

CircHIPK3 aggravates myocardial ischemia-reperfusion injury by binding to miRNA-124-3p

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Abstract. – OBJECTIVE: To elucidate whether circHIPK3 could inhibit proliferation and induce apoptosis of cardiomyocytes *via* binding to miRNA-124-3p, thus aggravating myocardial ischemia/reperfusion (I/R) injury.

MATERIALS AND METHODS: CircHIPK3 expression in HCM cells simulated with myocardial I/R was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Influences of circHIPK3 on myocardial injury marker levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) in the *in vitro* model of myocardial I/R were evaluated using the relative commercial kits. The regulatory effects of circHIPK3 on proliferation, viability and apoptosis of simulated myocardial cells were examined by Cell Counting Kit-8 (CCK-8) assay and flow cytometry, respectively. Dual-Luciferase reporter gene assay was conducted to verify the binding of circHIPK3 to miRNA-124-3p. Finally, the roles of the circHIPK3/miRNA-124-3p axis in regulating apoptotic gene expressions and cardiomyocyte apoptosis were evaluated after myocardial I/R were explored.

RESULTS: CircHIPK3 was highly expressed in HCM cells simulated myocardial I/R related to those with normoxic treatment. The overexpression of circHIPK3 in simulated HCM cells increased levels of LDH, SOD and GSH-PX, whereas increased the MDA level. Inhibited proliferation and accelerated apoptosis were observed in simulated HCM cells overexpressing circHIPK3. Western blot analyses illustrated that circHIPK3 overexpression upregulated pro-apoptotic Bax, and downregulated anti-apoptotic Bcl-2. Subsequently, we confirmed the binding between circHIPK3 and miRNA-124-3p. Rescue experiments demonstrated that circHIPK3 overexpression reversed the protective effects of miRNA-124-3p on myocardial I/R and cardiomyocyte apoptosis.

CONCLUSION: CircHIPK3 inhibits proliferative ability and induces apoptosis of cardiomyocytes after myocardial I/R injury by binding to miRNA-124-3p, which may serve as a potential therapeutic target for I/R.

Key Words:

CircHIPK3, miRNA-124-3p, I/R, Proliferation, Apoptosis.

Introduction

Coronary atherosclerotic cardiopathy is a myocardial ischemic disease caused by atherosclerosis involving coronary artery or dysfunction of the coronary artery such as spasm¹. It has become a severe disease with high mortality and morbidity in developed countries, which threatens human life. Coronary atherosclerotic cardiopathy is also the major reason for chronic heart failure². Acute myocardial infarction results from acute occlusion of the epicardial coronary artery, leading to myocardial ischemia, hypoxia injury and even necrosis of the distal vascular endothelium³. In the case of uncorrectable myocardial ischemia and hypoxia injury due to coronary occlusion, it will lead to myocardial necrosis in the acute phase and subsequently, myocardial damage and fibrosis in the chronic phase. Cardiac function is seriously affected owing to the insufficient systolic and diastolic function of heart after myocardial fibrosis⁴. Ischemia-reperfusion (I/R) injury is caused by blood flow supply restoration in the lesioned tissues after a long-term period of ischemia and hypoxia. I/R aggravates the initial ischemic injury on both structures and functions of tissues, even resulting in irreversible damage⁵. I/R injury is

affected by multiple factors, including free radicals, intracellular calcium overload, neutrophilic leukocytosis, etc. During the process of I/R injury, cardiomyocyte apoptosis is induced by abundant oxygen free radicals, increased intracellular calcium ions, mitochondrial damage, inflammatory cell infiltration and other mechanisms^{6,7}.

