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EXPERIMENTAL STUDY

Silencing of Long noncoding RNA SOX2-OT relieves myocardial ischemia/reperfusion injury through up-regulating microRNA-146a-5p

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ABSTRACT

PURPOSE: Long noncoding RNAs (lncRNAs) are involved in the development of myocardial ischemia/reperfusion injury (MIRI). In this study, we aimed to explore the regulatory effect and mechanism of lncRNA SOX2-OT in MIRI.

METHODS: The expression levels of SOX2-OT and miR-146a-5p in OGD/R-treated H9C2 cells and in myocardial tissues of MIRI rats were measured by qRT-PCR. Cell viability was detected by MTT assay. The levels of IL-1β, IL-6, TNF-α, MDA, and SOD were measured by ELISA. The target relationship between SOX2-OT and miR-146a-5p was predicted by LncBase, and subsequently confirmed by DLR assay. The effects of SOX2-OT silencing on myocardial apoptosis and function were further validated in MIRI rats.

RESULTS: The expression of SOX2-OT was increased in OGD/R-treated H9C2 cells and myocardial tissues of MIRI rats. Silencing of SOX2-OT increased the viability and inhibited the inflammation and oxidative stress of OGD/R-treated H9C2 cells. SOX2-OT negatively regulated its target miR-146a-5p. Inhibition of miR-146a-5p reversed the effects of sh-SOX2-OT on increasing the viability, and on inhibiting the inflammation and oxidative stress of OGD/R-treated H9C2 cells. In addition, silencing of SOX2-OT alleviated myocardial apoptosis and improved myocardial function in MIRI rats.

CONCLUSIONS: Silencing of SOX2-OT relieved the apoptosis, inflammation, and oxidative stress of myocardial cells via up-regulating miR-146a-5p, contributing to the remission of MIRI *(Fig. 6, Ref. 33)*. Text in PDF *www.elis.sk*

KEY WORDS: SOX2-OT, miR-146a-5p, myocardial ischemia/reperfusion injury, inflammation.

Introduction

Myocardial ischemia-reperfusion injury (MIRI) is a kind of myocardial injury that is induced by initial scarcity of heart blood supply and subsequent perfusion and oxygenation (1). Since MIRI can induce the apoptosis and necrosis of cardiomyocytes, it contributes to adverse cardiovascular outcomes, such as malignant arrhythmia cardiac insufficiency, and even heart failure (2, 3). Until now, MIRI is still unavoidable following myocardial ischemia, cardiac surgery, and circulatory arrest, and there are still no efficacious strategies for protecting the heart against MIRI (4, 5). Therefore, discovering potential therapeutic targets for MIRI is urgently needed.

Long non-coding RNAs (lncRNAs) are critical regulators involved in the pathogenesis of diverse cardiovascular diseases, including coronary disease, myocardial infarction, and heart failure (6). Notably, more than 2000 lncRNAs have been discovered to be dysregulated in MIRI (7). In recent years, studies have determined that some lncRNAs exert protective role against MIRI, such as AK006774 (8), OIP5-AS1 (9), and PART1 (10), and on the contrary, some lncRNAs exert pathogenic role, such as KCNQ1OT1 (11), TUG1 (12), GAS5 (13), and MALAT1 (14). SOX2-OT is a specific lncRNA that functions an oncogene in cancers (15–17). Evidence has determined that SOX2-OT also plays an important role in myocardial infarction and ischemic heart failure. For example, SOX2-OT exacerbates hypoxia-induced cardiomyocyte injury in vitro (18). SOX2-OT inhibition suppresses the apoptosis, oxidative damage, and inflammation in oxygen and glucose deprivation-treated cells, and improves myocardial dysfunction in the model rats (19). However, the regulatory role of SOX2-OT in MIRI is rarely known.

The miRNA-lncRNA interaction network plays a pivotal role in the pathophysiological processes of human diseases (20). Previous studies have reported that SOX2-OT is involved in cardiovascular diseases through regulating specific miRNAs, such as SOX2-OT-miR-27a-3p in myocardial infarction (18), SOX2-OT-miR-455-3p in ischemic heart failure (19), and SOX2-OT-miR-215-5p in ischemic heart failure (21). MiR-146a-5p is known as a tumor suppressor (22) that also functions in the protection of

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ischemia-reperfusion (I/R) injury (23, 24). It has been reported that overexpression of miR-146a-5p inhibits autophagy and attenuates intestinal I/R injury in a mouse model (23). MiR-146a-5p protects human renal tubular epithelial cells from hypoxia-reoxygenation injury, contributing to the therapeutic effect of urine-derived stem cells on renal I/R injury (24). Therefore, we suspected that whether SOX2-OT is involved in MIRI through regulating miR-146a-5p.

In this study, the regulatory effects of SOX2-OT were analyzed on the viability, inflammation, and oxidative stress of oxygen and glucose deprivation/reperfusion (OGD/R)-treated cells, and on the myocardial apoptosis and function in MIRI rats. In addition, the regulatory relationship between SOX2-OT and miR-146a-5p was further determined *in vitro*. This study aimed to reveal potential therapeutic targets for MIRI.

