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# Inhibition of MiR-92a May Protect Endothelial Cells After Acute Myocardial Infarction in Rats: Role of KLF2/4

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
Manuscript Preparation E  
Literature Search F  
Funds Collection G

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**Background:** This study was designed to investigate the effects of microRNA-92 (miR-92), Kruppel-like factor 2 (KLF2), and Kruppel-like factor 4 (KLF4) on endothelial injury after acute myocardial infarction (AMI).


**Material/Methods:** Blood samples were collected from 50 AMI patients for detection of cardiac troponin I (cTnI), heart-type fatty acid-binding protein (H-FABP), and von Willebrand factor (vWF). The Sprague-Dawley rat models of AMI (n=30) were established by ligating their left anterior descending coronary artery. The cardiac markers of AMI patients and rat models were analyzed with enzyme-linked immunosorbent assay and immunohistochemistry. Human umbilical vein endothelial cells were processed into 5 groups: control, negative control, miR-92a inhibitors, miR-92a inhibitors + KLF2 small interfering RNA (siRNA), and miR-92a inhibitors + KLF4 siRNA. Cell proliferation and apoptosis were detected using MTT assay and flow cytometry. RT-PCR and Western blot were conducted to analyze KLF2 and KLF4 expressions.

**Results:** AMI patients exhibited significantly higher expression of both endothelial injury markers (e.g., cTnI, H-FABP, vWF) and miR-92a in blood samples, when compared with controls ( $P<0.05$ ). Model rats also had similar expressional tendencies, along with lower KLF2 and KLF4 expressions ( $P<0.05$ ). Further, it could be observed in cellular experiments that treatment of miR-92a mimics can further upregulate endothelial injury markers, and miR-92a and both KLF2 and KLF4 were downregulated by miR-92a mimics (all,  $P<0.05$ ). Also, the luciferase activity assay confirmed the direct binding of miR-92a to 3' UTR of KLF2/4.

**Conclusions:** MiR-92a was involved in the endothelial injury process after AMI and was able to suppress KLF2 and KLF4 expression.

**MeSH Keywords:** **Endothelial Cells • Kruppel-Like Transcription Factors • MicroRNAs • Myocardial Infarction**

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## Background

Myocardial infarction (MI), or coronary occlusion, is the most common cause of heart failure, contributing to a large number of deaths worldwide [1,2]. An MI results from coronary artery myocardial necrosis caused by acutely persistent ischemia and hypoxia, which tends to occur in patients with coronary atherosclerotic stenosis [3]. Generally, it is acknowledged that MI is closely related to oxygen radicals, vascular endothelial injury, and myocardial cell apoptosis [4]. Moreover, cardiomyocyte apoptosis and necrosis triggered by MI result in the loss of cardiomyocytes, which may further lead to a significant decrease in heart pumping and congestive heart failure [5]. Previous research has shown that timely reperfusion treatment for acute myocardial infarction (AMI), by shrinking the myocardial necrosis zone, may cause reperfusion injury [6–11]. Thus, it is vital to discover an effective, reperfusion injury-free tool for AMI prevention and treatment [12].

The vascular endothelium, the layer between the circulating blood and the vessel wall, sustains fluid shear stress (SS) by blood flow [13]. Therefore, vascular endothelial cell injury is the initial factor that triggers cardiovascular disease [14]. SS regulates the proliferation of endothelial cells by some unknown mechanisms related to several signal factors, such as platelet endothelial cell adhesion molecule-1 and vascular endothelial growth factor receptor-2 [15]. Moreover, both Kruppel-like factor 2 (KLF2) and Kruppel-like factor 4 (KLF4) have substantial influence on endothelial activation [16,17].

As suggested by recent studies that focused on the mechanism of MI, microRNAs (miRNAs) take an active part in the regulation of myocardial cell apoptosis [18,19]. miRNAs are small noncoding conserved RNA molecules that are constructed by around 22 nucleotides and that participate pivotally in regulating gene expression [20–22]. miRNAs modulate gene expression by binding to their target messenger RNAs in the 3' untranslated region (UTR) and this may affect the protein translation process [23,24]. Researchers also discovered that miRNAs are differently expressed in different diseases and they have regulation and control effects on the development of cardiovascular disease [12]. As a major cause of cardiovascular disease that is related to cell apoptosis, ischemia changes the manifestation pattern of miRNAs in cardiac tissues. Several miRNAs have exhibited their ability to regulate fibrosis after MI. Among these miRNAs, miR-92a is expressed not only in endothelial cells but also in cardiac fibroblasts and myocardial cells [13]. Previous research also indicated that endothelial miR-92a expression differs in regulation by SS and hypercholesterolemia, and up-regulation of miR-92a triggered by oxidized low-density lipoproteins (oxLDL) contributes to endothelial cell proliferation and atherosclerotic plaque formation [25]. Through improving neovascularization and enhancing functional recovery,

inhibition of miR-92a can significantly decrease cell apoptosis [26]. Therefore, miR-92a depressor seems to be a new approach for atheroprotective therapy [25]. Additionally, functions of miR-92a in regulating endotheliocyte activation have correspondence to modulation of KLF2, KLF4 [27,28]. KLF2 is a critical mediator for vascular formation and angiogenesis. The regulation of KLF2 is reduced once progenitor cells become aged or affected by diabetic conditions because of poor angiogenic cell capacity [29,30]. Apart from that, KLF4 is able to revert the programming of differentiated somatic cells and restore their pluripotent status [31]. Both KLF2 and KLF4 expressions dramatically increased since the blockade of miR-92a, which eliminates the impact of oxLDL and SS [25]. Although KLF2 and KLF4 play important parts in modulating miR-92a's function in regulating endothelial cell activation, no exact research has proved that miR-92a and KLF2/4 are associated with MI or endothelial injury.

Published studies mainly focus on the association between MI and cardiomyocyte death, including apoptosis and necrosis [32]. Existing experiments *in vitro* revealed that miR-92a has effects on some cardiac diseases, such as endothelial inflammatory, atherosclerosis, and ischemia-reperfusion injury [25,33]. Moreover, miR-92a inhibition can prevent some cardiac diseases and relieve their symptoms, whereas anti-inflammatory transcription factors of KLFs were likely to enhance cardiac functions [13,34]. Our study will further systematically clarify the effect of KLF2, KLF4, and miR-92a inhibitors on endothelial injury protection after AMI via *in vivo* and *in vitro* experiments.

## Material and Methods

### Ethics statement

All human tissue collections were agreed and authorized by the institutional ethics committee of Nanyang City Center Hospital and Second Affiliated Hospital of Nanjing Medical University, according to the Helsinki Declaration. Informed consent was obtained from patients before study commencement. All rat experiments were carried out under the Guidance for Care and Usage of Laboratory Animals and were adopted by the National Cancer Institute Animal Care and Use Committee.

