Research Square

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The Protective Role of Exosomal circRNA-0008302 from Adipose-Derived Stem Cells (ADSCs) Against Myocardial Injury

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Research Article

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Abstract

Background

This study aims to explore the expression of circRNA-0008302 in adipose stem cells derived exosome (ADSCs-Exo), and to investigate the function as well as mechanism of circRNA-0008302 in H_2O_2-induced cardiomyocyte injury.

Methods

Differentially expressed circRNAs between ADSCs and ADSCs-Exo were identified by circRNA chip screening, and further validated by qRT-PCR. CircRNA-0008302 siRNA was used to silence circRNA-0008302 expression in M6200 mouse cardiac myocytes. M6200 cells were divided into four groups: M6200 group, M6200 + H_2O_2 group, M6200 + H_2O_2 + ADSCs-Exo group and M6200 + H_2O_2 + ADSCs-Exo$^{circ-0008302\ siRNA}$ group. Thereafter, cell viability was evaluated using CCK8 assay. Apoptosis and intracellular ROS levels were measured by flow cytometry. The expression level of miR-466i-5p was evaluated by qRT-PCR. Western blotting was performed to detect MsrA protein expression.

Results

The expression of circ-0008302 was significantly increased in ADSCs-Exo compared with that in ADSCs. Through the delivery of circ-0008302, ADSCs-Exo maintained the viability, inhibited the apoptosis and reduced ROS production in cardiomyocytes, thereby protecting cardiomyocytes from H_2O_2-induced oxidative injury. Mechanistically, circ-0008302 downregulated the level of miR-466i-5p, and thus promoted the expression of a miR-466i-5p target gene-MsrA in cardiomyocytes.

Conclusion

ADSC-secreted exosomes have a protective role in myocardial injury by delivering circ-0008302, which increased MsrA expression via targeting miR-466i-5p in cardiomyocytes.

Introduction

Cardiovascular disease is one of the leading causes of death and disability worldwide. For the patients with myocardial infarction (MI), although myocardial reperfusion treatments like thrombolysis or percutaneous coronary intervention (PCI) are commonly adopted(1), in many cases these treatments are inefficient for preventing the occurrence of myocardial injury(2).

Stem cells play important roles in the repair and regeneration of damaged tissue, mainly achieved by paracrine secretion of tissue-regenerative factors(3). Recent reports suggested that stem cell-derived

exosomes, which are membrane lipid nanovesicles with a diameter of 30–100 nm and serve as important mediators of intercellular signal transduction, contribute to tissue regeneration by delivering protective microRNAs (miRNAs) and proteins(4, 5).

Adipose-derived stem cells (ADSCs) are a type of stem cells which play pivotal functions in mediating tissue repair(6). ADSCs can secrete subcellular granules with a diameter of 20–100 nm, namely adipose stem cell-derived exosome (ADSCs-Exo). Compared with ADSCs, ADSCs-Exo has many advantages such as high stability as well as low antigenicity, and thus can provide a long-time protection. Therefore, ADSCs-Exo is comprehensively used in tissue damage-related disorders. Our previous studies have found that ADSCs-Exo can protect cardiomyocyte injury(7), but the mechanism of ADSCs-Exo's protection has not been clarified yet.

There are a wide variety of RNAs and proteins in ADSCs-Exo. Among them, circular RNAs (circRNAs) are endogenous non-coding RNA molecules, which have important regulatory roles in gene expression. CircRNAs are produced by back-splicing in which the 5’ end of a pre-mRNA upstream exon is non-colinearly spliced with the 3’ end of a downstream exon. Compared to miRNAs and mRNAs which have linear structure, circRNAs are resistant to exonuclease-mediated degradation since there is no 5’ cap and 3’ tail in their structure(8, 9). Hence, circRNAs are usually more stable than linear RNAs and thus can exert long-time biological effects. Mechanistically, circRNAs can act as ceRNAs to competitively influence the functions of miRNAs and their target genes in various pathological processes(10), including cardiovascular diseases. For example, circRNA_MFACR exacerbated myocardial infarction by upregulating MTP18 expression(11). CircRNA_NCX1 mediated myocardial injury by suppressing the function of miR-133a-3p and thus increasing the expression of CDIP1(12). CircRNA_000203 promoted cardiac hypertrophy by inhibiting miR26b-5p and miR-140-3p binding to Gata4(13). However, to date, the expression profile and therapeutic potential of ADSCs-Exo-derived circRNAs in cardiomyocyte injury are still elusive. In our present study, we screened differentially expressed circRNAs in ADSCs-Exo in order to explore the effects and mechanisms of ADSCs-Exo in cardiomyocyte injury. Hopefully this work can provide experimental basis for the clinical treatment and drug development of cardiomyocyte injury.

