Exosomes derived from human umbilical cord mesenchymal stem cells improve myocardial repair via upregulation of Smad7

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Abstract. It has been previously reported that exosomes derived from human umbilical cord mesenchymal stem cells (hucMSC)-exosomes exhibit cardioprotective effects on the rat acute myocardial infarction (AMI) models and cardiomyocyte hypoxia injury models *in vitro*, however the exact mechanisms involved require further investigation. The present study aimed to investigate the repair effects of hucMSC-exosomes on myocardial injury via the regulation of mothers against decapentaplegic homolog 7 (Smad7) expression. Compared with sham or normoxia groups (*in vivo* and *in vitro*, respectively), western blotting demonstrated that Smad7 expression was significantly decreased in the borderline area of infraction myocardium and in H9C2(2-1) cells following hypoxia-induced injury. Additionally, microRNA (miR)-125b-5p expression was markedly increased using reverse transcription-quantitative polymerase chain reaction, but was reversed by hucMSC-exosomes. Trypan blue staining and lactate dehydrogenase release detection demonstrated that cell injury was significantly increased in the AMI + PBS and hypoxia group compared with in the sham and normoxia groups and was inhibited by hucMSC-exosomes. A dual luciferase reporter gene assay confirmed that Smad7 is a target gene of miR-125b-5p. In addition, miR-125b-5p mimics promoted H9C2(2-1) cell injury following 48 h exposure to hypoxia. Downregulation of Smad7 expression under hypoxia was increased by miR-125b-5p mimics compared with the mimic negative control, and hucMSC-exosomes partially alleviated this phenomenon. In conclusion, hucMSC-exosomes may promote Smad7 expression by inhibiting miR-125b-5p to increase myocardial repair. The present study may provide a potential therapeutic approach to improve myocardial repair following AMI.

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Introduction

Acute myocardial infarction (AMI) usually leads to myocardial injury and heart failure due to a sudden reduction in oxygen and blood supply (1), and is one of the leading cause of morbidity and mortality worldwide (2). The number of studies on stem cells as one of the potential therapeutic applications have increased markedly, and researchers have used various experimental models and human clinical trials to demonstrate that stem cells have the capacity to improve acute and chronic myocardial injury (3-6). Additionally, the majority of the beneficial effects of stem cell-based therapies have been attributed to paracrine effects (7,8). As an important macromolecular substance secreted by stem cells, exosomes contain numerous bioactive molecules and they can produce similar effects to stem cells (9,10). Our earlier studies demonstrated that human umbilical cord mesenchymal stem cell (hucMSC)-exosomes may reduce cardiomyocyte apoptosis, promote angiogenesis and improve cardiac function following AMI (11,12); however, the underlying mechanism remains to be investigated.

It is well established that mothers against decapentaplegic homolog 7 (Smad7), a vital downstream regulator in the transforming growth factor-β (TGF-β) signaling pathway, has critical roles in cell proliferation, differentiation, apoptosis and numerous other essential physiological activities (13,14). It can inhibit TGF-βI-induced phosphorylation of Smad2/3 and interfere with the interaction between other Smad proteins or receptors to inhibit downstream gene transcription (13). Recent studies suggested that Smad7 may also suppress the activation of nuclear factor-κB and inhibit hypoxia/reoxygenation (H/R)-induced cardiomyocyte apoptosis (15,16). Therefore, Smad7 is a major negative regulator in the TGF-βI signaling pathway. Chen *et al* (17) reported that Smad7 has a critical role in the development and function of the heart. Wang *et al* (18) demonstrated that decreased Smad7 expression contributes to cardiac fibrosis in the infarcted rat heart. In a previous study (11), the results demonstrated that hucMSC-exosomes improved myocardial repair; Smad7 expression was increased in hypoxia cardiomyocytes *in vivo* and *in vitro*. However, the molecular mechanism of

hucMSC-exosomes-induced upregulation of Smad7 expression is not yet understood.

MicroRNAs (miRNAs/miRs) are small non-protein-coding single-stranded RNAs that negatively control the expression of target genes by binding to the 3'-untranslated region (3'UTR) of target mRNAs (19). miRNAs have been reported to exhibit critical roles in a variety of cardiovascular diseases, including heart failure (20), myocardial hypertrophy (21), arrhythmia (22) and myocardial infarction (23). miR-125b-5p was previously reported to be upregulated in patients with AMI and may serve as a novel biomarker for the early diagnosis of AMI (24). Bie *et al* (25) demonstrated that miR-125b contributes to the proliferation and migration of cardiac fibroblasts. The present study reported that hucMSC-exosomes inhibit miR-125b-5p expression following myocardial injury. Furthermore, miR-125b-5p was predicted to a target Smad7 mRNA. Therefore, the present study investigated whether hucMSC-exosomes promoted Smad7 expression via the downregulation of miR-125b-5p to improve myocardial repair.

Materials and methods

Ethical statement. The present study was approved by the Laboratory Animal Management Committee of Jiangsu University (Zhenjiang, Jiangsu, China).

Cell culture and reagents. hucMSCs were isolated and cultured as previously described (26). The collection and use of hucMSCs was approved by the Ethics Committee of Jiangsu University (Zhenjiang, China). All individuals provided informed consent for the use of their umbilical cord in the present study. The umbilical cords were collected between June 2016 and March 2017 at the Obstetrics Department of the Affiliated Hospital of Jiangsu University (Zhenjiang, China). Maternal age distribution was from 25-32 years old. HucMSCs were cultured in low-glucose Dulbecco's modified Eagle's medium (L-DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. The rat embryonic cardiomyocyte cell line H9C2(2-1) and 293T cells were purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in high-glucose DMEM (H-DMEM; Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS at 37˚C with 5% CO_2.

