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MicroRNA-489 promotes cardiomyocyte apoptosis induced by myocardial ischemia-reperfusion injury through inhibiting SPIN1

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Abstract. – OBJECTIVE: To investigate whether microRNA-489 could promote cardiomyocyte apoptosis through targeting inhibition of SPIN1, thus participating in the development of myocardial ischemia-reperfusion injury.

MATERIALS AND METHODS: MicroRNA-489 expression in H9c2 cells induced with hypoxia/reoxygenation (H/R) was determined by quantitative real-time polymerase chain reaction (qRT-PCR). With microRNA-489 overexpression in H/R H9c2 cells, activities of lactate dehydrogenase (LDH), methane dicarboxylic aldehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) were detected using relative commercial kits, respectively. The regulatory effect of microRNA-489 on the proliferation and apoptosis of H/R H9c2 cells was assessed through cell counting kit-8 (CCK-8) assay and flow cytometry (FCM), respectively. Through conducting a dual-luciferase reporter gene assay, we evaluated the binding condition between microRNA-489 and SPIN1. Protein expressions of apoptotic genes in H/R H9c2 cells with microRNA-489 overexpression were determined by Western blot. Finally, the regulatory role of microRNA-489 in PI3K/AKT pathway was detected through Western blot.

RESULTS: QRT-PCR data showed that microRNA-489 was highly expressed in H/R H9c2 cells than those in normoxic control. Overexpression of microRNA-489 in H/R H9c2 cells increased the activities of LDH, MDA and GSH-PX, while decreased the activities of SOD. MicroRNA-489 overexpression markedly inhibited the proliferative rate but accelerated apoptosis of H/R H9c2 cells. Western blot results indicated that protein expressions of pro-apoptotic genes Bax and cytochrome C upregulated, whereas anti-apoptotic gene Bcl-2 downregulated after overexpression of microRNA-489 in H/R H9c2 cells. We confirmed that microRNA-489 could target to SPIN1 and inhibit its expression. After overexpression of microRNA-489 in H/R H9c2 cells, PI3K/AKT pathway was inhibited, which was further reversed by PI3K/AKT pathway agonist SC79. Besides, SC79 treatment also reversed the regulatory effect of overexpressed microRNA-489 on cellular behaviors of H/R H9c2 cells.

CONCLUSIONS: MicroRNA-489 inhibits the proliferation and accelerates apoptosis of cardiomyocytes after MIRI by targeting inhibition of SPIN1 via inactivating PI3K/AKT pathway. MicroRNA-489 may be a potential therapeutic target for MIRI.

Key Words:
MicroRNA-489, SPIN1, Ischemia-reperfusion, Apoptosis, PI3K/AKT pathway.

Introduction

Blood reperfusion in the ischemic myocardium further aggravates cardiomyocyte damage or even necrosis, which is well known as myocardial ischemia-reperfusion injury (MIRI)[1]. Ischemia is a pathological manifestation of insufficient blood supply due to blood supply disorders. Ischemic diseases can occur in various tissues and organs[2]. Reperfusion is to restore blood supply after tissue ischemia. Ischemia is not only the pathogenic factor of diseases, but also the condition of reperfusion[3]. Free radical damage, calcium overload, myocardial energy metabolism disorder, endothelial cell antioxidant system damage, related cytokines, and apoptosis are considered to be the reasons for IRI[4,5]. Particularly, apoptosis is a major cause of IRI, which is a complex process. Accumulation of

