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Circular RNA 0001273 in exosomes derived from human umbilical cord mesenchymal stem cells (UMSCs) in myocardial infarction

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Abstract. – OBJECTIVE: To investigate whether human umbilical cord mesenchymal stem cells (UMSCs) derived exosomes (exosome) can repair the heart after myocardial infarction (MI) by delivering circ-0001273 and its mechanism.

MATERIALS AND METHODS: Through the Sprague Dawley (SD) rat MI model was established, at the same time, we designed si-circ-0001273. Phosphate-buffered saline (PBS), exosome and si-circ-0001273-exosome were transplanted into ischemic hearts of rat, respectively. Through the echocardiography, hematoxylin-eosin staining (HE) method to detect the rat heart recovery. Meanwhile, H9c2 was treated with hypoxic serum-free serum to construct an *in vitro* apoptosis model to further explore the effect of circ-0001273 on myocardial cell apoptosis.

RESULTS: Compared with the exosome-treated group, the left ventricular ejection fraction (EF) and shortened fraction (FS) of the rat heart was remarkably reduced and the cardiac structure was more disordered in the si-circ-0001273-exosome-treated group. Meanwhile, *in vitro* TUNEL staining and flow cytometry detection, results showed that compared with the exosome co-culture group, the incidence of H9C2 cell apoptosis in the si-circ-0001273-exosome co-culture group was obviously increased.

CONCLUSIONS: Circ-0001273 can remarkably inhibit the occurrence of myocardial cell apoptosis in ischemic environment, promote MI repair, and provide a good reference for clinical treatment.

Key Words:
Human umbilical cord mesenchymal stem cells, Circ-0001273, Myocardial infarction, Apoptosis.

Introduction

Acute myocardial infarction (AMI) is one of the most common clinical emergencies due to rapid and persistent hypoxia and ischemia in coronary arteries caused by human or non-human factors[1]. Due to its high mortality and poor prognosis, it has posed a serious threat to people's health. At present, the treatment methods for MI are diversified, mainly including drug therapy, coronary artery bypass and interventional therapy, stem cell transplantation[2,3] and other drug therapy, coronary artery intervention and other routine treatment. Although to a certain extent, the pain of patients has been relieved, but they cannot fundamentally restore the damaged myocardium. New research shows that stem cell transplantation, as an emerging clinical frontier, can increase the number of active cardiac cells and promote angiogenesis to improve the blood supply to the heart, thus improving the damaged cardiac function[4,5]. As a kind of pluripotent stem cell-mesenchymal stem cells (MSCs) has a high degree of self-renewal, multi-direction differentiation potential, and a wide range of sources[6]. It can be rapidly amplified *in vitro* and is the first choice of seed cells for stem cell therapy. Clinical trials have indicated the good safety and effectiveness of MSC[7]. However, the problems of MSC themselves, such as low retention rate and low cell survival rate, are still the main obstacles faced by stem cells in clinical application[8]. Therefore, alternative treatment based on cell-free "stem cells" is the preferred strategy.

Exosomes are lipid membranous vesicles secreted by cells with a diameter of 30-100 nm. As a car-

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rier of biological information, exosomes play a certain role in physiological and pathological processes such as immune response[9], cell apoptosis[10], angiogenesis[11] and inflammatory response[12]. Exosome secreted by stem cells was found to reduce myocardial cell apoptosis[13] and promote the proliferation and differentiation of myocardial cells and vascular endothelial cells in the ischemic environment[14,15]. Exosome myocardial protection effects emerge slowly, at the same time, the mechanism of myocardial repair, angiogenesis is the focus of research. The role of heat shock protein-related pathways, apoptotic protein family regulation mechanism, non-coding RNA (ncRNA) transmission and regulation is also becoming more and more important[16], which provides a new biomolecular treatment for MI.

Circular RNA (circRNA) is a kind of endogenous ncRNA emerging after micro RNA (miRNA) and long non-coding RNA (lncRNA). It has gradually received widespread attention in recent years and is a hot spot in current RNA research. CircRNA is obviously different from linear RNA. CircRNA mainly exists in a circular structure and is not easily degraded by exonuclease. The expression is more stable, and the half-life is longer[17]. CircRNA is involved in the development of cardiovascular disease[18]. It plays a certain role, and it has great potential in regulating cardiovascular disease, suggesting that it will have broader application prospects in clinical diagnosis and treatment. Exosome contains more circRNA than its source cells, but there are few reports on the function of circRNA in exosomes, and there is almost no literature. Therefore, it will be of greater research value to explore the regulatory role of circRNA in exosome.