HucMSC-exosomes extraction, purification and characterization. The hucMSC-exosomes were extracted and purified as the previously described (11). L-DMEM containing 10% FBS was replaced with L-DMEM containing 10% exosomes-free FBS when cultured hucMSCs attained 80-90% confluence. The conditioned medium of hucMSC (hucMSC-CM) was collected following replacement with L-DMEM containing 10% exosomes-free FBS for 48 h and centrifuged at 300 x g for 20 min, 2,000 x g for 20 min, 10,000 x g for 30 min at 4˚C to remove dead cells and cellular debris. The hucMSC-CM was then concentrated using a 100 kDa molecular weight cutoff (MWCO) hollow fibre membrane (EMD Millipore, Billerica, MA, USA) at 1,000 x g for 30 min at 4˚C. The concentrated hucMSC-CM was loaded onto 5 ml 30% sucrose/D_2O cushions and ultracentrifuged at 100,000 x g for 2 h at 4˚C (optimal-90k; Beckman Coulter, Inc., Brea, CA, USA). The bottom of the cushion containing the hucMSC-exosomes was collected and washed three times with PBS using a 100 kDa MWCO centrifuge tube at 1,000 x g for 30 min at 4˚C. The hucMSC-exosomes were filtered through a 0.22 μm membrane filter (EMD Millipore,) and stored at -70˚C until use. The protein content of hucMSC-exosomes was determined using a bicinchoninic acid (BCA) protein assay kit (CWBiotech, Beijing, China). Nanoparticle tracking analysis (NTA) was performed for the analysis of particle size and particle concentration of hucMSC-exosomes using a digital microscope LM10 system (NanoSight; Malvern Instruments, Ltd., Malvern, UK). The cluster of differentiation CD9, CD63 and CD81 molecules that are frequently located on the surface of hucMSC-exosomes were analysed via western blotting.

Establishing the rat acute myocardial infarction and evaluation of cardiac function. The animal protocol was approved by the Animal Experimental Center of Jiangsu University (Zhenjiang, China). Healthy male Sprague-Dawley rats (220-250 g; 8-week-old) were used in the AMI model according to the previously reported method (11). The rats were purchased from the Animal Experimental Center of Jiangsu University and housed in a specific pathogen-free animal facility under constant temperature and humidity, and with a 12/12 h light/dark cycle with sufficient qualified food and water. In brief, Sprague-Dawley rats were anesthetized with 10% chloral hydrate (300 mg/kg; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) by intraperitoneal injection and were mechanically ventilated (Alcott Biotech Co., Ltd., Shanghai, China). The rat's thorax was opened, and the left anterior descending (LAD) coronary artery was quickly and accurately ligated with a 6-0 suture. The thorax was closed by tightening the double purse suture. The animals were randomly divided into three groups with 18 rats per group (6 rats for western blot analysis, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) at 2 days following surgery, and echocardiography at 4 weeks following surgery, respectively). AMI + hucMSC-exosomes group received hucMSC-exosomes (400 μg proteins) diluted in 200 μl PBS and the AMI + PBS group received 200 μl PBS. PBS or hucMSC-exosomes were injected via the tail vein immediately after LAD ligation. The sham group underwent neither LAD coronary artery ligation nor PBS/hucMSC-exosomes injection. The borderline area (the juncture of infract and non-infract site) of infraction myocardium was used for RT-qPCR and western blot analyses at 2 days after surgery. The tissue of the sham group was selected from near the LAD coronary artery. Echocardiography of left ventricular performance was assessed by a high-frequency colour ultrasound instrument (Vevo2100; VisualSonics, Toronto, ON, Canada) at 4 weeks after surgery.

Hypoxia experiments in vitro. To mimic ischemic injury and study whether hucMSC-exosomes protected H9C2(2-1) cells against hypoxic injury, three experimental groups were designed as follows: Hypoxia + exosomes group, H9C2(2-1) cells were cultured in 6-well plates at $2x10^5$ cells/plate in L-DMEM containing 0.2% FBS and hucMSC-exosomes (200 μg/ml) at 37˚C with 5% CO_2, 94% N_2 and 1% O_2 for 48 h;

Table I. Primer list.

miR	Forward (5'-3')	Reverse (5'-3')
miR-21-5p	GTGCAGGGTCCGAGGT	GTGCGTGTCGTGGAGTCG
miR-92a-3p	TATTGCACTTGTCCCGGCCTGT	GTGCGTGTCGTGGAGTCG
miR-125b-5p	GCTCCCTGAGACCCTAAC	GTGCGTGTCGTGGAGTCG
U6	TGCGGGTGCTCGCTTCGGCAGC	CCAGTGCAGGGTCCGAGGT

miR, microRNA.

Table II. List of predicted binding sites of Smad7 and the targeting miRs.

Smad7 3'UTR bp position	Smad7 3'UTR sequence	miR	miR sequence
1182-1189	5'-UGCUCACACUUUAAU **AUAAGCUA**-3'	rno-miR-21-5p	3'-AGUUGUAGUCAGAC**U AUUCGAU**-5'
1393-1399	5'-CAUUAUUUAUGUAUU **GUGCAAUG**-3'	rno-miR-92a-3p	3'-GUCCGGCCCUGUU **CACGUUAU**-5'
3721-3727	5'-GACCAGCGAGGGGCA **UCAGGGA**-3'	rno-miR-125b-5p	3'-AGUGUUCAAUCCC AG**AGUCCCU**-5'

Complementary binding sites are in bold. Smad7, mothers against decapentaplegic homolog 7; miR, microRNA.

hypoxia + PBS group, H9C2(2-1) cells were cultured under the same conditions with the hucMSC-exosomes replaced by isometric PBS; normoxia group, H9C2(2-1) cells were cultured under normoxic conditions throughout the experiments as the control.

Bioinformatics analysis. The potential targets of Smad7 were predicted using miRbase (http://www.mirbase.org/) and TarBase (http://www.microrna.gr/tarbase).

