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ORIGINAL ARTICLE
Ischemic Heart Disease

Bone Marrow Mesenchymal Stem Cell-Derived Exosomal miRNA-29c Decreases Cardiac Ischemia/Reperfusion Injury Through Inhibition of Excessive Autophagy via the PTEN/Akt/mTOR Signaling Pathway

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Background: Cardiac ischemia/reperfusion (I/R) injury will cause a large amount of cardiomyocyte loss and cascade reactions such as apoptosis, mitochondrial dysfunction, and excessive autophagy. Mesenchymal stem cells (MSCs) are promising therapeutic tools to replace damaged cardiomyocytes, but the underlying mechanism is still unknown.

Methods and Results: Exosomes contain many microRNAs and protein, which are believed to have multiple biological functions. This study explored the role of bone marrow MSCs (BMMSCs)-derived exosomes under different oxidation levels in heart protection and miRNA-related mechanisms. Exosomes extracted from BMMSCs contained a high level of miR-29c, and its expression level changed after cells were treated under hypoxia/reoxygenation (H/R) conditions. In vivo I/R experiments also confirmed an expression change of miR-29c, and PTEN-Akt-mTOR is one of the predominant pathways that regulate autophagic change during this process.

Conclusions: This study highlighted the role of miR-29c in regulating autophagy under cardiac I/R injury, which also extended existing mechanisms of a stem cell and its derivative to explore potential therapeutic interventions in ischemic heart diseases.

Key Words: Autophagy; Exosome; Ischemia/Reperfusion; miR-29c

Cardiovascular diseases (CVDs) and stroke are the primary cause of morbidity and mortality worldwide, according to the epidemiological analysis report newly published in *The Lancet* 2019.[1] Ischemia heart diseases (IHDs), which refer to myocardial infarction, ischemia/reperfusion (I/R), and heart failure, will cause a large amount of cardiomyocyte loss and reactive oxygen species generation.[2] Due to the limitation of cardiomyocytes' self-regeneration, transplantation of cardiac-derived cells after ischemic heart injury has already become a promising treatment.[3]

Different types of cells are reported to have cardioprotective effects after transplantation, which includes cardiomyocytes, cardiac progenitor cells, different types of stem cells such as mesenchymal stem cells (MSCs) and bone marrow stem cells.[4,5] Cell transplantation therapy can provide timely replenishment of dead cardiomyocytes, reduce infarct scar area, apoptotic cell death, and promotes angiogenesis after ischemic heart disease.[6] Besides all these advantages, there is still the limitation of clinical application of cell therapy, such as the massive amount of cells required for transplantation, immune rejection in allotransplantation, strict quality control of cell therapeutic products, and low cell residence ratio.[7] These prompt us to find new mechanisms that underline cell transplantation as a stable and effective method targeting IHDs.

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Accumulating evidence from preclinical studies using stem cell therapy has suggested paracrine effects of transplanted cells contribute a lot to its protective effects[8] because the effect of direct stem cell administration includes, but is not limited to, cell replacement. It has been shown that MSC-conditioned medium (MSC-CM) can enhance cardiomyocyte survival in vitro and angiogenesis in infarcted hearts.[9] Cytokines and cell-derived vesicles such as exosomes can be a cell-free approach that have advantages such as being a stable source and being immunogenicity free to treat heart diseases.[10,11] Furthermore, the therapeutic effects of MSC-derived exosomes were comparable among different tissue sources, which indicated all

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MSCs could secret protective exosomes.[12]

Exosomes are nano-vesicles (30–100 nm) derived from a cell, and have been reported to contain various mRNAs, microRNAs (miRNAs), proteins, and lipids to modulate biological processes in the target cells. Numerous studies have shown that exosomal miRNAs play a significant role in the myocardial protective mechanisms of the exosome; for example, miR-22 derived from MSC exosomes attenuates apoptosis in a rat myocardial I/R model,[13,14] and endothelial cells that originated in exosomal miR-126 and miR-210 have pro-survival effects on the cardiac progenitor cells.[15] Besides, miRNAs can also function as a biomarker of CVDs, and many miRNAs are profoundly enriched or decreased after ischemic heart diseases; their functions are still not fully understood.[16] Previous studies have found that miR-29 is highly expressed in MSCs-derived exosomes, and administration of exosomes significantly reduced the infarct area (IR) of the rat after MI.[17,18] And miR-29c is decreased after the hypoxia and reoxygenation treatment of neonatal rat cardiomyocytes (NRCM) and H9C2 cells, but the mechanisms are still unexplained.[19,20] Here, we used bone marrow-derived MSC exosomes to test its protective efficacy under different oxidational levels in a mouse I/R model and the microRNA-related mechanism. We reported that H/R-treated exosomes were less efficient than in normoxia condition, and it was related to the expression of exosomal miR-29c. As I/R promoted excessive autophagy, we found that miR-29c could decrease excessive autophagy by targeting the PTEN/AKT/mTOR pathway, and these effects could protect the heart from oxidative stress.