### Clinical samples

A total of 51 patients (33 males and 18 females, Nanyang City Center Hospital and Second Affiliated Hospital of Nanjing Medical University) were included in this study. All patients underwent emergent percutaneous coronary intervention and had had clinically significant ST-T changes with ongoing chest pain for less than 12 hours. Blood samples were collected to determine the peak values of cardiac markers. The control

**Table 1.** Clinical data on AMI patients and controls.

Parameters	AMI patients (n=51)	Controls (n=51)	P value
Age, median (range), y	59 (17–99)	59 (21–96)	0.58 <sup>a</sup>
Gender, male, n(%)	33 (64.7)	32 (62.7)	0.86 <sup>b</sup>
Body mass index (kg/m <sup>2</sup> )	26.59±3.96	25.76±4.15	0.05 <sup>a</sup>
Hemoglobin (g/ml)	13.45±2.23	14.06±2.15	0.01 <sup>a</sup>
Hypertension, n (%)	21 (41.2)	22 (43.1)	0.84 <sup>b</sup>
Diabetes, n (%)	13 (25.5)	12 (23.5)	0.55 <sup>b</sup>
Alcohol, n (%)	15 (29.4)	13 (25.5)	0.45 <sup>b</sup>
Smoking, n (%)	16 (31.4)	14 (27.5)	0.60 <sup>b</sup>
Family history, (%)	11 (21.6)	10 (19.6)	0.76 <sup>b</sup>

AMI – acute myocardial infarction. <sup>a</sup> Independent sample t-test; <sup>b</sup> Chi-square test were used.

group consisted of 51 healthy volunteers (32 males and 19 females) obtained from a national observation study on cardiovascular risks. All clinical characteristics of patients are presented in Table 1.

#### Animal model

Forty Sprague-Dawley rats (Laboratory Animal Center of Nanyang City Center Hospital and Second Affiliated Hospital of Nanjing Medical University), with an average age of 2 to 3 months and weight between 225 to 350 g, were randomly allocated to the control (n=10) or model group (n=30). An AMI model was established by ligating the left anterior descending coronary artery (LAD) of rats in the model group and rats in the control group were treated with a sham operation [35].

During the AMI surgery, negative control (NC), miR-92a mimics, or miR-92a inhibitors (1 µg vector, mimics, or inhibitors, with a total of 60 µL solution) were injected into 3 areas of myocardium near the LAD. The rats were then divided into 4 groups: control group (10 rats with sham operation with vector injected); model group (10 rats in the model group with NC injected); miR-92a mimics group (10 rats in the model group with miR-92a mimics injected); miR-92a inhibitors group (10 rats in the model group with miR-92a inhibitors injected). All rats were killed 3 days after LAD ligation and their left atriums collected for further analysis.

#### Biochemical blood analyses

Blood samples were collected from patients or rats for analyzing endothelial injury markers including cardiac troponin I (cTnI), heart-type fatty acid-binding protein (H-FABP), and von Willebrand factor (vWF). These markers were tested using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) and the corresponding reagent kits (R&D, USA).

#### Hematoxylin-eosin staining and immunohistochemistry

After the surgery, rats in each group were killed via xylazine hydrochloride injection. Tissues from infarct regions in the left atrium were harvested and treated with 4% paraformaldehyde. Then 5-µm thick paraffin sections were embedded and stained with hematoxylin-eosin assay. Samples were observed and inspected microscopically and measured at 5 random sites.

Immunohistochemical analysis of KLF2 and KLF4 in cardiac tissues was performed using the EnVision 2-step method [36]. Rabbit polyclonal anti-KLF2 or anti-KLF4 antibody (Zhongshan Biology Company, Beijing) was diluted 100 times.

#### Cell culture and lentivirus transfection

Human umbilical vein endothelial cells (HUVECs) were purchased from the Institute of Biochemistry and Cell Biology (Shanghai). Cells were cultured in endothelial growth medium with 5% CO<sub>2</sub> (Gibco, CA, USA) at 37°C.

Three groups of fragments separately containing miR-92a inhibitors, KLF2 small interfering RNA (siRNA), and KLF4 siRNA were cloned into the pCDH vector. Then, the pCDH vector was co-transfected with other packaging plasmids into cells using Lipofectamine® LTX kit (Invitrogen, CA, USA), and the viral particles therein were collected 48 h after transfection.

HUVECs were infected with 5 groups of recombinant lentivirus and 8 µg/mL polybrene: control group (cells with no transfection), NC group (cells transfected with NC), miR-92a inhibitors group (cells transfected with miR-92a inhibitors), miR-92a inhibitors + KLF2 siRNA group (cells co-transfected with miR-92a inhibitors and KLF2 siRNA), and miR-92a inhibitors + KLF4 siRNA group (cells co-transfected with miR-92a inhibitors and KLF4 siRNA).

**Table 2.** Primer sequences for luciferase reporter experiments.

Gene		Primer sequence
KLF2 3' UTR	Sense	5'-GTCATGGGGGTGTGGGGTTCCAGGAAAT-3'
	Antisense	5'-ATTTCTGGAACCCACACCCCATGAC-3'
KLF2 3' UTR mutation	Sense	5'-GTCATGGTATTTAAGGGTCCAGGAAAT-3'
	Antisense	5'-ATTTCTGGAACCCCTAAAATACCATGAC-3'
KLF4 3' UTR	Sense	5'-CGCACTAGTATCCACGTAGTGGATG-3'
	Antisense	5'-CTAAAGCTTGCTCTCACTCCAACACTAC-3'
KLF4 3' UTR mutation	Sense	5'-AATGCCAAGGGGTGTTTGTGGATATCAG-3'
	Antisense	5'-CTGATATCCACAACCACCCCTTGGCATT-3'

UTR – untranslated region.

**Table 3.** Primer sequences of GAPDH and miR-92a for implementation of RT-PCR.

Gene		Primer sequence
GAPDH	Sense	5'-TGGTATCGTGAAGGACTCAT-3'
	Antisense	5'-GTGGGTGTCGCTGTTGAAGTC-3'
miR-92a	Sense	5'-CTGTCCTGTATTGAGCACTGGTCTATGG-3'
	Antisense	5'-AAGACATTAGTAACCCACCCCATCC-3'

GAPDH – phosphoglyceraldehyde dehydrogenase; RT-PCR – real time-polymerase chain reaction.

### Luciferase activity assay

The 3' UTR of KLF2 or KLF4 containing miR-92a binding sites was amplified through polymerase chain reaction (PCR) with prime sequences shown in Table 2 and was cloned into the downstream of the psiCHECK™-2 luciferase vector (Promega, USA) and named KLF2/4 3' UTR. The binding site was mutated using the GeneTailor™ Site-Directed Mutagenesis System (Invitrogen, USA) and the resultant mutant 3' UTR was cloned into the same vector and named KLF2/4 3' UTR mutation.

