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Original Article

MiR-146b protects cardiomyocytes injury in myocardial ischemia/reperfusion by targeting Smad4

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Abstract: MicroRNAs, a class of small and non-encoding RNAs that transcriptionally or post-transcriptionally modulate the expression of their target genes, have been implicated as critical regulatory molecules in many cardiovascular diseases, including ischemia-/reperfusion-induced cardiac injury. In the present study, we report on the role of miR-146b in myocardial I/R injury and the underlying cardio-protective mechanism. Antagomir-146b was used to explore the effects of miR-146b on cardiac ischemia/reperfusion injury (30 min ischemia followed by 180 min reperfusion). As predicted, miR-146b overexpression significantly reduced the infarct size and cardiomyocytes apoptosis and release of creatine kinase and lactate dehydrogenase. In addition, miR-146b attenuated H9c2 cell apoptosis. Furthermore, Smad4 was predicted and verified as a potential miR-146b target using bioinformatics and luciferase assay. In summary, this study demonstrated that miR-146b plays a critical protective role in cardiac ischemic injury and may provide a new therapeutic approach for the treatment of myocardial I/R injury.

Keywords: Ischemia/reperfusion, miR-146b, Smad4, apoptosis

Introduction

Ischemic heart disease (IHD) is one of the leading causes of death worldwide. According to the WHO, 7,400,000 deaths worldwide (13.2% of all deaths) resulted from IHD in 2012. The outcomes of IHD are usually attributable to the detrimental effects of acute myocardial ischemia/reperfusion injury (IRI). Myocardial IRI results in numerous deleterious consequences such as cardiomyocyte death and myocardial infarction. Therefore, possible molecular approaches of protecting the heart against IRI must be adopted to enhance resistance to cell death and increase the repair capacity of ischemic tissues. Recent evidence suggests that molecular targets such as microRNAs (miRs) may participate in modulating the survival and recovery of cardiomyocytes during IRI [1].

miRs are hairpin-derived RNAs 20-24-nucleotide-long, which posttranscriptionally repress the expression of target genes, usually by binding to the 3'UTR of messenger RNA (mRNA) [2]. miRs are involved in virtually all aspects of cardiac biology, from the development and cell lineage specification of different cell populations within the heart to the survival of cardiomyocytes under stress conditions. As a consequence, various miRs have been recently recognized as powerful mediators and contributing factors in the pathogenesis of many cardiac disorders [3]. For example, upregulation of miR-214 expression protects cardiomyocytes from IRI by attenuating Ca^{2+} overload-induced cardiomyocyte death [4]. Modulation of miR-499 and miR-24 expression may also suppress cardiomyocyte death by preventing oxidative stress and the expression of the proapoptotic protein Bim, respectively [5, 6]. Another specific microRNA, miR146b, has recently aroused attention in various biological processes. It has been demonstrated that miR-146b may promote invasion and metastasis of osteosarcoma [7]. Nevertheless, miR-146b-5p has been shown to

function as a tumor suppressor by targeting TRAF6 and predicts the prognosis of human malignant gliomas [8]. These studies suggest that miR-146b has different behaviors in the progression of carcinogenesis. Recently, miR profiling studies have revealed the alteration of miR146b expression in patients with atrial fibrillation [9]. In our study, the expression of miR-146b was downregulated in myocardial ischemia/reperfusion (I/R) rats. Therefore, it is necessary to elucidate the possible roles of miR-146b in I/R-induced cardiac injury.

In the present study, we investigated the role of miR-146b during cardiac IRI in myocardial I/R rat and hypoxia/reoxygenation (H/R) injured cardiomyocytes. We found that miR-146b was able to attenuate myocardial infarct size and apoptotic rates during IRI by targeting Smad4 and modulating the expression of c-fos and c-JUN in the myocardium. Therefore, miR-146b may provide a new insight to develop an effective pharmacological or genetic agent aimed at the molecular target.

