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MiR-93 inhibition ameliorates OGD/R induced cardiomyocyte apoptosis by targeting Nrf2

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Abstract. – OBJECTIVE: To identify the expression changes of microRNA 93 (miR-93) in oxygen-glucose deprivation/reoxygenation (OGD/R) injury in cardiomyocytes and its mechanism of mediating OGD/R and inducing apoptosis.

MATERIALS AND METHODS: Primary cardiomyocytes were extracted and OGD/R model in cardiomyocytes was established *in vitro*. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the expressions of miR-93, and Western blot assay was applied to measure the protein levels of nuclear factor erythroid 2-related factor 2 (Nrf2) and caspase-3. Flow cytometry was utilized to examine the cardiomyocyte apoptosis rate.

RESULTS: The apoptosis rate was increased after OGD/R in cardiomyocytes, accompanied by remarkable rise of miR-93 expression. After transfection of miR-93 antagomir, the apoptosis rate of cardiomyocyte induced by OGD/R was down-regulated, and the expression of cleaved caspase-3 was decreased. Meanwhile, the results of qRT-PCR and Western blot showed that the levels of Nrf2 mRNA and protein expression were up-regulated after the miR-93 level was inhibited, and luciferase reporter assay affirmed that Nrf2 was a target molecule for OGD/R-induced apoptosis mediated by miR-93.

CONCLUSIONS: miR-93 mediates OGD/R-induced hypoxia/reoxygenation injury apoptosis in cells by targeting Nrf2.

Key Words:
miR-93, OGD/R, Nrf2.

Introduction

Acute myocardial infarction is one of the major causes of death from cardiovascular diseases. It is crucial to open the infarct-related arteries and restore the blood supply to ischemic myocardium in time to save injured myocardium. Studies, however, have found that if myocardial ischemia has lasted for some time, the recovery of blood perfusion may lead to aggravated damage to myocardial structure and function as well as the occurrence of myocardial stunning, decreasing cardiac function and leading to malignant arrhythmia, etc. This process is known as cardiac ischemia/reperfusion injury. In recent years, more and more attentions have been paid to the regulatory role of microRNA (miRNA) in cardiac ischemia/reperfusion injury, and it has been confirmed that multiple miRNAs play a vital role in this problem. Research conducted by Ren et al[1], for instance, suggested that the expression of miR-320 in cardiac ischemia/reperfusion injury in rats was significantly reduced, and miR-320 played an important role in the cardiac ischemia/reperfusion injury in rats by means of regulating its target gene, heat-shock protein 20 (Hsp20). MiR-320 knockdown could rise the expression of Hsp20 and lower the cardiomyocyte apoptosis induced by cardiac ischemia/reperfusion injury[1]. In addition, injecting miRNA-1, miRNA-21 and miRNA-24 into mice with cardiac ischemia/reperfusion injury caused by non-heat shock, could reduce the myocardial infarct size[2]. Some studies have reported that the expression of miR-93 is up-regulated in a variety of tumor tissues[3,4], and the decreased expression of miR-93 can ameliorate cerebral ischemia-reperfusion injury[5]. There are fewer studies on the role of miR-93 in cardiac ischemia/reperfusion injury. Therefore, this research investigated the expression changes of miR-93 in oxygen-glucose deprivation/reoxygenation (OGD/R) injury in cardiomyocytes at the cellular level and its mechanism of mediating OGD/R and inducing apoptosis.

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Materials and Methods

Cell Extraction and Main Reagents

Primary cardiomyocytes were extracted according to a previous report[6]. The cardiomyocyte culture media were purchased from HyClone (Logan, UT, USA); fetal bovine serum (FBS) was bought from Invitrogen (Carlsbad, CA, USA), and trypsin from Gibco (Rockville, MD, USA), penicillin-streptomycin solution and collagenase type II from Invitrogen (Carlsbad, CA, USA), 5-bromine-2-deoxyuridine (BrdU) from Gibco (Rockville, MD, USA). Flow cytometry assay kits were purchased from BD Company (Franklin Lakes, NJ, USA); goat serum and fluorescein isothiocyanate (FITC)-conjugated secondary goat-anti-mouse antibody were bought from Beijing Zhongshan Golden bridge Biotechnology Co., Ltd. (Beijing, China), and 4,6-Diamino-2-Phenyl Indole (DAPI) from Sigma-Aldrich (St. Louis, MO, USA). The primers of miR-93 and U6 were purchased from Applied Biosystems (Foster City, CA, USA); miR-93 antagomir, miR-93 agomir and negative control miRNA were from Shanghai GenePharma Co., Ltd. (Shanghai, China); Lipofectamine 2000 transfection reagent was bought from Invitrogen (Carlsbad, CA, USA), and Dual Luciferase Reporter Assay system from Promega Company (Madison, WI, USA).