HUVECs stored in 48-well plates were co-transfected different groups of substances: one group was co-transfected with the combination of 200 ng pGL3-control luciferase reporter, 10 ng pRL-TK vector and miR-92a vector, while the miR-92a vector was replaced with the NC vector in the other group. The transfected cells were analyzed by the Dual-Luciferase® Reporter Assay System (Promega) 48 h after transfection.

### Cell proliferation and apoptosis assay

MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) assay was used to evaluate cell proliferation. Approximately  $3 \times 10^3$  cells were cultured in 96-well plates, incubated for 48 h, and stained with 0.5 mg/mL MTT for 4 h. We discarded the supernatant and added 200  $\mu$ L dimethylsulfoxide to dissolve precipitate. Samples were measured at 490 nm with an ELISA reader.

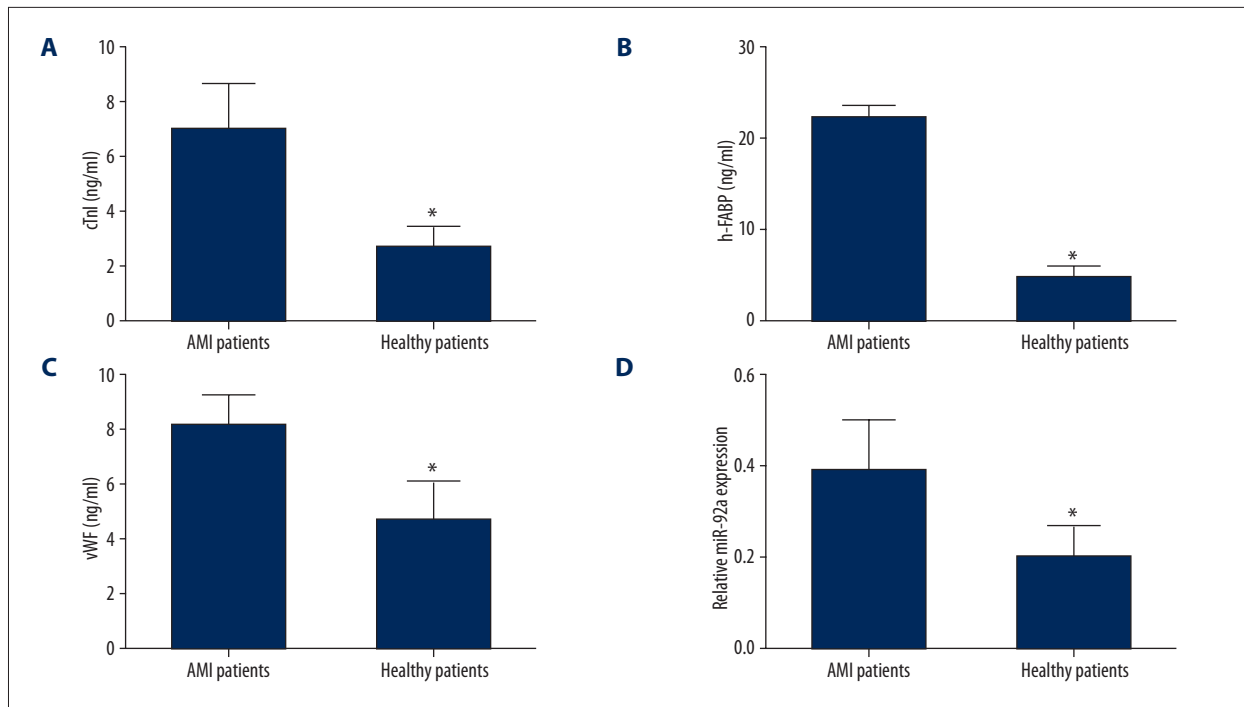
Cell death was induced by 18-h hypoxia in HUVECs of 5 groups. Apoptosis rates of samples were calculated after cells were stained with Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences) in flow cytometry.

### RNA isolation and RT-PCR

Total RNA was isolated from tissues and cells using the TRIzol® reagent (Invitrogen, Germany) under the instruction of manufacturer. The ReverTra Ace® qPCR RT Kit (Toyobo, Japan) was used to transcribe the total RNA into cDNA and RT-PCR was carried out using THUNDERBIRD SYBR® qPCR Mix (Toyobo, Japan) with the instrument of CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The primer sequences were listed in Table 3. The expression level of target genes was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and was calculated using the method of  $2^{-\Delta\Delta CT}$ .

### Western blot

Tissues and cells were harvested and lysed using radio immunoprecipitation assay buffer. Total protein was separated and calculated in line with the Bradford method [37]. Then total protein was denatured in boiled water and transferred onto polyvinylidene fluoride membranes after the process of sodium dodecyl sulfate-polyacrylamide gel electrophoresis was completed. The membranes were blocked in Tris Buffered Saline With Tween® with 5% skim milk for 1 h and were treated with primary antibodies against KLF2



**Figure 1.** Quantitative data of cTnI (A), H-FABP (B), vWF (C), and miR-92a (D) in patients in the AMI group and controls. Data were presented as mean ±SD. \*  $P < 0.05$  versus patients in the AMI group.

**Table 4.** Mean levels of endothelial injury markers and miR-92a in serum sample of AMI patients and controls.

Parameters	AMI patients (n=51)	Controls (n=51)	P value
cTnI, ng/ml	6.97±1.69	2.64±0.8551	<0.001 <sup>a</sup>
h-FABP, ng/ml	22.18±1.63	4.62±1.26	<0.001 <sup>a</sup>
vWF, ng/ml	8.17±1.14	4.75±1.42	<0.001 <sup>a</sup>
miR-92a	0.39±0.11	0.20±0.07	<0.001 <sup>a</sup>

cTnI – cardiac troponin I; H-FABP – heart-type fatty acid binding protein; vWF – von Willebrand factor. <sup>a</sup> Independent sample t-test.

and KLF4 (1:800 dilution, Zhongshan Biology Company, Beijing) at 4°C overnight. After the membranes were washed, they were incubated with secondary antibodies (horseradish peroxidase-conjugated goat antigoat, 1:2000 dilution, Zhongshan Biology Company, Beijing). Finally, samples along with reduced GAPDH as the endogenous control were processed with enhanced chemiluminescence and quantified by Lab Works 4.5 (Mitov Software).

