

## Original Article

# miR-124 regulates myocardial ischemia reperfusion injury

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**Abstract:** Objective: To study the role of miR-124 in myocardial ischemia-reperfusion induced cardiomyocyte injury by targeted regulation of beclin 1 (BECN 1). Method: The rat model of myocardial ischemia-reperfusion was established. The rats were divided into the miR-124 group (injected with miR-124 inhibitor), the MIRI group (not treated), and the miR-NC group (injected with miR-NC). Meanwhile, blank group and sham operation groups were set up. LDH and CK in the five groups were detected by ELISA at 12 h after operation. The myocardial infarct size of each group was measured by multimedia color pathological analysis. Then, an *in vitro* cell model was established. First, the rat cardiomyocytes were isolated, extracted and cultured; then miR-124 was transfected into the cells, the test group cells were transfected with miR-124, and the miR-NC were transfected into cells. NC cells were set as negative control group, and cells not transfected were set as blank control group. Then, an *in vitro* cell model was established. First, the rat cardiomyocytes were isolated, extracted and cultured, then cells transfected with miR-124 and miR-NC were set as test group and negative control group, and cells not transfected were set as blank control group. The relative expression of miR-124 in rat myocardium as well as in cardiomyocytes transfected with miR-124 were measured. Western blot was applied to analyze the relative expression levels of Beclin-1 and LC3B proteins in cardiomyocytes and flow cytometry was used to analyze the apoptosis of cells. Results: There were no significant differences in serum CK and LDH levels between the blank group, the sham operation group and the miR-124 group after modeling ( $P>0.05$ ), but those of the MIRI group and the miR-NC group were significantly higher than that in the blank group, the sham operation group and the miR-124 group ( $P<0.05$ ). The miR-124 in the myocardial tissue of the blank group, the sham operation group and the miR-124 group were significantly lower than those of the MIRI group and the miR-NC group ( $P<0.05$ ). There was no significant difference in Beclin 1 mRNA expression among the five groups ( $P>0.05$ ). The myocardial infarct size of the miR-124 group was significantly smaller than that of the MIRI group and the miR-NC groups ( $P<0.05$ ). For *in vitro* results, the expression of miR-124 after transfection in the myocardial cells of the test group was significantly higher than that in the blank group and the negative control group ( $P<0.05$ ). The expression levels of Bcln 1 and LC3 autophagy in the cardiomyocytes of the test group were significantly lower than those of the blank and negative control groups ( $P<0.05$ ). The apoptosis rate of cardiomyocytes in the test group was also significantly higher than that in the blank group and the negative control group ( $P<0.05$ ). Conclusion: miR-124 is highly expressed in MIRI and may reduce the level of autophagy and promote apoptosis through competitive binding of BECN 1, which may be an important target for MIRI treatment in the future.

**Keywords:** miR-124, BECN 1, myocardial ischemia

### Introduction

Acute myocardial infarction (AMI) is a severe ischemic heart disease due to persistent ischemia and hypoxia of the coronary arteries [1, 2]. Currently, AMI is principally treated by thrombolysis. However, treatment intervention will lead to reperfusion of the persistently ischemic cardiomyocytes, resulting in the partial loss of

heart function or the apoptosis of cardiomyocytes and aggravates the conditions of the patients: resulting in the so-called myocardial ischemia-reperfusion injury (MIRI) [3]. Studies on the mechanism of MIRI discovered that the major causes of MIRI may include mitochondrial injury, the increase of reactive oxygen species, and autophagy regulated by Beclin 1 gene [4, 5].