Materials and methods

Cell culture and transfection

Rat cardiomyocytes H9c2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Australia) supplemented with 10% fetal bovine serum (FBS, Gibco, Australia) and 1% penicillin–streptomycin solution at 37°C with 5% CO_2. H9c2 cells were plated in a 6-well plate at a concentration of 3×10^5 cells per well and transfected with scramble mimics and miR-146b mimics (GenePharma, Shanghai, China) at a final concentration of 40 nM following the manufacturer's instructions.

I/R injury model

Adult Sprague-Dawley (SD) rats (280±20 g) were randomly divided into sham group, I/R group, antagomiR control (Riobio, Guangzhou, China) treatment group and antagomiR-146b (Riobio, Guangzhou, China) treatment group. Rats were anesthetized intraperitoneally with 1% pentobarbital sodium at a dosage of 40 mg/kg. The hearts were exposed through a left thoracotomy in the fourth intercostal space following skin incision. The left anterior descending (LAD) coronary artery was ligated with a 6-0 silk suture. Visual observation (cyanosis) and continuous ECG monitoring was applied to confirm the successful establishment of the I/R injury model. After 30 min of myocardial ischemia, the coronary artery was reperfused by releasing the knot. Following reperfusion for 180 min, the rats were sacrificed and the hearts were harvested. The sham surgery animals underwent the same procedures without the occlusion of the LAD. The rats in the treatment group were given a tail vein injection of antagomiR control and antagomiR-146b (2 μg/time) 24 h and 12 h before the left coronary artery ligation.

H/R of H9c2 cardiomyocytes

Forty-eight hours after transfection of the miR-146b mimics and scramble mimics, H9c2 cells were maintained in medium containing 1% FBS (low serum medium) followed by exposure to hypoxia (94% N_2, 5% CO_2 and 1% O_2) for 6 h. Subsequently, the medium was removed and maintained in 10% FBS-containing medium in an environment with 95% air and 5% CO_2 for reoxygenation for 12 h. Cells under normoxic conditions served as a control.

Infarct size determination

At the end of the reperfusion, the rat LAD coronary artery was re-occluded, and 3 mL 1% (wt/vol) Evans blue dye solution (Sigma, MO, USA) was injected into the left ventricle. The hearts were harvested and refrigerated at -20°C for 30 min, and then the left ventricle was transversely cut into 1 mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TCC, Sigma, MO, USA) at 37°C for 30 min to distinguish the ischemic and infarcted tissue. Following staining, the slices were fixed in 4% paraformaldehyde solution. The non-infarcted areas with blue staining were designated as viable, and the infarcted areas without staining were designated as non-viable. Finally, after the areas of the ventricle were weighed separately, the AAR and infarct size were calculated, and the infarct size was expressed as a percentage of the AAR [10].

Lactate dehydrogenase (LDH) and creatine kinase (CK) measurement

The blood samples were centrifuged at 1000 r/min, 4°C for 15 min, and the serum was extracted and stored at -80°C for the subsequent analysis. The LDH and CK activities in the

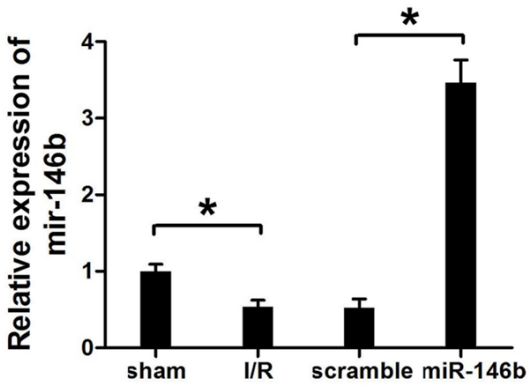

Figure 1. The expression of miR-146b was significantly downregulated in I/R myocardial tissue. qPCR was used to detect the expression of miR-146b in myocardial of rats subjected to ischemic/reperfusion. The data were presented as mean ± SD; the significance was indicated as *P<0.05.

serum were measured using commercial kits (Jiancheng, Nanjing, China) following the instruction of the manufacturers.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from heart tissues using the TRIzol reagent (Invitrogen, CA, USA). Then, 1 μg of RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase with oligo-dTs. Real-time quantitative PCR was performed on a IQ50 realtime system (Biorad, CA, USA) using a SYBR Green Master Mix (TIANGEN, Beijing, China) in accordance with the manufacturer's instructions. MicroRNA-146b levels were quantified with the $2^{(-\Delta\Delta ct)}$ method, which was normalized to the snRNA U6. All of the samples were analyzed in triplicate.