### Statistical analysis

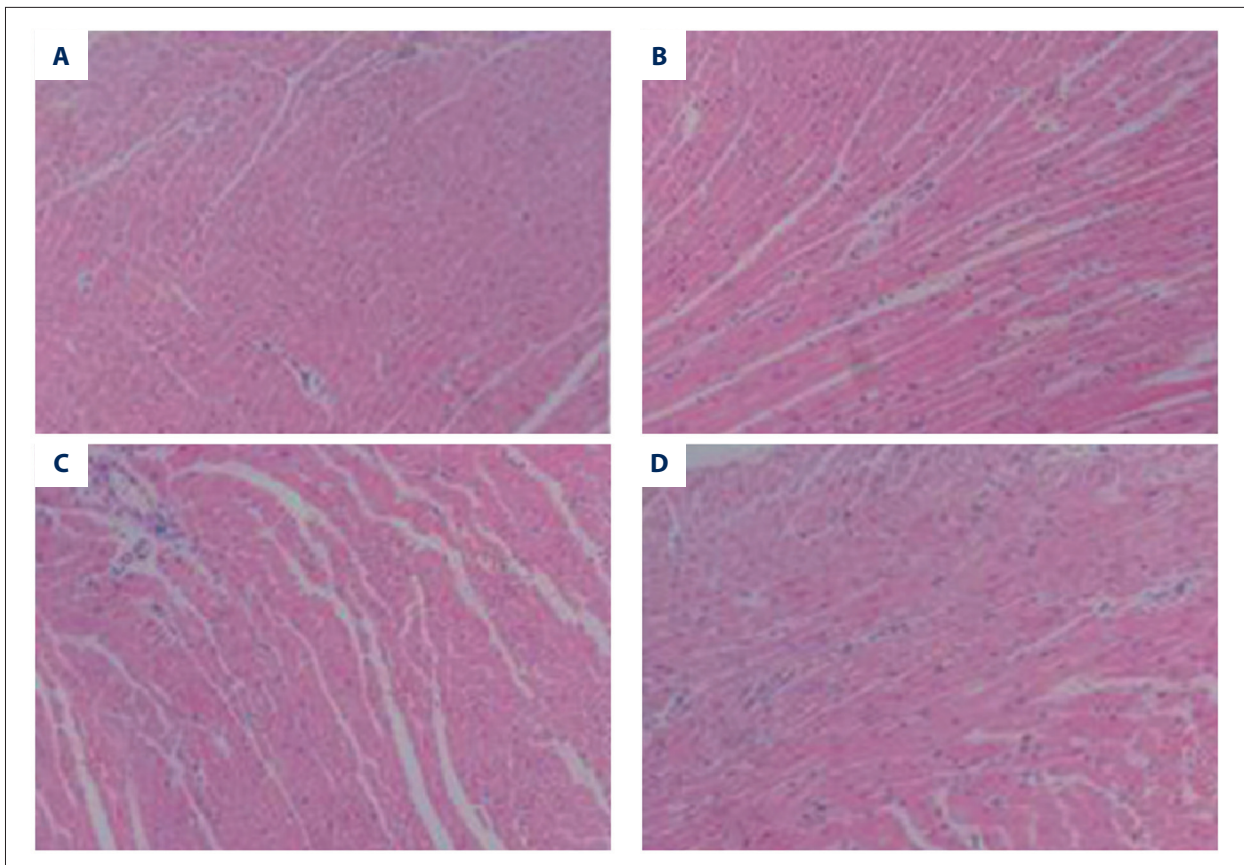
All statistical results were obtained using SPSS 18.0 software (Chicago, IL, USA). Data were presented in the form of mean ± standard deviation (SD). Two-tailed *t* test or 1-way analysis of variance was used to assess between-group comparisons, whereas the chi-square test was used for investigating the association between categorical variables.  $P < 0.05$  suggested evidence for statistical significance.

## Results

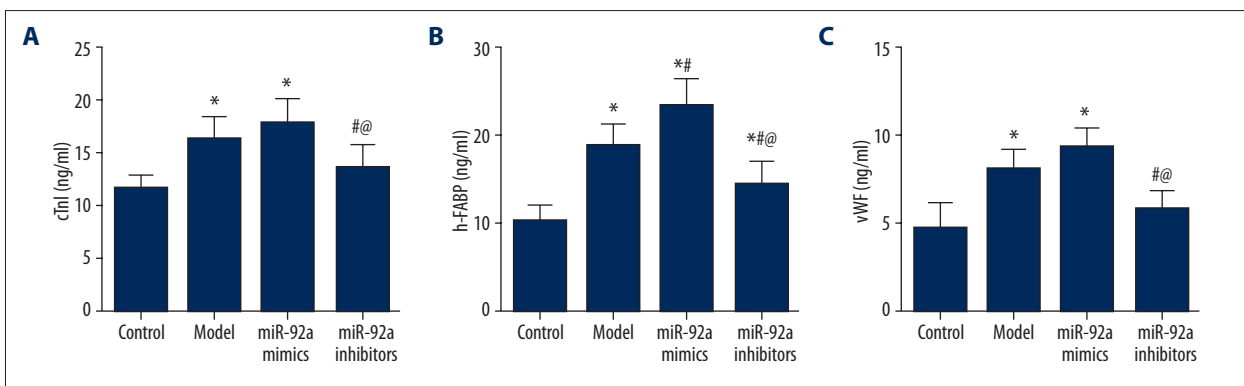
### AMI patients had higher endothelial injury marker and miR-92a expression

Table 1 shows the clinical characteristics of patients in the AMI group and of those in the control group in this study. No significant differences were detected in terms of age, sex, diabetes, alcohol consumption, smoking status, or family history of AMI between the 2 groups. By contrast, there were remarkable differences between the AMI group and the control group in terms of body mass index and hemoglobin level ( $P < 0.05$ ). Similarly, the expression of endothelial injury markers, including cTnI, H-FABP, vWF, and miR-92a, were significantly up-regulated in the AMI group, compared with the control group (all,  $P < 0.05$ , Figure 1, Table 4).





**Figure 2.** Hematoxylin-eosin staining of atrial tissues in groups of control (A), model (B), miR-92a mimics (C), and miR-92a inhibitors (D) under microscope ( $\times 400$ ).

