Knockdown of miR-665 Protects Against Cardiomyocyte Ischemia/Reperfusion Injury-Induced ROS Accumulation and Apoptosis Through the Activation of Pak1/Akt Signaling in Myocardial Infarction

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Summary

Myocardial infarction (MI) is one of the major causes of death worldwide, and the therapeutic strategies of MI are still limited. In this study, we investigated the function of miR-665 in MI. In the present study, an ischemia/reperfusion (I/R) rat model and a hypoxia/reoxygenation (H/R)-induced H9c2 cell model were successfully established to mimic the MI for in vivo and in vitro studies. The concentrations of lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB), tumor necrosis factor alpha (TNF-α), IL-6, and reactive oxygen species (ROS) were then measured. Moreover, cell viability and apoptosis were detected by MTT assay, TdT-mediated dUTP nick end labeling (TUNEL), and PI/FITC-annexin V assay. The binding of miR-665 and Pak1 was determined by luciferase assay. miR-665 was upregulated in I/R rats, and the overexpression of miR-665 significantly increased LDH, CK-MB, TNF-α, IL-6, and ROS concentrations and induced cell apoptosis, while knockdown of miR-665 had opposite results. Consistent with in vivo results, miR-665 induced cell apoptosis and ROS generation in H/R-treated H9c2 cells. More importantly, Pak1 was the target gene of miR-665, and knockdown of miR-665 depressed the accumulation of ROS and cell apoptosis by targeting Pak1 and promoting the phosphorylation of Akt, whereas knockdown of Pak1 could attenuate the protection of miR-665 inhibitor in H/R-treated H9c2 cells. Therefore, knockdown of miR-665 protects against cardiomyocyte ischemia/reperfusion injury-induced ROS accumulation and apoptosis through activating Pak1/Akt signaling in MI. In general, understanding the biology and modulation of miR-665 may have the potential to counteract the development of MI.

Key words: Pak1/Akt signaling pathway, Reactive oxygen species

Recently, myocardial infarction (MI) is one of the common causes of death worldwide, and the rates of recurrent myocardial infarction and mortality are still high.1,2 Prolonged myocardial ischemia leads to loss of cardiac cells and cardiac dysfunction.3 Mitochondria are easy to injure during the processes of ischemia/hypoxia, causing excessive oxidative stress and apoptosis of myocardial cells.4 Cardiomyocytes are terminally differentiated, and preventing the loss of cardiomyocytes has become a major challenge in the treatment of MI.5 Although a study has demonstrated that MI could be ameliorated by regulating HIF-1α, the investigations about the pathogenesis of MI and the treatment of MI are limited.6 Therefore, it is necessary to explore the therapeutic strategies of myocardial infarction.

MicroRNA (miRNA), an endogenous single-stranded RNA with about 18-20 nucleotides, could regulate gene expression by targeting the 3’-untranslated regions (UTRs) of mRNA. More than 60% of human genes are regulated by miRNAs.7,8 Previous studies have reported that miRNA contributes to the regulation of physiological and pathological processes, including cell apoptosis, proliferation, and migration.9,10 Moreover, increasing evidence has proved that different miRNAs are deregulated in MI and might be considered potential therapeutic targets for the development of myocardial ischemia. The suppression of miR-16 protects against acute myocardial infarction.11 The inhibition of miR-124 reduces cardiomyocyte apoptosis in MI.12 Indeed, more investigations about the function of miRNAs in MI are needed.

MiR-665 serves as a novel small molecule involved in regulating the pathological process of various diseases.
A recent study has demonstrated that miR-665 promotes apoptosis and colitis in inflammatory bowel disease by inhibiting XBP1 and ORMDL3. Moreover, miR-665-3p regulates inflammation and apoptosis by targeting ATG4B in intestinal ischemia/reperfusion (I/R). However, the role of miR-665 in myocardial ischemia still remains unclear. P21-activated kinases (Paks) are a family of serine/threonine kinase, which is activated by cell division control protein 42 (Cdc42) and Ras-related C3 botulinum toxin substrate (Rac1). Egom EE has proved that Pak1 contributes to I/R injury in in vivo experiment and points that it might be a new therapeutic target for I/R. Loss of Pak1 results in phosphorylation of myosin and inhibits the recovery of cardiac function after I/R. Pak1/Akt signal activity is enhanced by fingolimod (FTY720), suggesting the protective effect of Pak1 on rat heart. Nevertheless, the mechanism of miR-665 in myocardial I/R injury remains unknown.