Western blot assays

Total proteins were extracted with RIPA lysis buffer containing protease inhibitors (Sigma, MO, USA). The protein concentrations were examined using the BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). The protein lysates were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore, MA, USA). After blocking in 5% milk at room temperature for 2 h, the membranes were incubated with primary antibodies, including mouse anti-SMAD4, anti-c-fos, anti-c-jun and anti-GAPDH antibodies (Abcom, Cambridge, England) overnight followed by incubation with secondary anti-IgG HRP-conjugated antibodies for 2 h. Finally, the protein bands were visualized using the enhanced chemiluminescence (ECL) reagent (Thermo Fisher, MA, USA). GAPDH was used as an internal control.

Flow cytometry

Myocardial apoptosis was assessed with the Annexin V-FITC and propidium iodide (PI) apoptosis kit (BD, NJ, USA) according to the manufacturer's instructions. After dual-staining with Annexin V-FITC and PI, the apoptotic cells were immediately analyzed and quantified by flow cytometry (BD, NJ, USA).

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL)

The hearts were fixed in 10% paraformaldehyde, embedded in paraffin, and cut into 4-μm thickness sections. Apoptosis of the heart tissues was performed by TUNEL assay with an in situ cell death detection kit (Roche, IN, USA) according to the instructions of the manufacturer. After TUNEL staining, the sections were incubated with DAPI solution to stain nuclei for 5 min. The apoptotic nuclei were stained green and the normal nuclei were stained blue.

Luciferase assay

The H9c2 cells were co-transfected with miR-146b mimics with reporter gene containing wild-type or mutated 3'UTR sequence of SMAD4. After transfection for 48 h, the cells were harvested, and the luciferase intensity was measured using a Dual Luciferase Reporter Gene Assay kit according to the manufacturer's instructions (Promega, USA). The renilla luciferase intensity served as a control to normalize the firefly luciferase intensity.

Statistical analysis

The data are expressed as mean ± SEM. The significance of the results was analyzed using a student's t-test. A value of $P<0.05$ was considered as a significant difference.

Results

MiR-146b was downregulated in myocardial of I/R rats

To investigate the potential role of miR-146b involved in I/R injury, quantitative real-time PCR was used to detect the expression levels of miR-146b in myocardial I/R rats. The miR-