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free radicals and intracellular Ca^{2+} elevation are the leading causes of cardiomyocyte apoptosis induced by ischemia-reperfusion. Endogenous antioxidants are inactivated or deficient due to the hypoxic ischemic environment. The accumulation of adenosine triphosphate (ATP) metabolites in ischemic and hypoxic myocardial tissue, thereafter, produces a large number of oxygen free radicals[6]. Extremely unstable oxygen free radicals immediately lead to glucose and lipid peroxidation, protein denaturation, enzyme inactivation, thus resulting in DNA strand broken and apoptosis. MicroRNAs (miRNAs) are small, endogenous, single-stranded, non-coding RNAs, consisting of approximately 22 nucleotides. They are involved in the regulation of gene expressions[7]. MiRNAs are considered to be negative regulators in gene expressions by complementary pairing with the 3'UTR of target mRNA[8]. About one-third of genes are currently regulated by miRNAs[9]. Recent studies have shown that some miRNAs are highly expressed in the cardiovascular diseases, showing a certain regulatory function, which may be utilized as therapeutic targets[10-13]. MicroRNA-489 is a recently discovered gene that is associated with tumorigenesis. It has been found that microRNA-489 is lowly expressed in various malignancies, such as colon cancer, non-small cell lung cancer, and breast cancer[14-16]. MicroRNA-489 exerts an anti-cancer effect through regulating tumor cell proliferation, migration, epithelial-mesenchymal transition (EMT), and chemotherapy resistance. Overexpression of microRNA-489 induced apoptosis of glioma cells[17]. However, the specific role of microRNA-489 in cardiomyocytes after MIRI has not been well elucidated. This study investigated the potential influence of microRNA-489 on biological behaviors of H/R-induced cardiomyocytes and its underlying mechanism.

Materials and Methods

Cell Culture and H/R Induction

Embryonic rat cardiac-derived H9c2 cells were obtained from ATCC (American Type Culture Collection) (Manassas, VA, USA). These cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Sigma-Aldrich, St. Louis, MO, USA) containing 10% FBS (fetal bovine serum, Invitrogen, Carlsbad, CA, USA). For H/R induction, H9c2 cells were placed in a humidified chamber equilibrated with 5% CO_2 and 95% N_2 for 4 h, followed by reoxygenation with 5% CO_2 and 95% air for 3 h in DMEM containing 10% FBS. After 24 h of reoxygenation, cells were harvested for the following use. H9c2 cells under the normoxic environment were collected as control.

Transfection

Cells with a good viability were seeded in 6-well plates and incubated in 1.5 mL of serum-free medium with 500 μL of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 24-48 h. Transfection plasmids (microRNA-489 mimics, siRNA-SPIN1 and negative control) and PI3K/AKT pathway agonist SC79 were provided by GenePharma (Shanghai, China).

RNA Extraction

Cells were lysed in 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) and thoroughly mixed with chloroform. After centrifugation at 4°C, 12,000 rpm for 10 minutes, the precipitate was incubated with isopropanol for another centrifugation. Finally, the precipitate was washed with 75% ethanol, air-dried and dissolved in diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China). RNA sample was quantified, purified and preserved in a -80°C refrigerator.

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

The extracted RNA was reversely transcribed on ice to obtain complementary deoxyribose nucleic acid (cDNA). The reverse transcript template was diluted to a final concentration of 10 ng/μL in DEPC water. QRT-PCR was carried out in accordance with the instructions of the SYBR Green PCR Kit (TaKaRa, Dalian, China) with a total reaction system of 10 μL. Parameters of qRT-PCR were: Pre-denaturation at 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Primers of microRNA-489, SPIN1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were as follows: MicroRNA-489, F: 5′-ACACTCCAGCTGGGGTGACATCACATA-3′, R: 5′- TGGTGTCGTGGAGTCG-3′; SPIN1, F: 5′-GCAATGGCCCTGTTACCCA-3′, R: 5′-TGTCCGCTAAGTGTGCATCG-3′; GAPDH, F: 5′-GGAATCCACTGGCGTCTTCA-3′, R: 5′-GGTTCACGCCCATCACAAAC-3′; U6, F: 5′-ACACTCCAGCTGGGGTGCTCGCTTCGGCAGCACA-3′, R: 5′-AGGGTCCGAGGTATTC-3′.

Determination of Levels of Lactate Dehydrogenase (LDH), Methane Dicarboxylic Aldehyde (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

Cells in the logarithmic growth phase were cultured in a 96-well plate at a density of 4×10^3/mL with 100 μL per well. Five replicate wells were set in each group. Viability was tested at 0 h, 24 h, 48 h, and 72 h after seeding, respectively. Two hours before the test, 10 μL of cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) solution was added to each well and incubated at 37°C for 1 h. The wavelength at 450 nm was detected by a microplate reader. The absorbance values of each well were measured for three independent tests and the proliferation curve was plotted.