Recently, it has been found that circ-0001273 is highly expressed in oral squamous cell carcinoma and other disease models, which can promote cell growth. However, its role in damaged myocardial repair, cardiac protection and regeneration and its regulatory mechanism have been rarely reported. Therefore, this study intends to explore the influence of circ-0001273 in exosome on myocardial repair and regeneration after MI, and further study the molecular mechanism of its regulation.

Materials and Methods

Experimental Animals and Preparation of MI Models

This investigation was approved by the Animal Ethics Committee of Qingdao Central Hospital Animal Center. Sprague Dawley (SD) rats, males, 8-12 weeks old, 220-240 g, used for making MI model, purchased from Huafukang Biotechnology, and then raised in the Animal Experiment Center of Qingdao Central Hospital. SD rats were fasted for 12 h before operation, and anesthetized by pentobarbital sodium (Tianpu Biochemical Pharmaceutical, Guangzhou, China) 3.5 mg/100 g body weight after intraperitoneal injection. The rats were fixed in the supine position on the operating table, and the hair on the chest was cut. The left margin of the sternum was disinfected by iodophor 3 mm. Cut the skin longitudinally (the upper boundary was the line connecting the posterior edges of the forelimbs and the lower boundary was the fifth intercostal space) to expose the heart. Then, we found the left anterior descending coronary artery from the pulmonary cone and the left atrium, and then the left anterior descending branch of the rat was ligated with the prepared 7-0 line (Jing Ke, Hefei, China), the chest cavity was closed after surgery, and adjacent ribs were sutured continuously. An electrocardiogram was performed to determine whether the model of MI rats was successful, and anti-infective treatment was performed for 7 days. Except that the sham operation group was not ligated, all the surgical procedures were consistent with the MI group. After the operation, rats were returned to their cages and kept under constant temperature (22±2°C) and constant humidity (55±5%).

Echocardiography

Rats were anesthetized with 4% sodium pentobarbital by intraperitoneal injection at a dose of 3.5 mg/100 g, and the epicardial area was depilated. Staff of the Animal Experiment Center of Suzhou University used high-frequency ultrasound imaging system (Nuohai Life Science, Shanghai, China) to detect cardiac function indicators: Left Ventricular End Diastolic Volume (EDV), Left Ventricular End Systolic Volume (ESV), Left End Ventricular Diastolic Diameter ((LVIDd), Left Ventricular Diastolic Diameter (LVIDs), Left Ventricular Mass (LV Mass) Shortening rate (LVFS), left ventricular ejection fraction (LVEF). The calculation formula was LVEF = (EDV - ESV)/EDV $_*$ 100%, LVFS = (LVIDd - LVIDs) / LVIDd $_*$ 100%. Each parameter was measured three times and the average value was recorded.

Hematoxylin-Eosin Staining (HE)

Heart tissue was taken and fixed with 10% paraformaldehyde for 48 h, then embedded in paraffin, and then paraffin sections with a thick-

ness of 5 μm were made, and then stained with a HE staining kit (Jian Cheng, Nanjing, China), and the morphological changes of the heart tissue were observed under a microscope (Olympus, Tokyo, Japan).

Cell Culture and Cell Processing

The complete medium required for the growth of human umbilical cord mesenchymal stem cells (UMSCs) (American Type Culture Collection, Manassas, VA, USA) was composed of 4 components: α-minimum Eagle's medium (α-MEM) basic medium (Life Technology, Wuhan, China), 20% fetal bovine serum (FBS; Life Technology, Wuhan, China), and double antibodies (100 U mL penicillin and 0.1 mg / mL, streptomycin; Life Technology, Wuhan, China), L-glutamine (Life Technology, Wuhan, China). The complete medium required for H9c2 cell (American Type Culture Collection, Manassas, VA, USA) growth consists of 4 components: Dulbecco's Modified Eagle's Medium (DMEM; Life Technology, Wuhan, China) basic medium, 10% FBS, double antibody (100 U/mL penicillin and 0.1 mg/mL, streptomycin), L-glutamine. The cells were all cultured in a constant temperature incubator at 37°C and 5% CO_2. The cell culture medium was changed every 2 days. The cells in the hypoxic treatment group were cultured at 37°C in a cell incubator containing 50% CO_2 and 1% O_2. Construction of H9C2 apoptosis model: the experiment was divided into three groups: control, exosome treatment group, and si-circ-0001273 + exosome treatment group. When the cell density reaches about 60% -70%, replaced the DMEM complete medium with DMEM serum-free medium, and added the same amount of exosome to the medium and put them into a hypoxic incubator. After 48 h incubation, cells were collected.