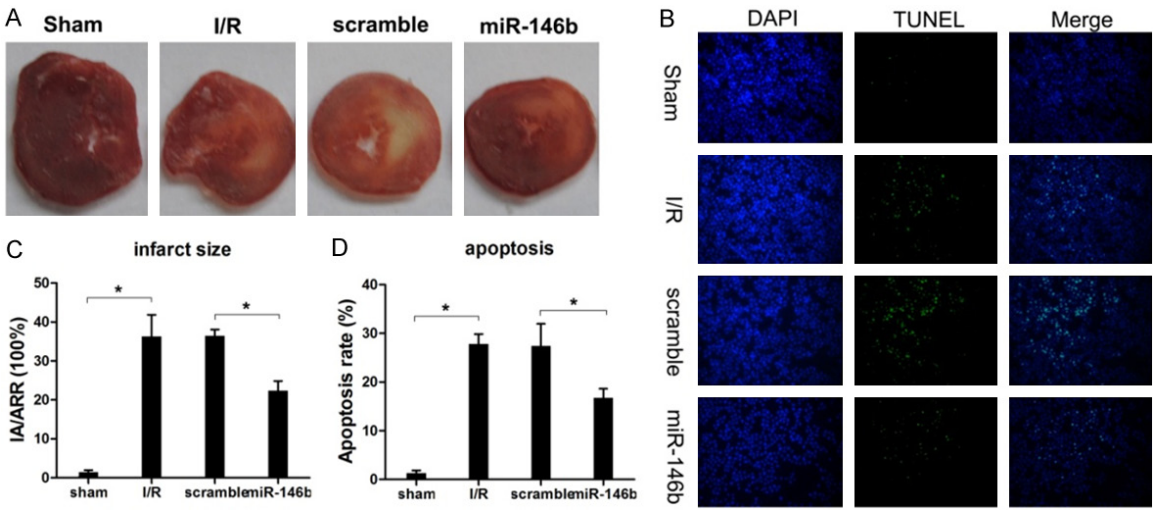

Figure 2. miR-146b decreased myocardial infarction and apoptosis in ischemia/reperfusion rats. A. The infarct size was evaluated using Evans Blue and TTC staining. Dark blue area: nonischemic tissue; red-stained area: area at risk; white area: infarcted tissue. B. TUNEL assay was performed to detect the myocardial apoptosis. Apoptotic nuclei were stained green, and normal nuclei were stained blue. C. The infarct size normalized to the area at risk. AAR: area at risk; IA: infarct area. D. The apoptosis rate of myocardial in each group. The data were presented as mean ± SD, the significance was indicated as *P<0.05.

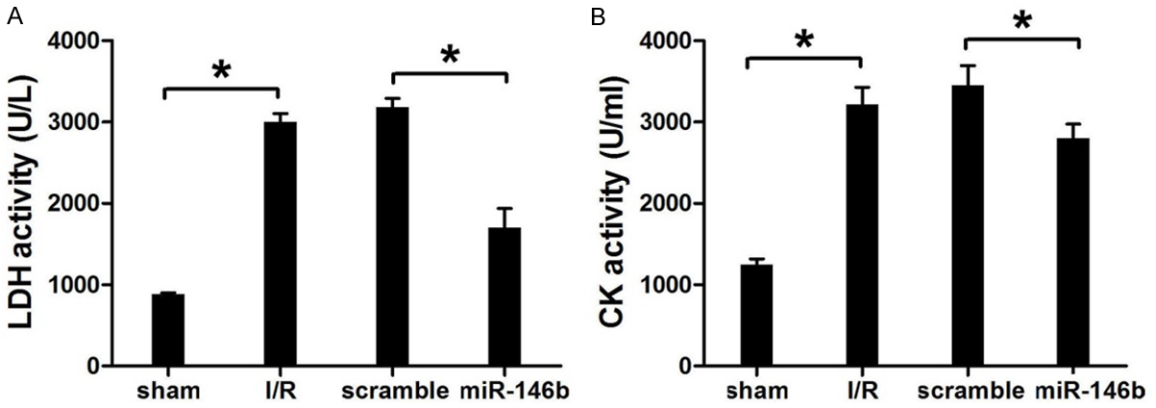

Figure 3. Effects of miR-146b on serum creatine kinase and lactate dehydrogenase level activity after IR injury in rats. A. The activity of serum creatine kinase in each of the groups. B. The activity of serum creatine kinase in each of the groups. The data were presented as mean ± SD, and the significance was designated as *P<0.05.

146b was downregulated in myocardial of I/R rats. The treatment of antagomir-146b elevated the expression of miR-146b by more than 3-fold (**Figure 1**).

miR-146b decreased myocardial infarction and apoptosis in ischemia/reperfusion rats and suppressed the activity of LDH and CK