In the present study, we established an I/R rat model and a hypoxia/reoxygenation (H/R)-treated H9c2 cell model to mimic the I/R in vivo and in vitro studies. We then explored that the inhibition of miR-665 could protect cardiomyocytes from oxygen-induced reactive oxygen species (ROS) accumulation and apoptosis via targeting Pak1 in MI. This exploration may contribute to the discovery of new therapeutic targets for MI.

**Methods**

**Animals and coronary ligation model:** Animal experiments in this study were performed according to the Guidelines for the Care and Use of Laboratory Animals and approved by Animal Ethics Committee of Qilu Hospital of Shandong University (approval no. KYLL-2019 (KS)-363). Male Sprague Dawley rats (30 rats, 240-270 g) were purchased from Nanjing Junkebio Co. Ltd. The animals were kept at a temperature of 23 ± 1°C for 12 hours light/12 hours dark cycle. During the experiments, the rats were given an adequate supply of food and water. The rats were randomly divided into six groups (n = 5/group): sham, I/R, I/R + agomiR NC, I/R + agomiR-665, I/R + antagomiR NC, and I/R + antagomiR-665.

The rats were anesthetized with the mixture of xylocaine (10 mg/kg) and ketamine (60 mg/kg). A left thoracotomy was performed between the third and fourth ribs to expose the heart. The antagomiR NC, antagomiR-665, agomiR NC, or agomiR-665 (Guangzhou RiboBio Co. Ltd, China) was injected through the apex of the heart to the left ventricle, and the aorta and pulmonary artery were clamped for 10 seconds. Rats were injected with the same volume of saline. The left anterior descending coronary artery was then looped around by 5-0 suture, and the chest cavity was closed with 5-0 suture. Sham rats received the same surgical procedure without tying the thread. The area of myocardial infarct size (IS) was determined through trypan blue staining. Afterward, the hearts were frozen for the following experiments.

**Cell culture and transfection:** The H9c2 cells were purchased from the American Type Culture Collection (ATCC, USA) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; 12491-015 Gibco, USA) containing 10% fetal bovine serum (FBS; F9423, Sigma, USA) and 1% penicillin/streptomycin (PS, 15140122, Gibco, USA) at 37°C in 5% CO2 air. The cells were then transfected with miR-665 mimic, NC-mimic, NC-inhibitor, or miR-665 inhibitor using Lipofectamine 2000 (7222551, Invitrogen, USA) for 24 hours. The sequences were as follows: NC-inhibitor, CAGUACUGUAGUGAUACTT; miR-665 inhibitor, AGGGGCCUCAGCCUCCGU; NC-mimic, CCCUGUGCCAGGUGGA; miR-665 mimic, AGACAGAGGUCCAAGAATT; and antisense 5'-UUUUCUUCUAAGAAUUTT-3'.

The H9c2 cells were transfected with si-Pak1 by using Lipofectamine 2000. The sequence of si-Pak1 was sense 5’-GGCGAUCCUAAGAAUUTT-3’ and antisense 5’-UUUUCUUCUAAGAAUUTT-3’.

For the H/R procedure, the H9c2 cells were incubated with hypoxic conditions (5% CO2, 1% O2, and 94% N2) for 12 hours and were then cultured under normoxic conditions for 6 hours to achieve reoxygenation.

**Automatic biochemical analysis:** The concentrations of creatine kinase (CK-MB) and lactate dehydrogenase (LDH) in serum were examined by CK-MB (NAC-act.) (CK7946, RANDOX Laboratories, UK) and Cytotoxicity LDH Assay Kit (WST-8, Beyotime, China).

**Enzyme-linked immunosorbent assay (ELISA):** The concentrations of TNF-α and IL-6 in serum and cells were tested by TNF-alpha (rat) ELISA Kit (K1052-100, Biovision, USA), Human TNF-α ELISA Kit (CS-ELISA 1826, TSZ, US), Rat IL-6 ELISA Kit (ab100772, Abcam, UK), and Human IL-6 ELISA Kit (ab178013, Abcam, UK) as the instruction described.

