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MiR-145-5p alleviates hypoxia/reoxygenation-induced cardiac microvascular endothelial cell injury in coronary heart disease by inhibiting Smad4 expression

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Abstract. – OBJECTIVE: To investigate the effects and mechanism of miR-145-5p on hypoxia/reoxygenation (H/R)-induced cardiac microvascular endothelial cell (CMEC) injury in coronary heart disease (CHD).

PATIENTS AND METHODS: Patients with CHD (n=96) and healthy volunteers (n=96) were enrolled, and H/R injury model of CMECs was established. The expression of miR-145-5p and mothers against decapentaplegic homolog 4 (Smad4) mRNA in cells was quantified with reverse transcription polymerase chain reaction (RT-PCR). Then, miR-145-5p mimics and Smad4 inhibitor were transfected into CMECs. Cell counting kit-8 (CCK-8) was employed for proliferation detection, flow cytometry for apoptosis detection, and Western Blot for measuring the expression of apoptosis-related proteins and Smad4 protein.

RESULTS: The expression of serum miR-145-5p in patients with CHD was significantly lower than that in healthy individuals. The area under the curve (AUC) of miR-145-5p in diagnosing CHD was 0.894, and the expression of miR-145-5p was negatively correlated with that of Smad4 ($p<0.05$). Over-expression of miR-145-5p promoted the proliferation, inhibited the apoptosis, and reduced inflammatory responses and oxidative stress in H/R-injured CMECs. Moreover, miR-145-5p might negatively regulate the expression of Smad4 in CMECs. Dual-Luciferase reporter assay determined the targeting relation between miR-145-5p and Smad4.

CONCLUSIONS: MiR-145-5p is lowly expressed in patients with CHD, and its over-expression effectively alleviates H/R-induced CMEC injury by inhibiting Smad4.

Key Words:
MiR-145-5p, Smad4, Coronary heart disease, Cardiac microvascular endothelial cell injury.

Introduction

Coronary heart disease (CHD) is a common disease with increasing prevalence and younger trend in age following the changes of social environment and living habits[1,2], and it is also one of the leading causes of disease-induced death at present[3]. The primary pathology of CHD is reported to be endothelial cell dysfunction[4]. Cardiac microvascular endothelial cells (CMECs) are closely related to cardiovascular survival and maintain cardiac function by regulating heart metabolism and contractility; moreover, CMECs are injured by reperfusion of blood flow earlier than cardiomyocytes both temporally and spatially[5,6]. Therefore, it is of great significance to find out the mechanism to reduce hypoxia/reoxygenation (H/R) injury in CMECs.

MicroRNA (miRNA) is a single-stranded non-coding RNA with a length of approximately 21-23 nucleotides. Several miRNAs[7] are found to be closely related to cardiovascular diseases and exert regulatory effects on the growth of the heart. Consistently, Yang et al[8] reported that miR-126 attenuates H/R-induced CMEC injury. MiR-145-5p is a miRNA that plays a vital role in breast cancer[9] and bladder cancer[10]. In addition, it is capable of regulating inflammatory response and apoptosis of cardiomyocytes[11]. Moreover, miR-145-5p, which is markedly suppressed after myocardial ischemia-reperfusion injury, is believed to play a key regulatory role in the proliferation of vascular smooth muscle cells[12]. However, the effect and mechanism of miR-145-5p on CMECs still remains unknown. In this study, we determine the targeting relation between miR-145-5p and

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Smad4 through bioinformatics prediction. Smad4 has also been proved to play an important role in cardiomyocyte apoptosis[13].

Therefore, we speculate that miR-145-5p alleviates H/R injury in CMECs by regulating the expression of Smad4.

Patients and Methods

General Data

Ninety-six patients with CHD (51 males and 45 females) who were admitted to our hospital from January 2016 to January 2019 and average aged (55.12 ± 5.37) years old were included as study group. Another 96 healthy volunteers receiving physical examination during the same period were selected as control group. Exclusion criteria: patients diagnosed with CHD by coronary angiography. Exclusion criteria: patients undergoing coronary stent surgery; patients with malignant tumors, severe liver and kidney dysfunction, peripheral vascular diseases, or serious infectious diseases. All patients and their families agreed to participate in the research and signed the informed consent form. The investigation was approved by the Jiaozhou Central Hospital of Qingdao Ethics Committee.