The infarct area of hearts was measured using TTC and Trypan blue staining. Histomorphometric analysis showed the IA/AAR (ischemic area/area at risk) ratio was significantly increased in the I/R group compared to the sham group, which indicated the success of the I/R model establishment (**Figure 2A**). Treatment of antagomir-146b significantly reduced the IA/AAR ratio in comparison with the antago-scramble group (**Figure 2C**). To further investigate the protective effect of miR-146b in myocardial ischemia/reperfusion, a TUNEL assay was applied to determine the myocardial apoptosis (**Figure 2B** and **2D**). In agreement, treatment of antagomir-146b significantly reduced the myocardial apoptosis compared to the I/R and antago-scramble group. In addition, as **Figure 3** showed, treatment of antagomir-146b significantly

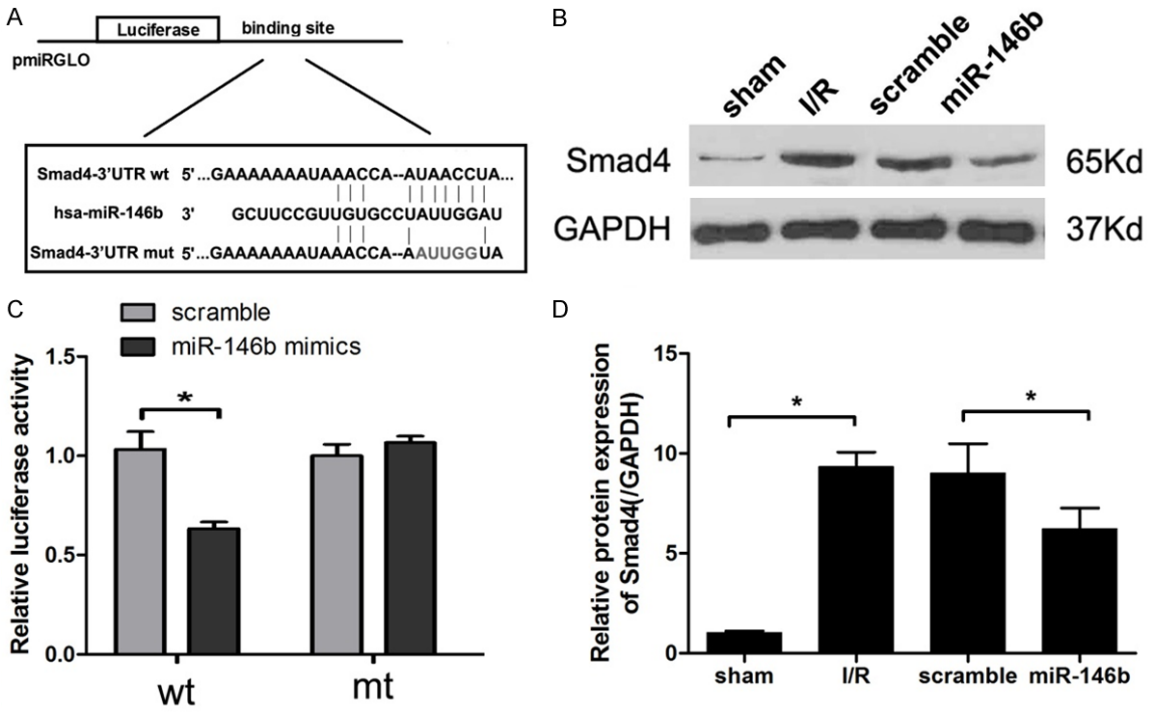

Figure 4. MiR-146b direct target Smad4. A. Sequence alignment between miR-146b and the 3'UTRs of Smad4 of rat. B, D. Western blot was used to investigate the expression of Smad4 in each group. C. A luciferase reporter assay was performed in H9c2 cells using the pGL3 reporter vector fused with either Smad4 wild-type 3'-UTR or Smad4 3'-UTR mutant. MiR-145 significantly decreased the activity of luciferase gene fused with Smad4 wild-type 3'-UTR but had no effect on the activity of luciferase fused with Smad4 3'-UTR mutant. The data are presented as mean ± SD, and significance is designated as *P<0.05.

suppressed the activity of LDH and CK compared to the sham group.