Methods

Exosome Isolation

We isolated and purified BMMSCs from the mouse. Exosomes isolated from normoxia and hypoxia/ reoxygenation (O_2 <1%, hypoxia 48 h, reoxygenation 24 h) cultured BMMSCs were simplified as Nor-exo and Hypo-exo. The collected BMMSC medium was centrifugated at 300 g for 0.5 h and 2,000 g for 0.5 h next to remove suspension cells, and 10,000 g 0.5 h to remove cellular debris, then finally 100,000 g 1.5 h twice while washed by PBS. And the final product was resuspended with PBS for further analysis. The above centrifugate conditions were all at 4 degrees.

Animal and Exosome Treatment

Left anterior descending (LAD) coronary artery ligation and reperfusion was performed on 10-week-old male C57BL/6 N mice. After randomly dividing the mice into 3 groups: I/R+PBS, I/R+Nor-exo, and I/R+Hypo-exo, mice received sodium pentobarbital (50 mg/kg i.p.) before surgery. A thoracotomy was performed between the second and third ribs, and the LAD was ligated by a 7-0 Prolene suture for 30 min and reperfusion for 24 h occurred so that the scar area could be assessed. In total, 20 μg of exosomes were resuspended in 20 μL PBS and injected in 2 sides of the border zones right after LAD coronary ligation. In sham surgery, only the chest was opened, but no ligation of the LAD was carried out. After 24 h of reperfusion, mice underwent another thoracotomy and LAD ligated again while Evans blue was injected into the femoral vein, and then TTC staining was applied to the isolated heart. The left ventricle (LV) area, IR, and area at risk (AAR) was determined by computerized planimetry. The percentage of the AAR/LV and IR/AAR was calculated.

The Ethics Committee of Biomedicine from the The First Hospital of Jilin University approved this study, which also conformed to the revised Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH) (Publication Nos. 85-23, (1996)).

Quantitative Real-Time RT-PCR of miRNA

Total RNA of exosomes was isolated by using the miRNeasy Micro Kit (Qiagen). For reverse-transcription into cDNA, the miRcute Plus miRNA Kit (Tiangen Biotech) was used. The housekeeping genes (U6) was used for normalization. The sequence to maintain the q-PCR experiment of miR-29c was CGCGCTAGCACCATTTGAAAT/CAGTGCGTGTCGTGGAGT. The sequence used for antimir studies (anti-29c, GATTTCAAATGGTGCT) could inhibit both miR-29b and miR-29c.

Western Blot

Total proteins were extracted using tissue lysis buffer, and the protein concentration was determined by a BCA protein assay. Protein lysates (10 μg) for each sample were separated by SDS-PAGE and then transferred to a PVDF membrane. The band density of specific proteins was quantified after normalization with the density of GAPDH.

Measurement of Autophagic Level

The cells were infected with adenovirus expressing LC3-GFP according to the manufacturer's protocol, and continued to be incubated for 24–48 h. Cells were transfected with miR-NC or miR-29c after the cells were infected with adenovirus for 24 h. After fixation with 4% paraformaldehyde for 15 min, the cells were incubated with Hoechst 33342 for 10 min. And LC3-positive puncta were viewed with a confocal laser scanning microscope and counted in 40× fields.

Luciferase Reporter Assay

The full-length of 3'UTR PTEN containing the predicted miR-29c binding sites were amplified by PCR and cloned into the downstream section of the dual-luciferase reporter gene in psiCHECK2 (Progema, USA) vectors. A site-directed Gene Mutagenesis Kit (Beyotime, China) was used to generate a recombinant PTEN-mutation vector. The constructed luciferase reporter plasmids, PTEN-wt or PTEN-mut, were cotransfected with miR-29c or miR-negative control into 293A cells using Lipofectamine 3000 reagent (Invitrogen, USA). After 36 h transfection, the luciferase activity was determined using the Dual-Luciferase Reporter Gene Assay Kit (Beyotime, China). The Renilla luciferase activity was regarded as the normalization.