CircRNA is a class of RNA molecules that do not have a 5' end cap and a 3' end poly(A) tail, forming a closed loop structure by covalent bonds⁸. Classical RNA detection methods can only distinguish linear RNA molecules containing poly(A) tails. Due to the specific structure, circRNA has not been identified by analysis of transcriptome polyadenylate RNA⁹. In recent years, circRNAs are sequenced after removing ribosomal RNA and linear RNA in the total RNA. Biological functions of circRNAs are involved in the whole process of gene regulations, including mRNA transcription and splicing, as well as RNA degradation and translation¹⁰. CircHIPK3 shows a high abundance in glioma, osteosarcoma, hepatocellular carcinoma, etc.¹¹⁻¹³, which is mainly originated from the second exon of HIPK3. There are three spliceosomes of circHIPK3, namely circHIPK3, circHIPK3.1 and circHIPK3.2. However, only circHIPK3 is highly abundant and has special functions in cells. Nevertheless, the regulatory mechanism of circHIPK3 in myocardial I/R injury has not been clarified.

Materials and Methods

Cell Culture

Human-derived cardiomyocytes HCM were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). HCM cells were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 100 µg/mL streptomycin (Gibco, Grand Island, NY, USA), and maintained in a 37°C, 5% CO₂ incubator.

Simulation of I/R in HCM

In vitro simulation of myocardial I/R injury was induced by oxygen and glucose deprivation (OGD) and reperfusion (OGD/R) in HCM cells. Briefly, 3×10⁵ HCM cells were cultured in a 35 mm dish and incubated for 24 h. After serum starvation and glucose deprivation, cells were maintained in a humidified chamber equilibrated with 5% CO₂ and 95% N₂ for 10 h. Subsequently, cells were cultured in DMEM

containing 10% FBS and 4.5 mg/mL glucose for 2 h and transferred in the chamber equilibrated with 5% CO₂ and 95% air for 24 h. HCM cells cultured in normoxic condition were considered as controls.

Cell Transfection

Cells with good viability were seeded in 6-well plates and incubated in 1 mL of serum-free medium containing 100 µL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 24-48 h. CircHIPK3-O₂, circ-HIPK3-siRNA, miRNA-124-3p mimics, inhibitor and negative control were provided by Genesharma (Shanghai, China).

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. Cell lysis was mixed with chloroform, centrifuged and the precipitate was incubated with isopropanol. After centrifugation, the precipitate was washed with 75% ethanol, air dried and diluted in diethyl pyrocarbonate (DEPC) water (Sangon, Shanghai, China). The extracted RNA was subjected to reverse transcription using the Revert Aid First Strand complementary deoxyribose nucleic acid (cDNA) Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA) and amplified by SYBR® Green Master Mix (TaKaRa, Otsu, Shiga, Japan). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as the internal reference. Primer sequences were as follows: Circ-HIPK3, F: 5'-TATGTTGGTGGATCCTGTTCCGGCA-3'; R: 5'-TGGTGGGTAGACCAAGACTTGTGA-3'; MiRNA-124-3p, F: 5'-CGGTAAGGCACGCGTGA-3'; R: 5'-AGTGCGAACTGTGGCGAT-3'; Bax, F: 5'-CACAACTCAGCGCAAACATT-3'; R: 5'-ACAGCCATCTCTCTCCATGC-3'; Bcl-2, F: 5'-GAAGCACAGATGGTTGATGG-3'; R: 5'-CAGCCTCACAAGGTTCCAAT-3'; GAPDH, F: 5'-GGAATCCACTGGCGTCTTCA-3'; R: 5'-GGTTCACGCCCATCACAAAC-3'; U6, F: 5'-AGAGAAGATTAGCATGGCCCCTG-3'; R: 5'-ATCCAGTGCGGGTCCGAGG-3'.

Determination of Levels of Lactate Dehydrogenase (LDH), Malondialdehyde (MDA), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GSH-PX)

Commercial LDH, MDA, SOD and GSH-PX determination kits were obtained from Sangon

Biotech (Shanghai, China). Transfected cells were harvested for determining these levels based on the manufacturer's recommendations.