Cardiomyocytes are highly and terminally differentiated cells. Their digestion and degradation are achieved principally by intracellular autophagy, which also provides the cardiomyocytes with stable energy so as to maintain the normal function of the heart. However, excessive autophagy will not only impact the normal function of cardiomyocytes, but also lead to the death of the cells. Ischemia and hypoxia not only cause death of cardiomyocytes, but also a certain degree of inflammatory response and stress response. In addition, studies on autophagy of cells have displayed that autophagy aroused by hypoxia would reduce the apoptosis of cells [6]. However, although autophagy induced by early hypoxia will decrease the apoptosis of cells, persistent hypoxia will still cause massive death of cells. Studies on MIRI displayed that the removal of Beclin 1 gene would reduce apoptosis of cardiomyocytes in MIRI [7]. Many studies have shown that miRNAs are associated with autophagy of cells, for example, one previous study found that miR-124 could affect apoptosis and autophagy of neuronal cells by regulating dopamine in patients with Parkinson's disease and also impact autophagy of cells by down-regulating the Beclin 1 gene [8]. We found by bioinformatics analysis that miR-124 and Beclin 1 genes possessed binding sites at the 3'UTR region, and that Beclin 1 played a very critical role in inducing the autophagy of cardiomyocytes. Although the studies on the role of miR-124 in MIRI are rare, we still speculate that miR-124 may work in the MIRI-induced cardiomyocyte injury by regulating the Beclin 1 gene.

In order to prove our hypothesis and to find new directions in the treatment and prevention of cardiovascular disease, we studied the role of miR-124 in MIRI-induced cardiomyocyte injury by regulating the Beclin 1 gene. The details of the study are as follows.

### Materials and methods

#### *Experimental materials and animals*

Fifty healthy male SD rats at the age of 8 weeks were selected. All the rats were purchased from Shanghai SLAC Laboratory Animal Co., Ltd (license No.: SCXK (HU) 2012-0002) and the body weight of the rats was (190.42±10.57) g. The rats were fed in a well-ventilated environment at constant temperature (22°C). The light

and dark alternated every 12 hours. The rats were allowed to eat and drink freely. The sodium pentobarbital, serum lactate dehydrogenase (LDH) and creatine kinase (CK) kits were purchased from Nanjing Institute of Biomedical Engineering. The rabbit anti-human beclin 1 (BECN 1) monoclonal antibody was purchased from Cell Signaling, USA; the rabbit anti-human  $\beta$ -actin monoclonal antibody was purchased from Proteintech Group, Inc., Wuhan; the BCA protein assay kit was purchased from Shanghai Biotechwell; the real-time qPCR instrument was purchased from BioRad, USA; the CytoFLEX LX flow cytometer was purchased from Beckman, USA; the DMEM culture media was purchased from Gibco, USA; the fetal bovine serum (FBS) and trypsin were purchased from Hyclone, USA; the Trizol reagent was purchased from Applide Invitrogen, USA; the PCR kit and minScript reverse transcription kit were purchased from TaKaRa, Dalian; the miR-124 mimics, miRNA NC and internal reference U6, as well as the  $\beta$ -actin primers were designed and synthesized by Gene Pharma, Shanghai; the CANnexin V-FITC/PI apoptosis detection kit was purchased from KeyGEN Biotech, Jiangsu.

#### *Animal model establishment*

The rats were randomly divided into 5 groups including the blank group, the sham group, the MIRI group, the miR-124 group and the miR-NC group with 10 rats respectively in each group. Twenty-four hours before surgery, 10  $\mu$ l of miR-124 inhibitor was injected into five intramyocardial sites in the miR-124 group. In the miR-NC group, 10  $\mu$ l of miR-NC inhibitor was administered with the same method. While the rats in the blank group were fed routinely without any treatment. At first, all 40 rats were all injected with 10% chloral hydrate (3.5 ml/kg) for anesthesia. The rats were connected to an ECG sensor by their limbs, skin was prepared and disinfected, they were operated on with thoracotomy under sterile condition. After thoracotomy, the pericardium of the rats in MIRI group, miR-124 group and MIRI group were opened to expose the heart, and the heart was kept perpendicular to the left coronary vein. Afterwards, the left coronary artery was ligated at the place of 2 mm under the auricula sinistra with a suture line. After 60 min, the ligature was removed to recover the blood supply. The rats in the sham group were not ligated arterially after the thora-

**Table 1.** Sequence table of related primers

Factor	Upstream primer	Downstream primers
miR-124	5'-TAAGGCACGCGGTGAAG-3'	5'-GTGCGTGTGCTGGAGTC-3'
Beclin 1	5'-TTCAAGATCCTGGACCGAGTGAC-3'	5'-CGTCTTCAGAGACAGCCAGGAG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
$\beta$ -actin	5'-CCCATCTATGAGGGTTACGC-3'	5'-TT-TAATGTCACGCACGATTC-3'

cotomy. Twelve hours after operation, LDH and CK of the rats in all 5 groups were detected by ELISA. After the rats were sacrificed, the myocardial infarct size of the rats in each group was measured by multimedia color pathological analysis.