RNA isolation and detection. Total RNA of tissues and cell lines was extracted with TRIzol Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Bulge-loop™ miRNA qRT-PCR Primer Sets (one RT primer and a pair of primers for each set) specific for miR-21-5p, miR-92a-3p and miR-125b-5p (Table I) were designed by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). RT-qPCR was performed by using the Bulge-Loop miRNA qRT-PCR Starter kit (R11067.1; Guangzhou RiboBio Co., Ltd.) according to the manufacturer's protocols. The reaction was performed at 95˚C for 10 min, followed by 40 cycles of 95˚C for 2 sec, 60˚C for 20 sec and 70˚C for 10 sec. The relative expression levels of miRNA were analysed using the $2^{-\Delta\Delta Cq}$ method (27). The U6 small nuclear RNA served as the internal reference to normalize the expression of miRNA.

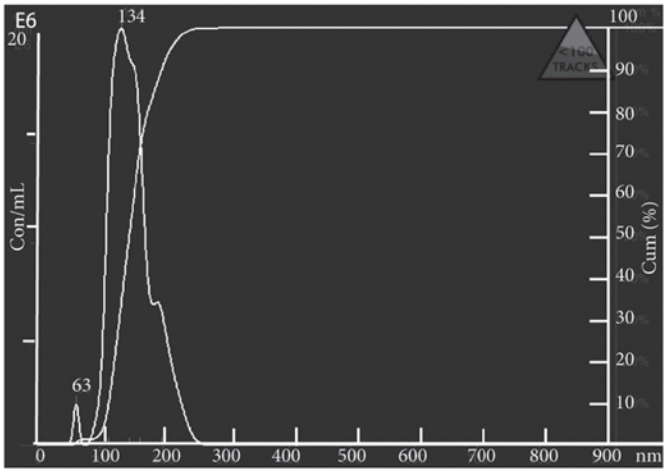
Transient transfection. miR-125b-5p mimics (25 nM) (5'-UCC CUGAGACCCUAACUUGUGA-3') and mimic negative control (MNC; 25 nM; 5'-UUGUACUACACAAAAGUA CUG-3') were synthesized and purified by Guangzhou RiboBio. 293T cells and H9C2(2-1) cells were transfected with Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The cells were undertaken to extract protein, isolate RNA or hypoxia experiments following transfection for 24 h.

Trypan blue staining. H9C2(2-1) cells were cultured in hypoxia conditions *in vitro* as described the above. Cell viability was analysed by trypan blue (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). A total of 100 μl cell suspension (10^4 cells) and 100 μl 0.4% trypan blue solution were mixed well, then incubated for 1 min at room temperature; 10 μl of the mix was collected to carefully fill the haemocytometer chamber. Cells were counted according to the protocols of the haemocytometer. Viable cells remained unstained, non-viable cells were stained blue.

Lactate dehydrogenase (LDH) release detection. To evaluate the membrane integrity of H9C2(2-1) cells with various treatments, the concentration of LDH in the culture media was measured with a Lactate Dehydrogenase Assay kit (Shanghai Rongsheng Biotech Co., Ltd., Shanghai, China) using the continuous monitoring method according to the manufacturer's protocols.

Dual-luciferase reporter gene assay. To verify the association between miR-125b-5p and Smad7, the pmirGLO dual-luciferase reporter vector (Promega Corporation, Madison, WI, USA) was employed to construct a 3'UTR wild-type reporter vector of Smad7. Additionally, a 3'UTR mutant reporter vector of Smad7 lacking miR-125b-5p binding site was also constructed. 293T cells were transiently co-transfected with Smad7 3'UTR wild (or mutant) reporter vector (200 ng) and miR-125b-5p mimics (25 nM) using Lipofectamine® 2000

A



B

Bin centre (nm)	Percentile undersize (%)
10	0.000
30	0.000
50	0.324
70	1.389
90	2.557
110	15.361
130	40.772
150	65.041
170	81.035
190	90.000
210	96.377
230	99.260
250	99.950
270	100.000

C

hucMSC-exosomes	Protein concentration (mg/ml)	Total concentration (particles/ml)
1	4.39	5.62×10^{10}
2	3.23	3.74×10^{10}
3	2.98	3.16×10^{10}

D

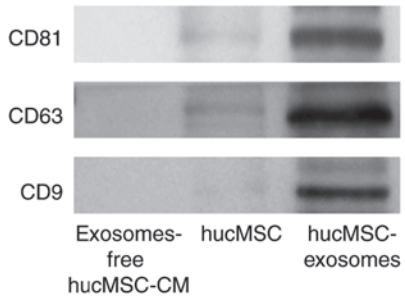


Figure 1. Characterization of hucMSC-exosomes. (A) Particle size distribution and (B) particle concentration were analysed by nanoparticle tracking analysis. (C) Protein concentration and particle concentration of hucMSC-exosomes in various batches. (D) Expression levels of CD9, CD63 and CD81 in purified hucMSC-exosomes were detected by western blotting. CD, cluster of differentiation; hucMSC, human umbilical cord mesenchymal stem cell.

reagent. The cells were collected 24 h after co-transfection to measure the luciferase activity using a Dual-Luciferase Reporter Assay system (E1910; Promega Corporation) and normalized to that of *Renilla* luciferase activity according to the manufacturer's protocols.