Flow Cytometry (FCM)

Transfected H9c2 cells for 48 h were digested, washed and resuspended in 240 μL of 1×Binding buffer. Cell density was adjusted to 2×10^5/mL. A total of 5 μL of Annexin V-APC and 10 μL of 7-AAD were added and cells were maintained in dark for 30 min. Before cell apoptosis determination using FCM, cells were diluted in 260 μL of 1×Binding buffer.

Dual-Luciferase Reporter Gene Assay

SPIN1 3'UTR containing the wild-type or mutant-type sequences of the microRNA-489 target binding sites was cloned into the luciferase reporter vector, respectively. It was co-transfected with microRNA-489 mimics in H9c2 cells, followed by luciferase activity determination.

Western Blot

Total protein was extracted from cell lysis, quantified and electrophoresed. After transferring on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA), they were incubated with primary antibodies at 4°C. At the other day, membranes were incubated with the corresponding secondary antibody for 2 h. Bands were exposed with the enhanced chemiluminescence. Integral optical density was analyzed by gel imaging analysis system (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical Analysis

Data were analyzed by using Statistical Product and Service Solutions (SPSS) 20.0 statistical software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). Quantitative data were represented as mean ± standard deviation ($\bar{x} \pm s$) and analyzed by the t-test. $p<0.05$ was considered as statistically significant.

Results

MicroRNA-489 Was Highly Expressed in H/R H9c2 Cells

QRT-PCR data showed that microRNA-489 was highly expressed in H/R H9c2 cells than that in normoxic control (Figure 1A). Subsequently, microRNA-489 expression was found to be markedly upregulated in H/R H9c2 cells transfected with microRNA-489 mimics, suggesting the sufficient transfection efficacy (Figure 1B). By transfection of microRNA-489 mimics, activities of LDH, MDA and GSH-PX elevated, while activities of SOD decreased in H/R H9c2 cells, showing the aggravated myocardial injury (Figure 1C-1F).

Overexpression of microRNA-489 Inhibited Viability But Accelerated Apoptosis of H/R H9c2 Cells

We overexpressed microRNA-489 in H/R H9c2 cells and examined the effect of microRNA-489 on cell proliferation. CCK-8 assay results showed that cardiomyocyte proliferation was significantly attenuated after transfection of microRNA-489 mimics (Figure 2A). Meanwhile, FCM data showed a higher apoptotic rate of H/R H9c2 cells after microRNA-489 overexpression (Figure 2B). Expressions of apoptotic genes were determined at both mRNA and protein levels. Identically, pro-apoptotic genes Bax and cytochrome C increased, whereas anti-apoptotic gene Bcl-2 decreased in those transfected with microRNA-489 mimics (Figure 2C). Protein levels of Bax, cytochrome C and Bcl-2 yielded the same changes as their mRNA levels (Figure 2D). Those results showed that overexpression of microRNA-489 in H/R H9c2 cells inhibited cell proliferation and induced apoptosis.