#### *Detection of miR-124 and Beclin 1 mRNA by RT-PCR*

Five rats were randomly selected in each group and sacrificed. The execution followed animal ethics. The myocardial tissues of each group of rats were taken out, cut into pieces and ground, Trizol reagents were added, the RNA was extracted and the purity and concentration of RNA were determined with ultraviolet spectrophotometer. One  $\mu$ g of total RNA was used to synthesize the cDNA according to the instructions of reverse transcription kit. Two  $\mu$ l of synthesized cDNA was used for qPCR. The reaction conditions of miR-124 were as followings: pre-denaturation at 95°C for 10 min, 95°C for 15 s and 60°C for 60 s; 40 cycles in total. The reaction conditions of Beclin 1 mRNA were as followings: pre-denaturation at 95°C for 30 s, 95°C for 5 s and 60°C for 35 s; 40 cycles in total, and then 95°C for 15 s and 60°C for 1 min. The expression of miR-124 was detected with U6 as the internal reference and the expression of Beclin 1 mRNA was determined with  $\beta$ -actin as the internal reference. The primer sequence is shown in **Table 1** and the relative expression of the gene was expressed with  $2^{-\Delta\Delta CT}$ .

#### *Primary isolation and culture of cardiomyocytes of rats*

The rats in the blank group were anesthetized. The hearts of the rats were removed, placed in a culture dish containing PBS, homogenized and put in a DMEM medium containing 10% FBS. The resulting substance was shaken for 5 min at 37°C, the supernatant was discarded, 10 ml of the collagenase digestive serum was added, and shaken for 1 min, and the supernatant was discarded again. Six ml of collagenase

digestive serum was added followed by 15 min of stirring and the operation was repeated 2 times. The resulting solution was transferred to the centrifuge tube to be cen-

trifuged at 1000 rpm for 5 min and 10 ml of DMEM medium was again added to suspend the cardiomyocytes. The cells were cultured in an incubator containing 5% CO<sub>2</sub> at 37°C, and collected for subsequent experiment when the cells grew with adherence to 80%.

#### *Transfection of miR-124*

Before transfection, the cells were divided into the test group (transfected with miR-124), the negative control group (transfected with miR-NC) and the blank control group, and were inoculated to a 6-well plate with  $3 \times 10^5$  cells in each well. The lipofectamine 2000 and DNA were diluted and mixed following the instructions of Lipofectamine 2000 transfection kit, the resulting solution was set at room temperature for 5 min and mixed with the cells, and the transfection was performed under the condition of 37°C and 5% CO<sub>2</sub>. Twenty-four hours after the transfection, the expression of miR-124 and miR-NC transfected cardiomyocytes was determined by PCR, and the cells were collected for subsequent experiments.

#### *Expression detection of Beclin 1 protein and autophagy-related protein by western blotting*

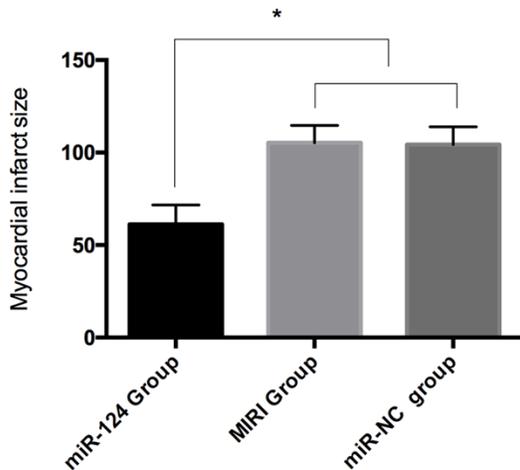
The transfected cells were flushed with PBS after discarding the culture solution, lysed with a cell lysate containing 200  $\mu$ l of protease inhibitor on ice and collected at 4°C as well as centrifuged at 13,000 $\times$  g for 15 min after lysis. After centrifugation, a 10% separation gel was prepared and the protein lysate was separated by electrophoresis. After separation, rabbit anti-human Beclin 1 antibody, p62, LC3 and  $\beta$ -tubulin mice monoclonal antibodies were added, and the resulting solution was incubated at 4°C overnight. The protein assay was performed with the absorbance values under ECL imaging development.