Methods

Cell culture and treatment

H9C2 cells, a rat cardiomyocyte cell line (European Collection of Cell Cultures, UK), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Weike Biotechnology, Shanghai, China) containing 100 U/mL Penicillin, 100 mg/mL Streptomycin, and 10 % fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C with 5 % CO_2. OGD/R model was induced in H9C2 cells by maintaining in "chemical ischemia" medium (140 NaCl, 5 $NaHCO_3$, 3.5 KCl, 1.7 $CaCl_2$, 1.25 $MgSO_4$, 0.43 KH_2PO_4; mmol/L unit; 20 Hepes; 7.2 to 7.4 pHe) for 6 h and then in normal medium for 24 h (24, 25).

Cell transfection

Lentivirus-packaged shRNAs targeting SOX2-OT (sh-SOX2-OT) and negative control (sh-NC) were constructed in Genechem (Shanghai, China). MiR-146a-5p mimic, inhibitor, and negative control (miR-NC) were also purchased from GenePharma. These agents were transfected into H9C2 cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) for 24 h according to the instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cells using a Trizol total RNA extraction kit (Vazyme Biotech, Nanjing, China), and was then reverse transcribed into cDNAs using a PrimeScript RT Reagent kit (Takara, Shanghai, China). qRT-PCR was performed using a SYBR Green Master Mix (Vazyme, Nanjing, China) on ABI7500 system (2016, Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. The primer sequences were shown as follows: SOX2-OT (forward, 5'-GTTCATGGCCTGGACTCTCC-3'; reverse, 5'-ATTGCTAGCCCTCACACCTC-3'); miR-146a-5p (forward, 5'-GTCGATGCAGCAAACCTCAGGGAA-3'; reverse, 5'-GCTCAGAAGCACACAAACAAAACT-3'); GAPDH (forward, 5'-TGTTCGTCATGGGTGTGAAC-3'; reverse, 5'-ATGGCATGGACTGTGGTCAT-3'). Relative expression level of SOX2-OT/miR-146a-5p was calculated by $2^{-\Delta\Delta Ct}$ method (25).

Measurement of cell viability

MTT assay was used to measure the cell viability. Simply, cells (2×10^5/well) were seeded into 96-well plates, and 10 mL MTT (5 mg/mL) was added into each well. After 4 h of culturing, cells were incubated with 150 μL dimethyl sulfoxide (DMSO) for another 15 min. The optical density (OD) at 570 nm was measured by a Microplate Reader (2016, Multiskan, Thermo Fisher Scientific, Waltham, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The contents of TNF-α, IL-1β, IL-6, malondialdehyde (MDA), superoxide dismutase (SOD), and lactate dehydrogenase (LDH) were measured using commercial kits (Jiancheng, Nanjing, China) following the manufacturer's instructions. Simply, the tissue or cell samples were homogenized and the supernatants were collected for specific antibody incubation. The OD value at 570 nm was measured by a Microplate Reader (Thermo Fisher Scientific).

Target prediction

The potential target miRNAs of SOX2-OT were predicted by LncBase. A total of 2007 targets were predicted, and 357 targets exhibited a score > 0.9. MiR-146a-5p is a target with a score of 0.973, which plays an important role on the protection of I/R injury (19–23). Since the regulatory relationship between SOX2-OT and miR-146a-5p in MIRI is unclear, miR-146a-5p was selected as a potential target of SOX2-OT for the following assays.

Dual-luciferase reporter (DLR) assay

The fragments of SOX2-OT containing the binding sites of miR-146a-5p were synthesized and cloned into luciferase reporter vector pmirGLO (LMAIBio, Shanghai, China) (pmirGLO-SOX2-OT wt). The corresponding pmirGLO-SOX2-OT mut was synthesized by cloning the fragments containing the mutated binding sites. H9C2 cells were co-transfected with pmirGLO-SOX2-OT wt/mut and miR-146a-5p mimics/ miR-NC for 48 h. After visualized using a DLR kit (Promega, Madison, WI, USA), the fluorescence intensity was measured by a Microplate Reader (Thermo Fisher Scientific). Relative fluorescence was finally calculated as the ratio of Fireny Luciferase/Renilla Luciferase.

Establishment of MIRI model in rats

Animal experiments were approved by the Ethics Committee of Jimo District People's Hospital (JY-2019014) in accordance with the Guide for the Care and Use of Laboratory Animals. Forty Sprague-Dawley (SD) rats (male, 180-200g, 6 weeks) purchased from Better Biotechnology Co., Ltd. (Nanjing, China) were randomly divided into MIRI (n = 30) and Sham groups (n = 10). MIRI rats were further randomized into MIRI (MIRI rats without treatment), MIRI + sh-NC, and MIRI + sh-SOX2-OT groups (n = 10 each group). Rats in MIRI + sh-NC, and MIRI + sh-SOX2-OT groups were intracoronarily injected with sh-NC and sh-SOX2-OT (2×10^{11} pfu/mL/body) at five days before modeling, respectively. MIRI model was established in rats as previously described (26). Simply, rats were anesthetized by intraperitoneal injection of 10 % urethane (U2500, Sigma, St. Louis, MO, USA) and anticoagulated by intraperitoneal injec-

tion of heparin sodium (Suzhou Yacoo Science, Suzhou, China). Left thoracotomy was then performed at the first to third intercostal space, and a 6-0 prolene suture was used for ligating the left anterior descending coronary artery (LAD). The LAD was ligated for 30 min and perfused for 3 h to induce MIRI. Similar surgical procedures, expect the ligation of LAD were performed in the Sham group.