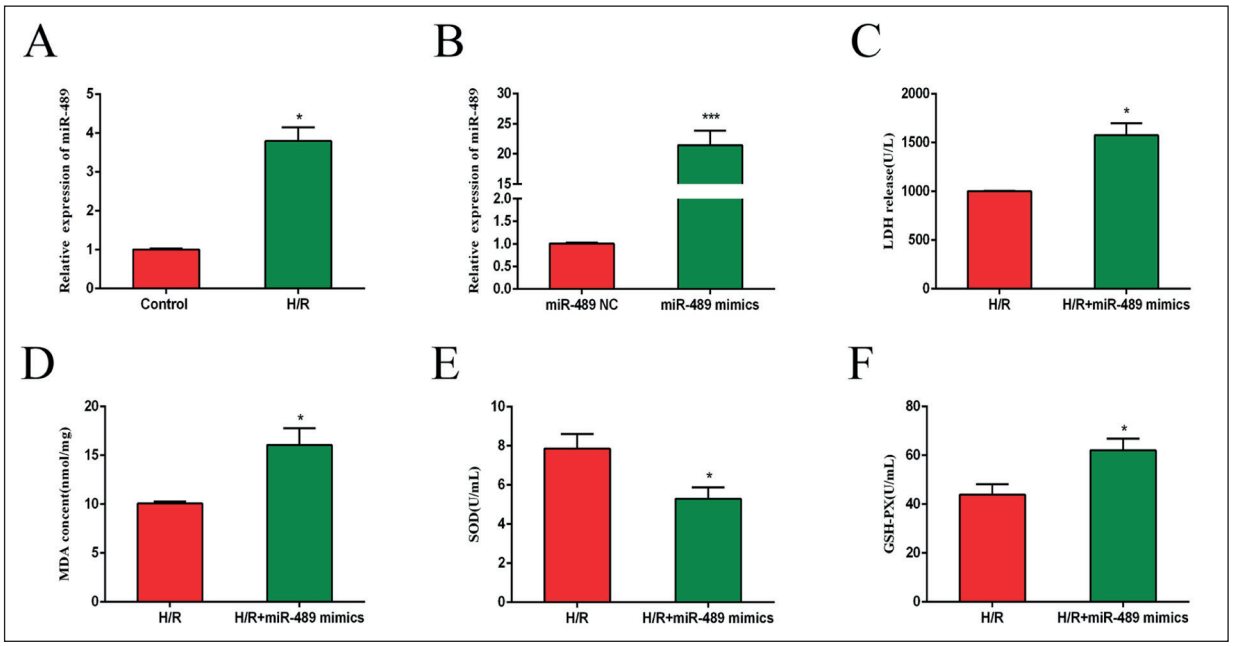

Figure 1. MicroRNA-489 was highly expressed in H/R H9c2 cells. ***A,*** MicroRNA-489 was highly expressed in H/R H9c2 cells. ***B,*** Transfection efficacy of microRNA-489 mimics in H/R H9c2 cells. ***C,*** LDH level in H/R H9c2 cells transfected with microRNA-489 mimics. ***D,*** MDA level in H/R H9c2 cells transfected with microRNA-489 mimics. ***E,*** SOD level in H/R H9c2 cells transfected with microRNA-489 mimics. ***F,*** GSH-PX level in H/R H9c2 cells transfected with microRNA-489 mimics. *$p<0.05$, ***$p<0.001$.