#### *Detection of cardiomyocyte apoptosis by Annexin V-FITC/PI double staining*

Cell apoptosis was detected by Annexin V-FITC/PI double staining together with flow cytometry.

**Table 2.** Expression of CK and LDH in serum of five groups of rats

Index	Blank group n=10	Sham operation group n=10	MiR-124 group n=10	MIRI group n=10	MIR-NC group n=10	F	P
CK (kU/L)	44.28±4.82	45.04±4.65	50.16±4.55	73.12±8.57	72.91±8.12	52.78	<0.001
LDH (U/L)	2576.11±132.12	2581.58±147.62	3158.69±126.37	6427.11±212.61	6386.13±214.35	1373	<0.001



**Figure 1.** Comparison of myocardial infarct size in rats. Note: \*Compared with the blank group and the negative control group, P<0.05.

The miR-124 and miRNA-NC transfected cardiomyocytes were inoculated into a 6-well plate with a density of  $3 \times 10^5$  cells/well, flushed twice with PBS after 24 h of incubation and 5  $\mu$ l of Annexin V-FITC was added after flushing. Ten  $\mu$ l of PI was added after 10 min of reaction at room temperature, and the incubation under darkness was kept for 20 min at room temperature, and finally cell apoptosis was detected with the flow cytometer. The experiment was repeated three times.

*Statistical methods*

In this study, the data was statistically analyzed with SPSS 18.0 software (Bizinsight (Beijing) Information Technology Co., Ltd) and the figures were plotted with GraphPad Prism 6 software. The counting data was compared with Chi-square test and the measurement data was expressed with mean  $\pm$  standard deviation. T test was used for analysis between two groups and one-way ANOVA with post hoc Bonferroni test was applied for comparison among groups. P<0.05 implied statistical difference.

**Results**

*Expression of serum CK and LDH*

After model establishment, the serum CK and LDH levels among the blank group, the sham group and the miR-124 group were not significantly different (P>0.05). However, the serum CK and LDH levels in the MIRI and miR-NC group were significantly higher than those in the blank group, the sham group and the miR-124 group (P<0.05) (Table 2).

*Myocardial infarct size*

The study measured myocardial infarct size in miR-124, MIRI, and miR-NC groups with myocardial infarction. The results showed that the myocardial infarct size of the miR-124, MIRI, and miR-NC groups were (61.19±10.47) mm<sup>2</sup>, (105.31±9.42) mm<sup>2</sup> and (104.33±9.56) mm<sup>2</sup>, respectively. The myocardial infarct size of the miR-124 group was significantly smaller than that of the MIRI group and the miR-NC group (P>0.05) (Figure 1).

*The expression of miR-124 and Beclin 1 mRNA in myocardial tissue*

The expression of miR-124 in the myocardial tissues of the blank group, the sham group and the miR-124 group were significantly lower than that in the MIRI group and miR-NC group (P<0.05). The expressions of Beclin 1 mRNA in the myocardial tissue among the five groups were not significantly different (P>0.05) (Table 3).

*Expression of miR-124 in transfected cardiomyocytes*

The expressions of miR-124 in the test group, the blank group and the negative control group were (5.72±1.13), (1.03±0.12) and (1.03±0.11), respectively. The results displayed that the expression of miR-124 in the test group was significantly higher than those in the blank group and the negative control group, and the

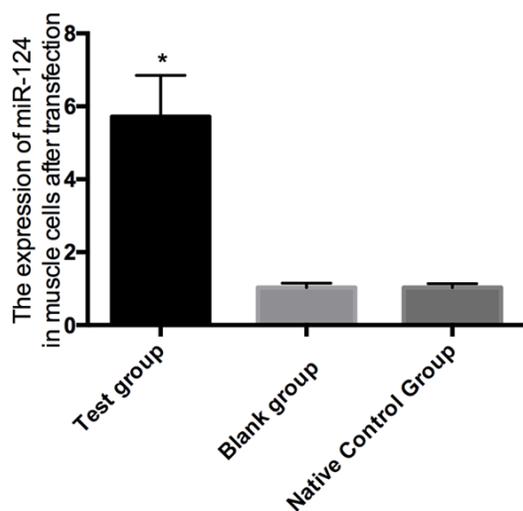
## Role of miR-124 in Cardiomyocyte injury