Collection and Identification of Exosome

Took the P3 generation UMSCs in good growth state, cultured the cells for 48 h with α-MEM complete medium containing 10% Exo-free FBS, then, collected the culture supernatant and centrifuged at 2000 g for 20 min. Discarded the cell debris and added 1/2 volume of exosome separation reagent to the cell supernatant for concentration, turned it upside down to mix thoroughly, and concentrated in a 4°C refrigerator overnight. On the second day, centrifuged the concentrated mixture for 1 h and discarded the supernatant. And resuspend the exosome pellet with an appropriate amount of 1×phosphate-buffered saline (PBS), and stored at -80°C.

Flow Cytometry

The concentration of exosome was measured by the bicinchoninic acid (BCA) protein concentration quantification kit (Jian Cheng, Nanjing, China), and 10 μg of exosome was used for flow cytometry identification. 10 μL of latex microsphere particles (Kaiji, Nanjing, China) were added to the 10 μg exosome suspension and incubated at room temperature for 15 min; 1× PBS was added to the Eppendorf (EP) tube to 1 mL, continued incubation at room temperature. After 2 h, added a certain volume of glycine (1 mol/L) (Kaiji, Nanjing, China) to the Eppendorf (EP; Hamburg, Germany) tube and incubated at room temperature for 30 min; centrifuged at 4 000 r/min for 3 min, and discarded the supernatant. After washing the exosome precipitation twice with 0.5% bovine serum albumin (BSA) solution, resuspend the exosome with 100 μL of 0.5% BSA solution, and then added 10 μL of CD63 antibody (FITC-labeled, Abcam, Cambridge, MA, USA), and incubated at 4°C for 30 minutes in the dark. 5% BSA solution was continued to wash the exosome precipitation twice, 200 μL 0.5% BSA solution was added to the exosome precipitation, and finally detected using a flow cytometer (Guava, Austin, TX, USA).

UMSCs-Exos Uptake

4 mg / mL Dil solution (molecular probe) was diluted with PBS (1: 2000) and cultured according to the manufacturer's instructions (Kaiji, Nanjing, China). Ultracentrifugation method was used to remove the excess dye in the labeled exosomes, and it was centrifuged at 100,000 × g for 1 h at 4°C. Then resuspended and washed three times in PBS. Dil-Exos was cultured with H9C2 cells for 24 hours, and then the cells were washed with PBS. The cells were fixed with 4% paraformaldehyde and observed with a fluorescence microscope (BIO, Minneapolis, MN, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Extracted the total RNA, and added 1 μg of total RNA and 11 μL of Nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA) to the RNase-free PCR tube, mixed and incubated at 85°C for 5 min to denature the RNA, and then immediately placed it on ice to prevent RNA refolding. Added 0.5 μL Oligo (dT) (Thermo Fisher

Scientific, Waltham, MA, USA), 0.5 μL Random primer (Thermo Fisher Scientific, Waltham, MA, USA), 2 μL 10 mM dNTP (Thermo Fisher Scientific, Waltham, MA, USA), RNase inhibitor (Thermo Fisher Scientific, Waltham, MA, USA), 5× buffer (Thermo Fisher Scientific, Waltham, MA, USA) and M-MLV (Thermo Fisher Scientific, Waltham, MA, USA) to this PCR tube, and perform reverse transcription after mixing: 30°C for 10 min, 42°C for 60 min, 85°C for 10 min. After reverse transcription was completed, the reaction conditions were set on a PCR instrument at 50°C for 2 min, 95°C for 2 min, 95°C for 15 s, 60°C for 32 s with 40 cycles, and the melting curve was analyzed at 60°C-95°C. Endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Thermo Fisher Scientific, Waltham, MA, USA) was used to standardize the data. The comparative threshold cycle (Ct) method, that was, the $2^{-\Delta\Delta Ct}$ method was used to calculate fold amplification. Primers used were shown in Table I.

Western Blotting Technology

We used protein lysis (Camilo Biological, Nanjing, China) to fully lyse the cells and extracted the total protein from the cells. Added protein loading buffer (Camilo Biological, Nanjing, China) and mixed for 10 minutes in boiling water. Took 15 μg protein samples, and the protein was separated using a 10% sodium dodecyl sulfate-polyacrylamide gel. Then, the dispersed protein was then transferred to a polyvinylidene difluoride (PVDF, Thermo Fisher Scientific, Waltham, MA, USA) membrane for 2 h. And blocked with 8% skim milk for 2 h at room temperature, and then the membrane was incubated with primary antibodies at 4°C overnight. Then added sheep anti-rabbit secondary antibody (Yifei Xue Biotechnology, Nanjing, China, 1:3000) and incubated at room temperature for 2 h. The enhanced chemiluminescence (ECL) kit (Yifei Xue Biotechnology, Nanjing, China) was used for chemiluminescence development, and ImageJ software was used for semi-quantitative analysis. Primary antibodies were as follows: (CD9, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; CD63, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; CD81, Abcam, Cambridge, MA, USA, Mouse, 1:2000; Calnexin, Abcam, Cambridge, MA, USA, Mouse, 1:5000).