Materials And Methods

Differential circRNA screening and expression verification

M6200 cells were divided into experimental group and control group, ADSCs group and ADSCs-Exo group, and the total RNA of the two groups were purified and labeled with fluorescence, and the labeled products were purified. after that, chip hybridization, washing and scanning to obtain the circRNA chip and then validate the circRNAs identified by Quantitative Reverse Transcription PCR (qRT-PCR).

Construction and validation of circRNA siRNA plasmid

The obtained ADSCs-Exosomes and the differentially expressed circRNA in the exosomes were divided into two groups: control plasmid; circ-0008302 siRNA1、circ-0008302 siRNA2 and circ-0008302 siRNA3、at

the same time ,validated the circRNAs identified by Quantitative Reverse Transcription PCR (qRT-PCR)

Effect of ADSCs-Exosomes and differentially expressed circRNA on H_2O_2 -induced cardiomyocyte injury

1、Detection of cell viability by CCK8

M6200 cells in logarithmic growth phase were plated into the 96-well plate with 100 μL DMEM medium per well. After cell adherence, the original medium was removed. According to the instruction, we treated the cells with CCK8 solution 10 μL/ well. and placed in an incubator and incubated for 4 hours. To get the cell viability, we measured the absorbance at 450 nm with a microplate reader.

2、Apoptosis of cells was measured by flow cytometry

M6200 cells were plated into 6-well plates and placed in a 5% CO2 cell incubator at 37℃ for 24 h. After cell adherence, and then the cells were treated according to the grouping principle for 24h, followed by centrifugation, washing and precipitation, and detected by flow cytometry. Finally, we used the CELL Quest software to analyze CELL apoptosis.

3、Detected the intracellular ROS levels, miRNA levels and MsrA expression levels

M6200 cells were plated into 6-well plates and placed in a 5% CO2 cell incubator at 37℃ for 24 hours. After cell adherence, and then the cells were treated according to the grouping principle for 24h, then digested with 0.25% trypsin without EDTA, and collected after termination of digestion. After centrifugation and washing, the ROS levels in each group were detected by flow cytometer. At the same time, qRT-PCR was also used to detect miRNA levels and WB was used to detect MsrA expression levels in cells.

Statistics

Quantitative data were obtained from the indicated number of experiments. The statistical significance of differences between multiple experimental groups was determined by one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

Results

Profiling the differentially expressed circRNAs.

ADSCs-Exo were obtained by using previously published methods(7). Firstly, we compared the circRNA expression profile between ADSCs and ADSCs-Exo using circRNA chip. Seven circRNAs with the most dramatic upregulation in ADSCs-Exo were selected from the differentially expressed circRNAs(Table 1).

Table 1 Differentially expressed circRNAs between ADSCs and ADSCs-Exo

circRNA-ID	hostgene	up/down
circ-0008302	*Ralgps2*	up
circ-0002468	*Mknk2*	up
circ-0002157	*Rnf217*	up
circ-0008982	*Serpine2*	up
circ-0008455	*Rgs7*	up
circ-0010012	*Cacnb4*	up
circ-0009075	*Hdac4*	up
circ0007490	*Dcc*	up

Among those, circ-0008302，host gene-*Ralgps2*，was reported to be highly expressed in the heart and played critical functions in cell proliferation, differentiation, and survival(14, 15); circ-0002468，host gene-*Mknk2*，could not design specific primers to detect; circ-0002157，host gene-*Rnf217*，is associated with poor prognosis of myeloma(16); circ-0008982，host gene- *Serpine2*，was reported to promote myocardial fibrosis(17, 18); circ-0008455，host gene-*Rgs7*，was induced in the dentate gyrus after ischemia(19); circ-0010012，host gene-*Cacnb4*，encodes a calcium channel subunit expressed in the heart and is important for myocardial contraction(20); circ-0009075，host gene-*Hdac4*，is related to myocardial exercise intensity and exercise could enhance HDAC4-NT levels(21); circ0007490，host gene-*Dcc*，is related to central nervous system dysfunction. The mutation of *Dcc* gene was reported to cause abnormal axon signal conduction(22). Based on the above backgrounds, we selected circ-0008302, circ-0010012 and circ-0007490 for the further validation by qRT-PCR.

Compared with the control group, the expression of circ-0007490 and circ-0010012 in ADSCs-Exo group was significantly decreased. In contrast, the expression of circ-0008302 was increased. Therefore, circ-0008302 was selected for the subsequent experiments (Figure 1).

Silencing circ-0008302 expression by siRNA.

To investigate the role of circ-0008302, we transfected M6200 mouse cardiac myocytes with circ-0008302 siRNA. Compared with control group, the expression of circ-0008302 was not significantly changed in cells transfected with control siRNA (siRNA-NC), while the expression of circ-0008302 was significantly decreased in cells transfected with circ-0008302 siRNA1-3, with siRNA2 having the most obvious effect, so the circ-0008302 siRNA2 was used in the subsequent experiments (Figure 2).

The effects of ADSCs-Exo and circ-0008302 in H_2O_2-induced cardiomyocytes injury.