Western blot analysis. Western blot analysis was conducted according to the previously described procedure (11). In brief, protein was extracted from hucMSC-exosomes, H9C2(2-1) cells and myocardial tissue using radioimmunoprecipitation assay buffer and phenylmethylsulfonyl fluoride (Invitrogen; Thermo Fisher Scientific, Inc.). The protein concentration was determined with a BCA protein assay kit (CWBiotech) according to the manufacturer's protocols. Equal quantities of protein (30 μg) were separated by 12% SDS-PAGE and subsequently transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk in 1X TBS-Tween for 1 h at room temperature and incubated with primary antibodies against CD9 (1:1,000; cat. no. ab92726; Abcam, Cambridge, UK), CD63 (1:500; cat. no. BS3474; Bioworld Technology, Inc., St. Louis Park, MN, USA), CD81 (1:11,000; cat. no. ab109201; Abcam), B-cell lymphoma 2 (Bcl-2; 1:2,000; cat. no. MAB8272; R&D Systems, Inc., Minneapolis, MN, USA), Bcl-2-associated X (Bax; 1:1,000; cat. no. 2772; Cell Signaling Technology, Inc., Danvers, MA, USA), Smad7 (1:1,000; cat. no. MAB2029; R&D Systems, Inc.) and GAPDH (1:2,000; cat. no. CW0100M; CWBiotech) at 4˚C overnight. Subsequently, membranes were washed three times with 1x TBS-Tween (10 min per wash) and then incubated with HRP-conjugated secondary antibodies (1:5,000, cat. no. BS13278, Bioworld Technology, Inc.; and 1:2,000, cat. no. CW0102S, CWBiotech) at 37˚C for 1 h. Then, membranes were washed three times with 1X TBS-Tween (10 min per wash). The signals were visualized using the Luminata Crescendo Western HRP substrate (EMD Millipore) and the results were analyzed by Image-Pro Plus version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. Statistical analysis was performed by Graph Pad Prism (v5.01; GraphPad Software, Inc., La Jolla, CA, USA). All experimental data *in vitro* were obtained at least three independent experiments and expressed as the mean ± standard deviation. A Student's t-test was used to compare experimental and relative control groups. Multiple comparisons were performed using one-way analysis of variance followed by the Newman-Keuls post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of hucMSC-exosomes. The particle size distribution and particle concentration of hucMSC-exosomes were recorded by NTA (Fig. 1A and B). The mean protein concentration and mean particle concentration of hucMSC-exosomes were 3.53 mg/ml and $4.17\text{x}10^{10}$ particles/ml, respectively (Fig. 1C). Western blot analyses indicated

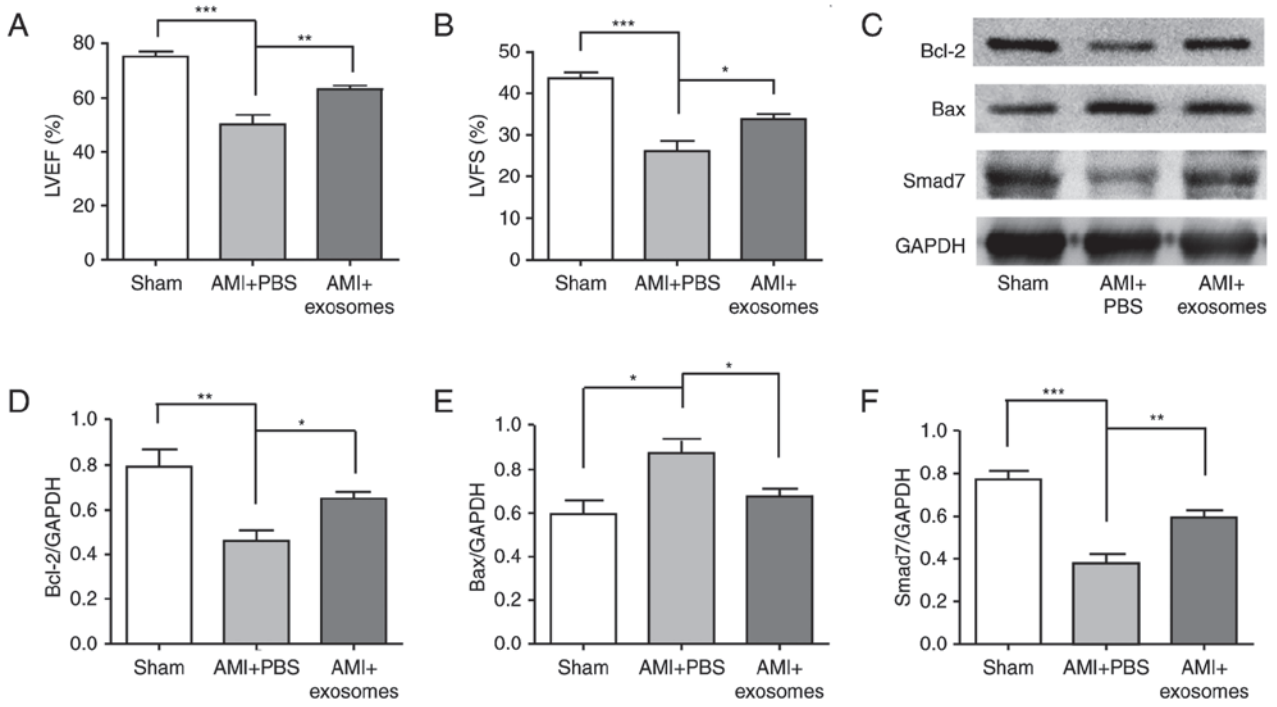


Figure 2. Cardiac systolic functions of AMI rats following various treatments and the cardioprotective effect of hucMSC-exosomes *in vivo*. Cardiac systolic function-associated parameters: (A) LVEF, (B) LVFS, (C) Western blot was used to detect markers and densitometry analysis was performed to semi-quantify, (D) Bcl-2, (E) Bax and (F) Smad7 protein expression in different groups. HucMSC-exosomes exhibited a cardioprotective effect *in vivo* and it may improve cardiac systolic function of AMI rats. *P<0.05, **P<0.01 and ***P<0.001. hucMSC, human umbilical cord mesenchymal stem cell; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening; AMI, acute myocardial infarction; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; Smad7, mothers against decapentaplegic homolog 7.

that purified hucMSC-exosomes expressed exosomal markers, including CD9, CD63 and CD81 (Fig. 1D).