Reagents and Materials

Rat CMECs (Institutes of Biomedical Sciences, Fudan University, Shanghai); monoclonal antibodies against Bax, Bcl-2, Caspase-3, and Smad4 (Cell Signaling Technology, Danvers, MA, USA); rabbit anti-human β-actin monoclonal antibody (Proteintech Group, Inc, Wuhan, China); bicinchoninic acid (BCA) protein assay kit (Well Biotechnology Co., Ltd., Shanghai, China); quantitative real-time polymerase chain reaction (qRT-PCR) instrument (BioRad, Irvin, CA, USA); Cytoflex LX flow cytometer (Beckman, Brea, CA, USA); Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA); fetal bovine serum (FBS) and trypsin (Hyclone, South Logan, UT, USA); Cell Counting Kit-8 (CCK-8; Sigma-Aldrich, St. Louis, MO, USA); TRIzol (Invitrogen, Carlsbad, CA, USA); qPCR and reverse transcription kits (TransGen Biotech, Beijing, China); Dual-Luciferase reporter gene assay kit (Solarbio, Beijing, China); primers of miR-145-5p, miRNA NC, internal references U6 and β-actin (GenePharma, Shanghai, China); Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis kit (KeyGEN Biotech Co., Ltd., Jiangsu, China).

Cell Culture and Modeling

CMECs were cultured in an incubator with DMEM containing 10% FBS at 37°C and 5% CO_2. When reaching 80% confluence, the cells were collected and allocated into blank control group, H/R group, miR-145-5p-mimics group, miR-NC group, Si-Smad4 group, and Si-NC group. Cells in blank control group were normally cultured, while others were cultured in a hypoxia incubator with serum-free and sugar-free medium at 37°C and 94% N_2 and 5% CO_2. After 24 h, the cells were transferred to a medium containing 10% FBS and incubated for 3 h at 37°C and 5% CO_2 to establish H/R models. Afterwards, the cells were assigned into blank group (normal CMECs), H/R group (untransfected injured CMECs), negative control group (injured CMECs transfected with miR-NC), miR-145-5p-mimics group (injured CMECs transfected miR-145-5p-mimics), Si-Smad4 group (injured CMECs transfected with Si-Smad4). The cells were transfected with Lipofectamine 2000 in accordance with the instruction kit.

Serum Samples and Reverse Transcription PCR (RT-PCR)

Fasting venous blood (5 mL) extracted from all patients on the next morning after admission were centrifuged at 1500 xg for 10 min. The sera were collected, and the cells were prepared into suspension. The total RNA in sera was extracted with TRIzol, and the purity and concentration were measured using an ultraviolet spectrophotometer. Then, 5 μg of total RNA taken from each group was reverse transcribed into cDNA. Reaction parameters: 37°C for 15 min, 42°C for 35 min, and 70°C for 5 min. Then, PCR amplification was carried out under the following conditions: pre-denaturation at 94°C for 45 s, denaturation at 94°C for 10 s, annealing and extension at 60°C for 45 s, for a total of 40 cycles. U6 was served as the internal reference of miR-216a-5p, and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as the internal reference of Smad4. $2^{-\Delta\Delta ct}$ was used to analyze the data. The primer sequences were shown in Table I.

Western Blot

The cultured cells were lysed with radio immunoprecipitation assay (RIPA) to obtain the total protein. The protein concentration measured with the bicinchoninic acid (BCA) was adjusted to 4 μg/μL. After separation with 10% of sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to a polyvinylidene difluoride (PVDF) membra-

Table I. Primer sequences.

Factor	Upstream sequence	Downstream sequence
miR-145-5p	5'-CAGTCTTGTCCAGTTTTCCCAG-3'	5'-TATGCTTGTTCTCGTCTCTGTGTGTC-3'
U6	5'-TGGAACGCTTCACGAATTTGCG-3'	5'-AGACTGCCGCCTGGTAGTTGT-3'
Smad4	5'-GGACCGGATTACCCAAGACACA-3'	5'-CTGCAATCGGCATGGTATGAAG-3
GAPDH	5'-GCATTGCCCTCAACGACCAC-3'	5'-CCACCACCCTGTTGCTGTAG-3'

ne, stained with Ponceau S staining solution, immersed in Phosphate-Buffered Saline with Tween 20 (PBST) for 5min. Afterwards, the membrane was washed, blocked with 5% skimmed milk powder for 2 h, and finally incubated overnight at 4°C with primary antibodies against Bax (1: 500), Bcl-2 (1: 500), Smad4 (1: 500), and β-Actin (1: 500). Subsequently, the membrane was washed again to remove the primary antibodies, and horseradish peroxidase(HRP)-labeled goat anti-rabbit secondary antibody (1: 1000) was added for a 1h incubation at 37°C. Next, the membrane was rinsed 3 times with Tris-Buffered Saline with Tween-20 (TBST) for 5 min each time. Excess liquid on the membrane was absorbed with a filter paper. The protein bands were developed in a dark room using the enhanced chemiluminescence (ECL) reagent.