**Table 3.** Expression of miR-124 and Beclin 1 mRNA in myocardium of five groups of rats

Index	Blank group n=10	Sham operation group n=10	MiR-124 group n=10	MIRI group n=10	MiR-NC group N=10	F	P
miR-124	1.04±0.10	1.02±0.09	0.95±0.07	2.12±0.43	2.09±0.41	48.58	<0.001
Beclin 1	0.99±0.09	0.98±0.08	1.01±0.12	0.92±0.21	0.94±0.20	0.602	0.660

**Table 4.** Expression of miR-124 in muscle cells after transfection

Factor	Test Group	Blank group	Negative control group	F	P
miR-124	5.72±1.13	1.03±0.12	1.03±0.11	50.63	<0.001



**Figure 2.** The expression of miR-124 in transfected cardiomyocytes of the test group was significantly higher than those of the blank group and the negative control group, and the differences were statistically significant ( $P<0.05$ ), which implied that the transfection was successful. Note: \*Compared with the blank group and the negative control group,  $P<0.05$ .

differences were statistically significant, which implied a successful transfection (**Table 4** and **Figure 2**).

### *Expressions of Beclin 1 protein and autophagy-related protein in each group after transfection*

The expressions of Bcln 1 and LC3 autophagy proteins in the test group were significantly lower than those in the blank and negative control groups (**Table 5** and **Figure 3**).

### *Apoptosis of cardiomyocytes after transfection*

The apoptosis rates of cardiomyocytes in the test group, the blank group and the negative

control group were ( $37.52\pm 0.98\%$ ), ( $17.55\pm 0.61\%$ ) and ( $18.71\pm 0.63\%$ ), respectively.

The apoptosis rate of cardiomyocytes in the test group

was significantly higher than those of the blank group and the negative control group, and the differences were statistically significant ( $P<0.05$ ) (**Table 6**, **Figure 4**).