HucMSC-exosomes improve cardiac systolic function, protected cardiomyocytes and upregulated the expression of Smad7. The cardiac systolic function was analysed using echocardiography at 4 weeks after AMI. Left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) reflected cardiac systolic function. Compared with sham group, LVEF and LVFS were both significantly reduced in AMI + PBS group (P<0.001), and were both significantly increased in AMI + exosomes group compared with in the AMI + PBS group (Fig. 2A and B). The cardioprotective effects of hucMSC-exosomes were analysed; Bax and Bcl-2 protein expression levels in myocardial tissue were detected by western blotting. Following LAD ligation for 48 h, Bcl-2 expression was significantly decreased in AMI + PBS group compared with the sham group; this phenomenon was reversed in the AMI + exosomes group. Additionally, Bax expression exhibited the opposite trend to Bcl-2 expression (Fig. 2C-F). Simultaneously, Smad7 expression levels were also significantly decreased in the AMI + PBS group compared with in the sham group, but increased in the AMI + exosomes group. To investigate the cardioprotective effects of hucMSC-exosomes *in vitro*, trypan blue staining of H9C2(2-1) cells was conducted following exposure to hypoxic conditions for 48 h. The number of viable cells was significantly increased in the hypoxia + exosomes group compared with hypoxia + PBS group (Fig. 3A). In addition, the release of LDH in H9C2(2-1) cells' culture media was detected. LDH release was significantly decreased in hypoxia + exosomes group compared with in the hypoxia + PBS group (Fig. 3B). Furthermore, Bax, Bcl-2 and Smad7 protein expression levels *in vitro* followed the same trends as observed *in vivo* (Fig. 3C-F).

HucMSC-exosomes inhibit miR-125b-5p expression in injured cardiomyocytes in vivo and in vitro. The present study reported that Smad7 expression was upregulated in hucMSC-exosomes to repair myocardial injury *in vivo* and *in vitro* (Figs. 2 and 3). Notably, Smad7 is a negative regulator of the TGF-β signaling pathway, which is involved in cardiac remodeling; miRNAs that are relevant to damage repair were investigated in the present study. To identify which miRNAs may regulate Smad7 expression, bioinformatics analyses were conducted using miRBase and TarBase. Three miRNAs (miR-21-5p, miR-92a-3p and miR-125b-5p) (Table II) were selected for further experiments.

As presented in Fig. 4, miR-21-5p, miR-92a-3p and miR-125b-5p expression were significantly altered the *in vivo* model after 2 days (sham group, AMI + PBS group and AMI + exosomes group) and in H9C2(2-1) cells exposed to normoxia or hypoxia for 48 h (normoxia, hypoxia + PBS and hypoxia + exosomes groups). However, only the alterations in miR-125b-5p expression were consistent *in vivo* and *in vitro*. Simultaneously, the results revealed that there was a negative association between Smad7 expression and miR-125b-5p expression.

miR-125b-5p directly targets Smad7. To confirm whether miR-125b-5p directly targeted the 3'UTR of Smad7, 293T cells were used to perform a dual-luciferase reporter gene assay.

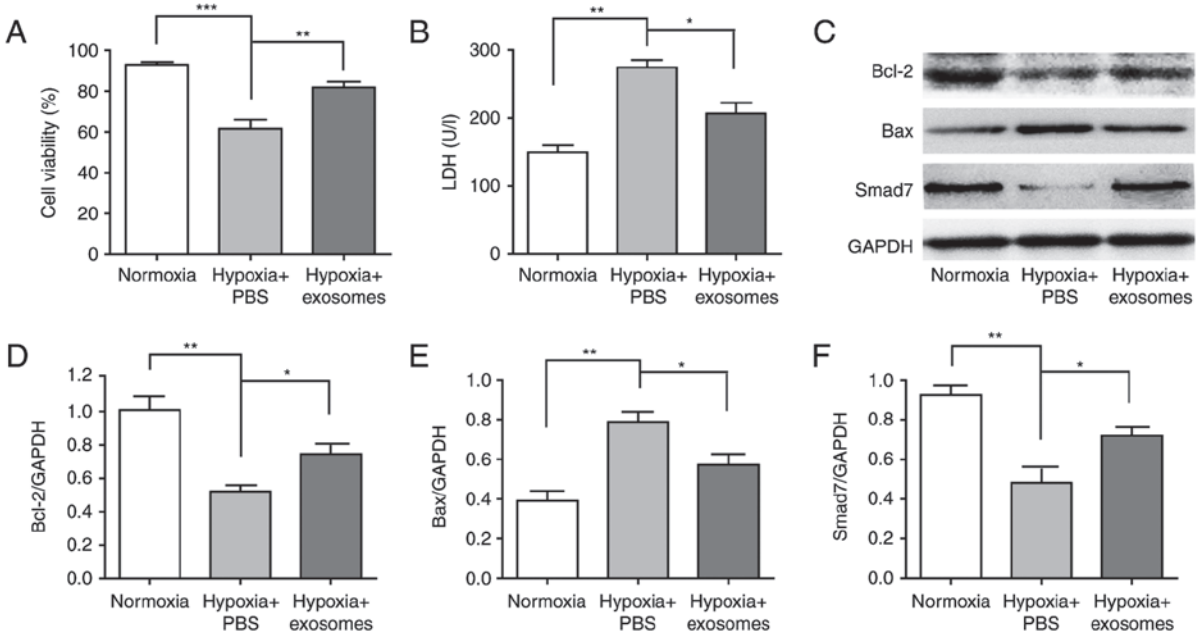


Figure 3. Cardioprotective effect of hucMSC-exosomes *in vitro*. (A) Cell viability was detected by trypan blue staining. (B) Release of LDH was detected with an LDH assay kit. (C) Western blotting and densitometry analysis of (D) Bcl-2, (E) Bax and (F) Smad7 expression in different groups. HucMSC-exosomes exhibited a cardioprotective effect *in vitro*. *P<0.05, **P<0.01 and ***P<0.001. hucMSC, human umbilical cord mesenchymal stem cells; LDH, lactate dehydrogenase; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; Smad7, mothers against decapentaplegic homolog 7.