Measurement of ventricular hemodynamic parameters

After modeling for two weeks, rats were anesthetized by intraperitoneal injection of 10 % urethane (10 mL/kg, Sigma-Aldrich). A pressure transducer was connected by inserting a cannula into the left ventricles through right common carotid artery. The left ventricular end diastolic pressure (LVEDP) and left ventricular systolic pressure (LVSP) were measured by a multi-lead physiological recorder (2017, Powerlab, ADInstruments Shanghai Trading, Shanghai, China). After measurements, rats were sacrificed by cervical dislocation.

TUNEL staining

Cell apoptosis in myocardial tissues was detected using a TUNEL kit (Beyotime, Shanghai, China). Simply, the tissue sections were incubated with DNase-free Proteinase K for 20 min, 3 % hydrogen peroxide for 10 min, TUNEL for 1 h, and then with Streptavidin-HRp for 30 min. After stained with diaminobenzidine and hematoxylin, the apoptotic cells were observed under a microscope (Olympus, Japan) at five randomly selected fields.

Statistical analysis

Data were presented as means ± standard deviation (SD) and analyzed by SPSS 20.0 (SPSS, Chicago, IL, USA). Student's t test was used for comparing differences between two groups. One-way ANOVA followed by Tukey's post hoc test (pairwise comparison) was used for comparing differences of multiple groups. p-value < 0.05 was considered as significantly different.

Results

Silencing of SOX2-OT increased the viability of OGD/R-treated H9C2 cells

The expression of SOX2-OT was significantly higher in OGD/R-treated H9C2 cells than that in controls ($p < 0.01$) (Fig. 1A). SOX2-OT was then silenced to evaluate the function of SOX2-OT in OGD/R-treated H9C2 cells. As expected, the expression of SOX2-OT in OGD/R-treated H9C2 cells was significantly decreased by the transfection of sh-SOX2-OT ($p < 0.01$) (Fig. 1B). MTT assay determined that the viability was lower in OGD/R-treated H9C2 cells than that in controls ($p < 0.01$). Notably, silencing of SOX2-OT (sh-SOX2-OT) significantly increased the viability of OGD/R-treated H9C2 cells ($p < 0.01$) (Fig. 1C).

Silencing of SOX2-OT inhibited the inflammation and oxidative stress of OGD/R-treated H9C2 cells

The contents of inflammatory factors, including IL-6, IL-1β, and TNF-α were higher in OGD/R group in comparison with those in control group ($p < 0.01$). The transfection of sh-SOX2-OT significantly decreased the contents of IL-6, IL-1β, and TNF-α in OGD/R-treated H9C2 cells ($p < 0.01$) (Fig. 2A–C). In addition, the content of MDA (an oxidative stress factor) was higher, and that of SOD (another oxidative stress factor) was lower in OGD/R-treated H9C2 cells than in controls ($p < 0.01$). The transfection of sh-SOX2-OT significantly reduced MDA content and increased SOD content in OGD/R-treated H9C2 cells ($p < 0.01$) (Fig. 2D and E).

MiR-146a-5p was a target of SOX2-OT

A special binding site between SOX2-OT and miR-146a-5p was predicted by LncBase (Fig. 3A). DLR assay showed that the luciferase activity was significantly decreased in cells co-transfected with SOX2-OT wt and miR-146a-5p mimics compared with cells co-transfected with SOX2-OT wt and miR-NC ($p < 0.01$) (Fig. 3B).

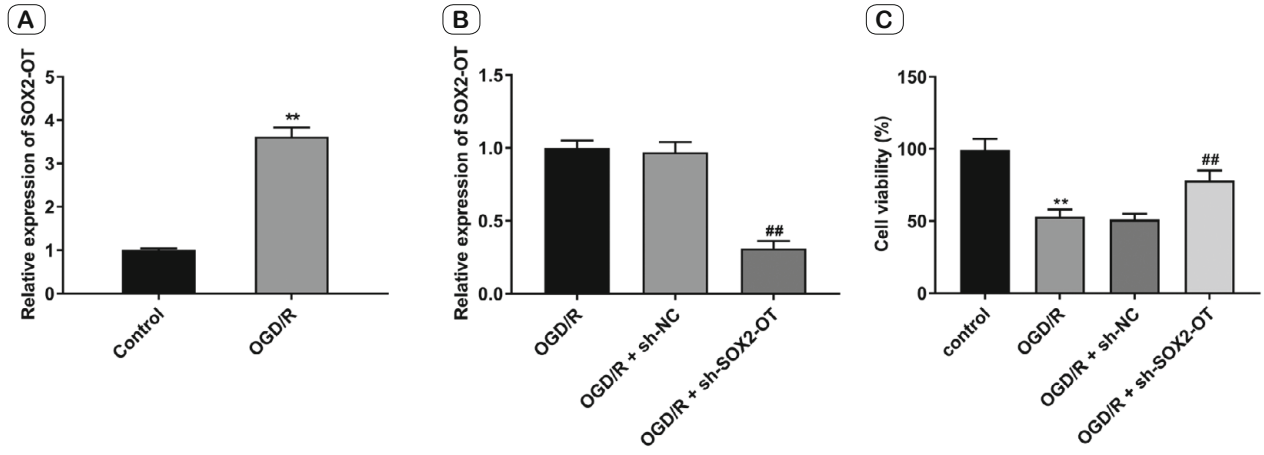

Fig. 1. Silencing of SOX2-OT increased the viability of OGD/R-treated H9C2 cells. A: The expression of SOX2-OT in OGD/R-treated H9C2 cells and control cells was detected by qRT-PCR. B: Knockdown efficiency of sh-SOX2-OT in OGD/R-treated H9C2 cells was verified by qRT-PCR. C: Cell viability was examined by MTT assay. **, compared with the control group, $p < 0.01$; ##, compared with the OGD/R + sh-NC group, $p < 0.01$.