To study the role of ADSCs-Exo and circ-0008302 in H_2O_2-induced cardiomyocyte damage, we tested the cell proliferation and apoptosis after different treatments. Compared with the control group, H_2O_2 treatment resulted in a reduction of viability of M6200 cardiac myocytes, which could be rescued by ADSCs-Exo. However, the protective role of ADSCs-Exo was abrogated by circ-0008302 knockdown (Figure 3A). Compared with the control group, H_2O_2 treatment increased cardiomyocyte apoptosis, while ADSCs-Exo treatment significantly reduced cell apoptosis. However, the anti-apoptotic role of ADSCs-Exo was significantly abrogated by circ-0008302 knockdown (Figure 3B-C). These results suggested that ADSCs-Exo could protect myocardium from ischemic injury, and this function is largely dependent on the circ-0008302 contained in ADSCs-Exo.

ADSCs-Exo can reduce the oxidative damage of cardiomyocytes through circ-0008302.

Cardiomyocyte damage caused by H_2O_2 is mainly due to the production of a large amount of ROS. Therefore, we tested whether ADSCs-Exo can reduce ROS production through circ-0008302 after H_2O_2 treatment of cardiomyocytes. Compared with the control group, H_2O_2 treatment increased the generation of intracellular ROS, which was reversed by ADSCs-Exo treatment. However, the knockdown of circ-0008302 abrogated the role of ADSCs-Exo in reducing intracellular ROS production (Figure 4A-B). Therefore, ADSCs-Exo prevents cardiac myocytes from oxidative damage, and this function is largely dependent on circ-0008302.

The protective effect of ADSCs-Exo and circ-0008302 is mediated by inhibiting the expression of miR-466i-5p to increase the expression of MsrA.

CircRNAs in exosomes often exert their effects by adsorbing miRNAs in cells. Therefore, we tried to find the downstream molecules of circ-0008302. According to the sequencing results, the circ-0008302 was predicted to bind to miR-466d-5p、miR-669f-3p、miR-466i-5p、miR-466b-3p and miR-466c-3p. Among those, miR-466d-5p and miR-466i-5p have more than one binding site. Through previous research results, we found that miR-466i-5p play crucial roles in various kinds of pathological processes including fibrosis(23), inflammation(24, 25), cancer(26) and metabolic disorders(27), while its function in cardiovascular diseases has never been investigated. Therefore, we selected miR-466i-5p for the following experiments.

Compared with the control group, the level of miR-466i-5p in cardiomyocytes treated with H_2O_2 was increased. ADSCs-Exo treatment significantly decreased the level of miR-466i-5p. However, the level of miR-466i-5p in ADSCs-Exo-treated cardiomyocytes was significantly increased by the knockdown of circ-0008302 (Figure 5A). The above results indicate that the efflux ADSCs-Exo of circ-0008302 can work by reducing the expression of miR-466i-5p induced by H_2O_2 challenge in cardiomyocytes. Finally, we explored the target gene of miR-466i-5p. It was predicted by Targets can database that the 3'-UTR of MsrA had the potential binding site of miR-466i-5p (Figure 5B). MsrA is an antioxidant factor that can negatively regulate NF-κB pathway and inhibit inflammatory response(28, 29). We then further validated the effect of miR-466i-5p on MsrA expression.

It can be seen from the results that compared with the control group, the level of MsrA in cardiomyocytes treated with H_2O_2 was decreased, ADSCs-Exo significantly reversed the effect of H_2O_2. Importantly, circ-0008302 knockdown significantly decreased MsrA expression in ADSCs-Exo-treated cardiomyocytes (Figure 5C-D). In summary, ADSCs-Exo serves as a positive regulator of MsrA expression in cardiomyocytes, this effect might be dependent on circ-0008302-mediated suppression of miR-466i-5p.

Discussion

Cardiovascular disease is the main cause of death worldwide(1), and myocardial infarction is the most serious type of CVD with poor prognosis. Even after percutaneous coronary intervention and surgical treatment, myocardial ischemia- reperfusion can still cause myocardial cell apoptosis, hypertrophy, or other kinds of tissue injuries. Previous studies have found that adipose-derived stem cells (ADSCs) have biological functions to promote the repair and regeneration of damage tissues(6).The purpose of our research is to investigate the effect of ADSC-Exo on myocardial injury and explore the potential mechanisms from circRNA perspective. The results showed that through the delivery of circ-0008302, ADSCs-Exo can maintain the viability, inhibit the apoptosis and reduce ROS production of cardiomyocytes, thus protecting cardiomyocytes from H_2O_2-induced oxidative injury.

