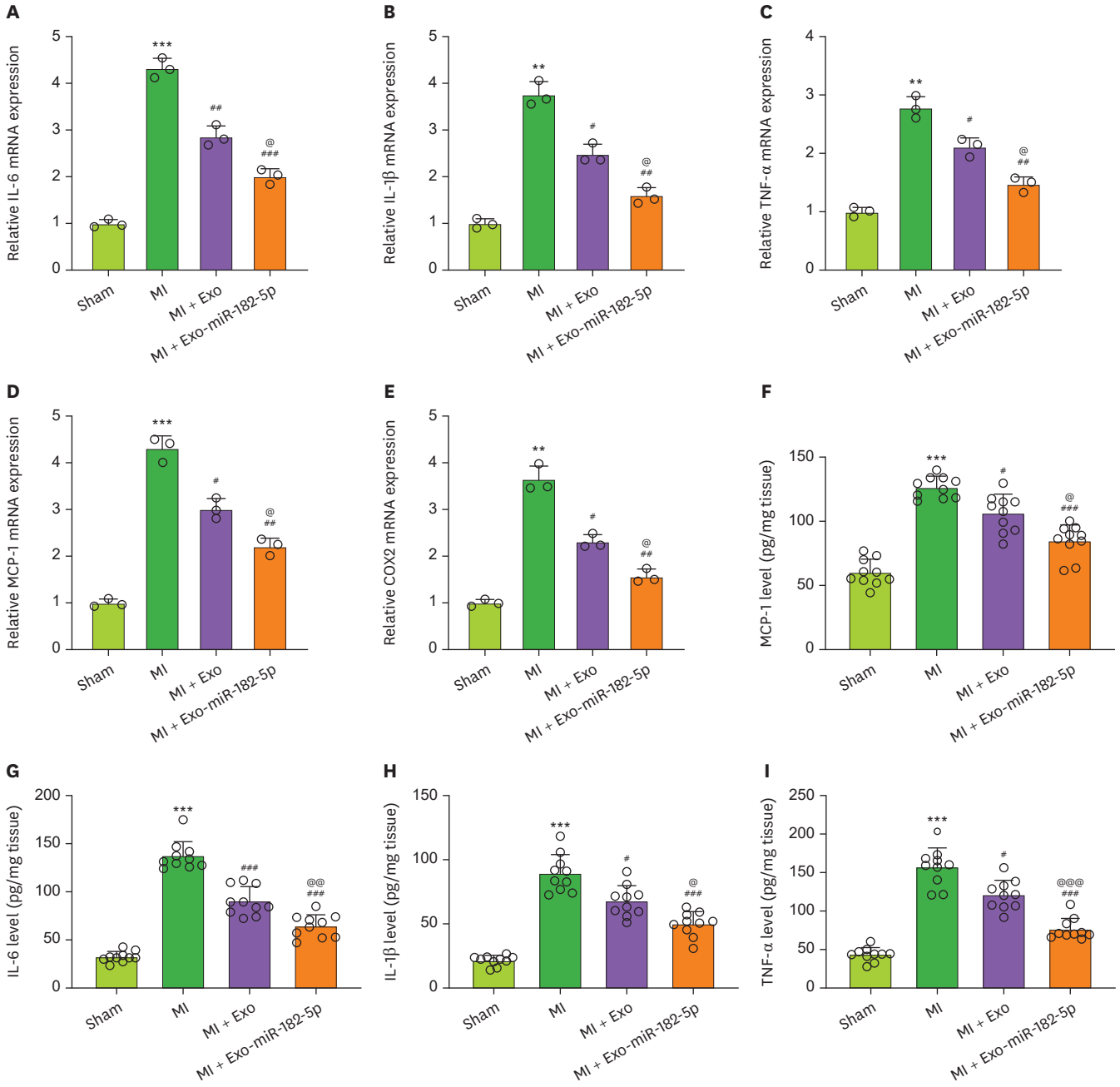

Figure 5. Effects of BM-MSCs derived exosome-mediated transfer of miR-182-5p on myocardial infarction induced inflammatory responses in mice. qRT-PCR was used to measure the mRNA expressions of IL-6 (A), IL-1β (B), TNF-α (C), MCP-1 (D), COX2 (E) in left ventricular tissues (n=3 [tissues form 10 animals were mixed] in each group). Effects of BM-MSCs derived exosome-mediated transfer of miR-182-5p on myocardial infarction induced upregulated levels of MCP-1 (F), IL-6 (G), IL-1β (H), TNF-α (I), in left ventricular tissues (n=10 [10 animals] in each group). One-way ANOVA followed Dunn's multiple comparisons test.
qRT-PCR, quantitative RT-PCR.
$^{**}p<0.01$, $^{***}p<0.001$ compared to sham; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to MI; $^{@}p<0.05$, $^{@@}p<0.01$, $^{@@@}p<0.001$ compared to MI + exosome group.