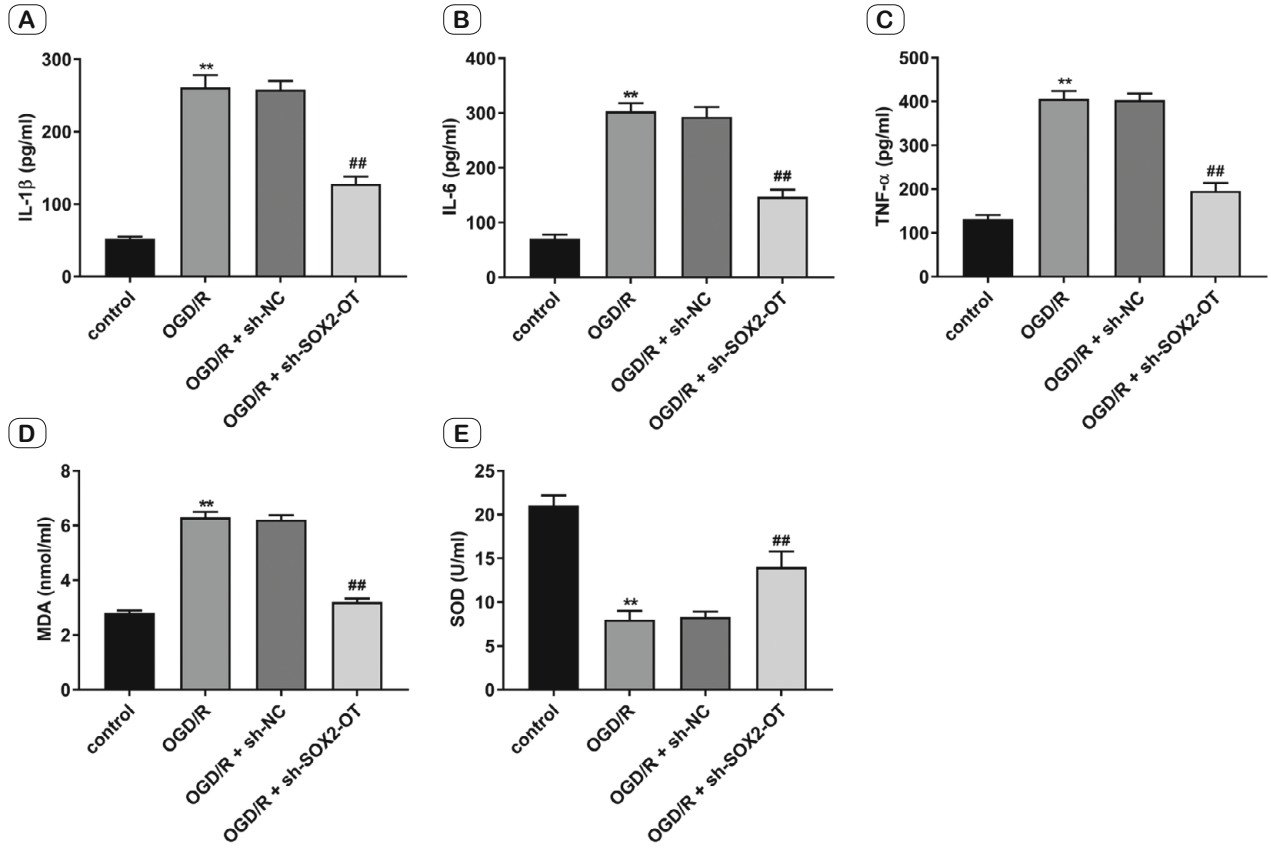

Fig. 2. Silencing of SOX2-OT inhibited the inflammation and oxidative stress of OGD/R-treated H9C2 cells. The contents of IL-1β (A), IL-6 (B), TNF-α (C), MDA (D), and SOD (E) were measured by ELISA. **, compared with the control group, p < 0.01; ##, compared with the OGD/R + sh-NC group, p < 0.01.