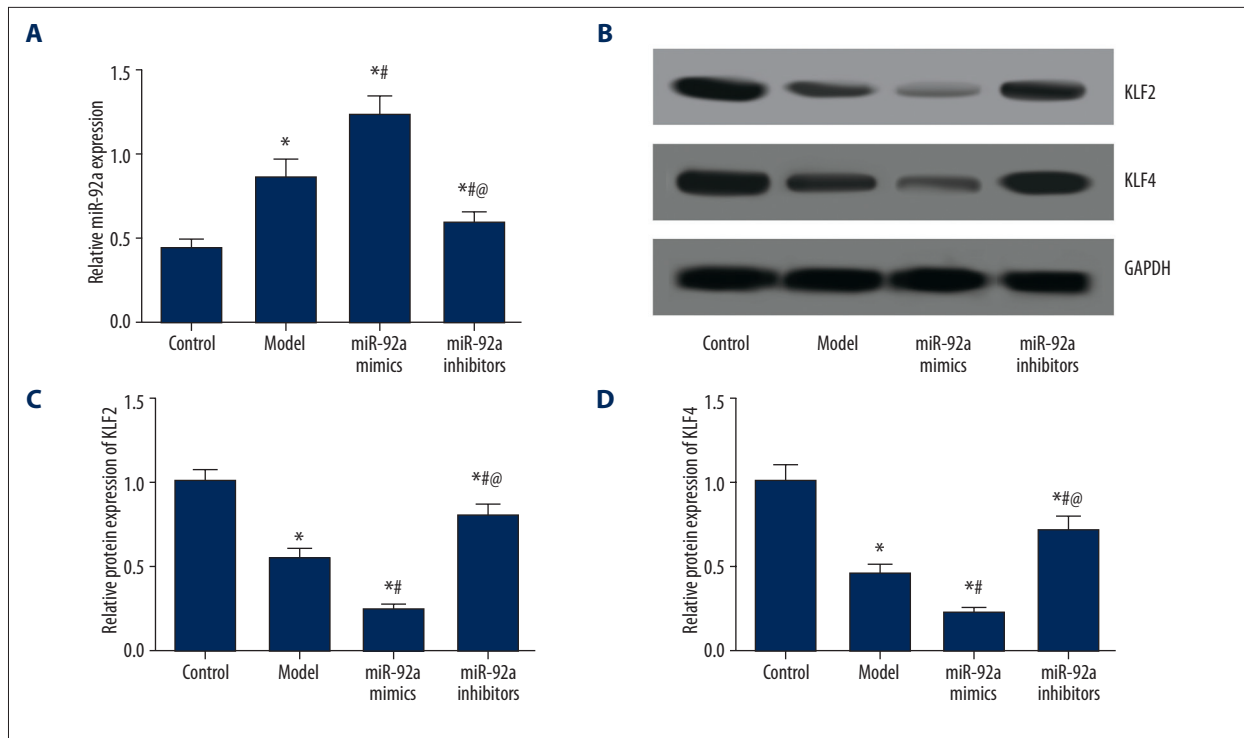


**Figure 3.** Quantitative expression levels of endothelial injury markers, including cTnI (A), h-FABP (B) and vWF (C) in different groups of rats (i.e., control, model, miR-92a mimics and miR-92a inhibitors). Data were presented as mean  $\pm$  SD. \*  $P < 0.05$  versus control group, #  $P < 0.05$  versus model group, @  $P < 0.05$  versus miR-92a mimics group.

### MiR-92a regulated endothelial injury marker and KLF2/4 expression in model rats

As shown in Figures 2–4 and Table 5, fibrous tissues were strained in cardiac tissues obtained from model rats, suggesting that the LAD ligation was involved in the disease. Compared with the control group, model rats had significantly higher

expression level of cTnI, H-FABP, vWF, and miR-92a and lower expression level of KLF2 and KLF4 (all,  $P < 0.05$ ). Treatment of miR-92a mimics can further up-regulate endothelial injury markers and miR-92a. Additionally, both KLF2 and KLF4 were down-regulated by miR-92a mimics and there were significant differences in expressions of H-FABP, miR-92a, KLF2, and KLF4 between the model and miR-92a mimics group (all,  $P < 0.05$ ).



**Figure 4.** Expressions of miR-92a, KLF2 and KLF4 in rats compared with different control groups (i.e. control, model, miR-92a mimics and miR-92a inhibitors). **(A)** Quantitative data of expression level of miR-92a in model rats. **(B)** Western blot analysis of KLF2 and KLF4 in model rats with GAPDH as the internal control. **(C)** Quantitative protein levels of KLF2 in model rats. **(D)** Quantitative protein levels of KLF4 in model rats. Data were presented as mean  $\pm$ SD. \*  $P < 0.05$  versus control group, #  $P < 0.05$  versus model group, @  $P < 0.05$  versus miR-92a mimics group.

**Table 5.** MiR-92a regulated the expression of endothelial injury markers and KLF2/4 in model rats.

Group	Control	Model	MiR-92a mimics	MiR-92a inhibitors
cTnl, pg/ml	11.67 $\pm$ 1.45	16.34 $\pm$ 2.12*	17.89 $\pm$ 2.35*	13.59 $\pm$ 2.25*#@
h-FABP, ng/ml	10.34 $\pm$ 1.78	18.70 $\pm$ 2.75*	23.34 $\pm$ 3.11*#	14.54 $\pm$ 2.36*#@
vWF, ng/ml	4.78 $\pm$ 1.41	8.12 $\pm$ 1.12*	9.24 $\pm$ 1.25*	5.87 $\pm$ 0.97*#@
miR-92a	0.44 $\pm$ 0.06	0.87 $\pm$ 0.10*	1.24 $\pm$ 0.12*#	0.59 $\pm$ 0.07*#@
KLF2	1.00 $\pm$ 0.11	0.45 $\pm$ 0.06*	0.22 $\pm$ 0.04*#	0.72 $\pm$ 0.08*#@
KLF4	1.00 $\pm$ 0.07	0.55 $\pm$ 0.05*	0.24 $\pm$ 0.04*#	0.80 $\pm$ 0.07*#@

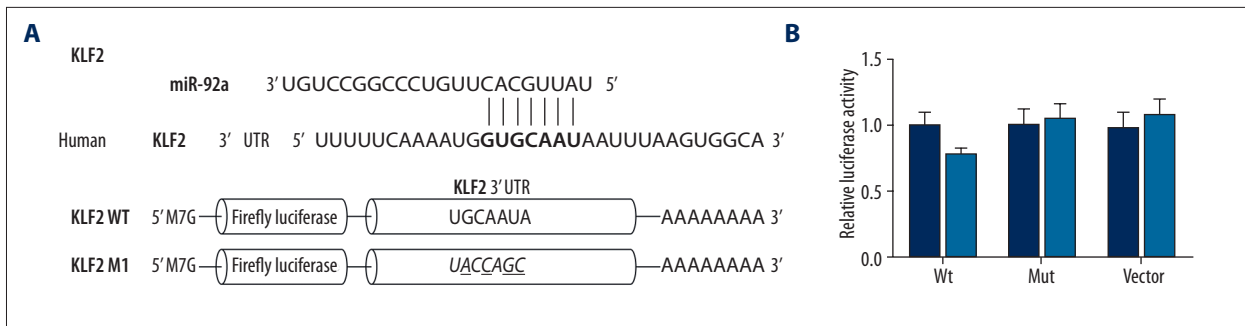
\*  $P < 0.05$  versus control group; #  $P < 0.05$  versus model group; @  $P < 0.05$  versus miR-92a mimics group.

Compared with the model and miR-92a mimics group, miR-92a inhibitors group had remarkably lower expression of endothelial injury markers (cTnl, H-FABP, and vWF) and miR-92a, as well as higher expression of KLF2 and KLF4 (all,  $P < 0.05$ ).