Annexin V Detects Apoptosis

Apoptosis was detected using Annexin V-FITC apoptosis flow cytometry kit (BD Pharmingen, Shanghai, China). 100 μL of 1× Binding Buffer was used to resuspend the cells to prepare a cell suspension. 5 μL of Annexin V-FITC antibody and 5 μL propidine iodide (PI) were added to the cell suspension, respectively, and stained at room temperature for 15 minutes in the dark, then added 400 μL of 1x Binding Buffer, mix gently, and then tested on the machine.

Terminal Dexynucleotidyl Transferase (TdT)-Mediated dUTP Nick End Labeling (TUNEL) Staining

TUNEL staining kit (Elabscience, Wuhan, China) was used to detect cell apoptosis. H9C2 cells in logarithmic growth phase were plated in 24-well plates at a density of $5X10^4$ / well. When the cell density reached about 60%, cultured with 400 μg/mL of exosome. After 48 h, the cells were fixed and permeabilized. About 50 μL of TdT incubation buffer was added to each well and incubated at 37°C in the dark for 1 h. After washing with a PBS for 5 minutes, 4',6-diamidino-2-phenylindole (DAPI) staining (Sigma-Aldrich, St. Louis, MO, USA) was performed, stained at room temperature for 5 min, and then observed the apoptosis of the cells by fluorescence microscopy (BIO, Minneapolis, MN, USA).

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA). Measurement

Table I. Real Time-PCR primers.

Gene name	Forward (5'>3')	Reverse (5'>3')
Circ-0001273	CCACCTCCAATGACAGACTT	CACACCTTAGCTGCTGACAC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

qRT-PCR, quantitative Reverse Transcription-Polymerase Chain Reaction.

data were expressed by mean ± SD (standard deviation), comparison between groups, and independent *t*-test. Comparisons between groups were performed using analysis of variance. The difference was statistically significant at $p<0.05$.

Results

Identification of UMSCs - Exos

To investigate the role of circ-0001273 in exosome derived from UMSCs, we first observed the morphology of exosomes through transmission electron microscope. The morphology of exosomes were round or oval with a bilayer membrane structure (Figure 1A). The source exosome was isolated and the expression of the marker CD63 protein molecule in exosome was identified by flow cytometry (Figure 1B). In addition, Western blotting results showed that the specific exosome surface markers CD9, CD63 and CD81 were positive (Figure 1C), and further confirmed the presence of exosomes.

Circ-0001273 Expression in Exosomes

The expression level of Circ-0001273 in UMSCs and its source exosome was detected by qRT-PCR, and the results showed that there was a positive correlation between the expression of circ-0001273 in UMSCs and exosomes. That is, when the expression level of circ-0001273 is higher in UMSCs, its corresponding exosome is also relatively higher, and at each time point, circ-0001273 in exosomes was higher than circ-0001273 in cells. Among them, when the circ-0001273 in UMSCs and exosome were at 12 h, the expression level was the highest (Figure 2A). However, since the amount of exosome secreted by UMSCs was relatively less at 12 h, we used 48 h as the culture time point of UMSCs in our study. In order to further investigate whether UMSCs-exos can be absorbed by H9c2 cells, Dil-exos was co-incubated with H9c2 cells *in vitro* and its absorption was observed by fluorescence microscope. After incubation for 12 and 24 h, Dil-exos was observed to have been absorbed by H9c2 cells and transferred to the cytoplasm (Figure 2B, 2C). Taken together, these data suggest that UMSCs-exos can be absorbed by H9c2 cells.