Establishment of OGD/R Injury Model in Primary Cardiomyocytes

The hypoxia/reoxygenation model in cardiomyocytes was established as the methods used by Ekhterae et al[7]. The culture medium without serum and glucose was used as the hypoxic solution. First, the hypoxic solution was equilibrated to saturate liquid for more than 2 h with gas mixture (95% N_2 and 5% CO_2); next, the normal culture medium of cardiomyocytes was discarded and the saturated hypoxic solution was filled in immediately. Later, the cardiomyocytes were placed in a hypoxia incubator (95% N_2 and 5% CO_2) at 37°C and cultured in anoxic condition for 10 hours. By this way, the hypoxia (ischemia) model was established. After the cardiomyocytes were cultured in hypoxia for 10 hours, the hypoxic solution was sucked out by a pipette and replaced with a culture medium containing glucose and 10% fetal bovine serum (FBS); then, the cardiomyocytes were cultured in normal conditions for 2 hours. By this way, the reoxygenation (reperfusion) model was established. The apoptosis rate was detected according to the instructions of flow cytometry assay kit.

Cell Transfection

After the extraction, the primary cardiomyocytes were added to the culture medium containing 10% FBS and incubated for 36 hours under routine conditions; transfection was conducted when the adherent density of the cells was about 70%. Lipofectamine® 2000 and Opti-MEM® culture media with low serum were used to transfect miRNA agomir, miRNA antagomir and its loading control (negative control miRNA), respectively; 48 hours later, the cells were collected to confirm the transfection efficiency or to implement other experiments.

Protein Extraction and Western blot

The cells were lysed and centrifuged at 12,000 g for 15 min, and the precipitate was discarded. Bicinchoninic acid (BCA) kit was utilized to quantify the protein concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for separation and polyvinylidene fluoride (PVDF) for transfer to membrane; the membrane was blocked in 5% skim milk for 1 h at room temperature. Nuclear factor erythroid 2-related factor 2 (Nrf2) rabbit-anti-rat monoclonal antibodies (1:1,000) (Abcam, Cambridge, MA, USA) and secondary goat-anti-rabbit antibody (1:2,000) (Abcam, Cambridge, MA, USA) were applied to identify the Nrf2 protein; Caspase-3 rabbit-anti-rat monoclonal antibodies (1:1,000) (Abcam, Cambridge, MA, USA) and secondary goat-anti-rabbit antibody (1:2,000) were used for identification of Caspase-3 protein. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control, of which the membrane was incubated with primary antibodies for 4 h and then washed for 6 min in Tris buffered saline tween (TBST) 5 times; then, it was incubated with secondary antibodies for 1 h and washed for 6 min in TBST 5 times. The moisture was dried by filter paper, and enhanced chemiluminescence (ECL) kit was added for color development.

Extraction of Total RNA and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

TRIzol kit (Gibco, Rockville, MD, USA) was utilized to extract the total RNA in cells, of which mRNA was taken as a template and reversely transcribed into complementary DNA (cDNA)

using reverse transcriptase. Then, with cDNA as the template, PCR amplification was performed so as to obtain the target genes and detect the gene expression. The Taqman miRNA reverse transcription kit (manufactured by Applied Biosystems, Foster City, CA, USA) was used for reverse transcription of the extracted total RNA. U6 was used as an internal normalized reference for miR-93 expressions, respectively. Expressions were normalized to endogenous controls and fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

Statistical Analysis

All the experimental results were presented as mean ± standard deviation (mean ± SD); Statistical Product and Service Solutions (SPSS Inc., Chicago, IL, USA) 13.0 software was used for data analysis; Student's *t*-test was conducted for data comparison between two groups, and analysis of variance (ANOVA) was adopted for data comparison among multiple groups by using Least Significant Difference (LSD). $p<0.05$ suggested that the difference was statistically significant.