**HE staining:** The tissues from rats were fixed in 4% paraformaldehyde, dehydrated, transparentized, embedded into paraffin, and cut into slices. The paraffin sections were dewaxed, dehydrated by gradient alcohol, embedded into paraffin, and cut into slices. The paraffin sections were dewaxed, dehydrated, transparentized, embedded into paraffin, and cut into slices. The area of myocardial infarct size (IS) was determined through trypan blue staining. Afterward, the hearts were frozen for the following experiments.

**Apoptosis assays:** The cell apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) with a Roche In Situ Cell Death Detection Kit (11684817910, Roche, UK). The H9c2 cells were transfected with si-Pak1 by using Lipofectamine 2000. The sequence of si-Pak1 was sense 5’-GGCGAUCCUAAGAAUUTT-3’ and antisense 5’-UUUUCUUCUAAGAAUUTT-3’.

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**HE staining:** The tissues from rats were fixed in 4% paraformaldehyde, dehydrated, transparentized, embedded into paraffin, and cut into slices. The paraffin sections were dewaxed, dehydrated by gradient alcohol, washed by phosphate buffer solution (PBS), incubated with hematoxylin staining solution for 10 minutes, and washed with ddH2O. The sections were then differentiated with 1% hydrochloric acid alcohol for 10 s, stained with eosin staining solution for 5 minutes, and washed by ddH2O. Thereafter, the stained sections were dehydrated by gradient alcohol and were incubated in xylene for 10 minutes. Finally, the sections were sealed and observed in a microscope (magnification, 200×).

**ROS detection:** The ROS levels were measured with dichlorodihydrofluorescein diacetate (H2DCFDA; D-399, Invitrogen, USA), and the DCF fluorescence signal was measured using a fluorophotometer.

**MTT assay:** The cell viability was evaluated through 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. The treated H9c2 cells were then plated on 96-well plates at the density of 2 × 104 cells/well and cultured in 5% CO2 at 37°C. A total of 20 μL MTT (5 mg/mL) was added to each well. After 4 hours, the supernatant was removed, and 100 μL DMSO was added to each well. After dissolving, the OD value of each well was measured at 490 nm wavelength with a microplate reader.
The H9c2 cells after different treatment were incubated with PI/ FITC-annexin V solution containing RNase A in the dark for 1 hour at room temperature and then washed with PBS. Apoptosis of cells was visualized by a flow cytometry, and the cell apoptosis rates were calculated.

**Dual luciferase-reporter assay:** The potential binding site of miR-665 and Pak1 was predicted by TargetScan (ht tp://www.targetscan.org/). Vectors containing wild-type or mutant miR-665 from the Pak1 3'-UTR (Pak1-WT or Pak 1-MUT) were obtained from GenePharma (Shanghai, China) and were co-transfected with miR-665 mimic into HEK293 cells. After 48 hours, the luciferase activities were measured by a Dual-Luciferase Reporter Assay Kit (FR201-02, TransGen, China).

**Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR):** The total RNA from the tissues or H9c2 cells was extracted by TRIzol reagent (10296-010, Ambion, USA). After evaluating the quality and integrity of acquired RNA, qRT-PCR was performed through PrimeScript reagent RT Kit and SYBR PrimeScript RT-PCR Kits (#RR037A, Takara, Japan). The mRNA level or target gene expression level was calculated with the 2^(-ΔΔCt) method, which was normalized by U6 or β-actin. The primers used in this study were as follows: U6-RT, GTCG TATCCAGTCAGGTTCCAGTCTGGATACGAC AAAATATGG; miR-665-RT, GTCGTATCCAGTCTGGAG GTCCGAGGTGCACTGGATACGACAGGGGC; miR-665-qPCR-forward, TGCGGACCAGGAGGCTGAG; U6-forward, TGGCGGTGCTCGCTTCGGCAGC; β-actin-forward, CGTGACATTAAGGAGAAGCTG; β-actin-reverse, CTAGAAGCATTTGCGGTGGAC; Pak1-forward, CGTGGCTACATCTCCCCATT; and Pak1-reverse, AGGC TTCTTCTTTGCGTCTCT.

**Western blot analysis:** A total protein was isolated from tissues or cells. Then, the equal protein was separated by 12% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; IPVH00010, Millipore, USA). Then primary antibodies containing cleaved caspase-3 human antibody (1:1000; Asp175, Cell Signaling Technology, CST, USA), cleaved caspase-9 rats antibody (1:1000; Asp353, CST, USA), cleaved caspase-9 human antibody (1:1000; Asp315, CST, USA), Bcl-2 antibody (1:500; sc-509, Santa Cruz Biotechnology, USA), Bax antibody (1:200; AB026, Beyotime, China), Pak1 antibody (1:500; GTX111208, GeneTex, USA), β-actin (1:500; sc-47778, Santa Cruz Biotechnology, USA), and the secondary antibody containing HRP-labeled Goat Anti-Rabbit IgG (H+ L) (1:8000; A0208, Beyotime, China) or HRP-labeled Goat Anti-Mouse IgG (H+L) (1:8000; A0216, Beyotime, China) were used.