CCK-8

Cell suspensions from each group were diluted and inoculated into 96-well plates at 1×10^3cells/100 μL/well. Three parallel wells were set for each group. The plate was cultured at 37°C and 5% CO_2, and each well was added with 10 μL of CCK-8 solution and inoculated for 4 h. The optical density (OD) value was measured at a wavelength of 450 nm, which indicated the proliferation of cells.

Flow Cytometry

The transfected cells digested with 0.25% trypsin were washed twice with PBS, and prepared into $1*10^6$/mL suspension with 100 μL of binding buffer. AnnexinV-FITC and PI were added sequentially, and the cells were incubated in the dark at room temperature for 5 min to detect the apoptosis with a FC500MCL flow cytometer. The experiment was repeated for 3 times to take the average value.

Detection of Inflammatory and Oxidative Stress Related Factors

The contents of interleukin-6 (IL-6) and IL-10 in cells were detected by enzyme-linked immunosorbent assay (ELISA). Superoxide dismutase (SOD) and myocardial malondialdehyde (MDA) were measured by hydroxylamine method and thiobarbituric acid (TBA) method, respectively. All operations were carried out in strict accordance with the kit instructions.

Dual-Luciferase Reporter Assay

TargetScan 7.2 predicted the downstream target genes of miR-145-5p. Smad4-3' untranslated region (3'UTR) wild type (Wt), Smad4-3'UTR mutant (Mut), miR-145-5p-mimics, and miR-NC were transferred into CMECs with a Lipofectamine™ 2000 kit, and Luciferase activity was measured by a Dual-Luciferase reporter gene assay kit (Promega, Madison, WI, USA) 48 h after transfection.

Statistical Analysis

Statistical Product and Service Solution 19.0 (IBM, Armonk, NY, USA) was used to carry out statistical analysis, and GraphPad 7 (La Jolla, CA, USA) was used for building graphs. Inter-group comparison was conducted with independent samples *t*-test, multi-group comparison with one-way analysis of variance (ANOVA), and post-hoc pairwise comparison with LSD-*t*-test. A value of $p<0.05$ was considered statistically significant.

Results

General Data

There was no significant difference in sex, age, and body mass index (BMI) between the two groups ($p>0.05$), indicating a comparability (Table II).

Expression of Serum miR-145-5p and Smad4 in Patients with CHD

RT-PCR showed that compared with control group, the expression of serum miR-145-5p in study group was significantly down-regulated ($p<0.05$), and Smad4 mRNA was significantly up-regulated ($p<0.05$). Correlation analysis found that the expression of serum miR-145-5p

Table II. General data.

Factor	Study group (n=96)	Control group (n=96)	t/x^2	p
Sex			0.021	0.885
Male	51 (53.13)	50 (52.08)		
Female	45 (46.88)	46 (47.92)		
Age (years)			0.083	0.773
≤55	49 (51.04)	51 (53.13)		
>55	47 (48.96)	45 (46.88)		
BMI (kg/m^2)	23.97±2.15	23.82±2.06	0.622	0.494
Smoking history			0.021	0.884
Yes	39 (40.63)	40 (41.67)		
No	57 (59.38)	56 (58.33)		
Drinking history			0.022	0.881
Yes	61 (63.54)	60 (62.50)		
No	35 (36.46)	36 (37.50)		
History of diabetes			0.027	0.870
Yes	26 (27.08)	25 (26.04)		
No	70 (72.92)	71 (73.96)		
History of hypertension			0.085	0.771
Yes	55 (57.29)	53 (55.21)		
No	41 (42.71)	43 (44.79)		
Triglyceride (mmol/L)	2.03±0.31	1.97±0.29	1.385	0.168
Total cholesterol (TC) (mmol/L)	4.58±0.45	4.49±0.41	1.449	0.149

was negatively correlated with that of Smad4 in patients with CHD (r=-0.777, $p<0.001$). The receiver operating characteristic (ROC) curve demonstrated that the area under the curve (AUC) of miR-145-5p in diagnosing CHD was 0.894 (Figure 1).