Results

MiR-93 was up-Regulated in OGD/R Model

RT-PCR was used to detect the expression levels of miR-93 in cardiomyocytes in control group and OGD/R group. The results showed that the miR-93 level was remarkably increased in OGD/R group, and the difference was statistically significant (Figure 1A). In addition, the levels of Nrf2 mRNA and proteins were down-regulated in OGD/R group, and the difference was statistically significant (Figure 1B-C).

MiR-93 Antagomir Reduced OGD/R-induced Apoptosis

In order to further explore the effects of miR-93 on OGD/R-induced cardiomyocyte apoptosis, functions of miR-93 antagomir transfected from cardiomyocytes was verified. As shown in Figure 2A, transfected miR-93 antagomir could significantly decrease the expression level of miR-93 in cardiomyocytes. Furthermore, the results of flow cytometry indicated that transfected miR-93 antagomir could remarkably reduce the apoptosis rate of cardiomyocytes (Figure 2B-C). Western blot results also confirmed that the expression of cleaved caspase-3 protein was down-regulated after transfection of miR-93 antagomir (Figure 3B-C), and the differences were statistically significant. It was found that the Nrf2 expression level was up-regulated significantly after the miR-93 expression was inhibited through the results of RT-PCR and Western blot assay (Figure 3A, C).

MiR-93 Regulated the OGD/R-Induced Cardiomyocyte Apoptosis by Inhibiting Nrf2 Expression

First, miR-93 was highly expressed in the cardiomyocytes by means of transfection of miR-93 agomir, and RT-PCR was used to verify its efficiency. The results showed that the expression of miR-93 after transfecting miR-93 agomir was remarkably increased compared with that of control group (Figure 4A). In order to prove

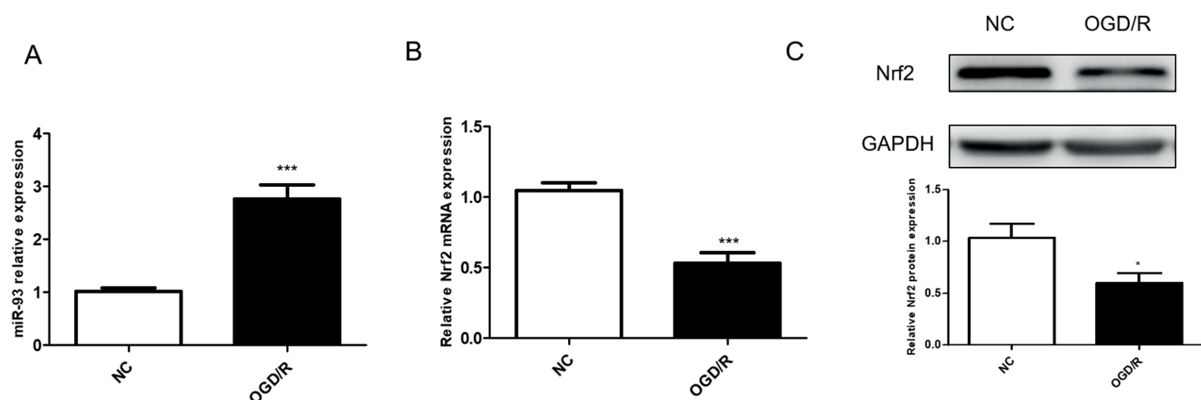

Figure 1. MiR-93 is up-regulated in OGD/R model. ***(A)*** It is detected by qRT-PCR that the expression of miR-93 is up-regulated in OGD/R group, n=6. ***(B)*** Results of qRT-PCR and Western blot ***(C)*** show that the levels of Nrf2 mRNA and proteins are significantly down-regulated in OGD/R group, n=6. $^{***}p<0.001$, $^{*}p<0.05$.