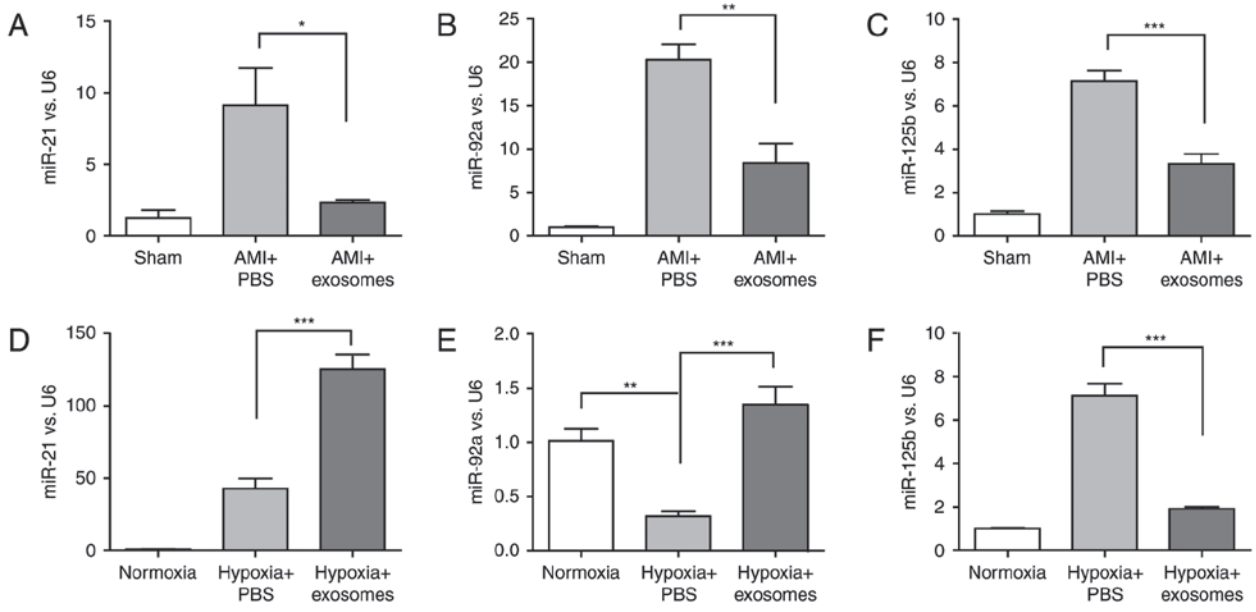


Figure 4. Expression of miRNAs in improving cardiomyocyte injury by hucMSC-exosomes *in vivo* and *in vitro*. Expression of (A) miR-21-5p, (B) miR-92a-3p and (C) miR-125b-5p were detected by RT-qPCR in rat myocardial tissue with different treatments. Expression of (D) miR-21-5p, (E) miR-92a-3p and (F) miR-125b-5p were detected by RT-qPCR in H9C2(2-1) cells with different treatments. miR-125b-5p was selected for further analysis. *P<0.05, **P<0.01 and ***P<0.001. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; AMI, acute myocardial infarction; exosomes, hucMSC-exosomes; hucMSC, human umbilical cord mesenchymal stem cells; miR, microRNA.

The 3'UTR of Smad7 was cloned downstream of a luciferase gene and the predicted binding site of miR-125b-5p in 3'UTR of Smad7 was mutated to generate a mutant plasmid (Fig. 5A). As presented, the overexpression of miR-125b-5p significantly decreased the luciferase activity, whereas transfection miRNA negative control did not (Fig. 5B). Additionally, miR-125b-5p mimics were transfected into 293T cells to verify that miR-125b-5p inhibited Smad7 expression. miR-125b-5p expression was significantly increased in 293T cells following the transfection miR-125b-5p mimics under normoxic conditions (Fig. 5C). Simultaneously, Smad7 protein expression was significantly decreased (Fig. 5D and E).

Subsequently, the cardioprotective effects of hucMSC-exosomes were further investigated. miR-125b-5p expression levels were also significantly increased following transfection with miR-125b-5p mimics in H9C2(2-1) cells for 24 h