Exosomes Promote MI Repair with Circ-0001273

When we collected exosome, we also extracted RNA from the ischemic myocardium of rats 1, 3, and 7 days after MI, and detected the expression of circ-0001273. It has significant statistical significance (Figure 3A). In order to investigate whether circ-0001273, which is highly expressed in exosome, has a therapeutic effect on MI, we designed siRNA and tested the expression level of circ-0001273 in UMSCs transfected with siRNA by qPCR. The results are shown in (Figure 3B): after siRNA treatment, the expression of circ-0001273 in UMSCs was significantly down-regulated. At the same time, we collected exosome secreted by siRNA transfected UMSCs, and detected the expression level of circ-0001273,

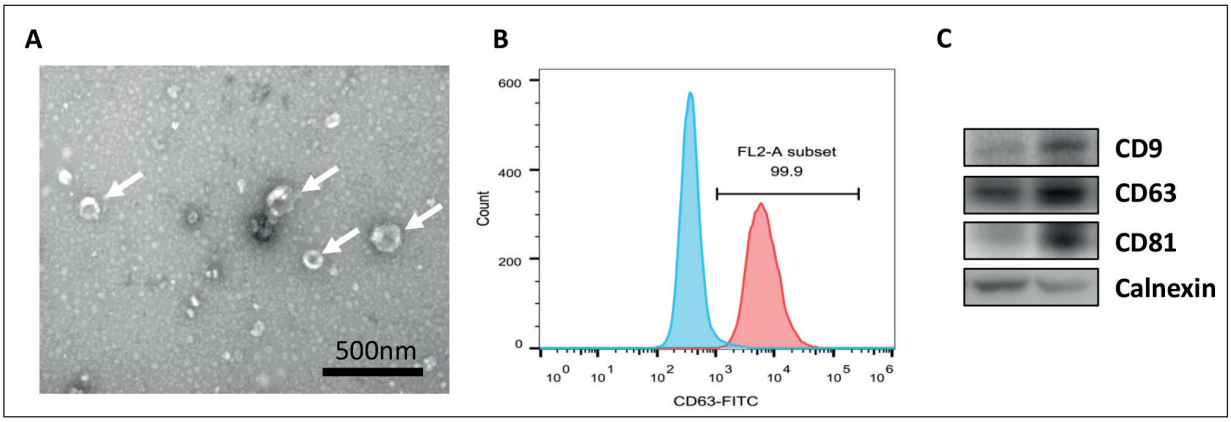

Figure 1. Identification of UMSCs – Exos. **A,** The morphology of exosomes were observed using TEM. The white arrow indicates exosome. Scale bar, 500 nm. **B,** CD63 expression on exosome surface was detected by flow cytometry. **C,** Western blot of specific exosome surface markers.