Studies have previously reported the potential efficacy of human umbilical cord mesenchymal stem cells (HUCMSCs) in repairing vascular injury(30, 31). Compare to HUCMSCs, ADSCs have some advantages because they are easier to obtain from large amounts of adipose tissue, and can rapidly expand in vitro. Exosomes, as a major paracrine component, are important mediators of intercellular signal transduction and play an important role in tissue repair by packaging and delivering RNA and protein. Compared to cells, cell-derived exosomes are safer and more convenient to deliver or storage. Due to these superiorities, ADSC-Exo has been widely used in the treatment of various diseases(32, 33).

In recent years, RNA therapy has emerged as a promising therapeutic option for cardiac regeneration and protection. As endogenous non-coding RNA molecules, circRNAs have been gaining increasing attention because they are resistant to exonuclease-mediated degradation and thus can provide a stable, long-time protection. In this work, we profiled the differentially expressed circRNAs between ADSCs and ADSCs-Exo, and identified circ-0008302 as a key mediator responsible for the protective role of ADSCs-Exo. In addition, the expression of circ-0007490 and circ-0010012 were also found to be significantly changed in ADSCs-Exo group. In contrast to circ-0008302, circ-0007490 and circ-0010012 was downregulated in ADSCs-Exo. Circ0007490 was reported to be involved in central nervous system dysfunction(22), while circ-0010012 was identified as a regulatory factor in Idiopathic Dilated Cardiomyopathy (IDC)(20). To date, their roles in the pathogenesis of myocardial injury remain known and deserve further investigation.

Mechanistically, circ-0008302 was found to target and inhibit miR-466i-5p expression. MiRNAs were found to be small (~ 22 nt) non-coding RNAs that negatively regulate the expression of protein-coding genes by inhibiting mRNA translation(34). MiRNA expression is associated with cardiac events, such as electrical signals, muscle contraction, heart growth and morphogenesis(35). Increasing evidence has

shown that changes in miRNA expression profiles are commonly associated with heart disease such as myocardial infarction and heart failure(36). Thus, manipulating miRNA might achieve therapeutic effects in these diseases(37). In this study, it was confirmed that ADSCS-Exo inhibited the expression of miR-466i-5p by delivering circ-0008302, and thus maintaining the expression of a miR-466i-5p target gene-MsrA in H_2O_2-challenged cardiomyocytes. It is noteworthy that although miR-466i-5p has the highest binding score, it is not the only predicted miRNA with circ-0008302-binding capacity. The functions of other candidates such as miR-466d-5p, miR-669f-3p, miR-466i-5p, miR-466b-3p or miR-466c-3p still need careful examination in the future.

As a target gene of miR-466i-5p, MsrA was reported to participate in antioxidant defense, thereby protecting the host from tissue damage caused by inflammation or chemicals(28, 29). Since the excessive inflammatory response is the common driven factor contributing to the pathogenesis of myocardial injury. We speculated that the circ-0008302-mediated upregulation of MsrA might mitigate myocardial injury by suppressing cardiac inflammation. Further investigation is required for validating the exact mechanisms of action of MsrA in the future.

Conclusion

ADSC-secreted exosomes have a protective role in myocardial injury by delivering circ-0008302, which increased MsrA expression via targeting miR-466i-5p in cardiomyocytes.

Study Limitations

It is only an experiment in vitro. Future experiments will be in vivo.

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Chengyan Hu involved in data analysis, figures, and manuscript writing; Xipeng Sun involved in manuscript writing; Yanyan Chu and Yanling Wang carried out experiments; Zhi Liu involved in conception and design, edits and final approval. All authors read and approved the final manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

Data available with Zhi Liu and Chengyan Hu.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors and Institute given consent to publish the research data.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