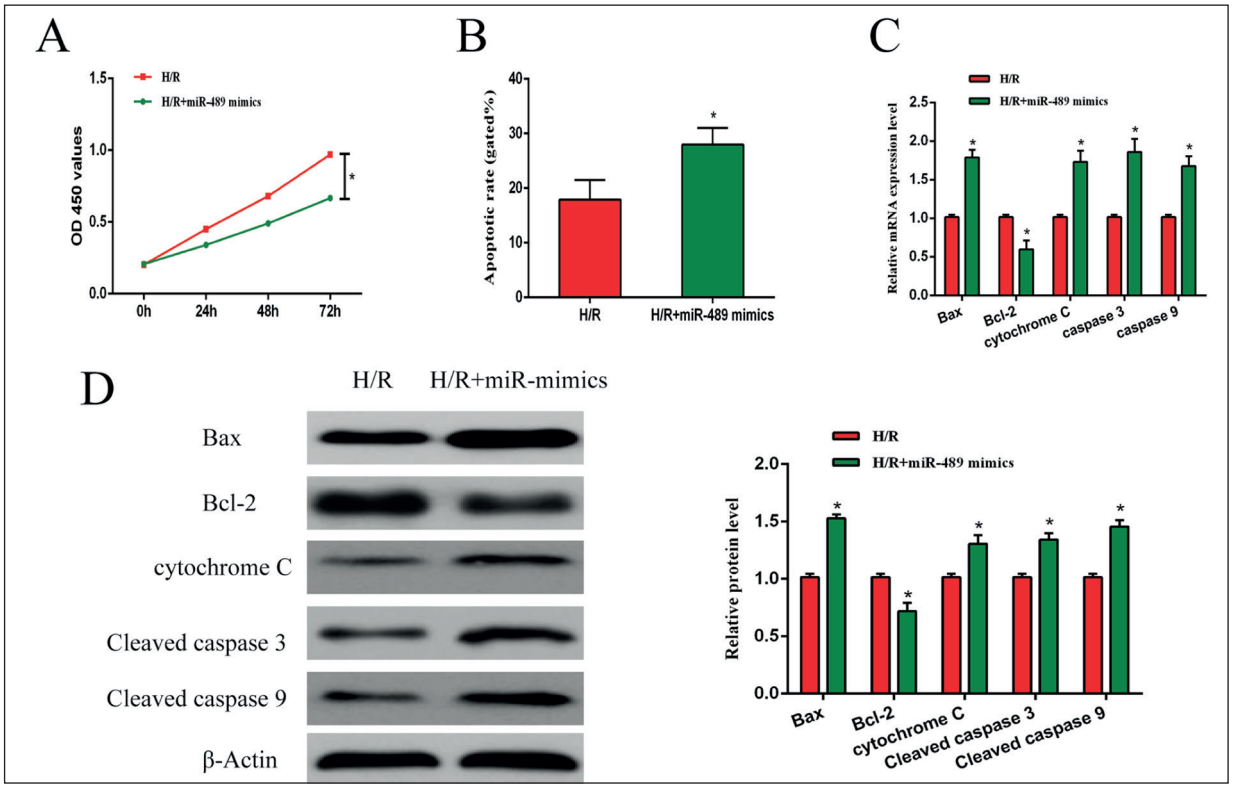

Figure 2. Overexpression of microRNA-489 inhibited viability but accelerated apoptosis of H/R H9c2 cells. ***A,*** CCK-8 assay showed that microRNA-489 overexpression inhibited proliferation of H/R H9c2 cells. ***B,*** FCM data showed that microRNA-489 overexpression accelerated apoptosis of H/R H9c2 cells. ***C,*** The mRNA levels of apoptotic genes in H/R H9c2 cells transfected with microRNA-489 mimics. ***D,*** The protein levels of apoptotic genes in H/R H9c2 cells transfected with microRNA-489 mimics. *$p<0.05$.

MicroRNA-489 Inhibited SPIN1 Expression

SPIN1 was the downstream target of microRNA-489 through bioinformatics prediction and function analyses. QRT-PCR results demonstrated a lower expression of SPIN1 in H/R H9c2 cells than that in normoxic control (Figure 3A). The binding condition was then verified by dual-luciferase reporter gene assay (Figure 3B). Overexpression of microRNA-489 downregulated SPIN1 expression in H9c2 cells, showing a negative correlation between them (Figure 3C). To further elucidate the potential role of SPIN1 in MIRI regulated by microRNA-489, siRNA-SPIN1 was constructed and verified its transfection efficacy (Figure 3D). Through CCK-8 assay and FCM, we found that knockdown of SPIN1 inhibited the cell viability but induced apoptosis of H/R H9c2 cells (Figure 3E and 3F). Similarly, knockdown of SPIN1 upregulated Bax and cytochrome C, and downregulated Bcl-2 at both mRNA and protein levels (Figure 3G and 3H). All the above results indicated that microRNA-489 may exert its biological effects by inhibiting the expression of SPIN1.

MicroRNA-489 Inhibited PI3K/AKT Pathway

Previous studies have shown that microRNA-489 exerted its biological effects through activating the PI3K/AKT pathway[18]. We found