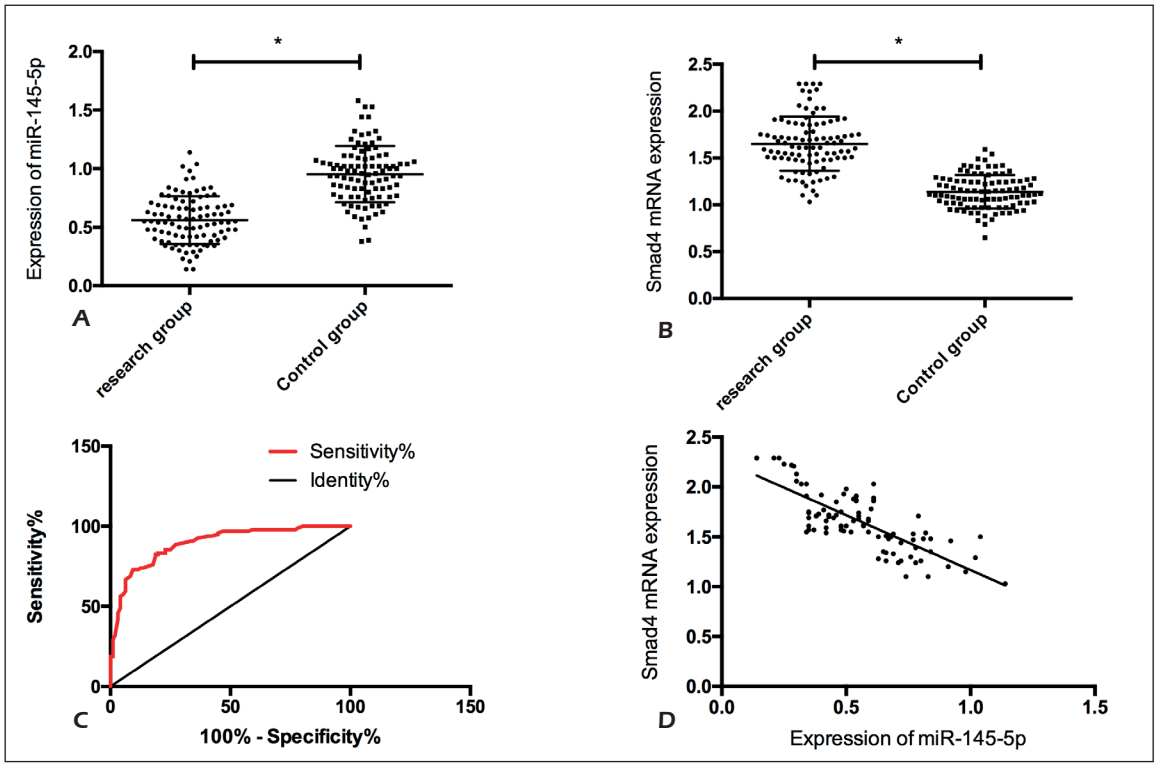

Figure 1. Expression of Serum miR-145-5p and Smad4 in Patients with CHD. **A**, Expression of serum miR-145-5p in patients with CHD. **B**, Expression of serum Smad4 in patients with CHD. **C**, ROC of miR-145-5p in diagnosis of CHD. D, Correlation analysis between miR-145-5p and Smad4, *indicated $p<0.05$.

Effects of MiR-145-5p on Injured CMECs

Models of H/R-induced CMEC injury were established to assess the effects of miR-145-5p. The injured CMECs showed significantly lower miR-145-5p expression than normal ones ($p<0.05$). Next, we transfected miR-145-5p-mimics into injured CMECs, and the results showed that compared with blank group, the proliferation of injured CMECs in H/R group and miR-NC group was significantly inhibited ($p<0.05$), the apoptotic rate was significantly increased ($p<0.05$), and the pro-apoptotic proteins (Bax and Caspase-3) were significantly up-regulated, anti-apoptotic protein (Bcl-2) was significantly down-regulated ($p<0.05$). Compared with H/R group and miR-NC group, the proliferation in miR-145-5p-mimics group was significantly increased ($p<0.05$), the apoptotic rate was significantly reduced ($p<0.05$), Bax and Caspase-3 were significantly down-regulated, and Bcl-2 was significantly up-regulated ($p<0.05$; Figure 2).