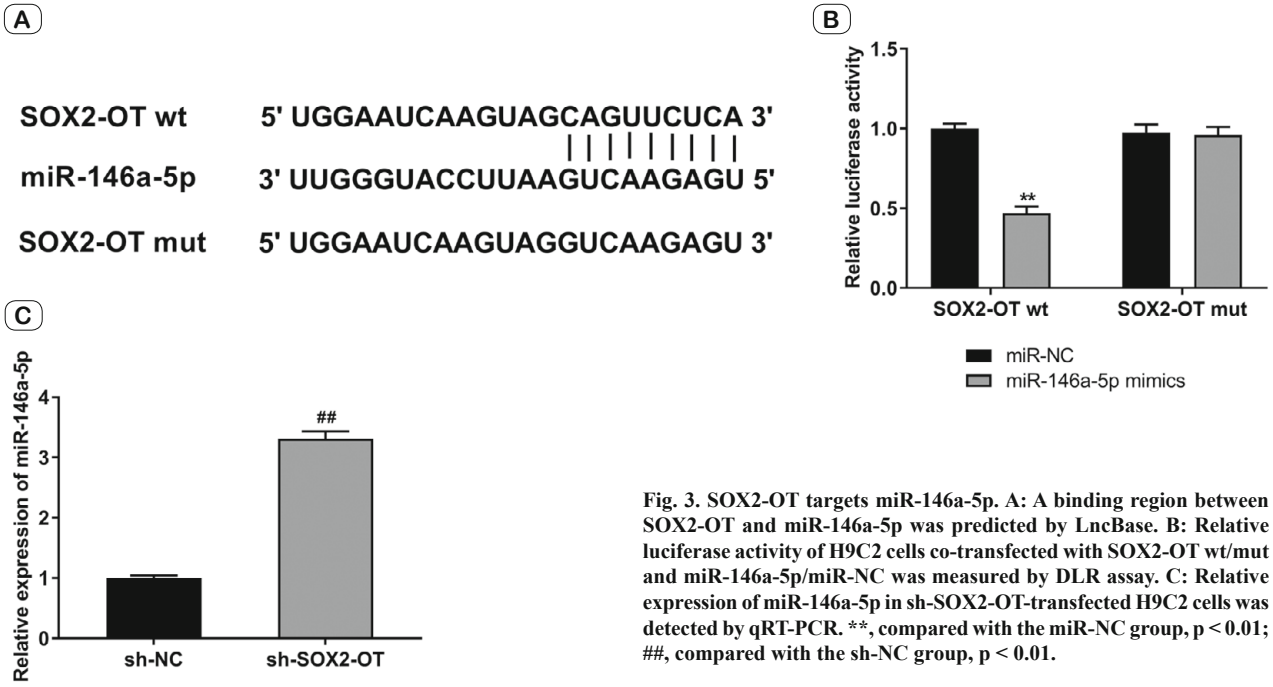

Fig. 3. SOX2-OT targets miR-146a-5p. A: A binding region between SOX2-OT and miR-146a-5p was predicted by LncBase. B: Relative luciferase activity of H9C2 cells co-transfected with SOX2-OT wt/mut and miR-146a-5p/miR-NC was measured by DLR assay. C: Relative expression of miR-146a-5p in sh-SOX2-OT-transfected H9C2 cells was detected by qRT-PCR. **, compared with the miR-NC group, p < 0.01; ##, compared with the sh-NC group, p < 0.01.

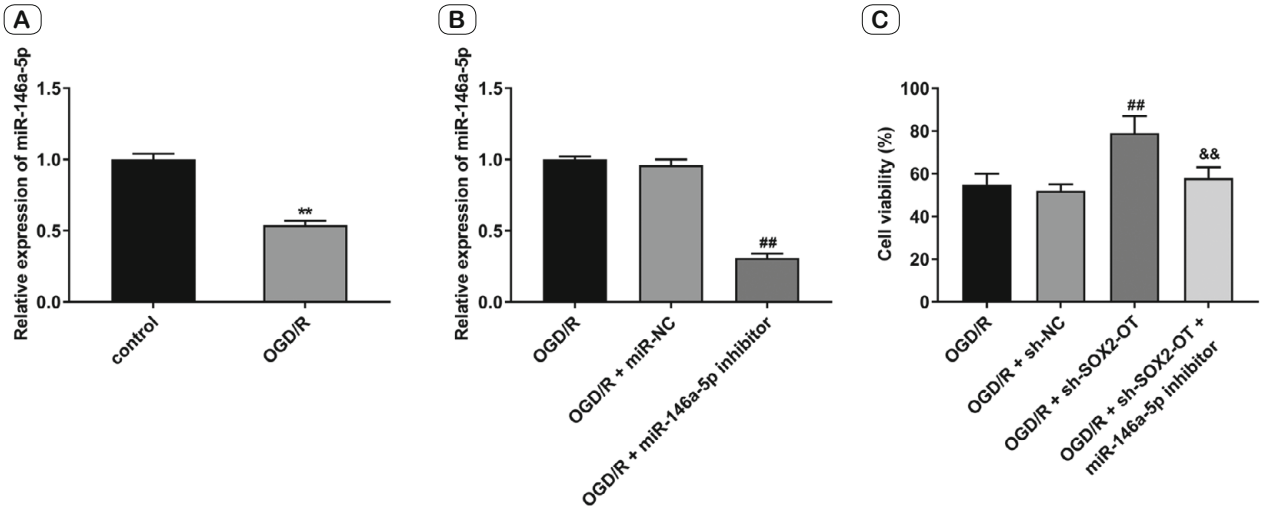

Fig. 4. Silencing of SOX2-OT increased the viability of OGD/R-treated H9C2 cells through regulating miR-146a-5p. A: Relative expression of miR-146a-5p in OGD/R-treated H9C2 cells and control cells was detected by qRT-PCR. B: Knockdown efficiency of miR-146a-5p inhibitor in OGD/R-treated H9C2 cells was verified by qRT-PCR. C: Cell viability was detected by MTT assay. **, compared with the control group, p < 0.01; ##, compared with the OGD/R + miR-NC group, p < 0.01; &&, compared with the OGD/R + sh-SOX2-OT group, p < 0.01.