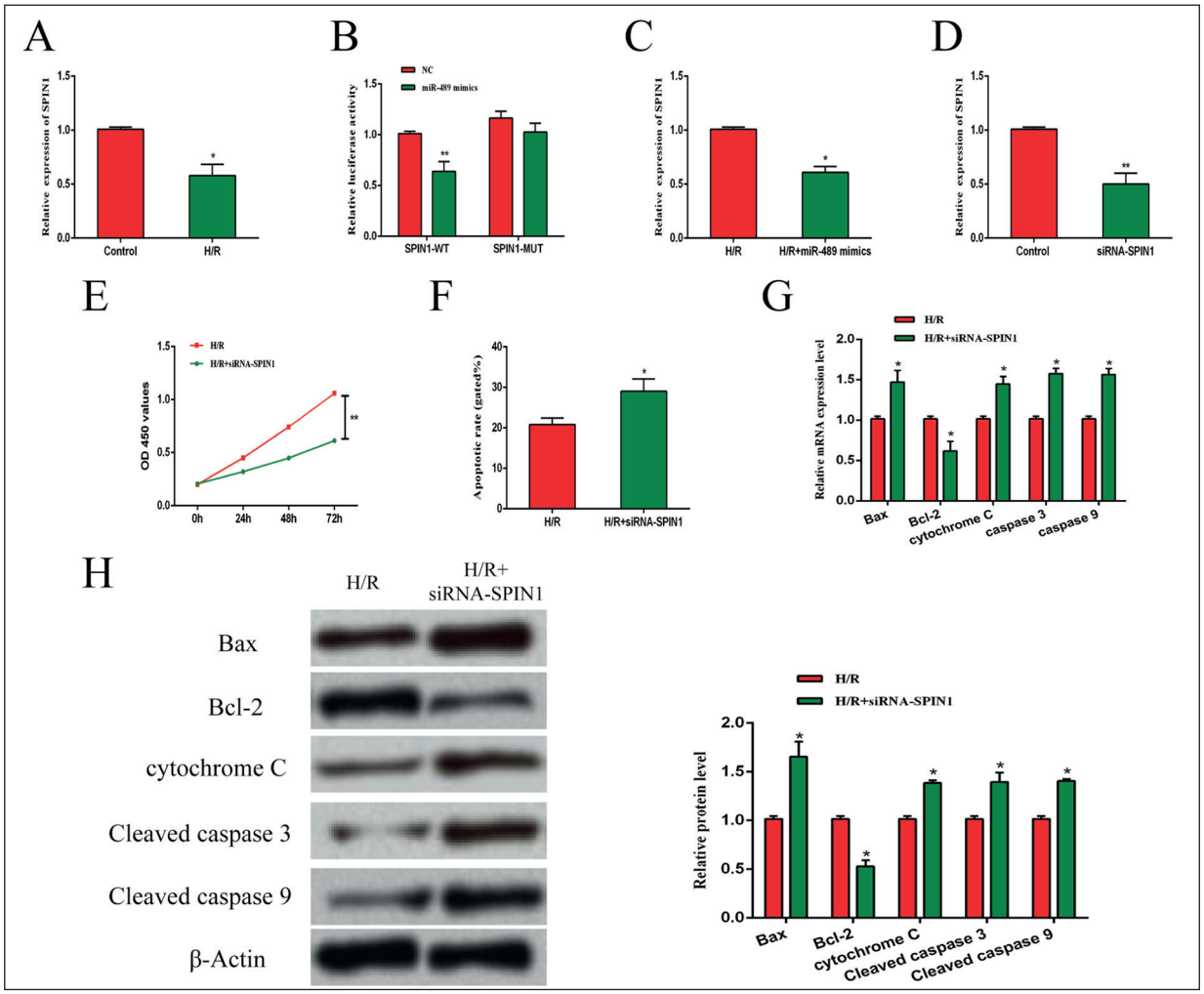

Figure 3. MicroRNA-489 inhibited SPIN1 expression. ***A,*** SPIN1 was lowly expressed in H/R H9c2 cells. ***B,*** Dual-luciferase reporter gene assay confirmed the binding between microRNA-489 and SPIN1. ***C,*** Expression level of SPIN1 in H/R H9c2 cells transfected with microRNA-489 mimics. ***D,*** Transfection efficacy of siRNA-SPIN1 H/R H9c2 cells. ***E,*** CCK-8 assay showed that SPIN1 knockdown inhibited proliferation of H/R H9c2 cells. ***F,*** FCM data proved that SPIN1 knockdown accelerated apoptosis of H/R H9c2 cells. ***G,*** The mRNA levels of apoptotic genes in H/R H9c2 cells transfected with siRNA-SPIN1. ***H,*** The protein levels of apoptotic genes in H/R H9c2 cells transfected with siRNA-SPIN1. *$p<0.05$, **$p<0.01$.

that the expression levels of p-AKT and p-mTOR remarkably decreased after overexpression of microRNA-489 by Western blot (Figure 4A). We suggested that the PI3K/AKT pathway was inhibited by overexpression of microRNA-489. Subsequently, H/R H9c2 cells were transfected with PI3K/AKT pathway agonist SC79. Interestingly, SC79 reversed the regulatory effects of microRNA-489 on apoptosis and the proliferation (Figure 4B and 4C). SC79 treatment in H/R H9c2 cells transfected with microRNA-489 mimics upregulated Bcl-2, and downregulated Bax, cytochrome C, cleaved caspase-3 and cleaved caspase-9 at both mRNA and protein levels, respectively (Figure 4D and 4E). These results demonstrated that microRNA-489 inhibits the cell proliferation and induces apoptosis of H/R-induced cardiomyocytes through inhibiting PI3K/AKT pathway.