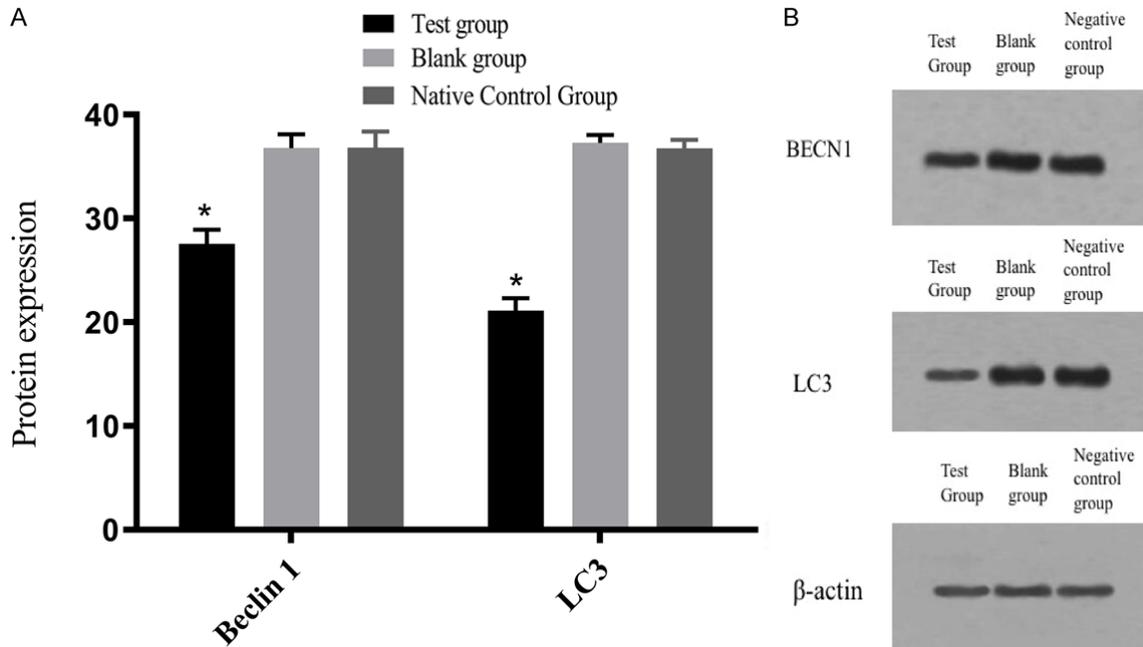
## Discussion

MiRNA is a highly conserved non-coding microRNA. A large number of previous studies displayed that the miRNA played an important role in the biological processes including cell proliferation and apoptosis [9-11]. In recent years, studies have shown that miRNA plays an important regulatory role in the apoptosis and differentiation of cardiomyocytes; for example, there was study on a MIRI mouse model discovering that miR-21 expression in fibroblasts increased significantly while PTEN protein expression in fibroblasts decreased significantly [12]. Autophagy is a process that degrades damaged organelles and proteins through lysosomes, in order to maintain intracellular homeostasis. The BECN 1 gene is a trigger for autophagy, and the expression elevation of BECN 1 protein will promote the increase of autophagy related protein-LC3. Some studies proposed that the action mechanism of BECN 1 in the ischemic myocardium may be that BCN3, after being activated will combine with Bcl-2, which is competing with BCN-2, and separate BECN 1 from the Bcl-2 complex so as to activate autophagy of the myocardium [13-16]. The bioinformatics analysis found that miR-124 and BECN 1 genes possessed binding sites at the 3'UTR region and we speculated that miR-124 may regulate MIRI by targeted regulation of the BECN 1 gene.

After establishment of the MIRI model, we found that serum CK and LDH in the blank group, the sham group and the miR-124 group were not significantly different ( $P>0.05$ ); however, serum CK and LDH in the MIRI group and

**Table 5.** Expression of Beclin 1 protein in muscle cells after transfection

Factor	Test Group	Blank group	Negative control group	F	P
Beclin 1	27.56±1.34	36.79±1.33	36.81±1.56	42.70	<0.001
LC3	21.12±1.21	37.27±0.79	36.76±0.81	276.4	<0.001



**Figure 3.** A. The expressions of Beclin 1 protein and autophagy-related protein in transfected cells of each group. The expressions of Bcln 1 protein and LC3 autophagy protein in the test group were significantly lower than those in the blank group and the negative control group ( $P<0.05$ ). B. Western blot results. Note: \*Compared with the blank group and the negative control group,  $P<0.05$ .

**Table 6.** Apoptosis of muscle cells after transfection

Factor	Test Group	Blank group	Negative control group	F	P
Apoptosis rate	37.52±0.98	17.55±0.61	18.71±0.63	654	<0.001

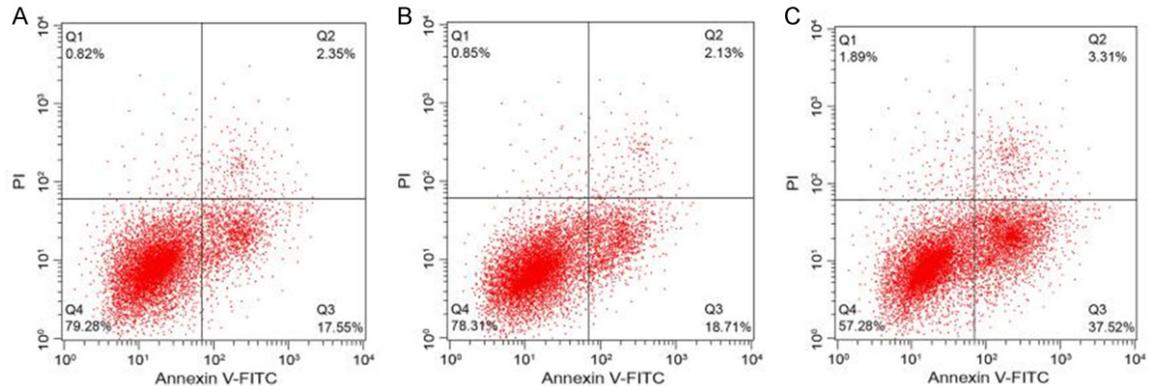
miR-NC were significantly higher than those in the blank group, the sham group and the miR-124 group, and the differences were statistically significant ( $P<0.05$ ). The increase of serum CK and LDH in the MIRI group suggested a successful model establishment. There were studies expressing that serum CK and LDH expressions increased because of the intracellular CK and LDH released into the blood after coronary artery ligation. The myocardial infarct size of the miR-124 group was significantly lower than that in the MIRI group and the miR-NC group ( $P<0.05$ ), indicating that decreasing the expression of miR-124 can significantly reduce the myocardial infarct size.