**Statistics analysis:** The data in this study were presented as the mean ± SD and performed using GraphPad Prism 8.0 (GraphPad Prism Software, USA). Statistical analyses were performed using Student’s t-test or one-way analysis of variance followed by Tukey’s post hoc test. P-values less than 0.05 were considered as significant.

**Results**

MiR-665 was upregulated and promoted myocardial infarction in I/R rats: RT-qPCR was used to detect the mRNA levels of miR-665 in heart tissues of I/R rats. As presented in Figure 1A, compared to sham rats, miR-665 was significantly upregulated in heart tissues of I/R rats. Moreover, miR-665 was downregulated in antagoniR-665 rats and upregulated in agomiR-665 rats compared with antagoniR-NC rats or agomiR-NC rats. To explore the importance of miR-665 in I/R injury, the infarct area of hearts was measured by HE and trypan blue staining. Compared with the sham rats, the IS was markedly increased in I/R rats, suggesting the success of I/R establishment. Overexpression of miR-665 following ischemia treatment significantly increased the IS compared with the agomiR-NC rats, and knockdown of miR-665 following ischemia treatment significantly reduced the IS compared with the antagoniR-NC rats (Figure 1B). In addition, the overexpression of miR-665 increased the concentrations of serum TNF-α, IL-6, LDH, and CK-MB, and knockdown of miR-665 decreased the concentrations of serum TNF-α, IL-6, LDH, and CK-MB (Figure 1C-F). Finally, the cardiomyocyte apoptosis was assessed by TUNEL assay, indicating that the overexpression of miR-665 significantly induced cardiomyocyte apoptosis compared with the agomiR-NC rats and that knockdown of miR-665 significantly inhibited cardiomyocyte apoptosis compared with the antagoniR-NC rats (Figure 1G). Therefore, miR-665 was upregulated and promoted MI in I/R rats.

MiR-665 induced cell apoptosis and ROS generation in H/R-treated H9c2 cells: To further explore the effect of miR-665 on MI, an H/R-treated H9c2 cell model was successfully established. As presented in Figure 2A, the miR-665 level was increased in H/R-treated H9c2 cells. The cell viability and proliferation were then determined by MTT assay. The miR-665 was upregulated in H/R-treated H9c2 cells, and overexpression of miR-665 decreased cell viability and proliferation; however, knockdown of miR-665 increased cell viability and proliferation in H/R-treated H9c2 cells (Figure 2B). Moreover, the concentrations of LDH and CK-MB were induced by miR-665 mimics but were inhibited by miR-665 inhibitor in H/R-treated H9c2 cells (Figure 2C and D). In addition, the mitochondrial ROS generation was significantly decreased by knockdown of miR-665 but was increased by the overexpression of miR-665 (Figure 2E). Next, the concentration of TNF-α and IL-6 was increased in H/R-treated H9c2 cells and was decreased by miR-665 inhibitor increased by miR-665 mimics (Figure 2F-G). More importantly, the cell apoptosis was examined by a PI/FITC apoptosis assay. As shown in Figure 2H, H/R treatment induced cell apoptosis in H9c2 cells. The miR-665 mimics aggravated cell apoptosis, but miR-665 inhibitor alleviated cell apoptosis in H/R-treated H9c2 cells. Finally, the apoptosis-related proteins containing Bax, Bcl-2, cleaved caspase-9, and cleaved caspase-3 were assessed through western blot. The protein levels of cleaved Bax caspase-9 and cleaved caspase-3 were promoted by H/R treatment or miR-665 mimics but were inhibited by miR-665 inhibitors, whereas the expression of Bcl-2 was opposite (Figure...
Figure 1. MiR-665 was upregulated and promoted myocardial infarction in I/R rats. A: Reverse transcription-quantitative polymerase chain reaction was used to detect the expression of miR-665 cardiomyocytes of different treated rats. B: HE (200×) and trypan blue-stained heart slices from the sample. Infarct size was calculated. C-F: The concentrations of serum LDH (C), CK-MB (D), TNF-α (E), and IL-6 (F) were measured. G: The TUNEL assay was used to determine the cardiomyocyte apoptosis (magnification, 200×). n = 5. **P < 0.01.