RNA Binding Protein Immunoprecipitation (RIP) Assay

RIP experiments were performed using the EZ-Magna RIP KIT (Millipore, MA, USA). Briefly, approximately 2×10^7 NRCM cells were pelleted and lysed in 100 μL RIP lysis buffer plus protease and RNase inhibitors. The cell lysates were incubated with IgG or Ago2 antibody, respectively. IgG was used as a negative control. After treating with proteinase K, the immunoprecipitated RNAs were extracted. The abundance of the miR-29c level was detected by a qRT-PCR assay.

In Vivo Delivery of miR-29c Mimic

All the in vivo experiments were performed on 10-week-old

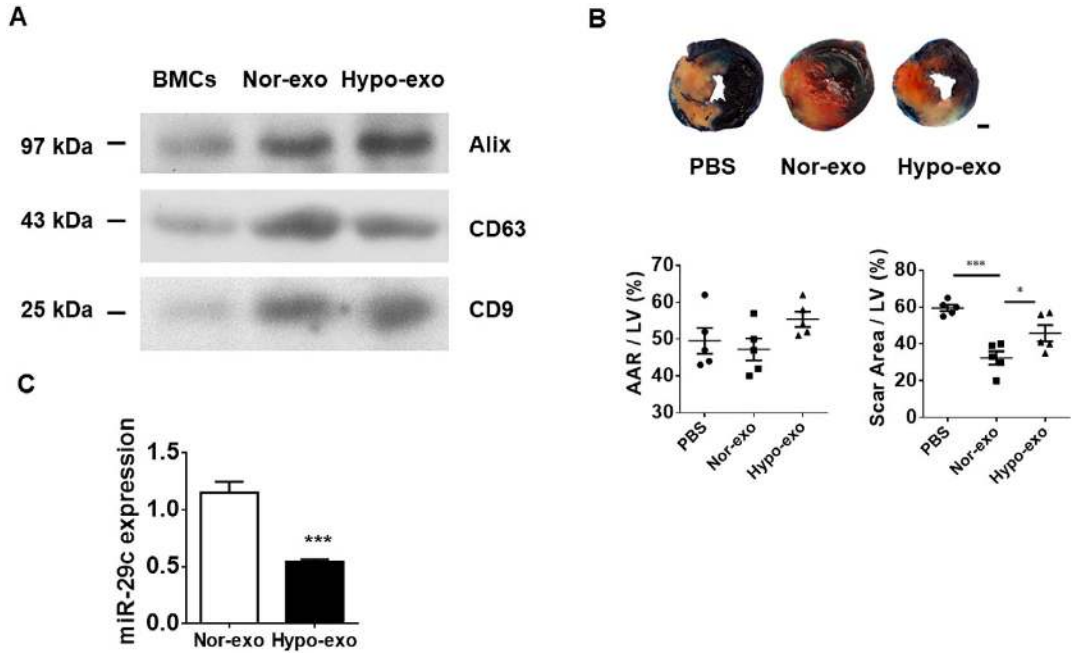

Figure 1. Exosomes identification and cardiac repair of bone marrow mesenchymal stem cells (BMMSCs)-exosomes to mice ischemia/reperfusion (I/R) injury. (**A**) BMMSCs were treated under normoxia and hypoxia/regeneration (H/R) conditions and exosomes were isolated and specific exosome markers, including Alix, CD63, and CD9, were expressed. (**B**) Representative cross-sectional images of I/R (ischemia 30 min, reperfusion 24 h) heart with TTC and Evan's blue dyes, and statistical data, N=5. Scale bars, 1 mm. (**C**) The expression level of exosomal micro RNA (miR)-29c was measured by qRT-PCR. N=3, *P<0.05; ***P<0.001.

male C57BL/6 N mice. Mice received sodium pentobarbital (50 mg/kg, i.p.) before surgery. A thoracotomy was performed between the second and third ribs. The dosages used per mouse were 50 ng miR-29c mimic, and every mouse received three injection points in the left ventricular anterior wall. After 24 h of injection, mice received thoracotomy again, and the mouse myocardial I/R model was established as described previously. After LAD was ligated for 30 min and reperfused for 24 h, the TTC/Evans staining was performed and then the hearts were harvested and analyzed.

Statistical Analysis

All values in this study were presented as mean±SEM. Statistical analysis was performed based on a Student's t-test, and differences between groups were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. All statistical analyses were performed using GraphPad Prism version 5.01 software. All experiments were repeated at least 3 times.