MiR-146b directly target SMAD4 in H9c2 cells

Luciferase activity assay was performed to illustrate the target of miR-146b. We cloned the 3'-UTR of SMAD4 downstream of a luciferase gene and mutated the predicted binding site of miR-146b in the 3'UTR of SMAD4 as a mutative plasmid (**Figure 4A**). As **Figure 4C** shows, overexpression of miR-146b significantly decreased the luciferase activity whereas transfection with the miR scramble did not. Western blot analysis also demonstrated that the miR-146 was able to regulate SMAD4 protein expression (**Figure 4B** and **4D**). These results suggest that SMAD4 is a direct target of miR-146b.

MiR-146b attenuated the apoptosis of H9c2 cells and the expression of c-Fos and c-Jun

Regarding the protective effect of miR-146b in I/R rats, we wondered whether miR-146b protected cardiomyocyte against SI/R-induced H9c2 cell apoptosis. As expected, transfection with miR-146b mimics decreased the apoptosis rate in the H9c2 cells after 6 h hypoxia and 12 h reoxygenation (H/R) (**Figure 5A**, **5C**). In addition, we investigated the expression of apoptosis relative protein c-fos and C-JUN. Interestingly, the expression of the two proteins was downregulated in the miR-146b mimics treatment group (**Figure 5B**, **5D** and **5E**).

Discussion

miRNAs regulate the expression of approximately one-third of the human genes. Studies have increasingly revealed the potential of miRNAs as diagnostic or prognostic biomarkers and therapeutic targets in the treatment of human disease [11, 12]. IHD is associated with the deregulation of multiple miRs that have significant effects on the regulation of ischemic injury and apoptosis of myocardiocytes following I/R injury [13-16]. For instance, upregulation of microRNA-135a protects against myocardial ischemia/reperfusion injury by decreas-