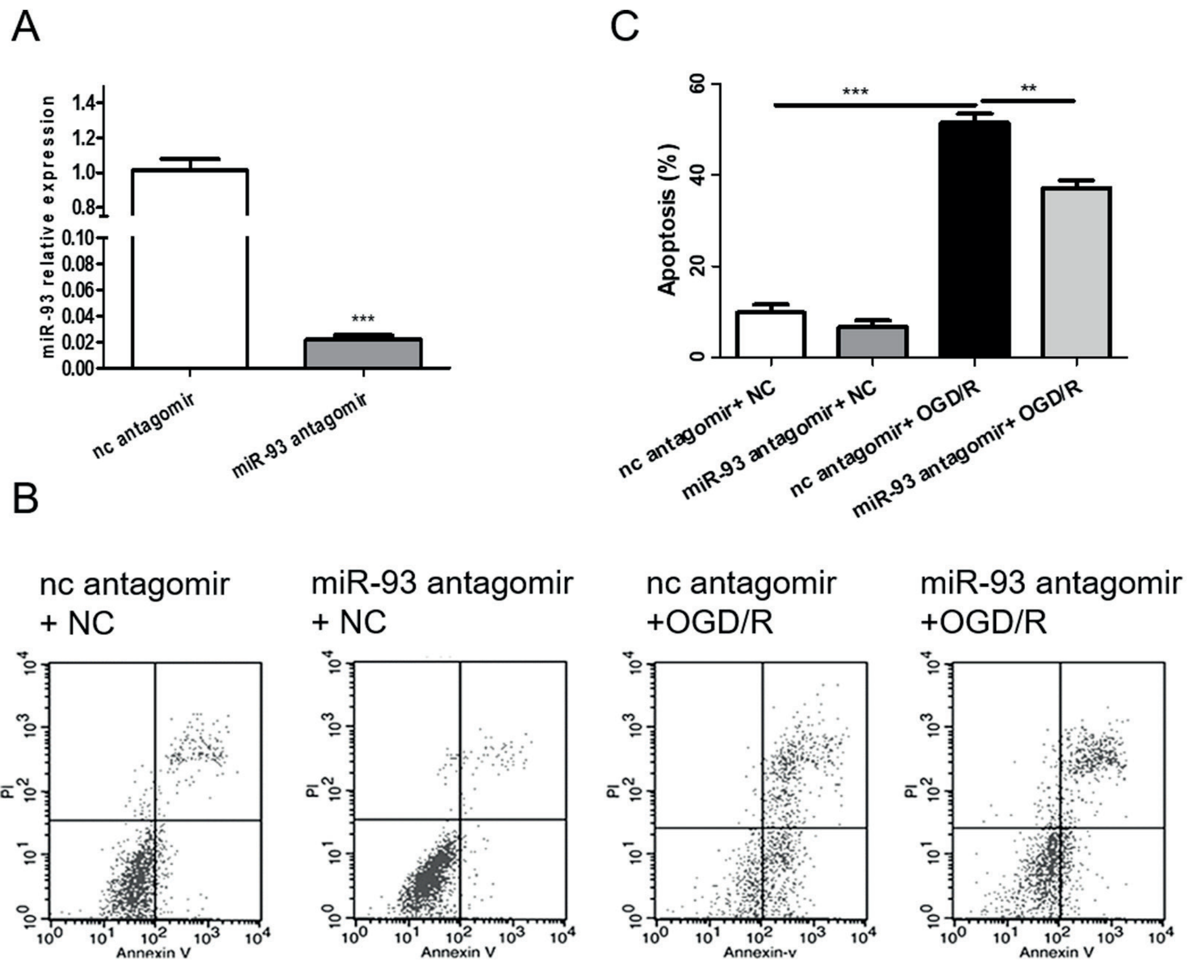

Figure 2. MiR-93 antagomir reduces OGD/R-induced apoptosis. ***(A)*** The transfection efficiency is verified using qRT-PCR 48 h after the miR-93 antagomir is transfected from cardiomyocytes, n=6. ***(B-C)*** Flow cytometry is utilized to measure the apoptosis rate after OGD/R in every group, n=4. $^{***}p<0.001$, $^{**}p<0.01$.

whether miR-93a regulated the OGD/R-induced cardiomyocyte apoptosis by directing against Nrf2, the possible miR-93 binding sites in 3'-untranslated regions (3'-UTR) of Nrf2 were analyzed firstly. Then, dual luciferase reporter vectors used to detect the 3'-UTR activity in Nrf2 were constructed according to the binding sites, and the dual luciferase reporter assay was performed through co-transfection (Figure 3B). The results indicated that by comparing with control group in which transfection of negative control (NC) was conducted, the signal intensities of luciferase carrying Nrf2 wild type reporter vectors were significantly decreased after transfection of miR-93 agomir, while there were no changes in those of luciferase carrying Nrf2 mutant reporter vectors (Figure 3C). All those demonstrated that Nrf2 was the target point for hypoxia/reoxygenation injury induced cardiomyocyte apoptosis mediated by miR-93.