Results

Delivery of BMMSC-Derived Exosomes Improves Heart Function After I/R, Exosomal miRNA-29c Changes in Hypo-Exo

To determine the protective effects of exosomes derived from BMMSCs, we first isolated exosomes as described in the Methods section. The exosome markers such as CD9, CD63, and Alix were analyzed by using Western blot (**Figure 1A**); the results showed the exosomes were isolated successfully with high efficiency, and the H/R treatment of cells did not change exosome abundance and integrity. Intramyocardial application of exosomes during mice in vivo I/R decreased infarcted size, which was partially rescued in hypo-exo treatment (**Figure 1B**). As early research has shown, exosome-derived miRNAs have multiple roles in cardiac diseases, such as pro-apoptosis, angiogenesis, and so on. After background investigation, we found that BMMSC exosomes contain a high level of miR-29c, which has been proven in H9C2 cells to have protective effects against oxidative stress.[21] After the H/R culture of BMMSCs, we isolated exosomes to analyze the expression level of miR-29c, and it was surprising to find that miR-29c decreased after H/R treatment (**Figure 1C**). These data demonstrated that MSC exosomes contained miR-29c, and its expression pattern changed under hypoxia conditions, which indicated its potential role involved in oxidative stress.

miR-29c Was Decreased After Myocardial I/R, and Overexpression of miR-29c Protected the Heart From I/R Injury

I/R injury is caused by significant oxidative stress and after the onset of heart diseases. To investigate whether the miR-29c expression pattern changes after hypoxic treatment of exosomes have a biological function, we established a mouse I/R model. We found that the expression level of miR-29c decreased after cardiac I/R (**Figure 2A**). And autophagy-related proteins, LC3 II and P62, increased dramatically after reperfusion (**Figure 2B**); excessive autophagy was proven to have detrimental effects during cardiac I/R,[22] and increased abundance of P62 protein

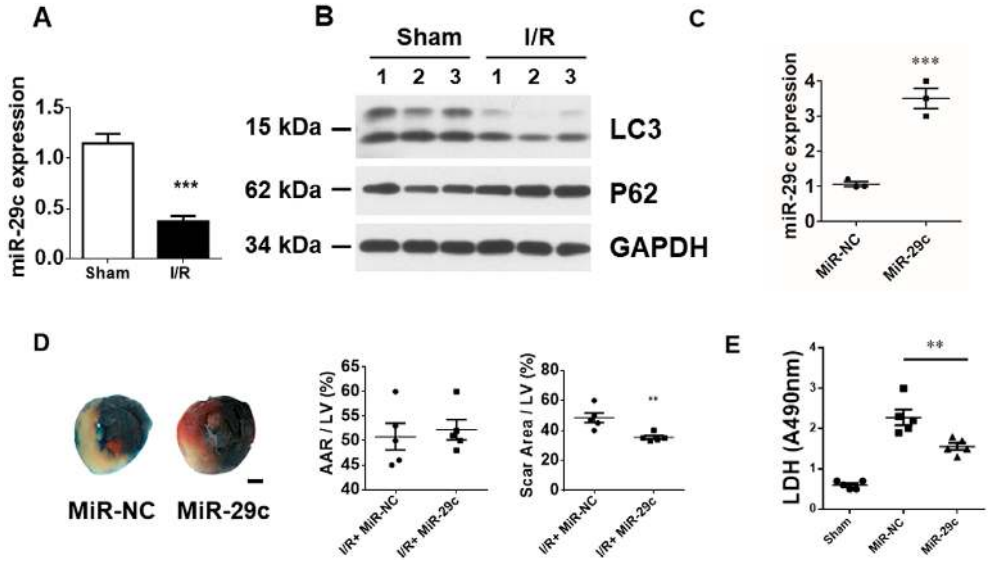

Figure 2. Micro RNA (miR)-29c expression and autophagy level change after ischemia/reperfusion (I/R); overexpression of miR-29c was protective. (**A**) The expression level of miR-29c after in vivo I/R was measured by qRT-PCR, N=5. (**B**) Western blot analysis of autophagy-related proteins, including LC3 and p62, N=3. (**C**) The expression level of miR-29c after injection of miR-29c in vivo was measured by qRT-PCR. (**D**) Scar area was detected by TTC and Evan's blue, N=5. (**E**) Detection of serum lactate dehydrogenase (LDH) level, N=5. **P<0.01; ***P<0.001 vs. miR-negative control (NC).