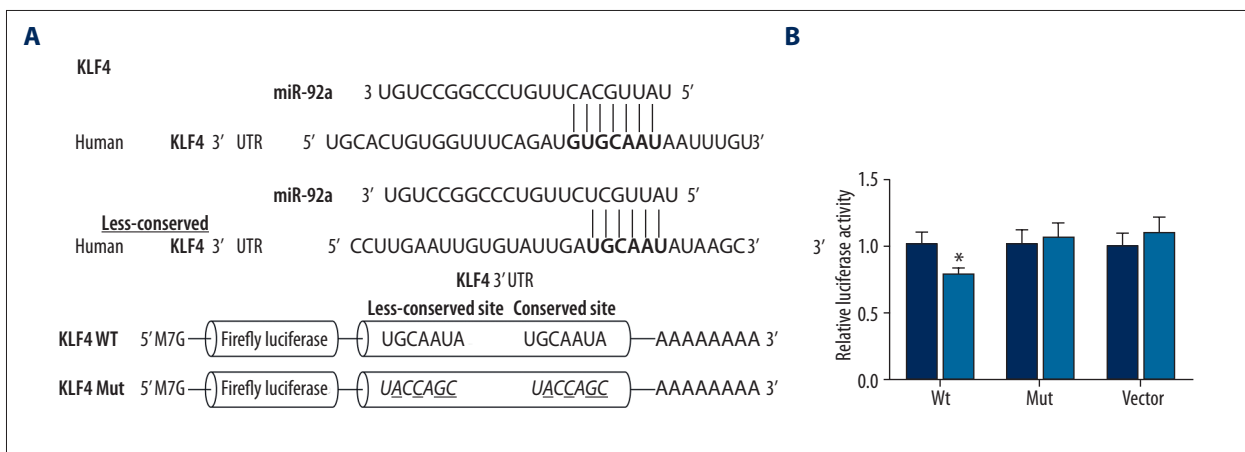
#### MiR-92a suppressed KLF2 and KLF4 expression by binding to their 3' UTR

One site in KLF2 3' UTR and 2 sites in KLF4 3' UTR were highly conserved to binding with miR-92a, which was consistent

with the search results of miRanda software (Figures 5A, 6A). Luciferase activity assay confirmed a direct interaction between miR-92a and KLF2/4 and provided evidence that miR-92a transfection could significantly decrease the relative luciferase activity in HUVECs when miR-92a bound to normal 3' UTR of KLF2/4 ( $P < 0.05$ ). Nonetheless, differences in luciferase activities between the NC and normal cells containing miR-92a within both groups of control and KLF2/4 mutation 3' UTR were not significant ( $P > 0.05$ ; Figures 5B, 6B, Table 6).



**Figure 5.** Binding sites prediction and results of luciferase report between miR-92a and KLF2. (A) Putative targets predicted by TargetScan. (B) Results of luciferase report. Data were presented as mean ±SD for 3 independent experiments. \*  $P < 0.05$  versus control group.



**Figure 6.** Binding sites prediction and results of luciferase report between miR-92a and KLF4. (A) Putative targets predicted by TargetScan. (B) Results of luciferase report. Data were presented as mean ±SD for 3 independent experiments. \*  $P < 0.05$  versus control group.

**Table 6.** Relative luciferase activity in wt KLF2/4, Mut KLF2/4 and vector groups.

Relative luciferase activity		Wt	Mut	Vector
KLF2	NC	1.00 ± 0.10	1.00 ± 0.12	0.98 ± 0.12
	Transfection group	0.78 ± 0.05*	1.05 ± 0.11	1.08 ± 0.12
KLF4	NC	1.00 ± 0.09	1.02 ± 0.14	1.02 ± 0.11
	Transfection group	0.65 ± 0.06*	1.01 ± 0.09	1.08 ± 0.09

Wt – wild type; Mut – mutation; NC – negative control. \*  $P < 0.05$  versus NC group.

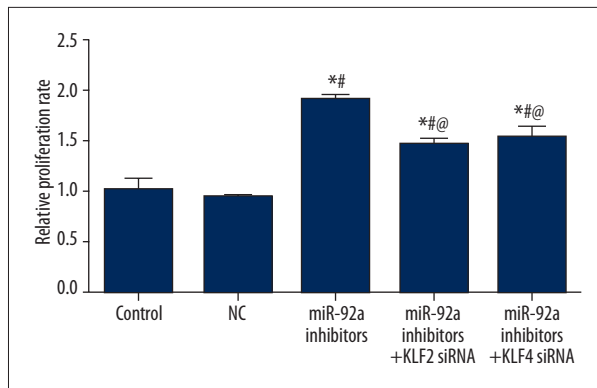
### Effects of miR-92a on HUVEC cell proliferation and apoptosis

As suggested by the proliferation rate of HUVECs, there was no significant difference between the control and NC group ( $P > 0.05$ ). Transfection of miR-92a inhibitors can significantly up-regulate the relative proliferation rate of HUVECs compared with the control and NC group ( $P < 0.05$ ). The effect of miR-92a inhibitors on the relative proliferation rate of HUVECs can be antagonized by siRNA of KLF2 or KLF4. The proliferation rates

of HUVECs in the miR-92a inhibitors + KLF2 siRNA and miR-92a inhibitors + KLF4 siRNA group were significantly lower than that in the miR-92a inhibitors group but still higher than those in the control and NC groups ( $P < 0.05$ ; Figure 7, Table 7). There was no significant difference in cell proliferation rate between groups of miR-92a inhibitors + KLF2 siRNA and miR-92a inhibitors + KLF4 siRNA ( $P > 0.05$ ; Figure 7, Table 7).

After HUVECs were treated with hypoxia, both the control and NC group experienced a high apoptosis rate with no significant





**Figure 7.** Effects of miR-92a inhibitors, KLF2 siRNA and KLF4 siRNA on proliferation of HUVECs estimated by MTT assay. Data were presented as mean  $\pm$ SD for 3 independent experiments. \*  $P < 0.05$  versus control group, #  $P < 0.05$  versus NC group, @  $P < 0.05$  versus miR-92a inhibitors group.

difference. Transfection of miR-92a inhibitors can significantly down-regulate the apoptosis rate of HUVECs compared with groups of control and NC ( $P < 0.05$ ). The effect of miR-92a inhibitors on the apoptosis rate of HUVECs can be antagonized by siRNA of KLF2 or KLF4. The apoptosis rates of HUVECs in groups of miR-92a inhibitors + KLF2 siRNA and miR-92a inhibitors + KLF4 siRNA were significantly higher than those of the miR-92a inhibitors group but still lower than those of the control and NC groups ( $P < 0.05$ ; Figure 8, Table 7). There was no remarkable difference in HUVEC cell apoptosis rate between miR-92a inhibitors + KLF2 siRNA and miR-92a inhibitors + KLF4 siRNA groups ( $P > 0.05$ ; Figure 8, Table 7).