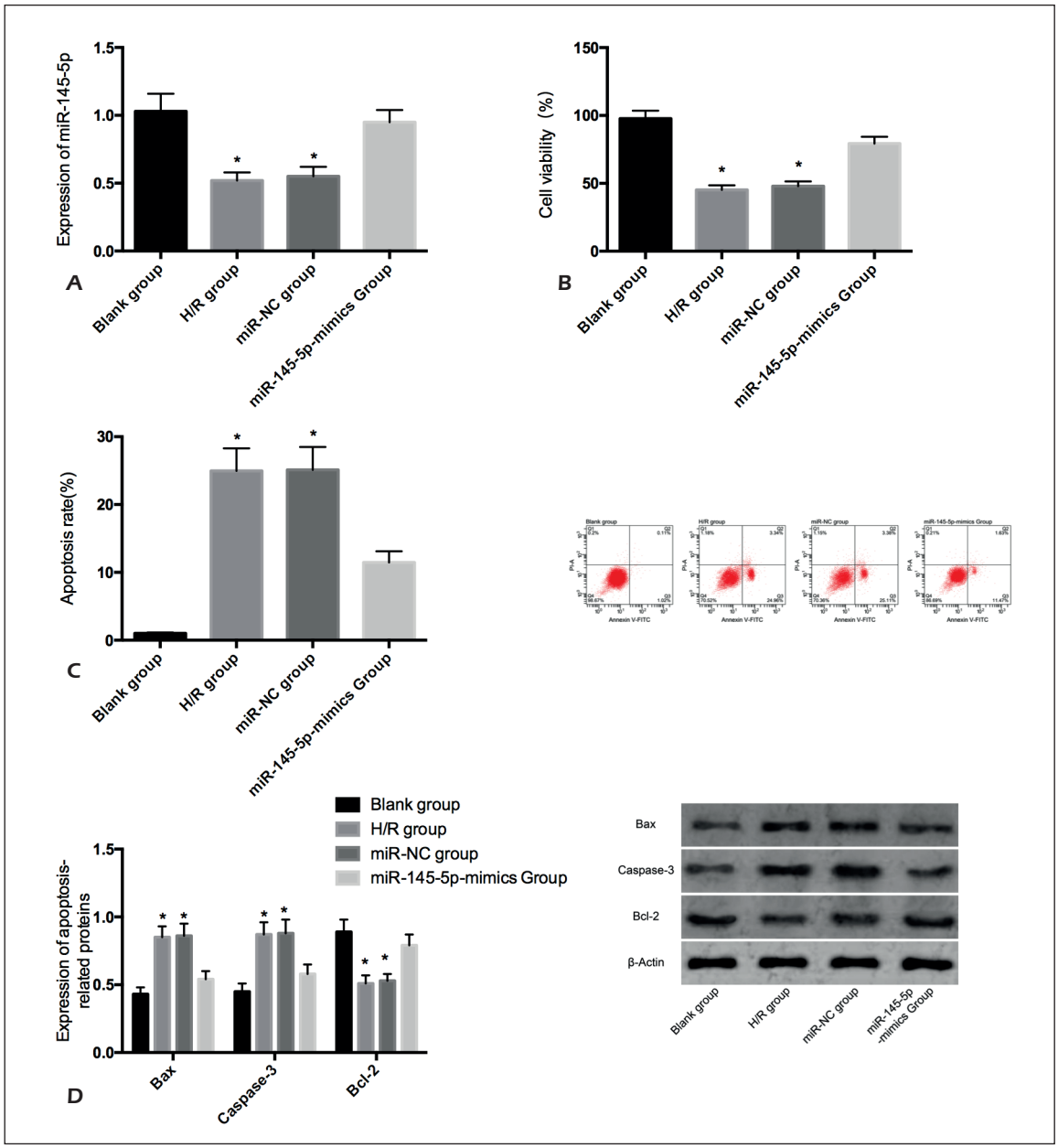

Figure 2. Effects of miR-145-5p on injured CMEC. **A**, Expression of miR-145-5p in CMECs. **B**, Effects of miR-145-5p on the proliferation of injured CMECs. **C**, Effects of miR-145-5p on apoptotic rate of injured CMECs. **D**, Effects of miR-145-5p on apoptosis-related proteins in injured CMECs, *indicated $p<0.05$.

Effects of Smad4 on Injured CMECs

The expression of Smad4 in injured CMECs was significantly higher than that in normal ones (p<0.05). We transfected Si-Smad4 into injured CMECs to observe the effects of Smad4. Compared with blank group, the proliferation of injured CMECs in H/R group and miR-NC group was significantly inhibited (p<0.05), the apoptotic rate was significantly increased (p<0.05), the pro-apoptotic proteins (Bax and Caspase-3) were significantly up-regulated, and the anti-apoptotic protein (Bcl-2) was significantly down-regulated (p<0.05). Compared with H/R group and miR-NC group, the proliferation in Smad4 group was significantly increased (p<0.05), the apoptotic rate was significantly reduced (p<0.05), Bax and Caspase-3 were significantly down-regulated, and Bcl-2 was significantly up-regulated (p<0.05; Figure 3).