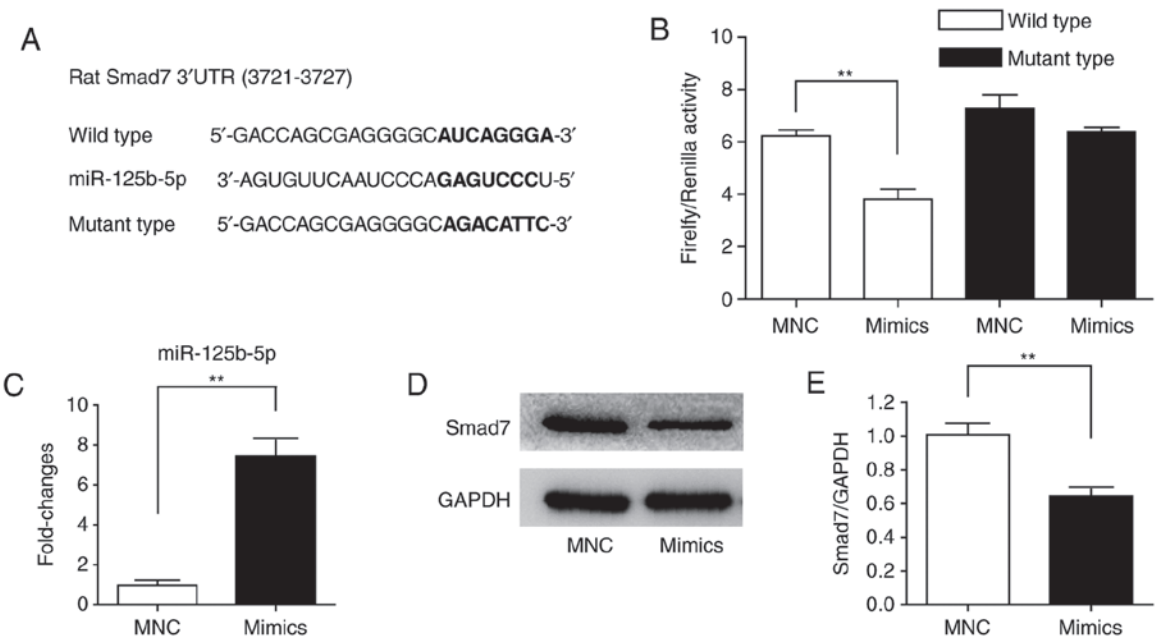


Figure 5. Identification the target gene association between Smad7 and miR-125b-5p. (A) Position and sequences of the predicted binding sites complementary to the seed region of miR-125b-5p in 3'UTR of Smad7 mRNA are presented in bold font. Wild-type, luciferase reporter vector contains the sequences of the predicted binding sites. Mutant type, mutant the predicted binding sites with the sequence in bold font. (B) Luciferase activity assay was performed by co-transfection of 293T cells with a luciferase reporter containing the 3'UTR of rat Smad7 with miR-125b-5p mimics. Luciferase activity was determined 24 h post-transfection. (C) Expression levels of miR-125b-5p were detected by reverse transcription-quantitative polymerase chain reaction in 293T cells following transient transfection miR-125b-5p mimics or MNC. (D) Expression levels of Smad7 were detected by western blotting and (E) semi-quantified according to the immunoblots of 293T cells following transient transfection miR-125b-5p mimics or MNC. **P<0.01; 3'UTR, 3'untranslated region; miR, microRNA; MNC, mimics-negative control; Smad7, mothers against decapentaplegic homolog 7.

(Fig. 6A). Subsequently, the transfected H9C2(2-1) cells were maintained under hypoxia for 48 h. Western blot analysis indicated hypoxia induced cell injury and downregulation of Smad7 expression; hucMSC-exosomes partially alleviated this phenomenon (Fig. 6B-E). The LDH assay revealed that LDH release was increased by miR-125b-5p mimics compared with the MNC + hypoxia group; hucMSC-exosomes also partially alleviated this phenomenon (Fig. 6F). According to these results, H9C2(2-1) apoptosis and injury were increased by hypoxia and miR-125b-5p, and hucMSC-exosomes protected H9C2(2-1) cells to attenuate this effect. These results indicated that miR-125b-5p may be a target gene of Smad7 and that hucMSC-exosomes may promote Smad7 expression via downregulation of miR-125b-5p to improve myocardial repair.

Discussion

An increasing number of studies have confirmed that paracrine effects served a key role in stem cell mediated myocardial repair (8,28-30). Exosomes are bioactive molecules secreted by stem cells that can reduce myocardial injury (9). Our previous studies also reported that hucMSC-exosomes may protect cardiomyocytes from injury, promote angiogenesis and improve cardiac function following AMI (11,12). However, the molecular reparation mechanism of hucMSC-exosomes remains unclear. In the present study, the effect of hucMSC-exosomes in repairing myocardial injury and possible mechanisms were further investigated. The results demonstrated that miR-125b-5p expression was increased in the borderline area of the infraction myocardium and in H9C2(2-1) cells following hypoxia injury, which indicated that hucMSC-exosomes may promote Smad7 expression via the inhibition of miR-125b-5p to improve myocardial repair.

Cell apoptosis and fibrosis have an important role in myocardial injury. Notably, TGF-β/Smad is a classical key cell signaling pathway involved in cardiac fibrosis and apoptosis during myocardial injury (31,32). Smad7, a major negative regulator that of TGF-β/Smad signaling, is be considered a protective protein during myocardial injury. Smad7 expression was reported to be decreased and associated with cardiac fibrosis in the infarcted rat heart (18). Zhang *et al* (15) reported that the upregulation of Smad7 may prevent myocardial apoptosis induced by H/R. Smad7 expression was significantly decreased in the borderline area of the infraction myocardium or H9C2(2-1) cells following 48 h of hypoxia in the present study. Following treatment with hucMSC-exosomes, Smad7 expression was increased compared with the AMI + PBS group or hypoxia + PBS group; simultaneously, myocardial injury and apoptosis were attenuated.

Using bioinformatics analysis, miR-21-5p, miR-92a-3p and miR-125b-5p were selected for verification using RT-qPCR. Changes in miR-21-5p and miR-92a-3p following AMI and exosome treatment demonstrated opposing results *in vitro* and *in vivo*, only miR-125b-5p exhibited consistent results *in vitro* and *in vivo*. These opposing results may be due to two potential factors: i) Myocardial tissue is different to H9C2(2-1) cells, it is a mixture of numerous cell types, in addition to cardiomyocytes, it also includes stromal cells, immune cells and other cells; ii) the environment is different between H9C2(2-1) cells *in vitro* with myocardial tissue *in vivo*, so the effects stimulus may vary to some extent; however, further investigation is required. The experimental results of miR-125b-5p were consistent *in vivo* and