Discussion

Previous studies[19] have found that apoptosis is the major reason for myocardial ischemia-reperfusion injury. As the main terminal cleavage enzyme during apoptosis, caspase-3 is involved in DNA repair and basal integrity. It can cleave PKCd and PKCq, whose overexpression can lead to apoptosis, fully demonstrating the crucial role of caspase-3 in apoptosis induction[20]. Bcl-2 prevents apoptosis through inhibiting mitochondrial rupture, caspase-3 activation and Bax cytotoxicity[21]. It is believed that the anti-apoptotic effect of Bcl-2 may be related to anti-oxidation effect, inhibition of calcium ion transmembrane flow and ion channel proteins[22]. Bax, a member of the Bcl-2 gene family, activates the pro-apoptotic protein caspase-9 and promotes apoptosis[23]. Overexpression of Bax antagonizes the protective effect of Bcl-2 in breast cells and respiratory epithelial cells[24]. Therefore, the Bax/Bcl-2 ratio accurately reflects the apoptotic regulatory effect of the Bcl-2 family[25]. SPIN1 belongs to the Spin/Ssty family and is a meiotic spindle protein. SPIN1 was first found to be highly expressed in ovarian cancer cells[26]. It regulates the stability of spindle tissue and chromosome, and exerts a crucial role in tumor progression. SPIN1 has been shown to