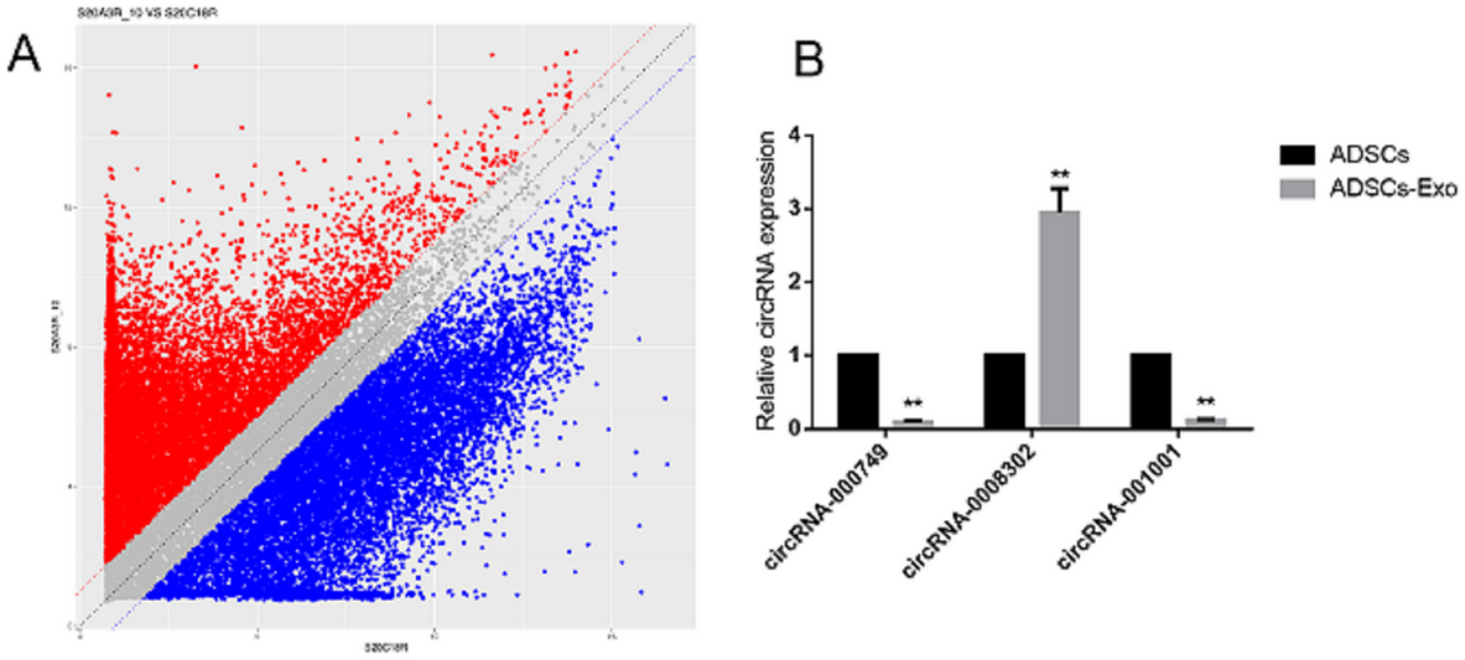

Figure 1

The expression of circRNAs in ADSCs and ADSCs-Exo. A. circRNA scatter plot: the red dot represents the up-regulated differential gene, fold change≥2 (Exo vs Control), blue represents the down-regulated differential gene. B. The expression of indicated circRNAs in ADSCs and ADSCs-Exo were evaluated by qRT-PCR,** p< 0.01 compared with the ADSCs group.