Cell Proliferation Assay

The cells in the logarithmic growth phase were seeded in a 96-well plate with 3×10^3 cells per well. At the appointed time points, 10 μ L of Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan) solution was added to each well and incubated at 37°C for 2 h. The wavelength at 450 nm was detected by a microplate reader.

Apoptosis Determination

The cells were washed with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) twice, digested and fixed in 70% cold ethanol for 30 min. The precipitate was incubated in 5 mL of Annexin V-fluorescein isothiocyanate (FITC; Beyotime, Shanghai, China) and 1 mL of Propidium Iodide (PI; 50 mg/mL). Finally, apoptosis was determined using EPICS XL-MCL FACSscan (Becton-Dickinson, Franklin Lakes, NJ, USA).

Dual-Luciferase Reporter Gene Assay

CircHIPK3 3'UTR containing the wild-type or mutant-type sequences of the miRNA-124-3p target binding sites was cloned into the luciferase reporter vector, respectively. It was co-transfected with miRNA-124-3p mimic into HCM cells for 48 h, followed by Luciferase activity determination.

Western Blot

Total protein was extracted from cell lysis, quantified and electrophoresed. After transferring on polyvinylidene difluoride (PVDF) mem-

branes (Millipore, Billerica, MA, USA), they were incubated with primary antibodies at 4°C. At the other day, the membranes were incubated with the corresponding secondary antibody for 2 h. Bands were exposed with the enhanced chemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA) and integral optical density was analyzed by gel imaging analysis system.

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 20.0 statistical software (SPSS Inc. Chicago, IL, USA) and Graph Prism 7 (GraphPad Software Inc., Jolla, CA, USA). Quantitative data are represented as mean $\pm$ standard deviation ($\pm s$) and analyzed by the *t*-test. $p < 0.05$ was considered statistically significant.

Results

CircHIPK3 is Upregulated in HCM Cells Simulated With I/R

CircHIPK3 expression in HCM cells simulated with myocardial I/R and those with normoxic treatment was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), which was markedly higher in the former (Figure 1A). Subsequently, transfection of the constructed circHIPK3-OE or circHIPK3-si greatly upregulated or downregulated circHIPK3 level in HCM cells, respectively (Figure 1B, 1C).

CircHIPK3 Overexpression Inhibited Proliferative Ability and Induced Apoptosis of HCM Cells

It is well known that LDH, MDA, SOD and GSH-PX are the markers of myocardial injury.

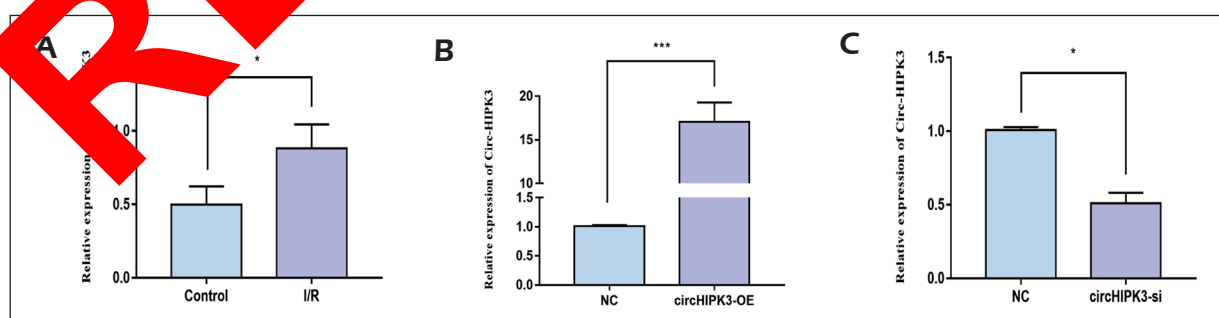


Figure 1. CircHIPK3 is upregulated in HCM cells simulated with I/R. **A**, CircHIPK3 expression was higher in HCM cells simulated with myocardial I/R and those with normoxic treatment. **B**, **C**, Transfection efficacy of the constructed circHIPK3-OE **B**, and circHIPK3-siRNA **C**, in HCM cells.