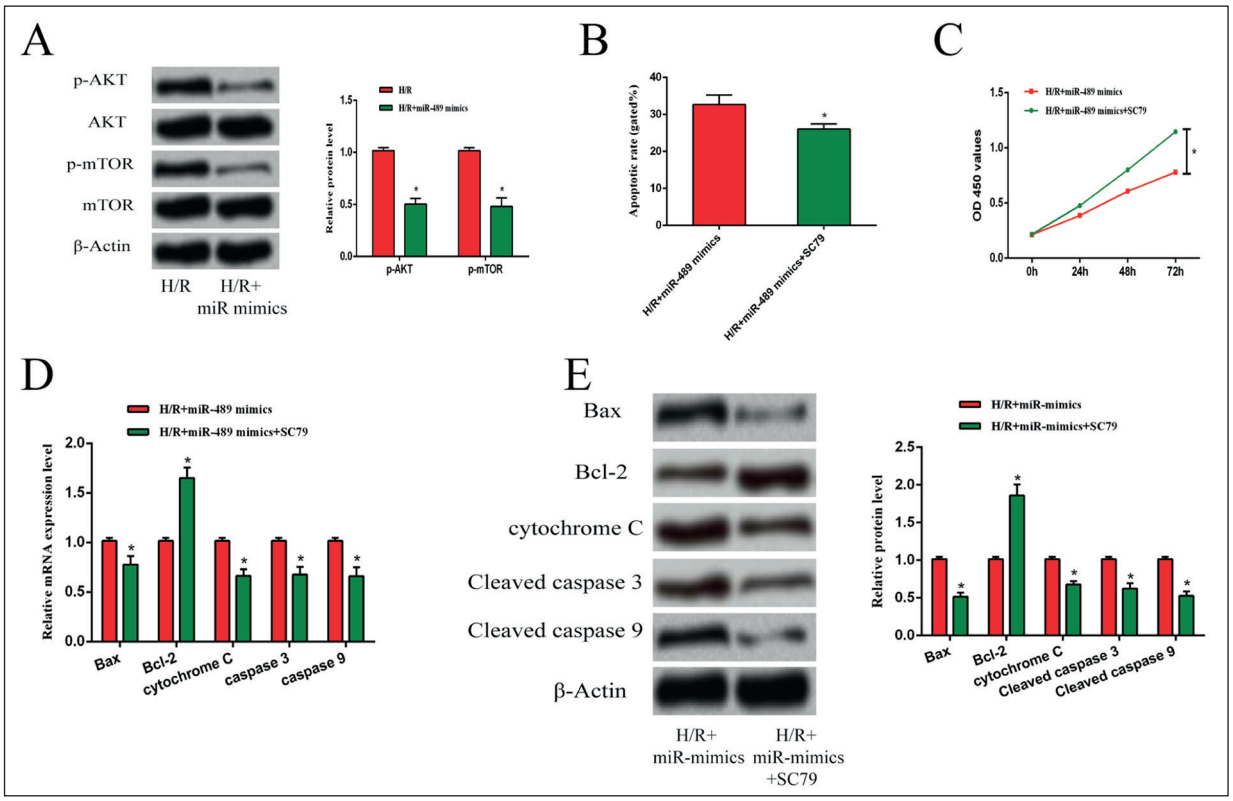

Figure 4. MicroRNA-489 inhibited PI3K/AKT pathway. ***A,*** Overexpression of microRNA-489 inhibited PI3K/AKT pathway. ***B,*** SC79 treatment reversed the promotive role of microRNA-489 in apoptosis of H/R H9c2 cells. ***C,*** SC79 treatment reversed the inhibitory role of microRNA-489 in proliferation of H/R H9c2 cells. ***D,*** SC79 treatment reversed apoptotic gene expressions regulated by microRNA-489. ***E,*** Activation of PI3K/AKT pathway inhibited pro-apoptotic gene expressions. *$p<0.05$.

regulate the progression of many cancers. For example, microRNA-489 increases chemosensitivity of breast cancer by inhibiting SPIN1-mediated PI3K-AKT pathway[27]. MiR-409 inhibited the progression of non-small cell lung cancer by direct targeting of SPIN1[28]. SPIN1 promoted the proliferation of ovarian cancer cells through activating Wnt/TCF-4 pathway, suggesting that SPIN1 may play a key role in tumor invasiveness[26]. In this study, we found that microRNA-489 was highly expressed in H/R H9c2 cells compared with normoxic control. After overexpression of microRNA-489 in H/R H9c2 cells, levels of myocardial injury markers (LDH, MDA, SOD, and GSH-PX) increased. Subsequently, we observed that microRNA-489 overexpression inhibited cell proliferation and induced apoptosis of H/R H9c2 cells. Besides, expression levels of apoptotic genes Bax and cytochrome C increased, whereas anti-apoptotic gene Bcl-2 decreased. Previous studies have shown that microRNA-489 promotes apoptosis through inhibiting SPIN1 expression[17]. We thereafter suspected that microRNA-489 might also exert a similar function in MIRI through the same mechanism. SPIN1 was highly expressed in H/R H9c2 cells compared with normoxic control. Meanwhile, SPIN1 was verified to bind to microRNA-489. However, the expression of SPIN1 was negatively regulated by microRNA-489. Furthermore, knockdown of SPIN1 in H/R H9c2 cells inhibited the proliferative potential, but accelerated apoptosis. At the same time, expression levels of Bax and cytochrome C upregulated, whereas Bcl-2 downregulated in H9c2 cells after knockdown of SPIN1. To elucidate the specific role of microRNA-489 in regulating biological behaviors of H9c2 cells, we detected the changes of PI3K/AKT pathway. Overexpression of microRNA-489 downregulated p-AKT and p-mTOR, indicating the inhibited PI3K/AKT pathway. Interestingly, the PI3K/AKT agonist SC79 reversed the regulatory effect of overexpressed microRNA-489 on the inhibited proliferation and accelerated apoptosis of H9c2 cells. Expression changes in Bax, cytochrome C and Bcl-2 were identically reversed by SC79 treatment. We may conclude that microRNA-489 is expected to be a therapeutic target for myocardial ischemia-reperfusion injury.

Conclusions

We detected in this study that microRNA-489 inhibits the proliferation and accelerates apoptosis of cardiomyocytes after MIRI by targeting inhibition of SPIN1 *via* inactivating PI3K/AKT pathway. MicroRNA-489 may be a potential therapeutic target for MIRI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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