Discussion

Apoptosis is closely related to the occurrence and development of many diseases, such as viral diseases, autoimmune diseases, ischemic heart diseases and cancers[8-11]. Therefore, research on apoptosis or anti-apoptosis mechanisms can not only help gain in-depth knowledge of the occurrence and development of various diseases, but also provide an important basis for clinical treatment. Moreover, it has been proved that a part of miRNA can promote or inhibit cardiomyocyte apoptosis by regulating the expressions of target genes related to apoptosis, thus participating in the occurrence

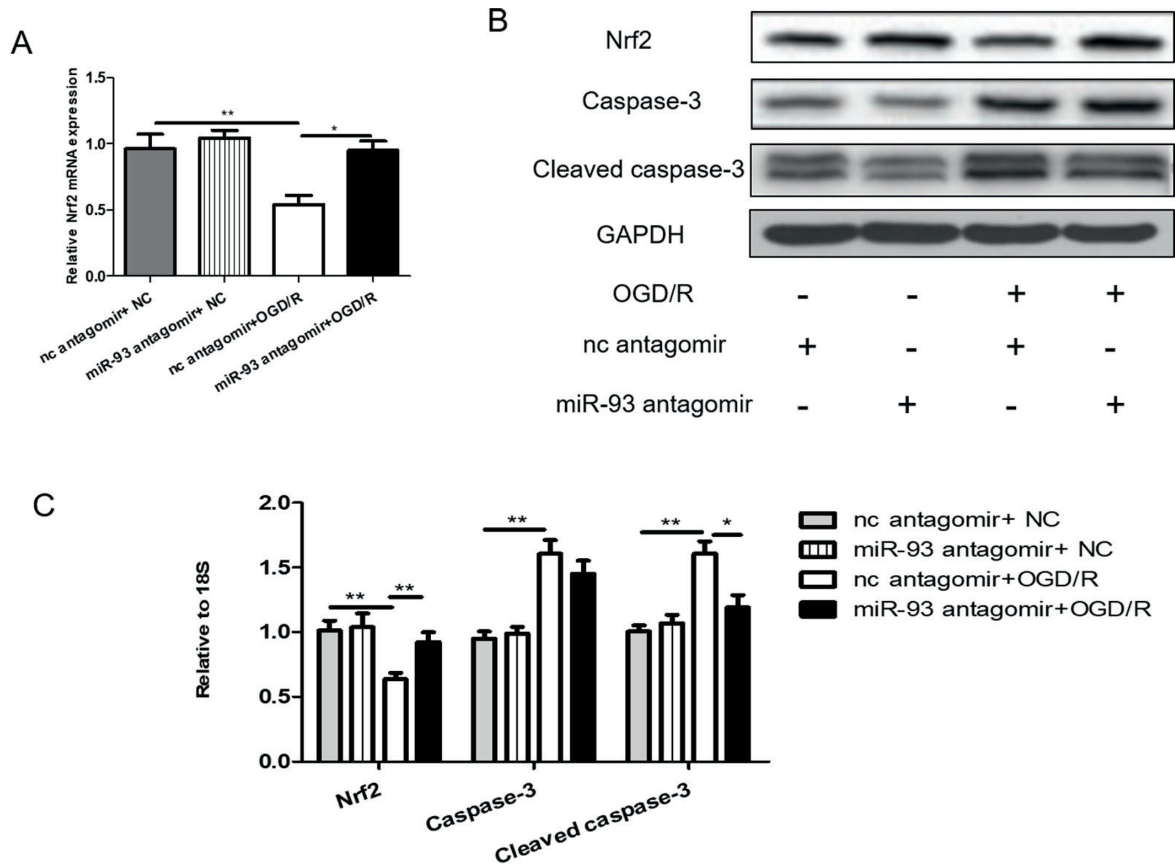

Figure 3. MiR-93 regulated the OGD/R-induced cardiomyocyte apoptosis by inhibiting Nrf2 expression. ***(A-B)*** 48 h after transfection of miR-93 antagomir from cardiomyocytes, qRT-PCR and Western blot are performed to examine the expressions of Nrf2 mRNA and proteins, n=6. ***(B-C)*** Western blot is used to detect the expression level of Caspase-3 protein after OGD/R in every group, n=6. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$.