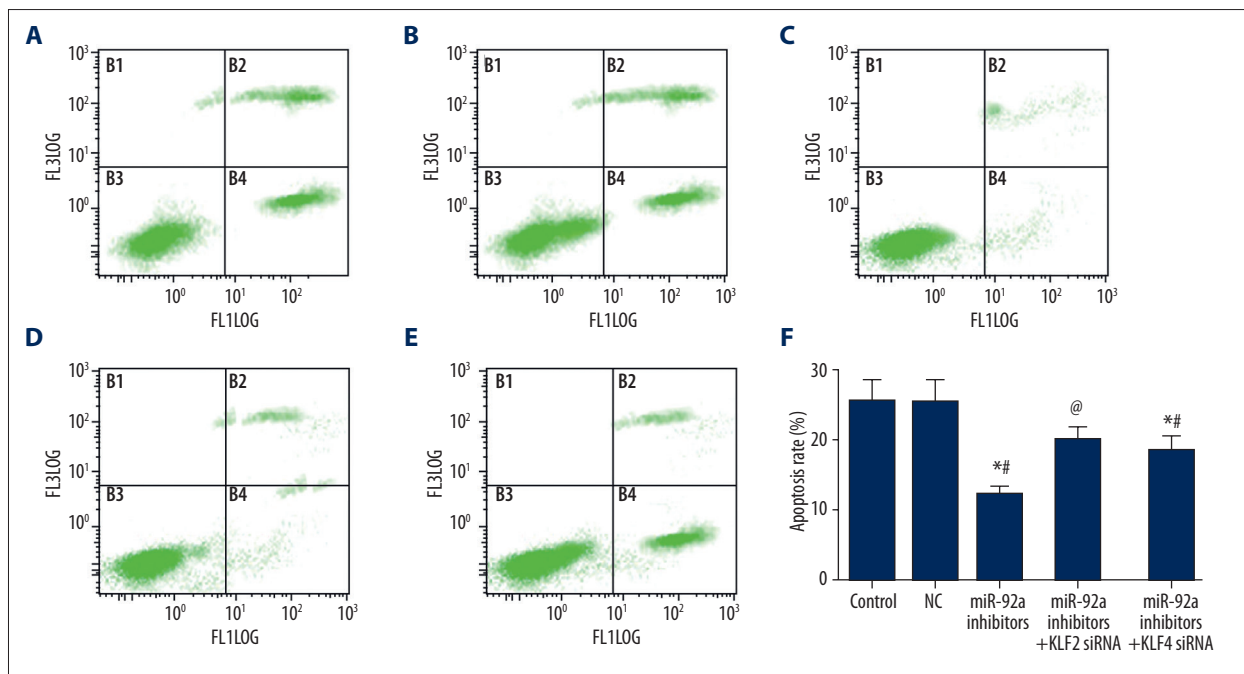
### MiR-92a regulated the expression of KLF2 and KLF4 in HUVECs

There was no significant difference in the expressions of miR-92a, KLF2, and KLF4 between the control and NC group

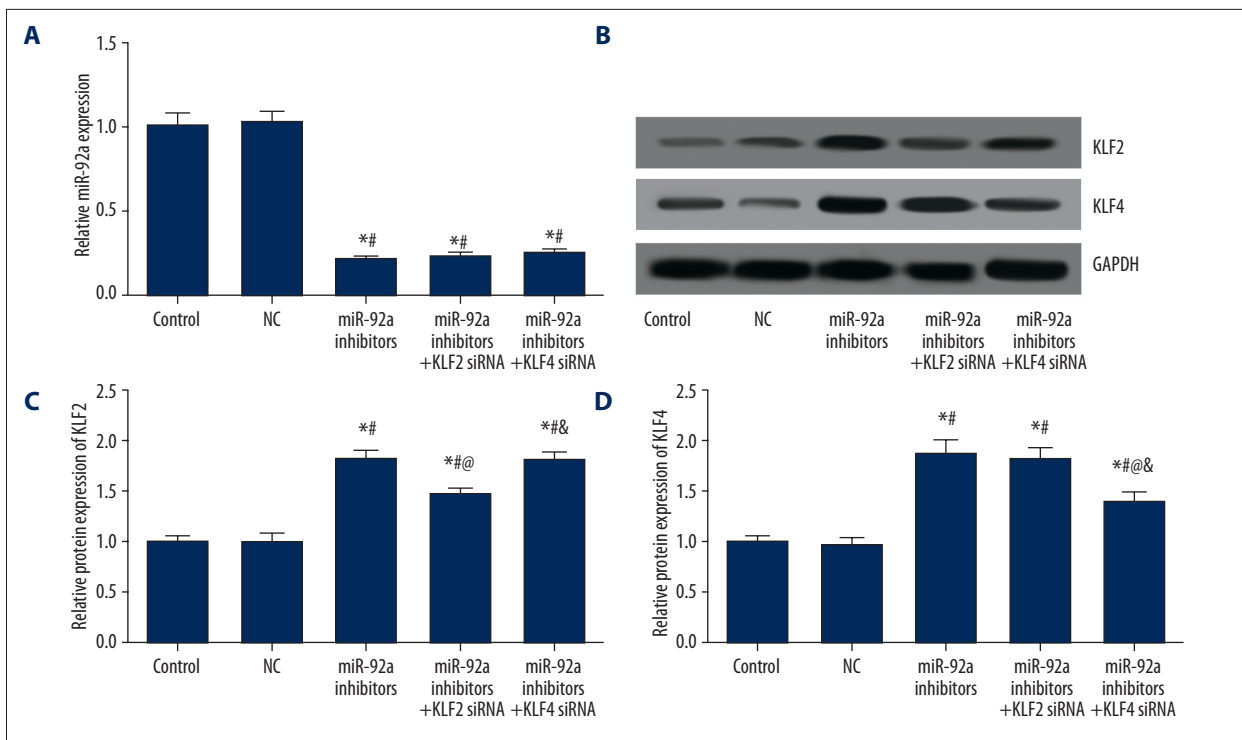
**Table 7.** Effects of miR-92a and KLF2/4 on proliferation and apoptosis of HUVEC cells.

Group	Control	NC	miR-92a inhibitors	miR-92a inhibitors + KLF2 siRNA	miR-92a inhibitors + KLF4 siRNA
Proliferation	1.00 $\pm$ 0.12	0.94 $\pm$ 0.03	1.90 $\pm$ 0.05*#	1.45 $\pm$ 0.07*#@	1.53 $\pm$ 0.11*#@
Apoptosis (%)	25.34 $\pm$ 3.22	25.27 $\pm$ 3.12	12.13 $\pm$ 1.12*#	19.87 $\pm$ 2.17@	18.23 $\pm$ 2.12*#

\*  $P < 0.05$  versus control group; #  $P < 0.05$  versus NC group; @  $P < 0.05$  versus miR-92a inhibitors group.



**Figure 8.** Apoptosis rate of endothelial cells in each group estimated by flow cytometry. Distribution of apoptotic endothelial cells in groups of control (A), NC (B), miR-92a inhibitors (C), miR-92a inhibitors + KLF2 siRNA (D), and miR-92a inhibitors + KLF4 siRNA (E). (F) Relative apoptosis rate of endothelial cells in each group. Data were presented as mean  $\pm$ SD for 3 independent experiments. \*  $P < 0.05$  versus control group, #  $P < 0.05$  versus NC group, @  $P < 0.05$  versus miR-92a inhibitors group.