have demonstrated that exosomes function as the primary paracrine factor participating in stimulating regeneration and inducing protection in cardiomyocytes (32-34). Several studies have described MSC-derived exosomes protect against myocardial diseases. Teng and colleagues (35) described that BM-MSCs-derived exosomes restrained the inflammatory response and stimulated neovascularization, then improved heart function after ischemic

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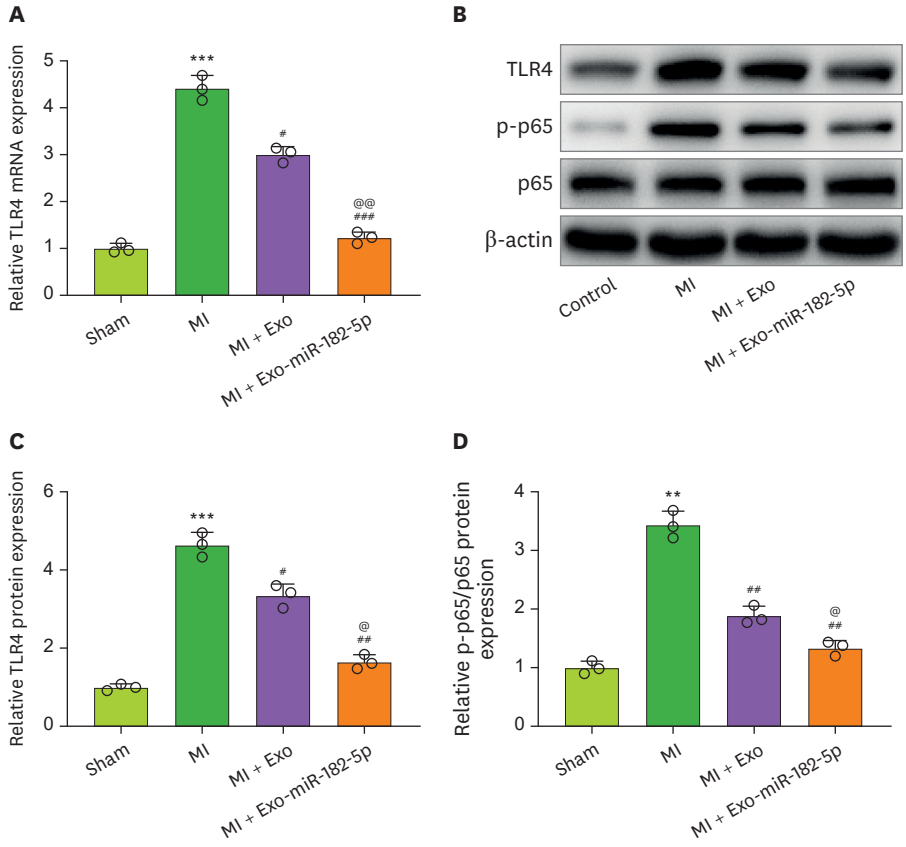

Figure 6. BM-MSCs derived exosome-mediated transfer of miR-182-5p inhibited TLR4/κB signaling in left ventricular tissues after myocardial infarction. qRT-PCR and Western blot were used to analyzed the mRNA (A) and protein expression of TLR4, p-p65 and p65 (B-D) in left ventricular tissues (n=3 [tissues form 10 animals were mixed] in each group). One-way ANOVA followed Dunn's multiple comparisons test.
qRT-PCR, quantitative RT-PCR.
$^{**}p<0.01$, $^{***}p<0.001$ compared to sham; $^{*}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to MI; $^{@}p<0.05$, $^{@@}p<0.01$ compared to MI + exosome group.

injury. Liu and colleagues (36) described that MSC-derived exosomes rescued injury as well as prevented cell apoptosis induced by myocardial ischemia/reperfusion. Similarly, we also found exosomes from BM-MSCs prevented H_2O_2-induced cell death and inflammation. In addition, administration of BM-MSCs-derived exosomes suppressed inflammation in cardiac tissues and promoted heart function in MI mice. Our study further confirmed the protective effects of BM-MSCs-derived exosomes on MI.