After the successful model establishment, we detected the expressions of miR-124 and BECN 1 mRNA in cardio-

myocytes of the five groups of rats and the result showed that the expression of miR-124 in the MIRI and miR-NC group was higher than those in the other three groups ( $P<0.05$ ), which indicated that the expression of miR-124 increased in the MIRI model group, but the expressions of BECN 1 mRNA among the five groups were not significantly different ( $P>0.05$ ). When detecting mRNA, it is hard to determine whether miR-124 regulates BECN 1; this may be because the expression of BECN 1 mRNA has not been directly affected during the short model establishment time.

Afterwards, we cultured and transfected the normal cardiomyocytes and the results dis-

## Role of miR-124 in Cardiomyocyte injury



**Figure 4.** Apoptosis of cardiomyocytes after transfection. A. Test group. B. Blank group. C. Negative control group.

played that miR-124 expression in the cardiomyocytes of the test group was significantly higher than those in the blank group and the negative control groups, and the differences were statistically significant ( $P < 0.05$ ), which implied successful transfection. We detected the expressions of Beclin 1 protein and autophagy-related protein in cardiomyocytes of the three groups and the results showed that the expressions of Beclin 1 protein and LC3 autophagy protein in the test group were significantly lower than those in the blank group and the negative control group and the differences were statistically significant ( $P < 0.05$ ). The results mentioned above indicate that the overexpression of miR-124 will decrease the expressions of Beclin 1 protein and LC3 autophagy related protein, and consequently inhibit the autophagy of cells. The result of cell apoptosis in the three groups showed that the apoptosis rate of cardiomyocytes in the test group was significantly higher than those in the blank group and the negative control group, and the differences were statistically significant ( $P < 0.05$ ), which implied that the autophagy inhibition would promote the apoptosis of cells. At present, the relationship between the miR and autophagy and apoptosis of cardiomyocytes is not reported yet, but some studies found that the miR-124 expression was negatively correlated with the expression of BECN 1 in non-small cell lung cancer, which was consistent with our conclusion [17-22]. There are rare studies on the relationship between miR and autophagy, but recent studies [23] have shown that miRNA plays a more and more important role in MIRI, and that most miRNAs will regulate genes by recognizing and binding to targets in the 3'UTR region. Studies have found that BECN 1 was also a direct target gene of miR-

30a, and overexpressed miR-30a will decrease cell autophagy induced by cisplatin, which indicated that miRNA plays an important regulatory role in autophagy. Further studies found that miR-30a would reduce the autophagy flux in chronic myelogenous leukemia by regulating BECN 1. In this study, the miR-124 levels increased significantly while the BECN 1 protein expression decreased significantly compared with the blank group and the negative control, which implied that miR-124 would inhibit autophagy in cardiomyocytes and consequently promote apoptosis of cells [24-26]. Therefore, we speculated that miR-124 may be an important marker of MIRI.

In summary, miR-124 was highly expressed in MIRI, and may decrease autophagy and promote apoptosis of cells by binding to BECN 1. MiR-124 may become an important target for MIRI treatment in the future. However, many problems are still not solved in the study of miRNA and autophagy. In this study, we did not investigate the changes of miR-124 during autophagy of cells, but some studies showed that the miRNA levels will fluctuate during autophagy induced by different conditions. In this study, a hypoxia model was not yet established to investigate the regulation of miR-124 on BECN 1 so as to prove the conclusions. It is expected that future scholars will study the direct relationship between miRNA and autophagy from multiple perspectives.

### Disclosure of conflict of interest

None.

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## Role of miR-124 in Cardiomyocyte injury

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## Role of miR-124 in Cardiomyocyte injury

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