**Figure 9.** Expression of miR-92a, KLF2, and KLF4 in various groups of HUVECs (i.e., control, NC, miR-92a inhibitors, miR-92a inhibitors + KLF2 siRNA and miR-92a inhibitors + KLF4 siRNA). **(A)** Quantitative data of expression level of miR-92a in HUVECs. **(B)** Western blot analysis of KLF2 and KLF4 in HUVECs with GAPDH as internal control. **(C)** Quantitative protein level of KLF2 in HUVECs. **(D)** Quantitative protein level of KLF4 in HUVECs. Data were presented as mean ±SD for 3 independent experiments. \*  $P < 0.05$  versus control group, #  $P < 0.05$  versus NC group, @  $P < 0.05$  versus miR-92a inhibitors group, &  $P < 0.05$  versus miR-92a inhibitors + KLF2 siRNA group.

**Table 8.** Effects of miR-92a inhibitors and KLF2/4 siRNA on expression of miR-92a and KLF2/4 in HUVEC cells.

Group	Control	NC	miR-92a inhibitors	miR-92a inhibitors + KLF2 siRNA	miR-92a inhibitors + LF4 siRNA
miR-92a	1.00±0.08	1.02±0.07	0.21±0.03 <sup>**</sup>	0.23±0.03 <sup>**</sup>	0.25±0.03 <sup>**</sup>
KLF2	1.00±0.07	0.99±0.09	1.82±0.09 <sup>**</sup>	1.45±0.08 <sup>**@</sup>	1.80±0.10 <sup>**&amp;</sup>
KLF4	1.00±0.07	0.97±0.08	1.87±0.15 <sup>**</sup>	1.83±0.11 <sup>**</sup>	1.39±0.11 <sup>**@&amp;</sup>

\*  $P < 0.05$  versus control group; #  $P < 0.05$  versus NC group; @  $P < 0.05$  versus miR-92a inhibitors group; &  $P < 0.05$  versus miR-92a inhibitors + KLF2 siRNA group.

( $P > 0.05$ ). Groups of miR-92a inhibitors, miR-92a inhibitors + KLF2 siRNA, and miR-92a inhibitors + KLF4 siRNA had significantly lower miR-92a expressions together with higher KLF2 and KLF4 expressions compared with groups of control and NC ( $P < 0.05$ ; Figure 9, Table 8). In addition, miR-92a inhibitors + KLF2 siRNA group had significant lower KLF2 expressions compared with groups of miR-92a inhibitors and miR-92a inhibitors + KLF4 siRNA. Besides that, miR-92a inhibitors + KLF4 siRNA group had significant lower KLF4 expressions compared with groups of miR-92a inhibitors and miR-92a inhibitors + KLF4 siRNA ( $P < 0.05$ ; Figure 9, Table 8).

## Discussion

Previous studies have noted the importance of miRNAs linked to cardiovascular diseases because miRNAs have been involved in a wide range of processes including the regulation of gene expressions [38]. Endothelial activation is an initial signal of the improvement of MI [39], and a strong relationship between miRNAs and endothelial injury has been reported in the literature [13,25,40,41]. By regulating the expression of KLF2 and KLF4 at the layer of transcriptional, posttranscriptional, and posttranslational modifications, endothelium-enriched miR-92a

mediates atherosusceptible endothelial cells [13,34]. Various long-term studies focusing on MI have reported that integrated delivery of miR-92a inhibitors alleviated MI symptoms and substantially enhanced neovascularization of ischemia-induced injury triggered by MI [25,33,40,42].

Other studies have reported the positive correlation between circulating microparticles (e.g., endothelial injury markers) and cardiovascular diseases (e.g., atherosclerosis stenosis and heart failure): the more severe the cardiovascular disorders, the more expression levels of microparticles [43,44]. Analogously, in the current study, significant increases in the expression level of cTnI, H-FABP, vWF, and miR-92a were observed in both patients in the AMI group and model rats. Besides that, declining miR-92a expression and its causative increased KLF2 level were found in the atheroprotective flow pattern, so that endothelial homeostasis could be maintained [13]. More recently, findings of Loyer et al. showed that miR-92a up-regulated with the progression of atherosclerotic lesions, and its changes were closely linked with the modulated expressions of KLF2 and KLF4 [13,25,41]. Our findings showed that the expression levels of cTnI, H-FABP, vWF, and miR-92a were significantly higher, whereas KLF2 and KLF4 expression levels were lower in patients in the AMI group and model rats, in comparison to healthy controls. Luciferase activity assay also confirmed the direct interaction between miR-92a and KLF2/4 with evidence that miR-92a transfection could significantly decrease the relative luciferase activity in HUVECs when miR-92a bound to normal 3' UTR of KLF2/4.

More importantly, our study provided evidence that the inhibition of miR-92a not only promoted endothelial activation but also enhanced cardiac cell proliferation and suppressed the apoptosis of cardiac cells after MI was triggered [25,40]. As suggested by other researchers, both KLF2 and KLF4 are involved in the protection and modulation of endothelial cells [13,45,46]. Further, KLF2 seemed to participate in modulating migration of smooth muscle cells and KLF4 appeared as a vital component in regulating invasion and apoptosis of esophageal cancer cells and chronic myeloid leukemia cells [47,48]. To sum up, it was assumed that KLF2/KLF4 expressions regulated by miR-92a were tightly linked with proliferation rate and apoptosis rate of endothelial cells. In response, we conducted a series

of experiments centering on HUVECs that were cultured in hypoxic conditions *in vitro* to simulate cell conditions induced by AMI [49–51]. We concluded that both miR-92a mimics and miR-92a inhibitors would affect proliferation and apoptosis of HUVECs by regulating the expression of KLF4 and KLF2. Moreover, the effect of miR-92a inhibitors on HUVECs can be antagonized by siRNA of KLF2/KLF4 [52,53].

Of note, this study may provide additional information for identifying new treatment targets of MI, since previous research did not cover the associations among miR92, KLF2, KLF4, and MI-related endothelial injuries. However, this study has some limitations. For instance, the research methods used in our study are not sufficient, which may lead to inconclusive or biased results. The sample size may not be adequate to provide representative results. Therefore, more research on this topic should be studied in order to ascertain the efficacy of anti-miR-92a treatment with respect to endothelial protection. Future studies may aim to discover factors other than siRNA that can enhance the effectiveness of miR-92 inhibitors.

## Conclusions

This study attested that miR-92a plays a crucial role in endothelial injury after AMI via targeting KLF2/4, which provided potential targets to alleviate clinically AMI symptoms and helped researchers better understand the mechanisms of endothelial injury. Nonetheless, we are still looking forward to further studies and more effective treatments for AMI based on our study.

## Disclosure of conflict of interest

None.

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