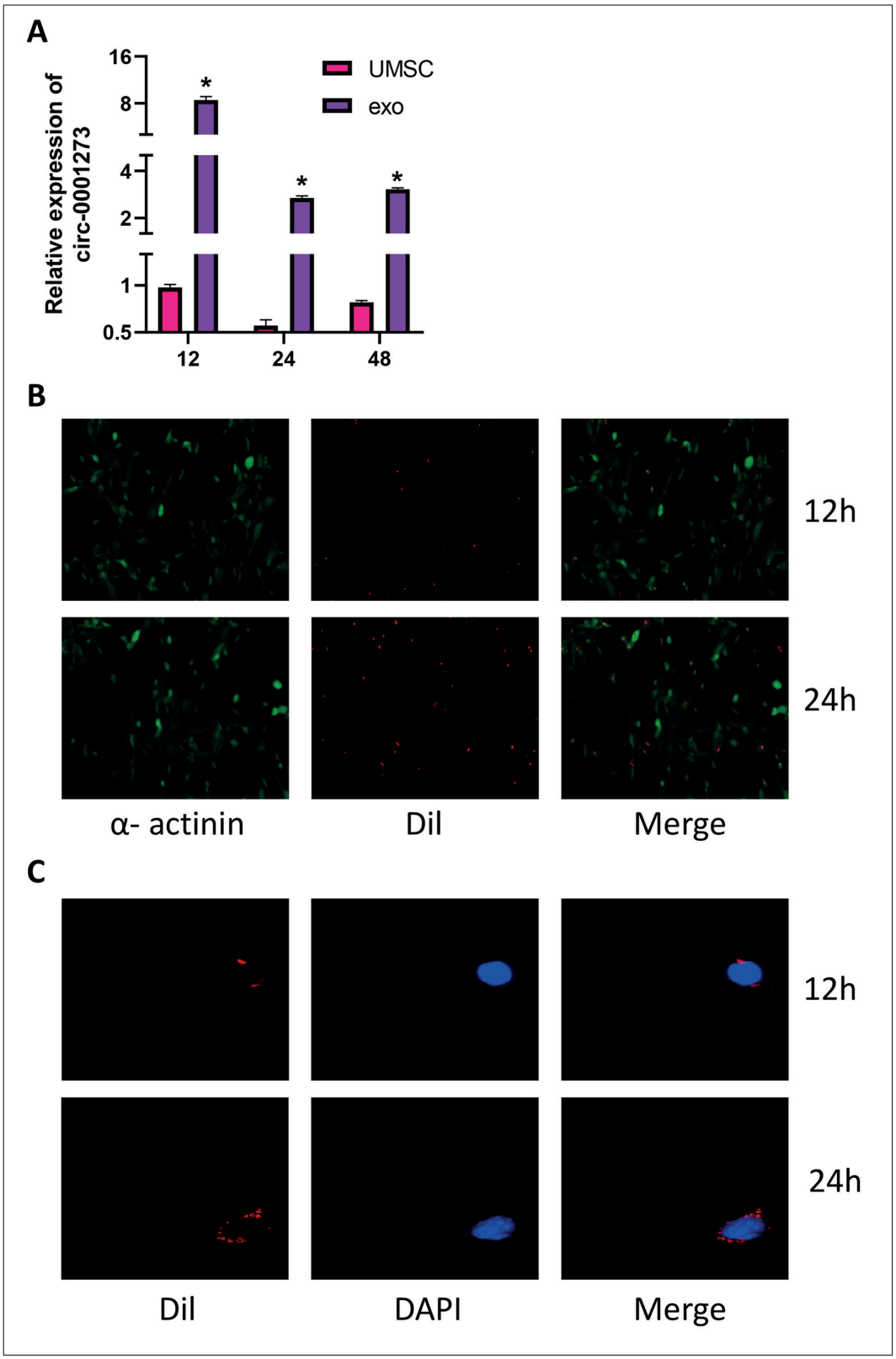

Figure 2. Circ-0001273 expression in exosomes. **A,** The expression of circ-0001273 in UMSCs and UMSCs-derived exosomes in 12 h, 24 h, 48 h. ("*" indicates that compared with the UMSCs-12h group $p < 0.05$). **B-C,** Uptake of DiI-labeled BMSC-Exos by H9C2 cells after 12 h and 24 h. (magnification: 40×).