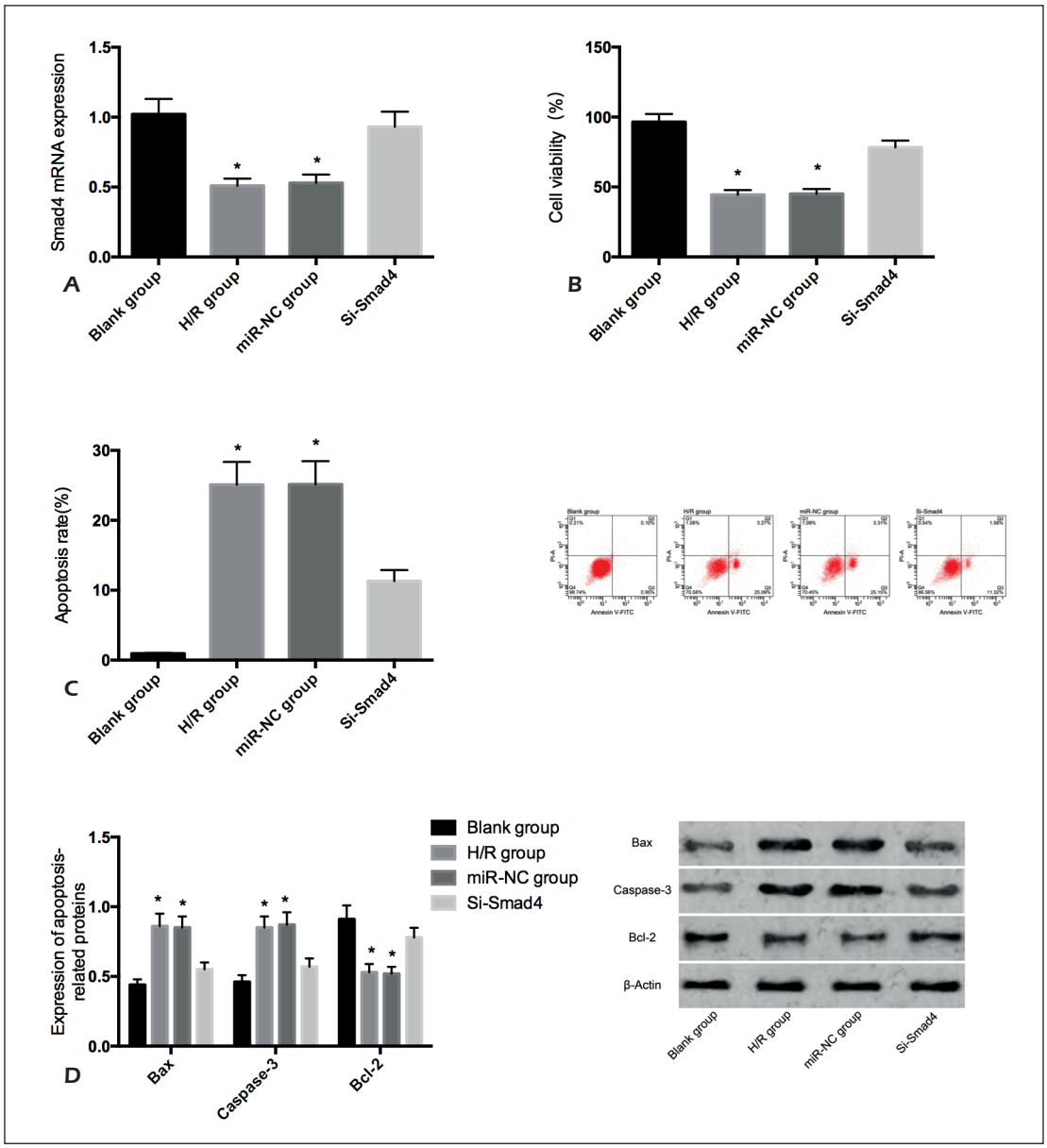

Figure 3. Effects of Smad4 on injured CMECs. **A**, Expression of Smad4 in CMECs. **B**, Effects of Smad4 on proliferation of injured CMECs. **C**, Effects of Smad4 on apoptotic rate of injured CMECs. **D**, Effects of Smad4 on apoptosis-related proteins in injured CMECs, *indicated p<0.05.

Effects of MiR-145-5p and Smad4 on Inflammation-Related Factors in CMECs

Compared with blank group, the contents of IL-6 and MDA in H/R group and miR-NC group were significantly increased, and the expression of IL-10 and SOD activity were significantly decreased ($p<0.05$). Compared with H/R group and miR-NC group, the contents of IL-6 and MDA in miR-145-5p-mimics group and Si-Smad4 group were significantly decreased ($p<0.05$), and the expression of Il-10 and SOD activity were significantly increased ($p<0.05$). Both over-expression of miR-145-5p and suppression of Smad4 alleviated H/R-induced inflammatory response and oxidative stress in CMECs (Figure 4).

Dual-Luciferase Reporter Assay

TargetScan 7.2 predicted downstream target genes of miR-145-5p to verify the targeting relation between miR-145-5p and Smad4, and the results showed that there were targeted binding sites between them. Therefore, Dual-Luciferase reporter assay was performed, manifesting that Luciferase activity of Smad4-3'UTR Wt was significantly reduced after miR-145-5p was over-expressed ($p<0.05$), but the Luciferase activity of Smad4-3'UTR Mut showed no change ($p>0.05$). Western Blot demonstrated that Smad4 protein expression in CMECs was significantly reduced after transfection of miR-145-5p-mimics ($p<0.05$; Figure 5).