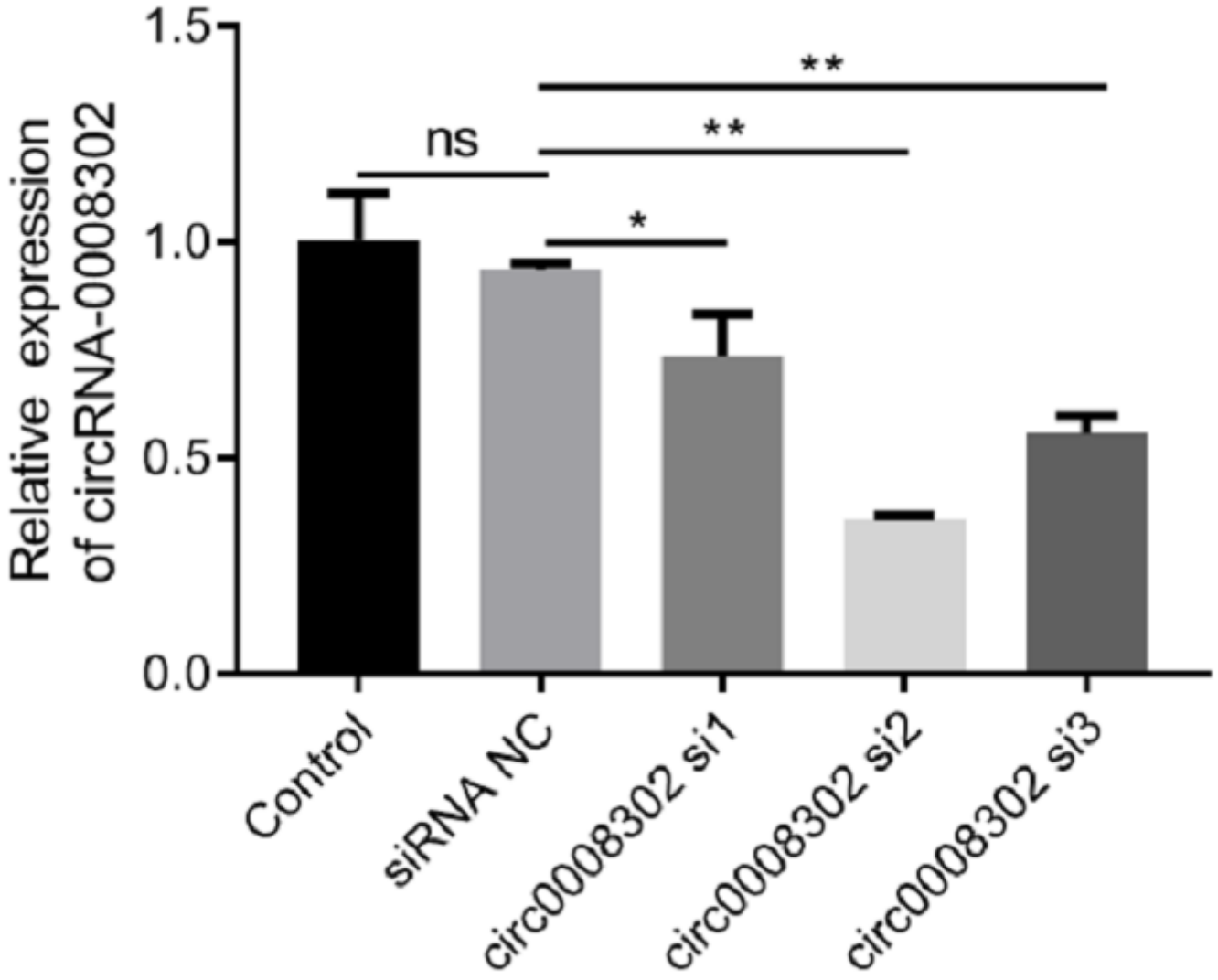

Figure 2

M6200 cells were transfected with circ-0008302 siRNA or control siRNA for 36 h. The expression of circ-0008302 in M6200 cells was evaluated by qRT-PCR. *P□0.05, **P□0.01, one-way ANOVA.