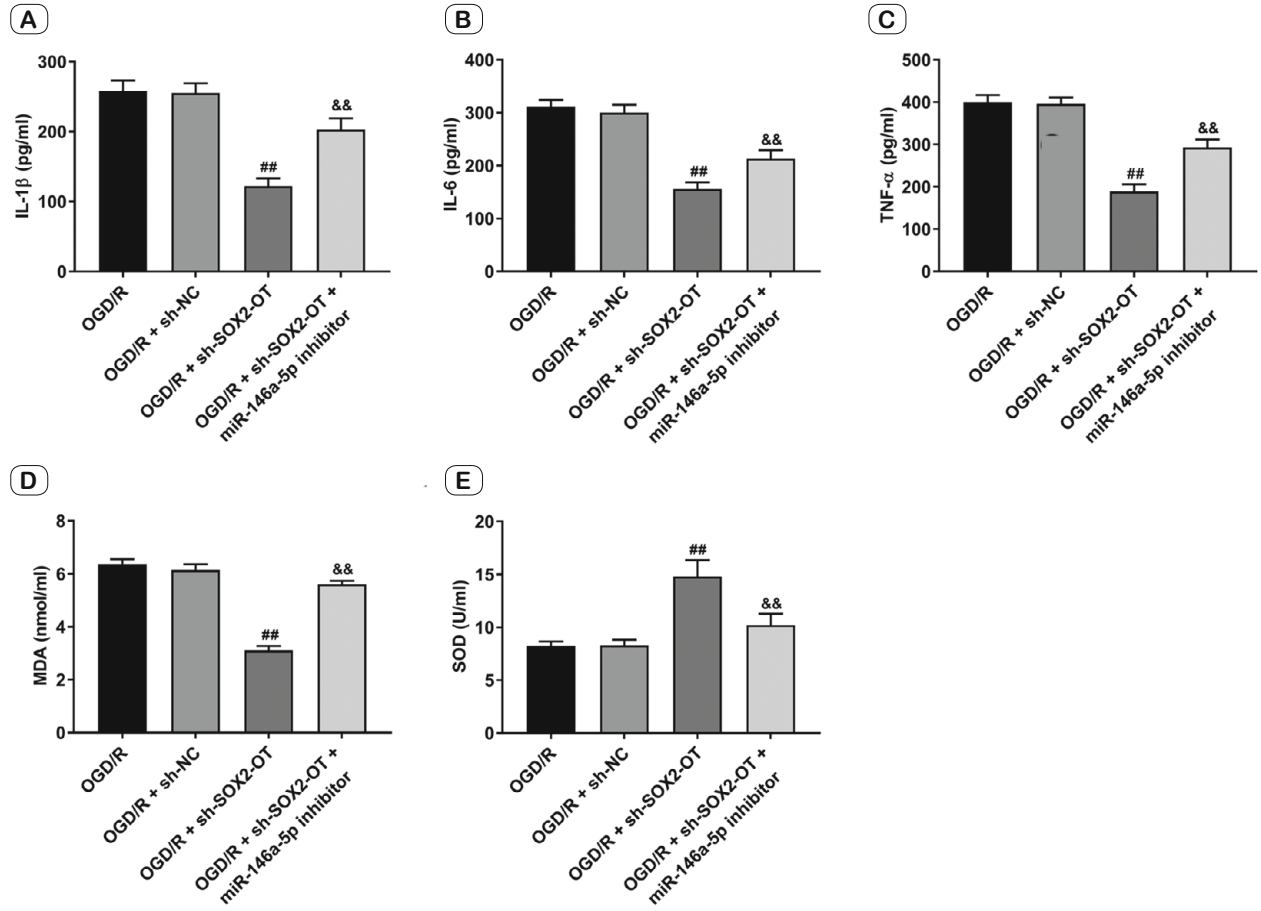

Fig. 5. Silencing of SOX2-OT inhibited the inflammation and oxidative stress of OGD/R-treated H9C2 cells through regulation of miR-146a-5p. The contents of IL-1β (A), IL-6 (B), TNF-α (C), MDA (D), and SOD (E) were measured by ELISA. ##, compared with the OGD/R + sh-NC group, p < 0.01; &&, compared with the OGD/R + sh-SOX2-OT group, p < 0.01.

In addition, silencing of SOX2-OT significantly increased the expression of miR-146a-5p in H9C2 cells ($p < 0.01$) (Fig. 3C).

Silencing of SOX2-OT increased the viability of OGD/R-treated H9C2 cells through regulating miR-146a-5p

Whether the regulatory effect of SOX2-OT on cell viability was related to miR-146a-5p was investigated. The expression of miR-146a-5p was significantly lower in OGD/R-treated H9C2 cells than that in the controls ($p < 0.01$) (Fig. 4A). The transfection of miR-146a-5p inhibitor significantly down-regulated miR-146a-5p in OGD/R-treated H9C2 cells ($p < 0.01$) (Fig. 4B). In addition, miR-146a-5p inhibitor also reversed the promoting effect of sh-SOX2-OT on the viability of OGD/R-treated H9C2 cells ($p < 0.01$) (Fig. 4C).