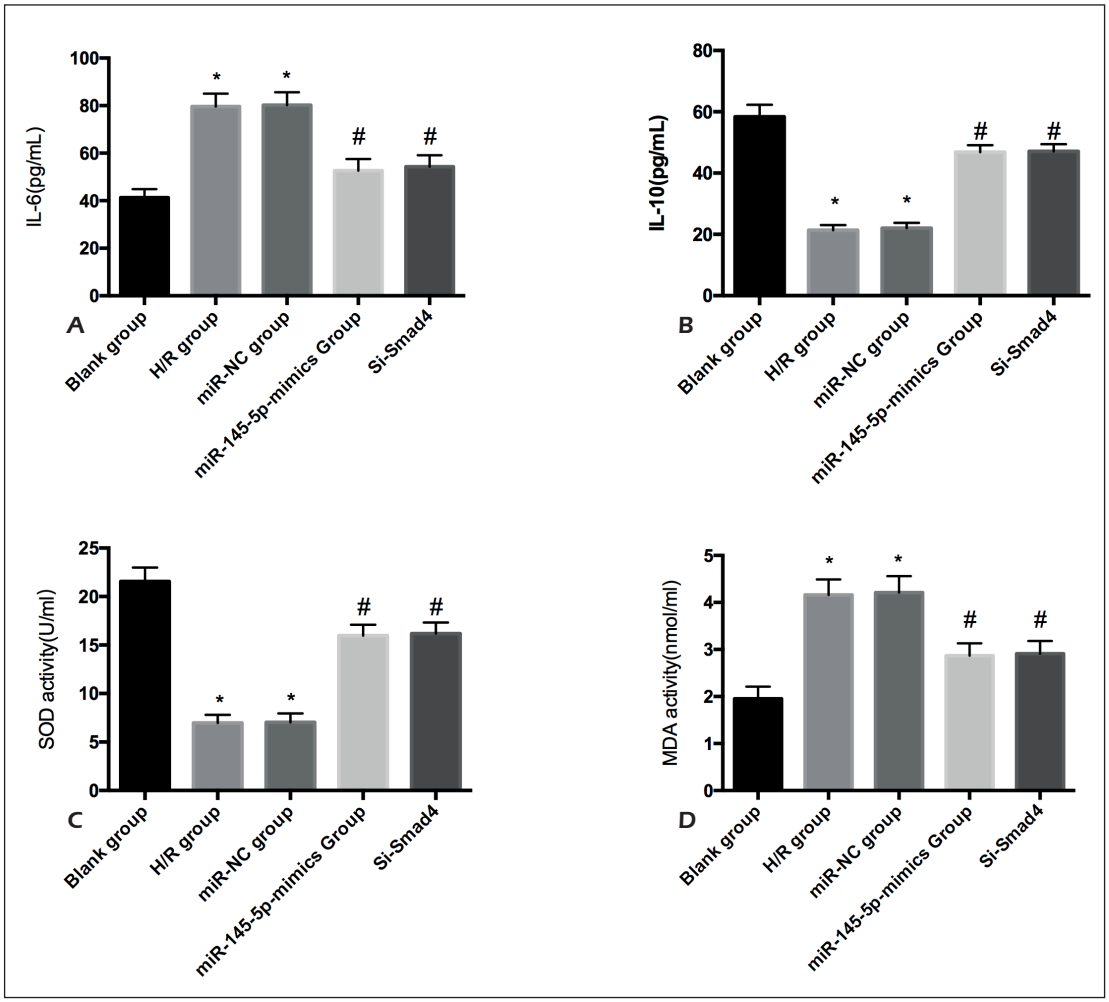

Figure 4. Effects of miR-145-5p and Smad4 on inflammation-related factors in CMECs. **A**, Effects of miR-145-5p and Smad4 on IL-6 expression. **B**, Effects of miR-145-5p and Smad4 on IL-10 expression. **C**, Effects of miR-145-5p and Smad4 on SOD expression. **D**, Effects of miR-145-5p and Smad4 on MDA expression,* indicated that compared with # and blank group, $p<0.05$.