Here, we determined their contents in simulated HCM cells with overexpression or knockdown of circHIPK3. Transfection of circHIPK3-OE reduced contents of LDH, SOD and GSH-PX, but enhanced SOD content. Conversely, transfection of circHIPK3-si obtained the opposite results (Figure 2A-2D). As the CCK-8 assay revealed, circHIPK3 overexpression attenuated the proliferative ability of simulated HCM cells, whereas circHIPK3 knockdown promoted HCM cells to proliferate (Figure 2E). Flow cytometry showed the accelerated apoptosis after circHIPK3 overexpression (Figure 2F). We further detected the relative levels of Bax and Bcl-2. Both mRNA and protein levels of Bax were upregulated in simulated HCM cells overexpressing circHIPK3, while Bcl-2 was downregulated (Figure 2G, 2H).

CircHIPK3 Could Bind to MiRNA-124-3p

Through bioinformatics prediction, we found the potential binding sequences between circHIPK3 and miRNA-124-3p (Figure 3A). Transfection efficacy of miRNA-124-3p mimics and inhibitor were first verified (Figure 3B). Furthermore, we tested the binding relationship between these two genes by the Dual-Luciferase reporter gene assay. Co-transfection of miRNA-124-3p mimics and circHIPK3-MUT markedly decreased the Luciferase activity. However, the Luciferase activity did not change in those co-transfected with miRNA-124-3p mimics and circHIPK3-WT (Figure 3C, 3D). We also detected miRNA-124-3p expression influenced by circHIPK3. Overexpression of circHIPK3 inhibited miRNA-124-3p, while