Silencing of SOX2-OT inhibited the inflammation and oxidative stress of OGD/R-treated H9C2 cells through regulating miR-146a-5p.

The regulatory mechanism of SOX2-OT on inflammation and oxidative stress relating to miR-146a-5p was further investi-

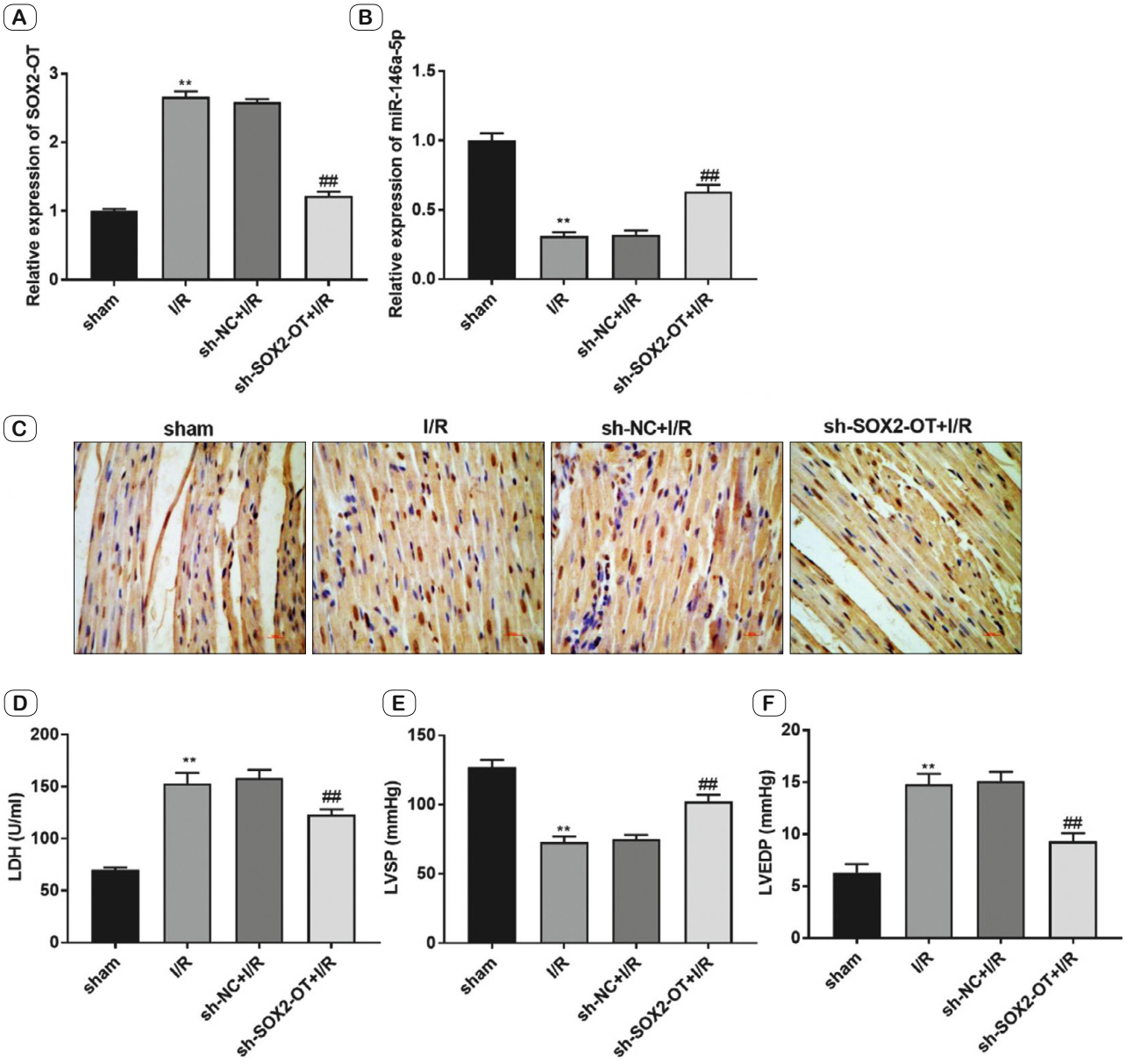

Fig. 6. Silencing of SOX2-OT alleviated MIRI of myocardial tissues in model rats. A: Relative expression of SOX2-OT in myocardial tissues was detected by qRT-PCR. B: Relative expression of miR-146a-5p in myocardial tissues was detected by qRT-PCR. C: TUNEL staining of apoptotic cells in myocardial tissues. D: LDH level in myocardial tissues. E: LVSp level. F: LVEDp level. **, compared with the sham group, $p < 0.01$; ##, compared with the MIRI + sh-NC group, $p < 0.01$.

gated. The contents of IL-6, IL-1β, and TNF-α in OGD/R-treated H9C2 cells were decreased by the transfection of sh-SOX2-OT in comparison with sh-NC ($p < 0.01$). MiR-146a-5p inhibitor significantly reversed the inhibiting effect of sh-SOX2-OT on inflammation (IL-6, IL-1β, and TNF-α) in OGD/R-treated H9C2 cells ($p < 0.01$) (Fig. 5A–C). In addition, the transfection of sh-SOX2-OT decreased MDA content and increased SOD content in OGD/R-treated H9C2 cells ($p < 0.01$). Silencing of miR-146a-5p significantly weakened the effects of sh-SOX2-OT on decreasing MDA and increasing SOD ($p < 0.01$) (Fig. 5D and E).