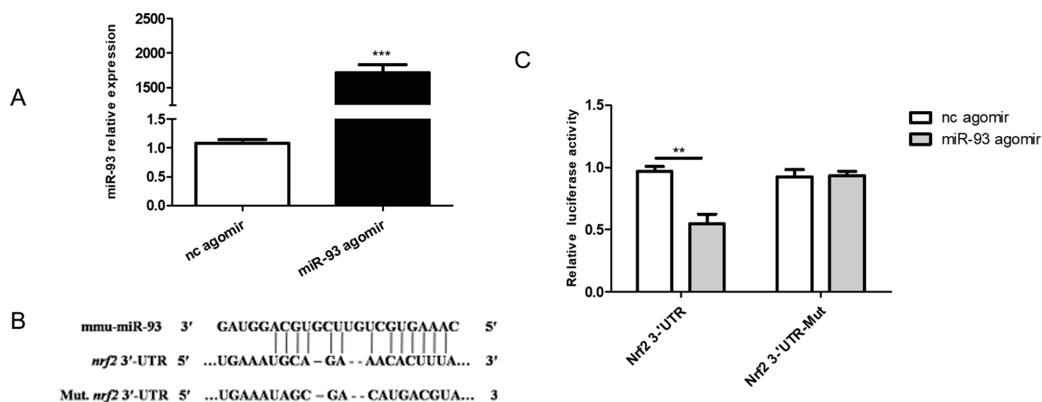

Figure 4. MiR-93 regulates activity of 3'-UTR in Nrf2. ***(A)*** Prediction of miR-93 binding sites in 3'-UTR in Nrf2. ***(B)*** Detection of 3'-UTR activity in Nrf2, n=4. $^{***}p<0.001$, $^{**}p<0.01$.

and development of acute myocardial infarction (AMI) and other ischemic heart diseases. Currently, studies have found that miR-93 plays a role in the occurrence, development, and drug-resistant processes of a variety of tumors. It is also involved in the physiological and pathological processes,

including senescence and apoptosis. Previous researches have discovered that down-regulation of miR-93 level can protect cerebral ischemia-reperfusion injury[5], but there is less research on myocardial ischemia-reperfusion injury. Some studies report that Nrf2 is a major pathway for regulating anti-oxidation and expression of phase II enzyme, which can alleviate cerebral ischemia-reperfusion injury and has neuroprotective effects[12,13]. Meanwhile, it can also reduce oxidative stress and protect cardiac ischemia injury[14]. The primary cardiomyocytes of rats were selected as the subject of this research, and the expression changes of miR-93 in OGD/R injury in cardiomyocytes and its mechanism of mediating OGD/R injury and inducing apoptosis were studied. The results indicated that the expression of miR-93 was increased in OGD/R group, accompanied by down-regulation of Nrf2 expression at the cellular level. After down-regulation of miR-93 expression through transfected miR-93 antagomir, the apoptosis rate was lowered, and the Nrf2 expression was up-regulated. In order to further verify whether miR-93 played its role by directing targeting Nrf2, luciferase reporter assay was performed. The results indicated that the signal intensities of luciferase carrying Nrf2 wild type reporter vectors were significantly decreased after transfection of miR-93 agomir by comparing with control group in which transfection of negative control (NC) was conducted, while there was no obvious changes in those of luciferase carrying Nrf2 mutant reporter vectors. The results confirmed that Nrf2 was the target point for cardiomyocyte hypoxia/reoxygenation apoptosis mediated by miR-93.

Conclusions

This research reveals the role of miR-93 in OGD/R-induced cardiomyocyte apoptosis and confirms that miR-93 mediates OGD/R-induced hypoxia/reoxygenation injury apoptosis in cells by targeting Nrf2.

Conflict of interest

The authors declare no conflicts of interest.

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