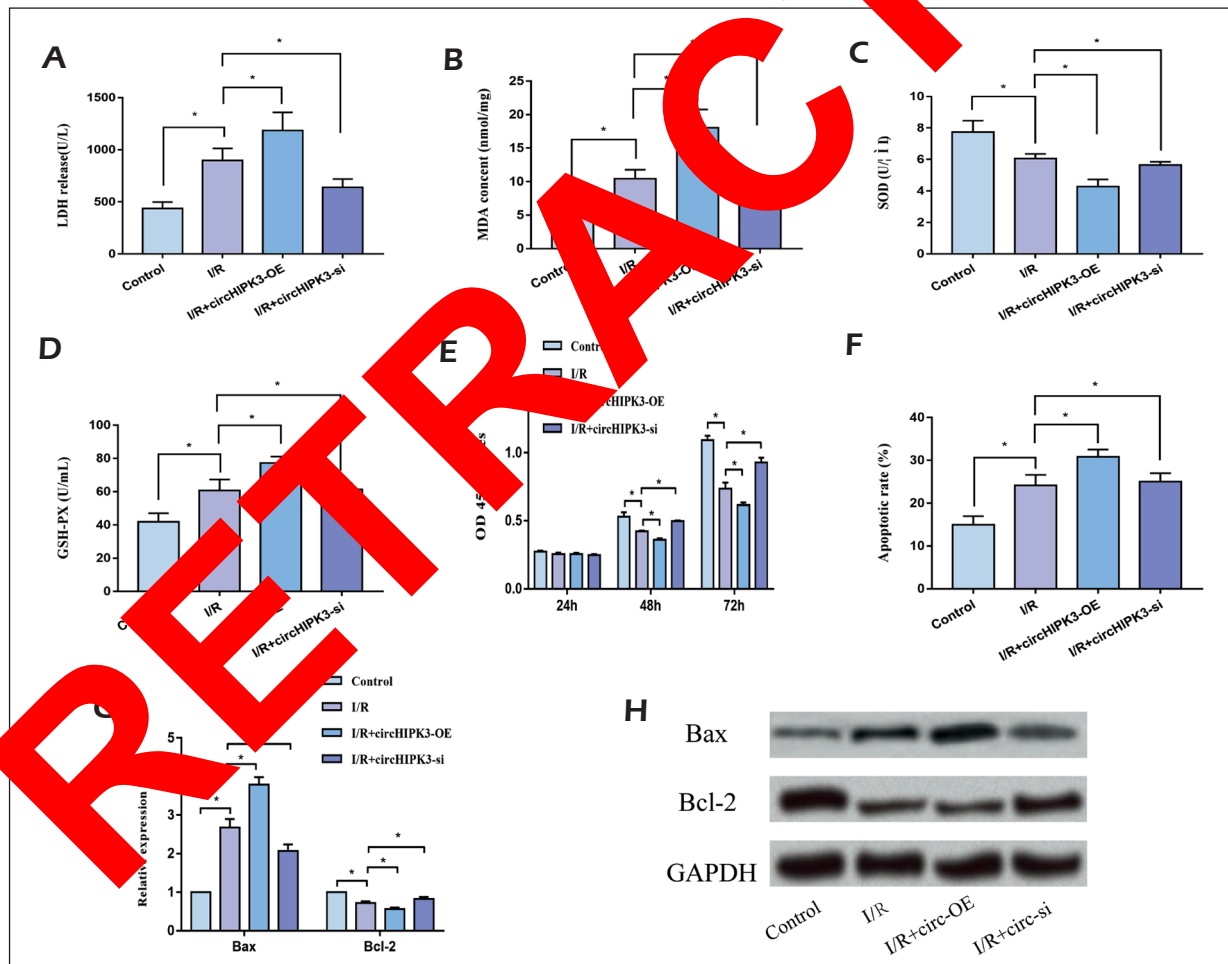


Figure 2. CircHIPK3 overexpression inhibited proliferative ability and induced apoptosis of HCM cells. HCM cells were untreated or treated with I/R, I/R+circHIPK3-OE or I/R+circHIPK3-si, respectively. **A-D**, Contents of LDH **A**, MDA **B**, SOD **C**, and GSH-PX **D**, in HCM cells. **E**, CCK-8 assay showed the proliferation in HCM cells. **F**, Flow cytometry showed the apoptosis in HCM cells. **G**, The relative levels of Bax and Bcl-2 in HCM cells determined by qRT-PCR. **H**, Protein levels of Bax and Bcl-2 in HCM cells determined by Western blot.