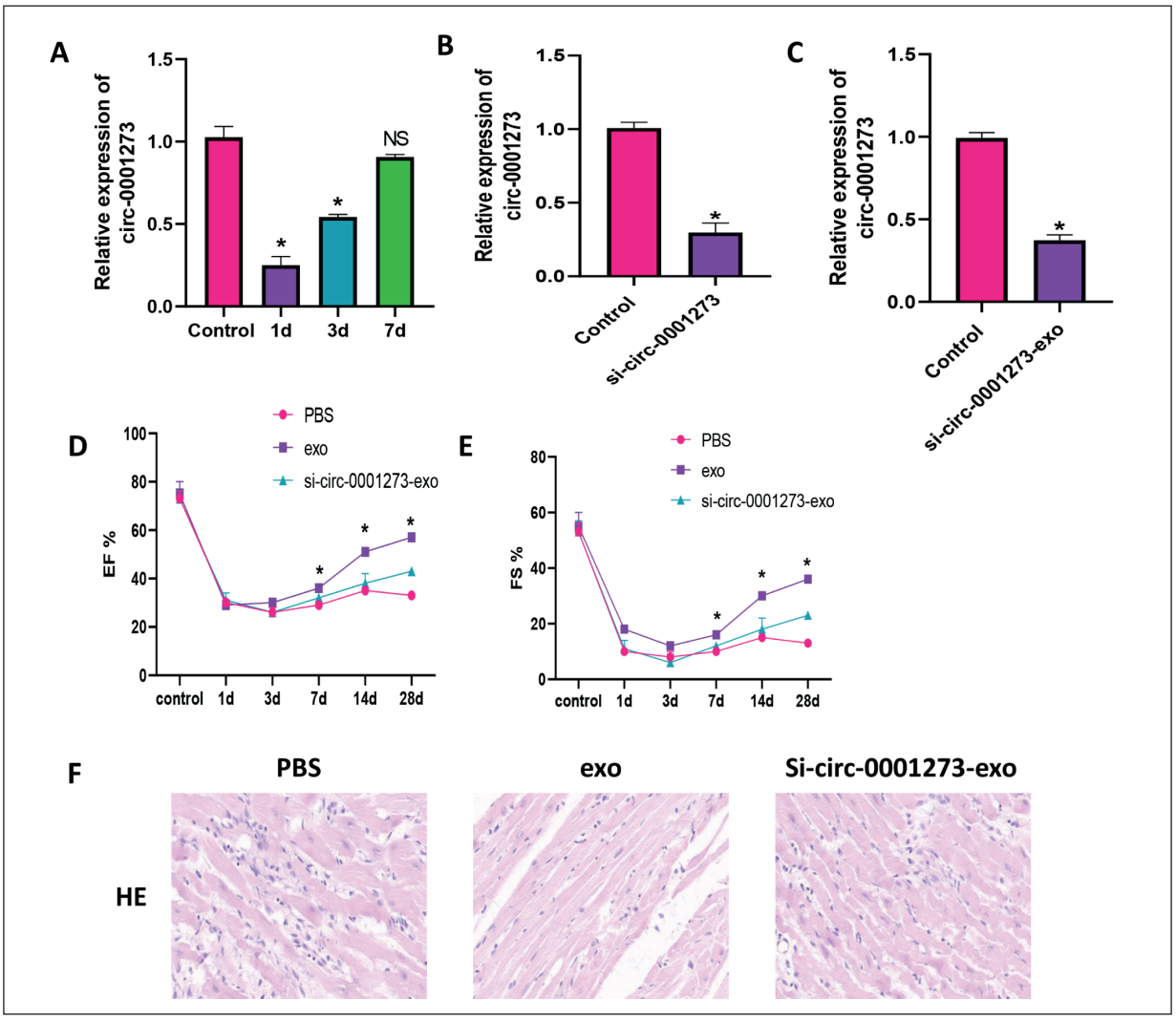

Figure 3. Exosomes promote MI repair with circ-0001273. **A,** PCR was used to detect changes in circ-0001273 expression levels before and after myocardial infarction at day 1,3,7 ("*" indicates that compared with the Control group $p < 0.05$). **B,** PCR was used to detect changes in circ-0001273 expression levels in control and si-circ-0001273 groups in UMSCs ("*" indicates that compared with the Control group $p < 0.05$). **C,** PCR was used to detect changes in circ-0001273 expression levels in control and si-circ-0001273 groups in UMSCs-derived exosomes ("*" indicates that compared with the Control group $p < 0.05$). **D-E,** The statistical results of left ventricular ejection fraction (EF) and shortening fraction (FS) were detected on day 1,3,7,14, and 28 after surgery ("*" indicates that compared with the PBS group $p < 0.05$). **F,** HE staining of myocardial tissues (magnification: 400×).

and found that the expression of circ-0001273 in exosome was also markedly reduced (Figure 3C). Next, we constructed a rat model of MI by injecting 100 μL PBS, 100μL exosome, si-circ-0001273 exosome (5 μg/L, 100 μ in total) into the myocardium, and 1, 3, 7, 14, 28 days after MI, the cardiac function of rats in different treatment groups was detected by ultrasound system. The results showed that there was no significant difference with LVEF and LVFS between the 3 groups before surgery and 1 day after MI. However, on the 7th day after MI operation, compared with the si-circ-0001273 treatment group and the PBS control group, the LVEF and LVFS of the exosome treatment group were obviously increased (Figure 3D and 3E). The HE staining results also showed that the heart structure of the si-circ-0001273 treatment group was more disordered and the cell edema was worse than that of the exosome

group (Figure 3F), indicating that exosome promoted MI repair by transmitting circ-0001273.

Circ-0001273 Repairs MI by Inhibiting Cardiomyocyte Apoptosis

To explore how circ-0001273 can play a role in repairing MI, we first detected the H9c2 cells transfected with si-circ-0001273 by qPCR, and the results showed that the expression of circ-0001273 in the si-circ-0001273 group was obviously reduced (Figure 4A). Next, PBS, exosome, and si-circ-0001273 exosome were co-cultured with H9c2 cells in a hypoxic environment for 48 h, and then the apoptosis of H9c2 cells was detected by TUNEL staining and flow cytometry. The results showed that compared with the exosome group, the protective effect of the si-circ-0001273 exosome group was obviously reduced (Figure 4B, 4C). It can be seen that circ-0001273 in exosome can promote the repair of MI by inhibiting the occurrence of myocardial cell apoptosis.

Discussion

Our experimental results show that circ-0001273 is more abundantly expressed in UMSCs and its derived exosome, and it is significantly down-regulated in rat heart after MI. So, we designed si-circ-0001273 to down-regulate UMSCs-derived exosome circRNA, the results show that exosome can inhibit cardiomyocyte apoptosis and promote repair and regeneration of damaged myocardium after MI by delivering circ-0001273.