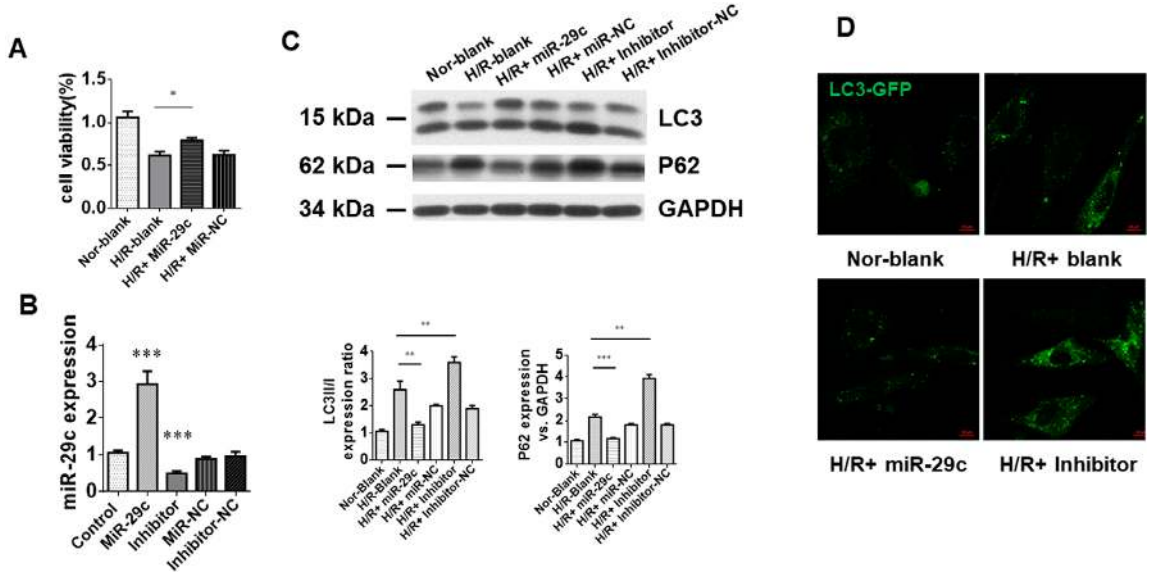

Figure 3. Overexpression of micro RNA (miR)-29c decreased the autophagy level and protected cell survival. (**A**) Cell survival was measured by CCK8, N=3. (**B**) The expression level of miR-29c after treatment with miR-29c; the inhibitor was measured by qRT-PCR, N=3. (**C**) Western blot analysis of autophagy-related proteins, including LC3 and p62, N=3. (**D**) Detection of LC3 dots. *P<0.05 vs. hypoxia/reoxygenation (H/R)-blank; ***P<0.001 vs. control.

caused autolysosome clearance block, which explained to some extent the harmful effect of autophagy after reperfusion. Preliminary work indicated that miR-29c could protect the cardiac cell from oxidative stress; to confirm that, we constructed a miRNA mimic to overexpression miR-29c (**Figure 2C**). We injected the mimic-29c, as well as mimic-NC, directly into the left ventricular wall of the mouse; after 24 h, we established the I/R model as mentioned in the methods section. Overexpression of miR-29c significantly reduced the scar area when analyzed with TTC and Evan's blue staining (**Figure 2D**), and decreased the level of serum lactate dehydrogenase (LDH, **Figure 2E**).