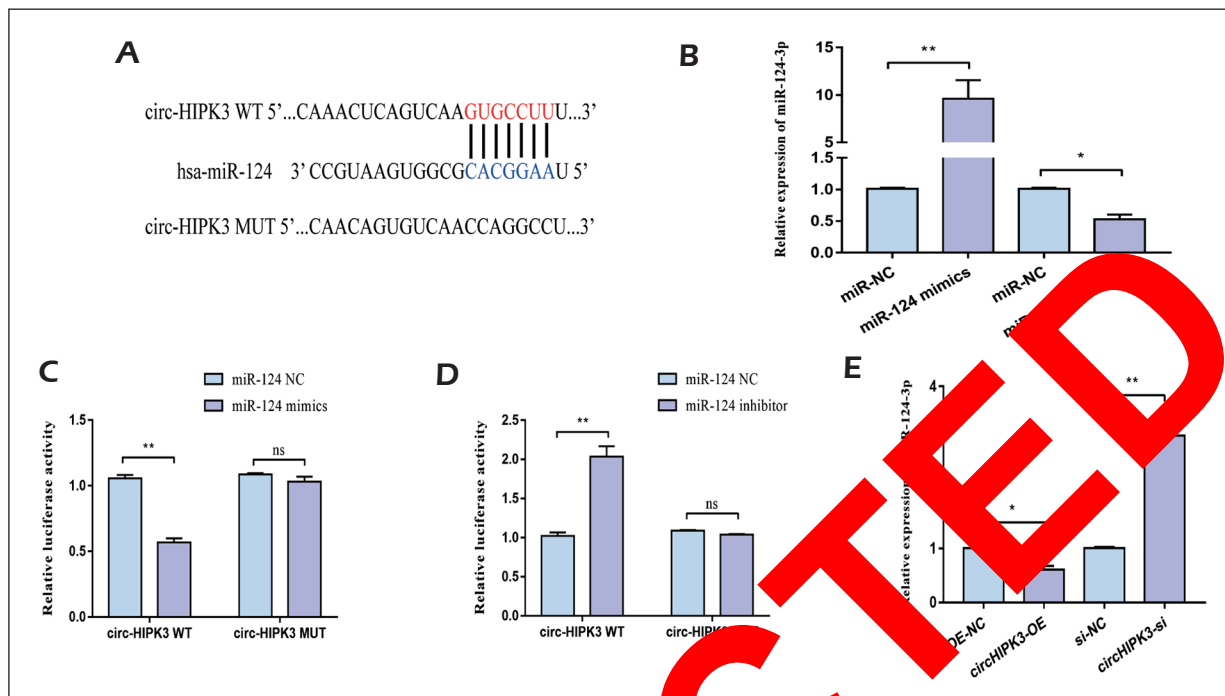


Figure 3. CircHIPK3 could bind to miR-124-3p. **A**, Potential binding sequence between circHIPK3 and miR-124-3p. **B**, Transfection efficacy of miR-124-3p mimics and inhibitor in HCM cells. **C**, **D**, Luciferase reporter gene assay showed that co-transfection of miR-124-3p mimics and circ-HIPK3 WT markedly reduced the Luciferase activity. However, the Luciferase activity did not change in those co-transfected with miR-124-3p mimics and circ-HIPK3 MUT. **E**, The overexpression of circHIPK3 inhibited miR-124-3p, while knockdown of circHIPK3 upregulated miR-124-3p level in HCM cells.

knockdown of circHIPK3 upregulated miR-124-3p level in HCM cells (Figure 3E).

CircHIPK3 Aggravated Myocardial I/R by Binding to miRNA-124-3p

Similarly, we investigated the role of miRNA-124-3p in myocardial I/R injury by measuring the levels of myocardial injury markers. The contents of LDH, SOD and GSH-PX increased by miRNA-124-3p overexpression in HCM cells, while LDH decreased. CircHIPK3 overexpression partially reversed the protective role of miRNA-124-3p in myocardial I/R (Figure 4A-4D). Next, the Wound-healing assay showed that miRNA-124-3p overexpression protected I/R-induced proliferation inhibition, but was further reversed by circHIPK3 overexpression (Figure 4E). Moreover, miRNA-124-3p overexpression slightly inhibited apoptosis in simulated HCM cells, which was reversed by circHIPK3 overexpression (Figure 4F). Therefore, we illustrated that circHIPK3 aggravated myocardial I/R injury by inhibiting miRNA-124-3p.

Discussion

Acute ischemic cardiovascular disease is caused by thrombosis or embolism formed in blood vessels and further leads to acute organ circulatory disorders¹⁴. Clinical manifestations of the ischemic cardiovascular disease include acute ischemic heart failure, varying degrees of angina and myocardial infarction¹⁵. Timely supply of oxygen to ischemic tissues and organs as soon as possible is the key for the treatment. Meanwhile, oxygen supply should be strictly controlled to avoid reperfusion injury resulted from oxygen free radicals and inflammatory factors^{16,17}. Apoptosis is an important pathological mechanism of I/R injury. ROS accumulation in the process of I/R promotes cardiomyocyte apoptosis through the mitochondrial apoptosis pathway. Aghdaei et al¹⁸ have confirmed that the Bcl-2 family can regulate apoptosis by mediating the expression levels of various anti-apoptotic and pro-apoptotic proteins. Pro-apoptotic proteins Bax and Caspase-3 activate the apoptotic pathway; whereas the anti-apoptotic pro-