Paracrine action is the main way for MSCs to exert therapeutic effects. Exosome, as one of the main components of paracrine, contains a variety of functional molecules such as mRNA, miRNA, circRNA, and protein. It also plays a critical role in signal transmission[19] and cell communication[20]. Many studies have reported that exosome derived from MSCs can promote cardiac repair after MI[21], but it is unclear whether exosome can completely replace MSCs for cardiac repair. The molecular mechanism of MSCs-derived exosome to mediate

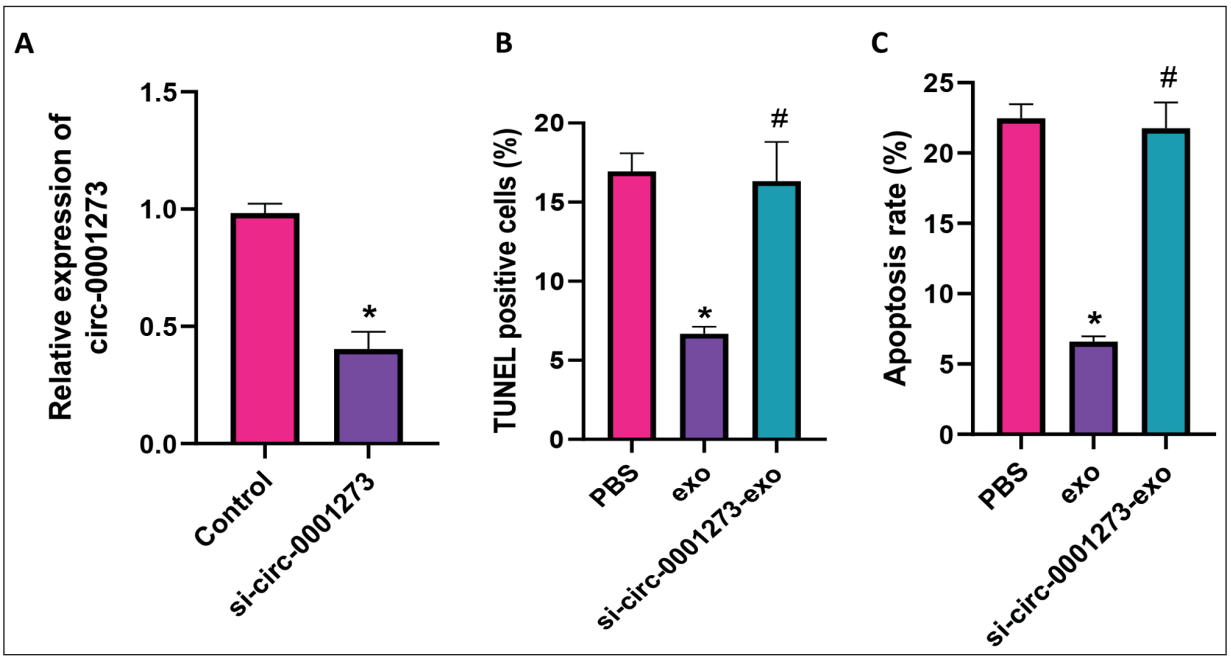

Figure 4. Circ-0001273 repairs MI by inhibiting cardiomyocyte apoptosis. **A,** PCR was used to detect changes in circ-0001273 expression levels in control and si-circ-0001273 groups in H9C2 cells ("*" indicates that compared with the Control group $p < 0.05$). **B,** Statistical results of TUNEL staining in H9C2 cells with PBS, exo, si-circ-0001273-exo. and ("*" indicates that compared with the PBS group, "#" indicates that compared with exo group $p < 0.05$). **C,** Statistical results of flow cytometary inH9C2 cells with PBS, exo, si-circ-0001273-exo ("*" indicates that compared with the PBS group, "#" indicates that compared with exo group $p < 0.05$).

heart repair needs to be further explored. CircRNA was thought to be a type of gene-splicing byproduct in the early days without actual effect, but it is now found to be related to many human diseases, such as osteoarthritis[22], autoimmune disease[23] and atherosclerosis[24]. CircRNA can absorb miRNAs in a sponge manner to prevent the inhibitory effect of miRNA on mRNA. Exosomes, especially in the regulation of circRNA, are blank. Our research direction just fills the gaps in this aspect of the mechanism and provides a new perspective for clinical investigation. Exosomes in combination with circRNAs for their peculiarity and high specificity can increase the potential use of both exosomes and circRNAs as markers of diagnosis and prognosis for many diseases. Exosome transportation was regarded as a reliable communication method between different cells. Our research showed that circ-0001273 functions as a protective factor in MI and has some special characteristics compared to other MI-related circ-RNA studies.

Conclusions

Summarily, our experimental results show that exosome derived from UMSCs, by delivering circ-0001273, inhibits myocardial cell apoptosis, promotes MI repair, and provides a good reference for cardiac tissue regeneration and repair and clinical application. However, this study did not involve the specific mechanism of circ-0001273 in inhibiting myocardial cell apoptosis and promoting cardiac repair and regeneration, which needs to be further explored in the future.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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