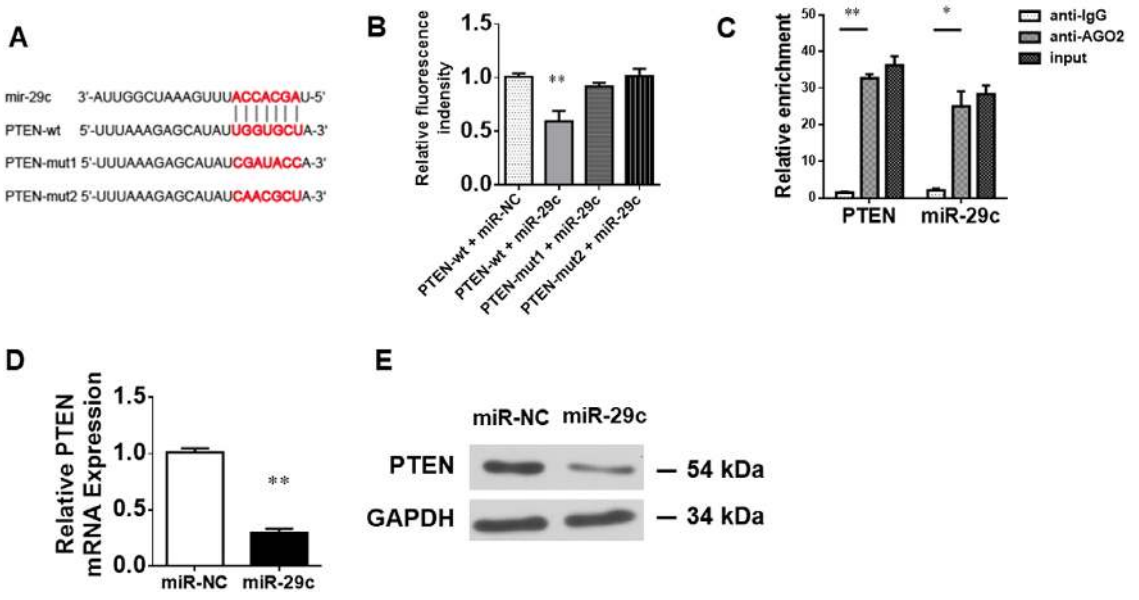

Figure 4. Micro RNA (MiR)-29c directly targeted PTEN. (**A**) Wild-type and mutant miR-29c binding sites in the 3'UTR of PTEN. (**B**) Dual-luciferase activity assay was performed at 36 h after 293A cells were cotransfected with PTEN-wt or PTEN-mut vector and miR-29C or miR-negative control (NC) mimic. (**C**) Endogenous interaction between PTEN and miR-29c in neonatal rat cardiomyocyte (NRCM) cells was confirmed by the RNA binding protein immunoprecipation assay (RIP) assay, followed by q-PCR analysis. (**D**) PTEN mRNA expression was assessed by q-PCR in NRCM cells after transfection with miR-29c or miR-NC mimic. (**E**) PTEN protein was examined by Western blot analysis. Data are presented as the mean±SEM of 3 independent experiments. *P<0.05; **P<0.01.

These data demonstrate that in our I/R model, miR-29c was significantly decreased and autophagy was overactive, whereas overexpression of miR-29c protected the heart from cardiac I/R injury.

Overexpression of miR-29c Decreased Excessive Autophagy Under Hypoxia Treatment, and Maintained Autophagy Flux

We then examined whether miR-29c has an effect on autophagy under H/R conditions using NRCM. After cells have been treated in 0.1% O_2 concentration for 4 h and had reoxygenation for 2 h, the cck8 results showed that overexpression of miR-29c could reduce cell death (**Figure 3A**, **Supplementary Figure**). To understand the relationship between miR-29c and autophagy, the present study downregulated miRNA-29c expression levels using an anti-miRNA-29c inhibitor. As presented in **Figure 3B**, the inhibitor suppressed miR-29c expression. In **Figure 3C**, LC3 II and P62 expression were suppressed by miR-29c overexpression, and downregulation of miRNA-29c further increased the autophagy level, compared with the H/R-blank control group. When cells were transfected with adenovirus LC3-GFP and had miR-29c expression levels manipulated, the WB results demonstrated that overexpression of miRNA-29c suppressed LC3 dots accumulation, whereas downregulation of miR-29c further aggregated LC3 dots compared with the hypoxia-blank control group (**Figure 3D**).

MiR-29c Directly Targeted PTEN

To investigate the underlying mechanism of miR-29c-induced cardiomyocyte protection, we predicted the target genes of miR-29c using the TargetScan (http://www.targetscan.org) and found that the PTEN gene constitutes a miR-29c binding domain on its 3′UTR. As shown in **Figure 4A**, in order to perform a miRNA target validation experiment, we constructed 3 luciferase vectors using a psiCHECK2 plasmid, including a wild-type PTEN 3′-UTR luciferase vector (PTEN-wt) and 2 vectors expressing a PTEN 3′UTR sequence with different mutated sites (PTEN-mt). Subsequently, we co-transfected cells with either a PTEN-wt or PTEN-mt vector and either miR-29c mimic or a negative control miRNA mimic (miR-NC). A dual-luciferase activity assay was carried out after 36 h of co-transfection. It showed that, compared to miR-NC, the fluorescence of the miR-29c mimic transfected with the PTEN-wt vector was downregulated significantly. However, the luciferase activity of the PTEN-mt did not differ from that of miR-NC (**Figure 4B**), suggesting that PTEN is one of the downstream targets of miR-29c. To further verify the endogenous binding between miR-29c and PTEN, we carried out a RIP assay in NRCM cells. The expression levels of miR-29c and PTEN mRNA were detected by using qRT-PCR. As shown in **Figure 4C**, expressions of miR-29c and PTEN mRNA were both significantly enriched in Ago2 complexes, suggesting the direct interaction between miR-29c and PTEN. As demonstrated by qRT-PCR and Western blot, after transfection of cells with miR-29c mimic, both PTEN mRNA (**Figure 4D**) and protein levels (**Figure 4E**) evidently decreased in NRCM cells. Thus, these results demonstrated that miR-29c targeted PTEN and negatively regulated its expression in NRCM cells.