It is well-described that exosomes could transfer functional miRNA and deliver the miRNA to cells (37). In present study, we overexpressed miR-182-5p in BM-MSCs and found the miR-182-5p were also enriched in exosomes derived from these BM-MSCs. MiR-182-5p targeted TLR4 and suppressed TLR4 expression. Consistently, we showed miR-182-5p targeted TLR4 and overexpressing miR-182-5p in BM-MSCs resulted in downregulation of TLR4 but not other TLRs in these cells. Furthermore, we detected that the exosomes-miR-182-5p could deliver the miR-182-5p to NMVMs, resulting in down-regulation of TLR4 in these cells. TLR4 has been implicated in the development of MI. In MI, DAMPs released from necrotic cardiomyocytes activate TLR4 (38). Activation of TLR4 signaling pathway induces inflammatory responses which lead to additional damage to myocardium. Inhibition of TLR4 has been shown to attenuate myocardial injury. Shimamoto and colleagues (7) utilized a TLR4 antagonist eritoran and found it significantly reduced inflammatory response and

ameliorated myocardial ischemia-reperfusion injury. Chong et al. (39) reported that C3H/HeJ mice with functional mutation of TLR4 had attenuation of myocardial infarction size after myocardial ischemia-reperfusion. In present study, we administrated exosomes-miR-182-5p to MI mice, and found exosomes-miR-182-5p but not normal exosomes suppressed TLR4 expression and inhibited the activation of TLR4/NF-κB signaling pathway. Furthermore, exosomes-miR-182-5p inhibited the inflammatory responses in heart tissues of MI mice, leading to the enhanced heart function. More importantly, exosomes overexpressing miR-182-5p exhibited better protective effects against MI than normal exosomes, suggesting miR-182-5p contributed to the protection against MI.

In conclusion, our study demonstrated that exosomes-miR-182-5p suppressed inflammation and ameliorated heart dysfunction in MI mice by targeting TLR4.

SUPPLEMENTARY MATERIALS

Supplementary Table 1
Oligonucleotide primer sequences for quantitative RT-PCR

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Supplementary Figure 1
miR-182-5p does not target TLR1, TLR2 or TLR5. BM-MSCs were transfected with miR-182-5p mimics or NC for 24 h. qRT-PCR was used to analyzed the mRNA expressions of TLR1 (A), TLR2 (B) and TLR5 (C).

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Supplementary Figure 2
BM-MSC derived exosome-mediated transfer of miR-182-5p attenuated H_2O_2 induced cell apoptosis in NMVMs. qRT-PCR was used to measure the mRNA expressions of Bax (A) and Bcl2 (B) in NMVMs. Data were shown as mean ± SD (n=3 in each group). One-way ANOVA followed Dunn's multiple comparisons test.

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Supplementary Figure 3
The delivery efficiency of miR-182-5p mediated exosomes *in vivo* was tracked by bioluminescence imaging. The 5 μg exosomes were injected intramyocardially at 5 spots around the MI region and normal mouse. After 3 days, representative bioluminescence images.

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Supplementary Figure 4
Representative echocardiography images for **Fig. 4**.

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Supplementary Figure 5

qRT-PCR was used to measure the mRNA expressions of iNOS in left ventricular tissues (n=3 [tissues form 10 animals were mixed] in each group, technical repeats). One-way ANOVA followed Dunn's multiple comparisons test.

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Supplementary Figure 6

NMVMs were transfected with TLR4 siRNA or it's NC for 24 h. qRT-PCR was used to measure the mRNA expressions of TLR4 (A). NMVMs were transfected with TLR4 siRNA for 24 h and then stimulated with 100 μM H_2O_2 for 12 h. (B) Cell viability was analyzed in NMVMs. (C) qRT-PCR was used to measure the mRNA expressions of Bax. (D) ELISA was used to measure the levels of IL-6. One-way ANOVA followed Dunn's multiple comparisons test.

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