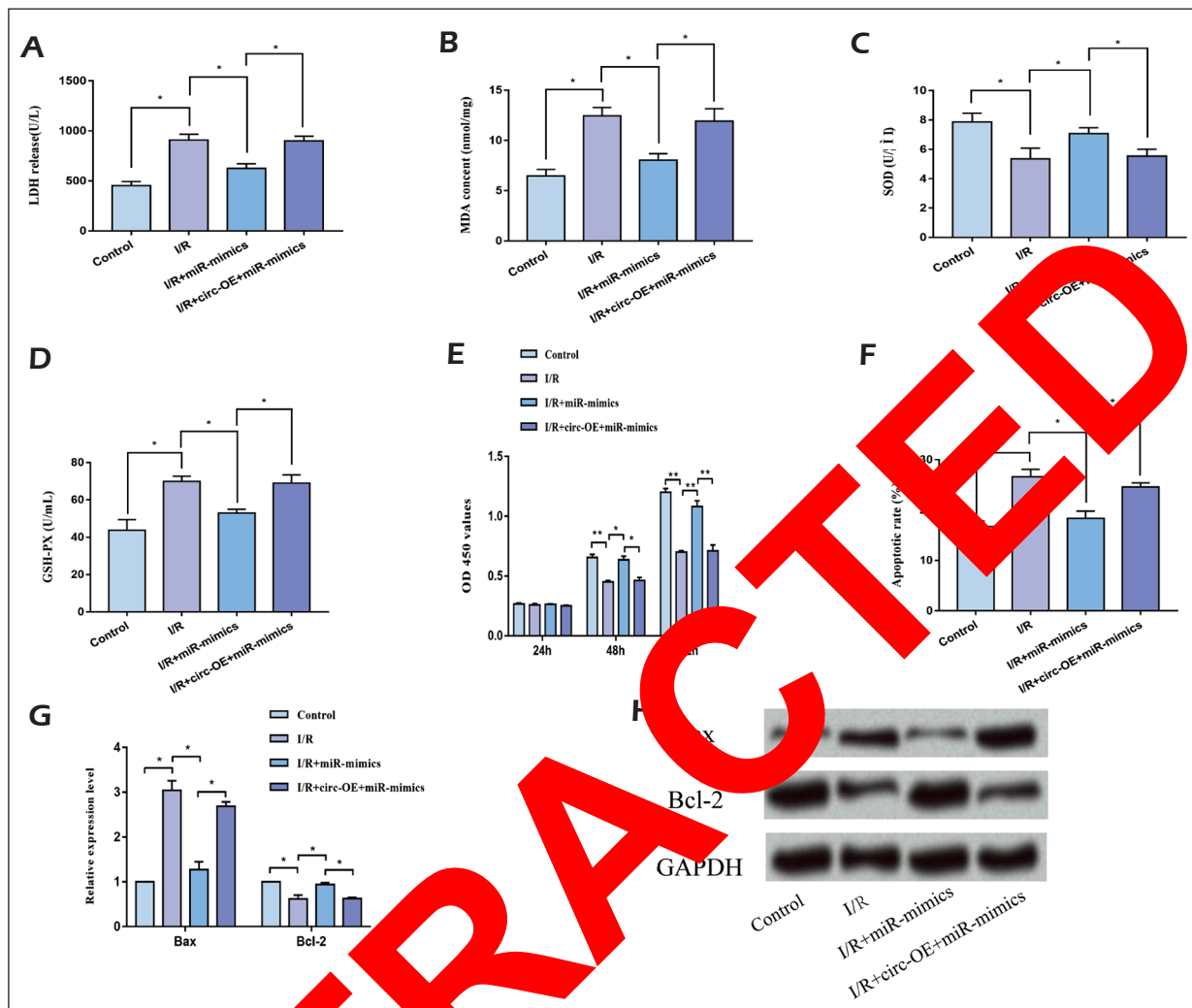


Figure 4. CircHIPK3 ameliorated myocardial injury by binding to miR-124-3p. HCM cells were untreated or treated with I/R, I/R+miR-mimics or I/R+circHIPK3-OE+miR-mimics, respectively. **A-D**, Contents of LDH **A**, MDA **B**, SOD **C**, and GSH-PX **D**, in HCM cells. **E**, WCK-8 assay assessed the proliferation in HCM cells. **F**, Flow cytometry showed the apoptosis in HCM cells. **G**, Relative levels of Bax and Bcl-2 in HCM cells determined by qRT-PCR. **H**, Protein levels of Bax and Bcl-2 in HCM cells determined by Western blot.

tein, exerts a composite role. Bcl-2 inhibits the release of Cytochrome C by blocking the opening of the mitochondrial permeability transition pore. Cytochrome C binds to the cytoplasmic protein Apaf-1, which ultimately inhibits caspase activation and apoptosis induction^{19,20}. Therefore, the ratio of Bcl-2/Bax determines apoptosis-induced cell death. Previous studies have demonstrated the role of circRNAs in cardiovascular diseases. For example, both Cdr1as and miR-7a are upregulated in the myocardium of myocardial infarction mice or hypoxic-preconditioning cardiomyocytes. The overexpression of Cdr1a promotes apoptosis of mouse

cardiomyocytes, which is reversed by miR-7a overexpression²¹. Cardiac-related circular RNA (HRCR) acts as the miR-223 sponge, directly binding to and inhibiting miR-223, thereby increasing ARC (apoptosis repressor with CARD) expression²². Hence, circHRCR/miR-223/ARC axis constitutes a new cardiac hypertrophy/heart failure regulatory pathway, which could be utilized as therapeutic targets. CircRNA000203 can promote the transformation of cardiac fibroblasts into myofibroblasts, and it is a potential therapeutic target for myocardial fibrosis in diabetic cardiomyopathy²³. In recent years, relevant ceRNAs in cardiovascular diseases have