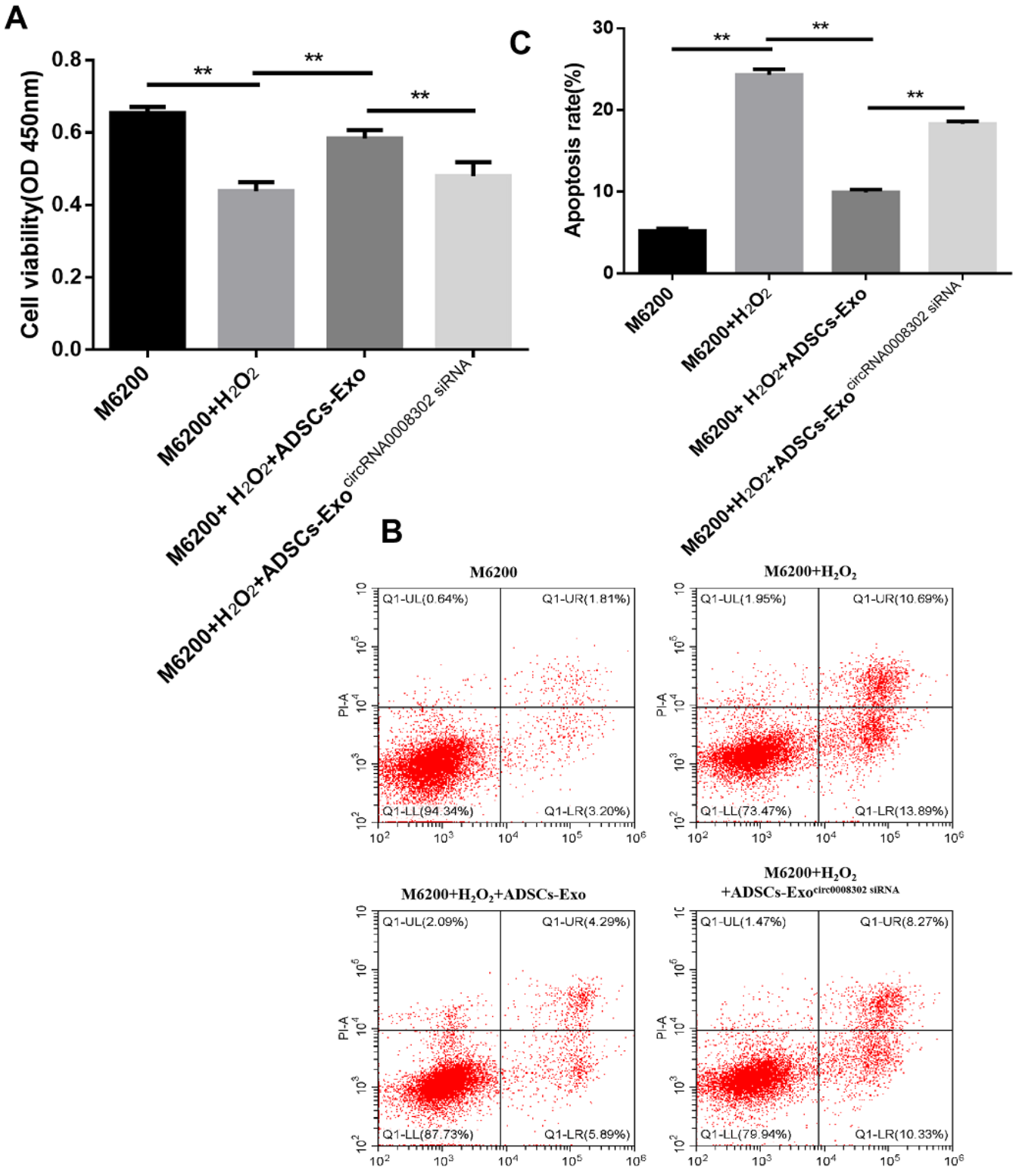

Figure 3

The effects of ADSCs-Exo and circ-0008302 in H_2O_2-induced cardio -myocytes injury. A.M6200 cells were treated with ADSCs-Exo in the presence of circ-0008302 siRNA or control siRNA for 36 h, followed by H_2O_2 for 24 h. The viability of M6200 cells was evaluated by CCK8 assay. B. The apoptosis of M6200 cells was evaluated by flow cytometry. C. The statistics of apoptosis results. **P□0.01, one-way ANOVA.

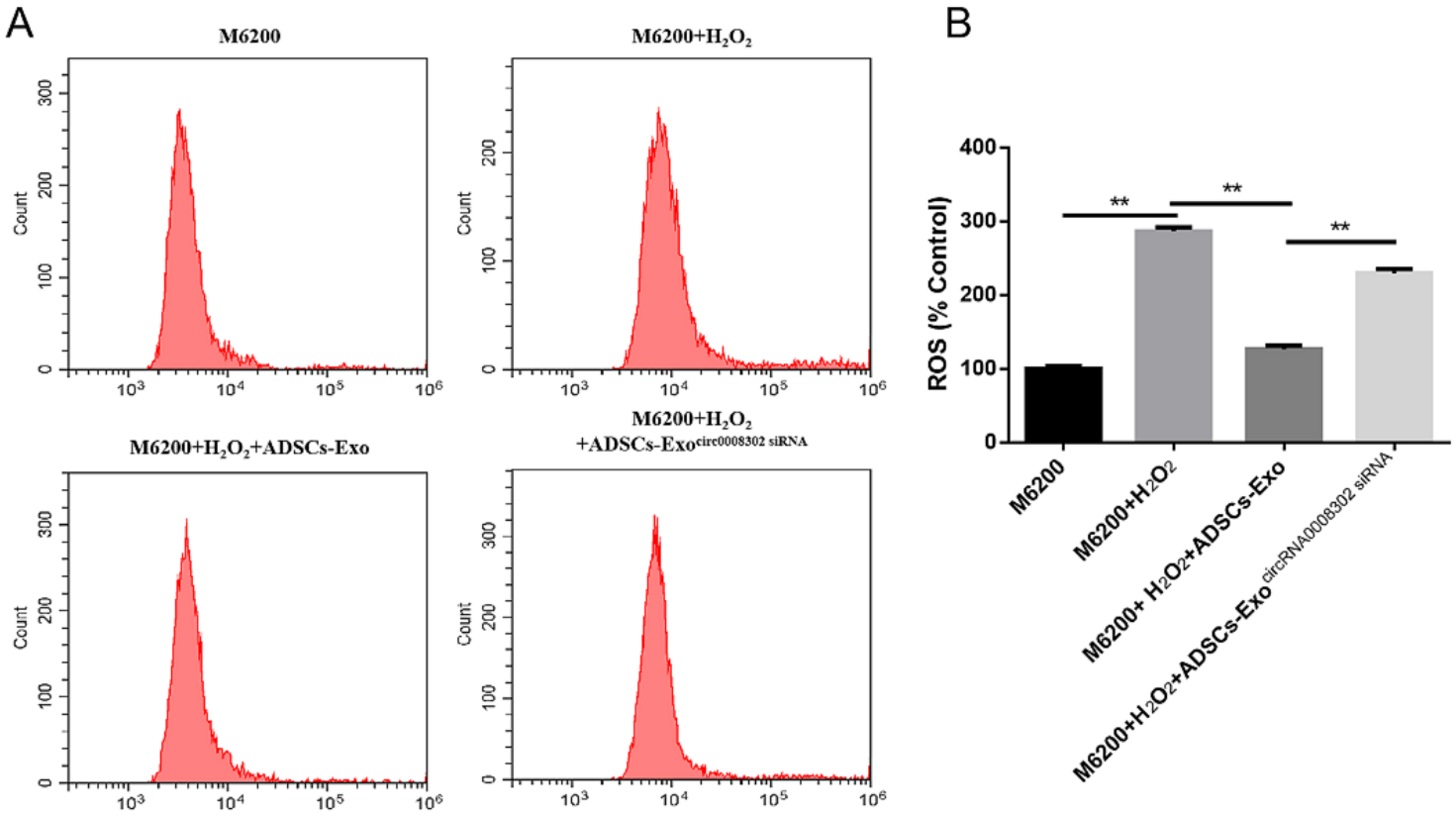

Figure 4

Detection of ROS production. A. M6200 cells were treated with ADSCs-Exo in the presence of circ-0008302 siRNA or control siRNA for 36 h, followed by H2O2 for 24 h. The ROS level in M6200 cells was evaluated by flow cytometry. B. Quantitative analysis of ROS production. **P□0.01, one-way ANOVA.