2I). Therefore, miR-665 induced cell apoptosis and ROS generation in H/R-treated H9c2 cells.

Pak1 was the target gene of miR-665: The levels of Pak1 were analyzed by TargetScan, and the results indicated that Pak1 is targeted by miR-665. There is a binding site between 3'-UTR of the Pak1 and miR-665. To verify this binding site, the putative miR-665 binding sequence from the Pak1 3'-UTR was cloned into the luciferase reporter plasmid (Figure 3A). HEK293 cells were co-transfected with miR-665 mimics or NC-mimic and Pak1-MUT or Pak1-WT, and the luciferase activity was then subsequently measured. It was observed that miR-665 mimic significantly inhibited luciferase activity in Pak1-WT cells, whereas NC-mimic did not. Moreover, both miR-665 and NC-mimic did not change the luciferase activity in Pak1-MUT cells (Figure 3B). To demonstrate that miR-655 targets Pak1 and inhibits endogenous Pak1 expression, the mRNA and protein levels of Pak1 were measured in H/R-treated H9c2 cells, which were transfected with miR-665 mimic, NC-mimic, NC-inhibitor, or miR-665 inhibitor. As presented in Figure 3C and D, miR-665 mimic significantly decreased the mRNA and protein levels of Pak1, whereas miR-665 inhibitor significantly increased the mRNA and protein levels of Pak1 in H/R-treated H9c2 cells. Finally, the expression level of Pak1 was determined by western blot in vivo and in vitro experiments. As shown in Figure 3E, H/R treatment and I/R treatment downregulated the expression of Pak1 in H9c2 cells and rats. Besides, antagomiR-665 increased the expression of Pak1 in rats compared with antagomiR-NC rats, and agomiR-665 decreased the expression of Pak1 in rats compared with agomiR-NC rats. Interestingly, the results from in vitro experiments were consistent with those from in vivo experiments. Therefore, Pak1 was the target gene of miR-665 and was regulated by miR-665 in vivo and in vitro studies.

Knockdown of miR-665 depressed H/R-induced ROS accumulation and apoptosis through the activation of Pak1/Akt signaling: To further confirm the role of Pak1 in H/R-treated H9c2 cells, the expression of Pak1 and the
MiR-665 PROMOTED ROS ACCUMULATION AND APOPTOSIS

Figure 2. MiR-665 induced cell apoptosis and ROS generation in H/R-treated H9c2 cells. A: The miR-665 level was detected by qRT-PCR. B: The cell viability was assessed by MTT assay. C, D: The concentrations of LDH (C) and CK-MB (D) were measured. E: Total intracellular ROS generation was assessed by DCF-DA assay and observed by a flow cytometric assay. F, G: The concentration of TNF-α (F) and IL-6 (G) was measured. H: The cell apoptosis was determined by a PI/FITC apoptosis assay. I: The apoptosis-related proteins containing cleaved caspase-3, cleaved caspase-9, Bax, and Bcl-2 were examined by western blot. n = 3. **P < 0.01.

Discussion

In this study, the miR-665 was observed to be...
Figure 3. Pak1 was the target gene of miR-665. A: The binding site of miR-665 and Pak1 was predicted by TargetScan. B: The binding of miR-665 and Pak1 was determined by luciferase assay. C, D: The mRNA (C) and protein (D) levels of Pak1 in miR-655 inhibitor/miR-665 mimic H9c2 cells were detected through qRT-PCR and western blot. E: The expression levels of Pak1 in I/R rats and H/R-treated H9c2 cells were examined by western blot. **P < 0.01.

upregulated in I/R rats, and overexpression of miR-665 significantly increased TNF-α, IL-6, LDH, CK-MB, and ROS concentrations and induced cell apoptosis but inhibited cell proliferation, while knockdown of miR-665 was opposite. From in vitro studies, miR-665 induced cell apoptosis and ROS generation in H/R-treated H9c2 cells. More importantly, Pak1 was proved to be a target gene of miR-665 based on luciferase reporter assay. Besides, miR-665 inhibitor could significantly depress the accumulation of ROS and cell apoptosis by targeting Pak1 and promoting the phosphorylation of Akt, whereas knockdown of Pak1 could inhibit the protection of miR-665 inhibitor in H/R-treated H9c2 cells. Thus, we could conclude that knockdown of miR-665 protects against cardiomyocyte ischemia/reperfusion injury-induced ROS accumulation and apoptosis through activating Pak1/Akt signal pathway in MI. This is the first study to investigate the function of miR-665 in cardiomyocytes I/R and to provide a primary foundation for future investigation of theories regarding MI-related diseases.