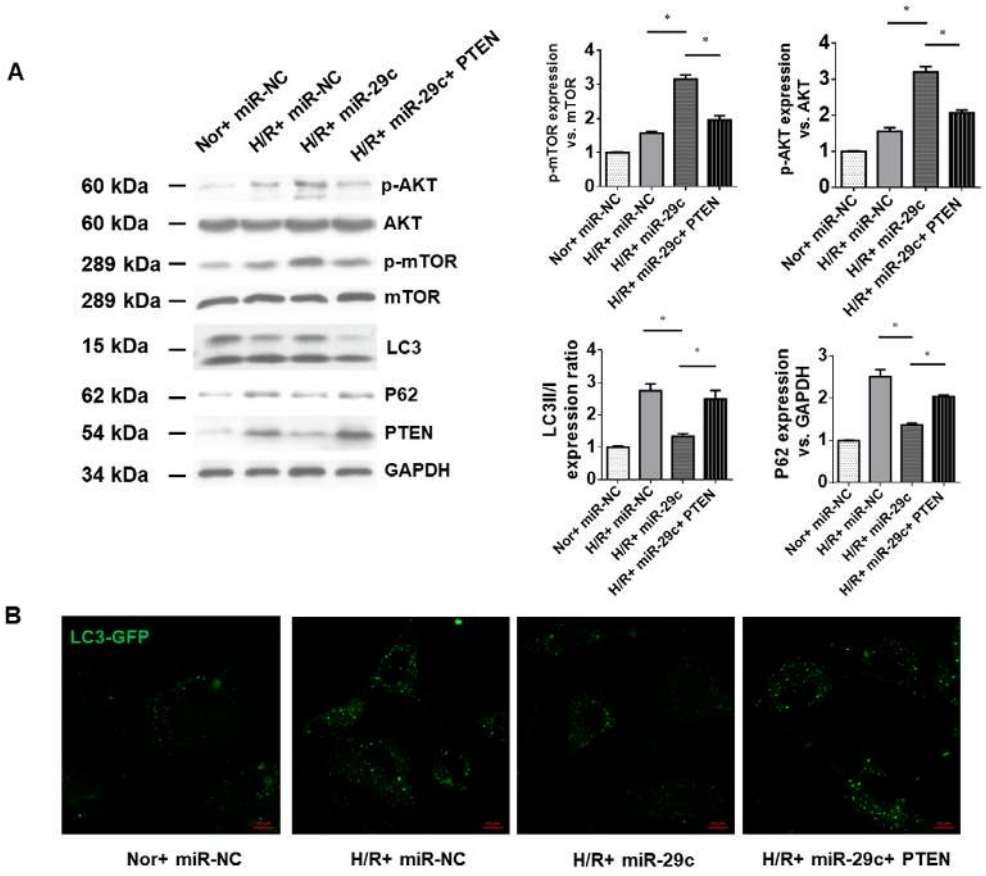

Figure 5. PTEN overexpression abolished the effect of miR-29c on cell autophagy in the hypoxia/reoxygenation (H/R) model. (**A**) Neonatal rat cardiomyocyte (NRCM) cells were transfected with miR-negative control (NC) or miR-29c mimic using lipofectamine 3000 or infected with adenovirus-PTEN. NRCM protein was collected at the end of each treatment and detected by Western blot analysis. (**B**) Detection of LC3 dots. Data are presented as the mean±SEM of 3 independent experiments. *P<0.05.

PTEN Overexpression Abolished the Effect of miR-29c on Cell Autophagy in H/R Model

To explore whether miR-29c inhibited H/R-induced excess autophagy via the PTEN/AKT/mTOR signaling pathway, we used adenovirus of PTEN and transfected miR-NC or miR-29c into NRCM. As shown in **Figure 5A**, overexpression of miR-29c significantly increased the protein levels of phosphor-AKT and phosphor-mTOR in the H/R model of NRCM cells. However, the high-expression of PTEN blocked the expression of phosphorylation-AKT and mTOR. Furthermore, the Western blot results showed that the LC3II/I ratio and the levels of the autophagy substrate P62 were decreased after miR-29c was transfected, whereas PTEN overexpression abolished this role. Moreover, we also transfected NRCM with adenovirus LC3-GFP and manipulated miR-29c expression levels; the results demonstrated that overexpression of miRNA-29c suppressed LC3 dots accumulation in H/R injury, while upregulation of PTEN aggregated LC3 dots compared with the control group (**Figure 5B**). These results indicated that overexpression of miR-29c decreased excessive autophagy by inhibiting PTEN and activating the AKT/mTOR signaling pathway during myocardial I/R.