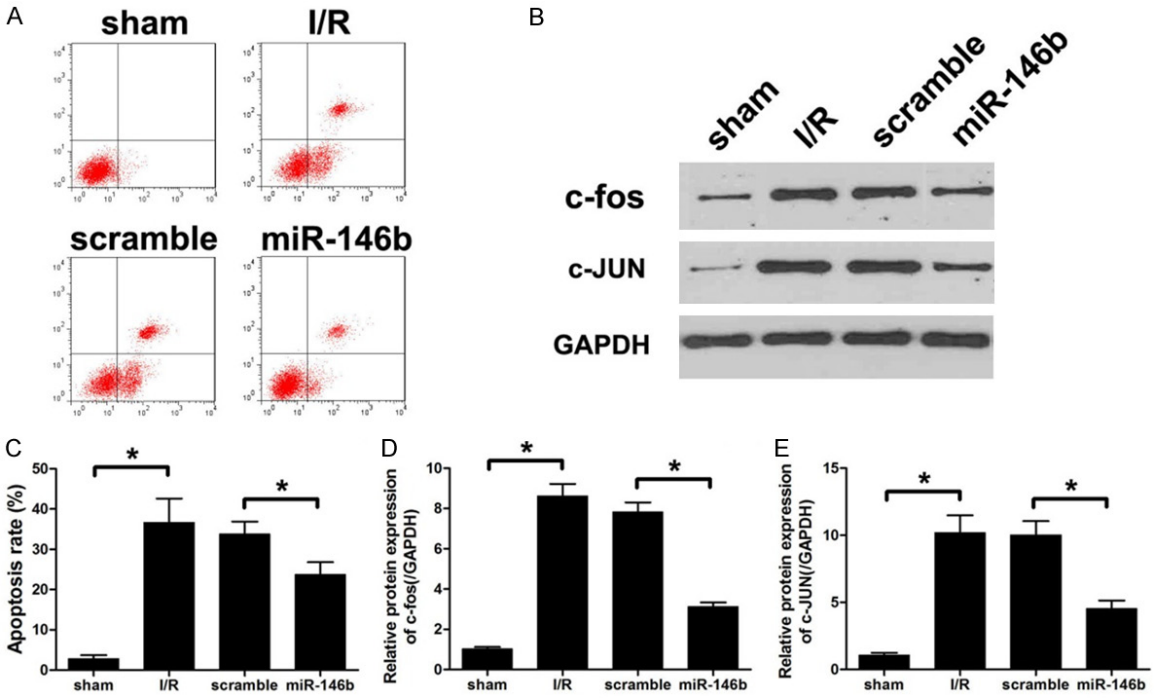

Figure 5. (A) H9c2 cell apoptosis was evaluated by flow cytometry. (B) Expression of c-Jun and c-Fos in each group. (C) A histogram of apoptosis rate in each group shown in (A). (D) Expression of c-Fos. (E) Expression of c-Jun. The data are presented as mean ± SD, and the significance was designated as *P<0.05.

ing TXNIP expression in diabetic mice [17]. MiR-29a and Let7 attenuated apoptosis by regulating IGF-1 after myocardial ischemia-reperfusion [18]. MicroRNA-141 regulates the expression level of ICAM-1 on endothelium to decrease myocardial ischemia-reperfusion injury [19]. Accordingly, it is reasonable to investigate the potential role of miRs during an episode of ischemic injury.

Previous reports have showed that miR-146b-5p may function as a tumor suppressor or promoter in different tumors [20-25]. Moreover, the miR-146 family, comprised of miR-146a and miR-146b, has been implicated in the regulation of immune cell signaling [26]. However, the role of miR-146b in myocardial ischemic injury remains limited. Li JW et al. demonstrated that MicroRNA-146b inhibition augments hypoxia-induced cardiomyocyte apoptosis, which indicated the potential protective effect of miR-146b in ischemia injury.

In the present study, we investigated the expression of mir-146b in ischemia/reperfusion of rat heart by using quantitative real-time RT-PCR analysis. The collection of mir-23a was downregulated significantly in the I/R group compared to the sham group. Thus, we treated the rats with antagomir-146b 24 h before surgery to determine whether miR-146b has a protective effect on ischemia injury. As expected, antagomir-146b treatment significantly promoted the expression of miR-146b in vivo and reduced the infarct area size.

It is well known that cardiomyocyte apoptosis contributes critically to cardiac I/R injury [27, 28]. In addition to working as a physiological regulator of cell density, apoptotic cell death is deemed to cause pathogenesis of diseases such as myocardial infarction. Accumulating investigation indicates that miRNAs play a critical role in apoptotic regulating not only in tumor cells but also in heart cells [29]. We observed that overexpression of mir-146b using antagomir-146b significantly suppressed I/R-induced myocardial apoptosis, which indicates that miR-146b exerts anti-apoptotic properties.

miRNAs modulate their biological functions via their multiple target gene mRNAs. In the current study, computational analysis suggested that SMAD4 may be a target of miR-146b. Moreover, it has been reported that miR-146b-5p regulates signal transduction by targeting

SMAD4 in thyroid cancer [21]. Luciferase activity assay verified our prediction that miR-146b directly targeted SMAD4 in H9c2 cells.

Smad4 is a pivotal transducer of signaling through the TGF-pathway. Smad proteins activated by receptors for TGF-β form complexes with Smad4. These complexes are translocated into the nucleus and regulate ligand induced gene transcription [30, 31]. It has previously been reported that mutations or deletions in the Smad4 gene correlate with the increased invasiveness or metastatic ability of various tumors [32, 33]. Smad4 overexpression increased apoptosis in bladder cancer cells, which indicated the potential of Smad4 for inducing apoptosis. It has been reported that Smad4 may act together with c-Jun and c-Fos to activate transcription in response to TGF-β. We detected the expression of c-Jun and c-Fos; interestingly, expression of the two proteins were downregulated by miR-146b. This mechanism of transcriptional activation by TGF-β, through functional and physical interactions between Smad4 and c-Jun-c-Fos, shows that Smad signaling and MAPK/JUN signaling converge at AP1-bingding promoter sites.

Disclosure of conflict of interest

None.

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