Current therapies for MI are limited and non-curative. MicroRNAs (miRNAs) are a series of noncoding RNAs at length of 22 nucleotides and regulate posttranscriptional gene expression by binding to 3'-UTRs of target genes. Nowadays, miRNAs have been proven to play a vital role in MI. MiR-125b-5p represses proapoptotic bak1 and klf13 in cardiomyocytes and protects the heart from AMI. MiR-19a/19b could reduce MI-induced cardiac damage and protect cardiac function. Despite the therapies of miRNAs in MI, there are still many miRNAs that act as a potential therapeutic target in MI. MiR-221-3p serves as a novel marker for early prediction of AMI. Given that miR-103a was increased in AMI patients, it might be a potential biomarker of MI and could be an index for the diagnosis of AMI. In the current study, we demonstrated that miR-665 was abnormally expressed in I/R-treated rats and might be related to the progression of MI; however, further investigations are needed to understand its mechanism.

MiR-665 is a novel miRNA and participates in multiple diseases. MiR-665 aggravates heart failure by suppressing coronary microvessel angiogenesis. In breast cancer, miR-665 promotes tumor metastasis by targeting to NR4A3. Prashad, et al. have proved that miR-665 inhibits murine neuroblastoma cell growth via targeting c-MYC and HDAC8. Recently, accumulating researches have proved that the recovery of oxidative stress homeostasis is considered to be a promising treatment strategy for cardiac remodeling and failure after AMI. In early stage, mitochondrial ROS production is a critical for IR
injury, which is the consequence of the interaction of a dysfunctional respiratory chain with oxygen.\(^{28-30}\) Moreover, apoptosis is the major cell death mechanism in IRI.\(^{31}\) In the present study, the miR-665 was observed to induce cell apoptosis and ROS generation in in vivo and in vitro studies, suggesting that miR-665 might be involved in MI via regulating cell apoptosis and ROS generation.

Pak1 stimulation could suppress the NOX2-dependent ROS production.\(^{32}\) Moreover, Pak1 promotes cellular survival and proliferation in breast and squamous NSCLCs.\(^{33}\) From in vivo study, silencing of Pak1 gene decreases proliferation in xenograft tumor through the p53/p21 pathway.\(^{34}\) In the present study, Pak1 was proved to be the target gene of miR-665. Interestingly, Pak1 promotes the proliferation and inhibits apoptosis via the Akt pathway.\(^{35}\) Similarly, silencing of Pak1 significantly decreased the phosphorylation of Akt and deactivated Akt signaling pathway. Given that miR-665 inhibitor could significantly depress the accumulation of ROS and cell apoptosis by promoting the phosphorylation of Akt, silencing of Pak1 could inhibit the protection of miR-665 inhibitor and depress the phosphorylation of Akt in H/R-treated H9c2 cells. Combining all these findings, we could conclude that miR-665 inhibitor inhibited ROS accumulation and apoptosis via targeting Pak1 and activating Pak1/Akt signaling in MI. Although miR-665 could regulate the ROS accumulation and apoptosis, whether it could modulate inflammatory cell recruitment or the respiratory oxidative burst in MI needs more efforts in the future.

In summary, we have demonstrated that knockdown of miR-665 protects against cardiomyocyte ischemia/reperfusion injury-induced ROS accumulation and apoptosis through activation of Pak1/Akt signaling pathway in MI. Collectively, understanding the biology and modulation of miR-665 may have the potential to counteract the development of MI.

**Disclosure**

Conflicts of interest: The authors declare that they have no conflicts of interests, and all authors should confirm its accuracy.
Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Authors' contributions: GQC and CZL conceived and designed the experiments, MMT and XQZ analyzed and interpreted the results of the experiments, and JHL performed the experiments.

Ethics approval and consent to participate: The animal use protocol listed below has been reviewed and approved by the Animal Ethical and Welfare Committee.

Informed consent: Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

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