been well studied. As a new post-transcriptional regulation model, ceRNA is involved in the regulation of physical or pathological processes. The expression level of ceRNA influences the occurrence and development of diseases. In this work, a higher level of circHIPK3 was observed in HCM cells simulated with I/R relative to those with normoxic treatment. The overexpression of circHIPK3 markedly decreased the levels of LDH, SOD and GSH-PX, but increased MDA level in simulated HCM cells. The opposite trends were yielded by circHIPK3 knockdown. Subsequently, we demonstrated that circHIPK3 overexpression attenuated proliferative ability and induced apoptosis in HCM cells. Western blot analysis and qRT-PCR identically showed the upregulated Bax and downregulated Bcl-2 in HCM cells overexpressing circHIPK3. It is reported that circRNA may exert its biological function by sponging miRNAs. Here, we observed the binding of miRNA-124-3p to circHIPK3. MiRNA-124-3p expression was negatively regulated by circHIPK3 in HCM cells. Functionally, miRNA-124-3p overexpression stimulated proliferative ability and inhibited apoptosis in HCM cells. Meanwhile, downregulated Bax and upregulated Bcl-2 were observed in HCM cells overexpressing miRNA-124-3p. More importantly, circHIPK3 overexpression reversed the protective effects of miRNA-124-3p on myocardial I/R and cardiomyocyte apoptosis. Therefore, we found that circHIPK3 may come a potential therapeutic target for myocardial I/R injury, but the specific mechanism still requires further studies.

Conclusions

CircHIPK3 inhibits proliferative ability and induces apoptosis of cardiomyocytes after myocardial I/R injury by binding to miRNA-124-3p, which may serve as a potential therapeutic target for myocardial I/R injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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