Silencing of SOX2-OT alleviated MIRI in myocardial tissues of model rats

A rat model of MIRI was established to evaluate the function of SOX2-OT *in vivo*. qRT-PCR showed that the expression of SOX2-OT was increased and the expression of miR-146a-5p was decreased in myocardial tissues of MIRI rats compared with those in sham rats ($p < 0.01$). The injection of sh-SOX2-OT significantly down-regulated SOX2-OT and up-regulated miR-146a-5p in MIRI rats ($p < 0.01$) (Fig. 6A and B). When compared with myocardial tissues in sham rats, there more apoptotic cells were observed in MIRI rats by TUNEL staining. The enhanced apoptosis of myocardial cells in MIRI rats was relieved by sh-SOX2-OT (Fig. 6C). In addition, sh-SOX2-OT also weakened the increasing of LDH and LVEDp and decreasing of LVSp in MIRI rats ($p < 0.01$) (Fig. 6D–F).

Discussion

MIRI is a serious myocardial injury following blood flow recovery, contributing to poor cardiovascular outcomes (5, 27). LncRNAs play important roles in the pathogenesis of MIRI through regulating a variety of physiological processes, such as oxidative stress, inflammatory response, cardiomyocyte apoptosis/autophagy/necrosis, mitochondrial dysfunction, and calcium overload (28). Among massive lncRNAs, SOX2-OT has been reported to be up-regulated in myocardial infarction and ischemic heart failure (18, 19, 29). However, the role of SOX2-OT in MIRI is rarely reported. In this study, SOX2-OT was found to be up-regulated in OGD/R-treated H9C2 cells and myocardial tissues of MIRI rats, indicating a possible pathogenic role in MIRI.

Researches have reported that up-regulated lncRNAs play important roles in MIRI progress, such as H19, HRIM, Gm4419, KCNQ1OT1, TUG1, GAS5, and MALAT1 (11–14, 30, 31). Previous studies based on *in vitro* experiments have reported that silencing of some specific lncRNAs is a potential strategy for the treatment of MIRI. For example, silencing of H19 increases cell viability, decreases inflammatory cytokines, and inhibits oxidative stress in OGD/R-treated cardiomyocytes (30). Silencing of HRIM inhibits the apoptosis and inflammation of OGD/R-treated cardiomyocytes (31). SOX2-OT is a potential therapeutic target in myocardial infarction and ischemic heart failure (18, 19), while its function in MIRI remains unclear. Here, SOX2-OT was silenced to evaluate the function of SOX2-OT in MIRI. Similarly with previous studies mentioned above, silencing of SOX2-OT increased cell viability and inhibited cell inflammation and oxidative stress in OGD/R-treated H9C2 cells. These *in vitro* findings indicate that SOX2-OT silencing is benefit to the treatment of MIRI. Furthermore, evidence has also determined the protective role of lncRNAs (such as H19, HRIM, and GAS5) silencing against MIRI *in vivo* (13, 30, 31). Similarly, this study revealed that silencing of SOX2-OT inhibited myocardial apoptosis and improved myocardial function in MIRI rats. Our findings illustrate that SOX2-OT is a potential therapeutic target for MIRI through regulating cell apoptosis, inflammation and oxidative stress.

LncRNAs can regulate their target miRNAs via competitive binding or sponge effects (32). There are many miRNA targets of SOX2-OT that have been determined in diverse cardiovascular diseases, including miR-942-5p in doxorubicin-induced cardiac muscle dysfunction (32), miR-27a-3p in myocardial infarction (18), miR-455-3p in ischemic heart failure (19), miR-215-5p in ischemic heart failure (21), and miR-2355-3p in ventricular arrhythmia associated with heart failure (33). In this study, miR-146a-5p was predicated to be a target of SOX2-OT, which was further identified by DLR assay. MiR-146a-5p plays an important regulatory role in I/R injury of different tissues. MiR-146a-5p inhibits autophagy of OGD/R-treated cells and attenuates intestinal I/R injury in mice (23). Li et al have found that urine-derived stem cells protect against renal I/R injury via producing exosomal miR-146a-5p (24). In this study, down-regulation of miR-146a-5p in MIRI rats was reversed by SOX2-OT silencing. To combine with the protective role of miR-146a-5p in I/R injury, we suspect that SOX2-OT silencing may relieve MIRI through up-regulating miR-146a-5p. This suspicion is subsequently confirmed by our following feedback experiments, evidenced by that silencing of miR-146a-5p reversed the effects of sh-SOX2-OT on inhibiting the inflammation and oxidative stress, and on promoting the viability of OGD/R-treated cells.

Conclusion

SOX2-OT was up-regulated and miR-146a-5p was down-regulated in OGD/R-treated cardiomyocytes and myocardial tissues of MIRI rats. Silencing of SOX2-OT inhibited the inflammation and oxidative stress and enhanced the viability of OGD/R-treated cardiomyocytes through up-regulating miR-146a-5p. Silencing of SOX2-OT also inhibited myocardial apoptosis and improved myocardial function in MIRI rats. However, the down-stream mechanisms of SOX2-OT-miR-146a-5p axis in MIRI are not fully discovered. Further research on detailed action mechanisms of SOX2-OT in MIRI is still needed.

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