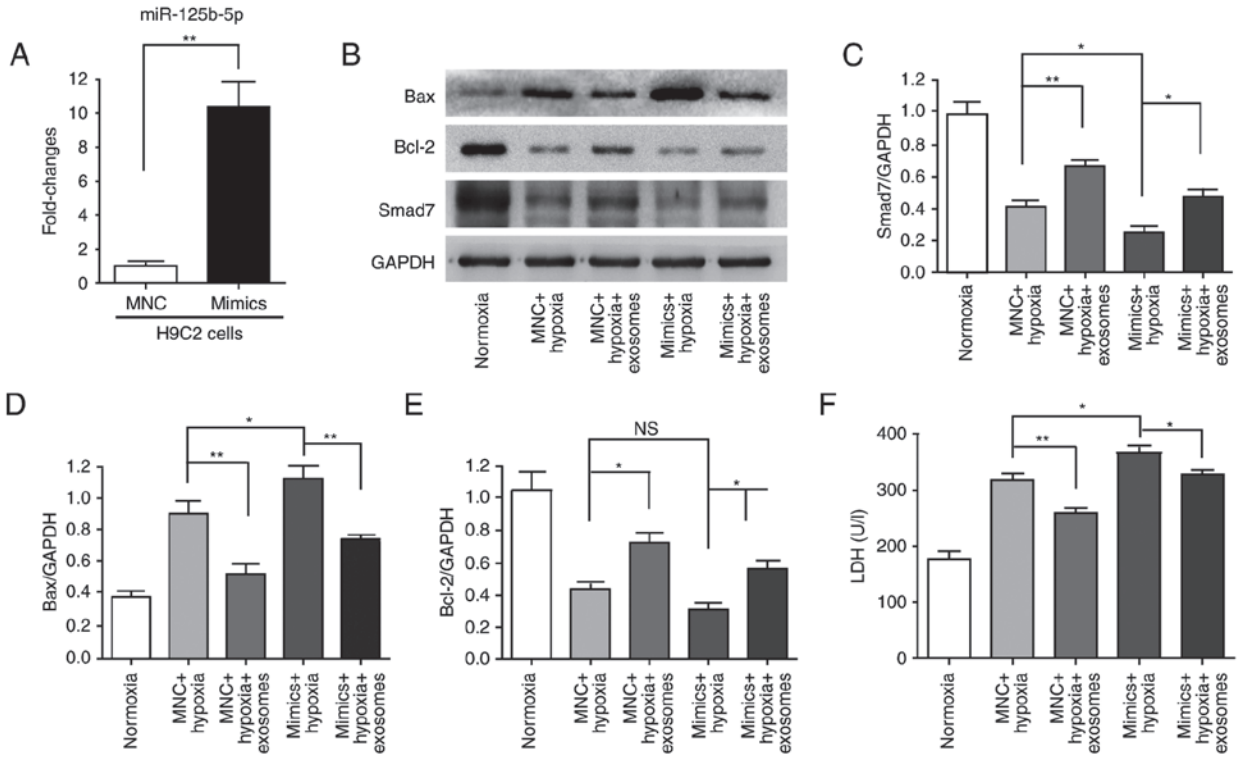


Figure 6. HucMSC-exosomes exhibits cardioprotective effects and increases Smad7 protein expression by inhibiting miR-125b-5p. (A) Expression levels of miR-125b-5p were detected by reverse transcription-quantitative polymerase chain reaction in H9C2(2-1) cells following transient transfection with miR-125b-5p mimics or MNC. (B) Western blotting was used to detect proteins and semi-quantification of (C) Smad7, (D) Bax and (E) Bcl-2 expression in H9C2(2-1) cells transfected with miR-125b-5p mimics were detected by western blotting and semi-quantified according to the immunoblots. (F) Release of LDH in H9C2(2-1) cells transfected with miR-125b-5p mimics was detected by LDH Assay kit. *P<0.05, **P<0.01; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; exosomes, human umbilical cord mesenchymal stem cells-exosomes; MNC, mimics-negative control; LDH, lactate dehydrogenase; Smad7, mothers against decapentaplegic homolog 7.

in vitro, thus it was investigated further. Smad7 is a target gene of miR-125b-5p, as confirmed by the dual luciferase activity assay; miR-125b-5p may inhibit Smad7 expression by binding to the 3'UTR. Studies on the effects of miR-125b have made great progress, particularly in cancer. It may act as a tumor suppressor gene and also as an oncogene; for example, miR-125b weakened the epithelial-mesenchymal transitions phenotype in hepatocellular carcinoma cells (33), whereas, it may also promote tumor growth and favor malignant progression (34). miR-125b-5p has gained increasing attention associated with cardiovascular disease. Busk and Cirera (35) reported that miR-125b was increased in a rat model of early hypertrophic growth of the left ventricle. Jia *et al* (24) reported that miR-125b-5p expression levels were higher in patients with AMI than in healthy subjects and higher expression levels may be of diagnostic value for early diagnosis of AMI. Therefore, it may be an important regulator of the development of cardiovascular disease.

In the present study, miR-125b-5p expression was significantly increased in the borderline area of the infraction myocardium and in H9C2(2-1) cells following hypoxia for 48 h, whereas Smad7 expression was decreased. Following treatment with hucMSC-exosomes *in vivo* or *in vitro*, this phenomenon revealed a significant change: miR-125b-5p expression was decreased while Smad7 expression was increased compared with in the AMI + PBS or hypoxia + PBS groups, respectively. To further investigate the effects of hucMSC-exosomes on miR-125b-5p/Smad7, H9C2(2-1) cells, transfected with miR-125b-5p mimics, were exposed to hypoxia for 48 h. The present study reported that Bax expression was markedly increased and Smad7 expression was further decreased compared with in the MNC + hypoxia group. Additionally, hucMSC-exosomes attenuated this phenomenon. However, some problems require further investigation. miR-125b-5p has numerous target genes; it is unclear whether Smad7 be the most important miR-125b-5p target gene in cardiomyocytes. In addition, how hucMSC-exosomes induce miR-125b-5p-mediated downregulation remains unknown. hucMSC-exosomes may contain a competing endogenous RNA, for example, long noncoding RNA, which may competitively bind miR-125b-5p to facilitate Smad7 expression in cardiomyocytes. However, this hypothesis requires further investigation in the future.

In conclusion, the findings of the present study demonstrated that hucMSC-exosomes may promote Smad7 expression by inhibiting miR-125b-5p to improve myocardial repair. The results of the present study provide novel insight into the mechanisms of myocardial repair following AMI.

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Availability of data and materials

All data and materials are available from the corresponding author on reasonable request.

Authors' contributions

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript. XLW and YYZ conducted the experiments and performed the statistical analysis. LS, YS and ZQL performed the statistical analysis. XDZ, CGX and HGJ designed the study. MW and WRX designed the study and performed the statistical analysis. WZ designed the study, provided financial support and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Laboratory Animal Management Committee of Jiangsu University (Zhenjiang, China). The collection and use of hucMSCs was approved by the Ethics Committee of Jiangsu University (Zhenjiang, China). All individuals provided informed consent for the use of their tissue in the present study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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