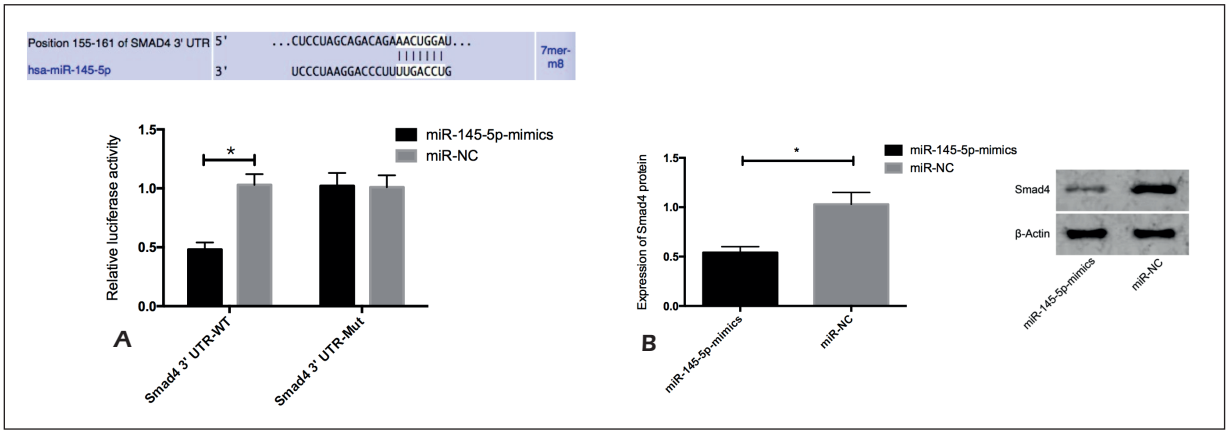

Figure 5. Determination of targeting relation. **A**, Dual-Luciferase reporter assay. **B**, Effects of miR-145-5p on expression of Smad4 protein, *indicated $p<0.05$.

Discussion

CHD has become one of the major diseases threatening human health, so the medical community has launched several studies[14,15] on its pathogenesis and preventive measures. Cardiac microvasculature is located at the end of circulation and regulates the blood in coronary arteries and myocardial perfusion. When ischemia-reperfusion occurs, CMECs will be damaged first[16]. MiRNAs are considered to be closely related to the physiological and pathological processes of cardiovascular diseases. Of note, Chiasson et al[17] reported that the lack of miR-1954 might lead to cardiac remodeling and fibrosis. Additionally, Ding et al[18] pointed out that miR-106a was a potential biomarker for patients with CHD.

In our study, we found that the expression of serum miR-145-5p in patients with CHD was significantly lower than that in controls, and the ROC curve indicated the high predictive value of miR-145-5p for the diagnosis of CHD. MiR-145-5p expression was also found to be down-regulated in breast cancer[19] and prostate cancer[20]. Subsequently, we established models of H/R-induced CMEC injury and over-expressed miR-145-5p. The results showed that the proliferation decreased and apoptosis increased in injured CMECs, however, the over-expressed miR-145-5p in CMECs restored the proliferation and apoptosis, suggesting that over-expression of miR-145-5p protected CMECs from H/R injury. Jin et al[21] on cardiovascular diseases revealed that up-regulating miR-145-5p protected human cardiomyocytes from injury induced by oxygen-glucose deprivation, which was similar to our findings. Inflammation, one of the non-negligible mechanisms in heart diseases, greatly affects the development and progression of CHD[22]. We explored the effects of miR-145-5p on inflammation and oxidative stress-related factors in injured CMECs, and the results showed that compared with normal CMECs, the levels of IL-6 and MDA in injured CMECs were significantly increased, and the expression of IL-10 and ROS activity were significantly decreased, which indicated the development of inflammatory responses. However, the over-expressed miR-145-5p significantly relieved inflammatory responses and oxidative stress, which suggested that miR-145-5p inhibited those responses in injured CMECs. However, the protective mechanism of miR-145-5p on injured CMECs is still unclear.

Smad4 is a key factor of TGF-β/Smad pathway mediating proliferation and apoptosis of many cell lines[23,24]. Cardiomyocyte Smad-dependent TGF-β signaling is crucial to maintain cardiac homeostasis[25]. In our study, miR-145-5p was found to have a negative regulatory effect on Smad4 expression through bioinformatics prediction and Dual-Luciferase reporter assay. Since Smad4 is closely related to cardiovascular diseases[26], we speculated that miR-145-5p may play a protective role on the injury of CMECs by regulating Smad4. Our study findings revealed that Smad4 expression in serum and injured CMECs of patient with CHD was significantly higher than those in healthy individuals, and it was negatively correlated with miR-145-5p expression. Knocking down the expression of Smad4 restored the proliferation in injured CMECs and significantly reduced the apoptosis, as well as inhibited the inflammatory responses and oxidative stress. We explored for the first time the role of Smad4 in injured CMECs since it has never been investigated.

Conclusions

In summary, miR-145-5p expression can effectively alleviate H/R-induced CMEC injury by inhibiting Smad4. However, there are still several limitations. First, whether miR-145-5p regulates other targets is unknown. Second, the downstream mechanism of Smad4 still remains unclear. Therefore, we will carry out basic trials to provide more data support for our conclusions.

Conflict of Interests

The authors declare that they have no conflict of interests.

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