Discussion

Ischemia/reperfusion injury occurs in all clinical scenarios where the blood flow to the tissues is discontinued and then restored. Myocardial I/R injury refers to the aggravation of ischemic myocardial injury after reperfusion.[23–25] It causes various myocardial damages such as arrhythmia, necrosis, and myocardial contractile dysfunction.[26] In recent years, I/R injury has become more common, with thrombolytic production of reactive oxygen species, calcium overload, excessive autophagy of cardiomyocytes, and inflammatory responses being essential pathways responsible for pathophysiological events after I/R injury.[27–29] Autophagy is a self-clearing process through the lysosomal degradation pathway, which is essential for survival and homeostasis as it removes dying cells and is accompanied by the activation of autophagy markers, p62 and LC3-II.[30,31] It has been reported that autophagy is considered an essential regulator of myocardial I/R injury during the I/R process,[32,33] suggesting that therapeutic manipulation of autophagy in the ischemic myocardium may benefit cardiac function. Autophagy has been reported to be commonly activated excessively in ischemic cardiomyocytes.[34,35] Therefore, the development of new cardioprotective strategies

through regulation of autophagy has clinical importance in treating myocardial ischemia and heart dysfunction.

miRNAs are a class of non-coding, single-stranded small RNA. miRNAs have been reported to be vital regulators of gene expression by binding to 3′UTR of target mRNA.[36,37] Furthermore, miRNAs act as essential modulators of autophagy.[38] Numerous studies show that their dysregulation also plays a vital role in the process of I/R, such as miR-429,[39,40] miR-24,[41,42] miR-17.[43,44] The exosome is a nanometer-sized lipid microvesicle derived in vivo and widely exists in body fluid such as blood, interstitial fluid, and urine.[45,46] The previous studies found that secretion of exosomes is closely associated with cellular physiological and pathological conditions.[47] Exosomes contain numerous types of active substances, especially miRNAs. According to recent studies, exosomes are considered as a new mechanism for miRNA transmission; they transmit different types of signaling molecules to the specific recipient cells and thus participate in the development of various diseases, including CVD.[48,49] For example, Xu et al[50] found that exosome-carried miR-30a can suppress myocardial apoptosis in myocardial I/R injury rats by reducing autophagy.

In our study, we found that exosomes extracted from BMMSCs contained a high level of miR-29c, and its expression level changed under H/R treatment. This suggested that miR-29c might have an endogenous regulation effect in the H/R model. However, previous research regarding miR-29c mainly focused on tumors; the role of miR-29c related to the myocardial I/R injury is largely unclear. Therefore, we sought to evaluate the importance of miR-29c in the mouse I/R injury model. The mouse, I/R model, was established by the ligation of the LAD coronary artery, and the myocardial infarction area in heart tissues was detected by Evans blue/TTC staining. We found that overexpression of miR-29c significantly reduced both myocardial infarction area and excess autophagy of I/R, thus protected the heart from I/R injury. We predicted the target genes of miR-29c using the TargetScan and found that the PTEN gene constitutes a miR-29c binding domain on its 3′UTR. To further verify the direct interaction between miR-29c and PTEN, we tested miR-29c-mediated binding of RNA-induced silencing complex (RISC) to PTEN mRNA using the RIP assay with the antibody against Ago2. The results showed that expressions of miR-29c and PTEN mRNA were both significantly enriched in Ago2 complexes. Systemic PTEN inhibitor or siRNA treatment in rats with contusive spinal cord injury was found to reduce autophagy and activate the Akt pathway.[51,52] Our results were consistent with previous studies. Another important finding was that the protective function of miR-29c could be reversed by PTEN overexpression in the NRCM H/R model. PTEN blocked the expression of phosphorylation-AKT, and phosphorylation-mTOR and upregulation of PTEN aggregated LC3 dots compared with the control group.

Conclusions

This study demonstrated that miR-29c could target the PTEN gene and activate the AKT/mTOR pathway, thereby partially blocking autophagy in cardiomyocytes and protecting the myocardial I/R injury. MiR-29c might serve as a novel therapeutic target for enhancing cardiac survival and function.

Disclosures

The authors declare no conflicts of interest. The authors report no relationships that could be construed as a conflict of interest.

Acknowledgement

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Supplementary Files

Please find supplementary file(s);
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