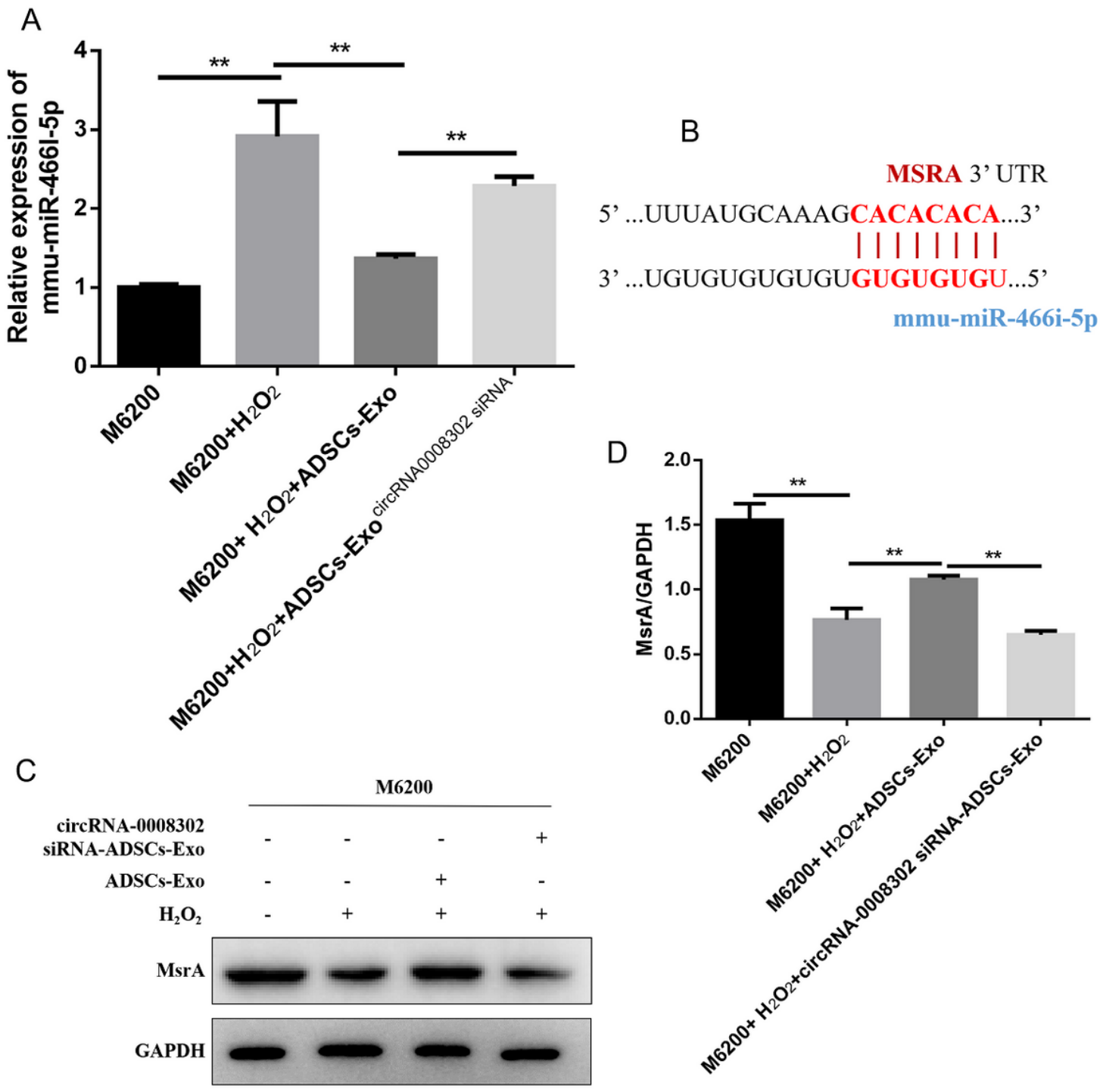

Figure 5

The role of exosomal circ-0008302 is through MiR-466i-5p targets MsrA in cardiomyocytes. A.M6200 cells were treated with ADSCs-Exo in the presence of circ-0008302 siRNA or control siRNA for 36 h, followed by H_2O_2 for 24 h. The expression of miR-466i-5p in M6200 cells was evaluated by qRT-PCR. B. The binding site between MsrA 3'-UTR and miR-466i-5p was predicted using Targets can database. C. M6200 cells were treated with ADSCs-Exo in the presence of circ-0008302 siRNA or control siRNA for 36 h, followed by H_2O_2 for 24 h. The expression of MsrA in cardiomyocytes was evaluated by Western blot, D. Statistical results of MsrA expression results, **P□0.01, one-way ANOVA.