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ORIGINAL ARTICLE

MiR-130a inhibition protects rat cardiac myocytes from hypoxia-triggered apoptosis by targeting Smad4

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Abstract

Background: Cardiomyocyte death facilitates the pathological process underlying ischaemic heart diseases, such as myocardial infarction. Emerging evidence suggests that microRNAs play a critical role in the pathological process underlying myocardial infarction by regulating cardiomyocyte apoptosis. However, the relevance of miR-130a in regulating cardiomyocyte apoptosis and the underlying mechanism are still uncertain.

Aim: We sought to explore the regulatory effect of miR-130a on hypoxic cardiomyocyte apoptosis.

Methods: The expression of miR-130a was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Cell survival was determined by the MTT assay. The lactate dehydrogenase (LDH) assay was performed to determine the severity of hypoxia-induced cell injury. Apoptosis was assessed via caspase-3 analysis. Protein expression level was determined by Western blotting. The genes targeted by miR-130a were predicted using bioinformatics and were validated via the dual-luciferase reporter assay system.

Results: We found that miR-130a expression was greatly increased in hypoxic cardiac myocytes, and that the downregulation of miR-130a effectively shielded cardiac myocytes from hypoxia-triggered apoptosis. In bioinformatic analysis the Smad4 gene was predicted to be the target of miR-130a. This finding was validated through the Western blot assay, dual-luciferase reporter gene assay, and qRT-PCR. MiR-130a inhibition significantly promoted the activation of Smad4 in hypoxic cardiomyocytes. Interestingly, knockdown of Smad4 markedly reversed the protective effects induced by miR-130a inhibition. Moreover, we found that the inhibition of miR-130a promoted the activation of transforming growth factor-β1 signalling. Blocking of Smad4 signalling significantly abrogated the protective effects of miR-130a inhibition.

Conclusions: The findings indicate that inhibition of miR-130a, which targets the Smad4 gene, shields cardiac myocytes from hypoxic apoptosis. This study offers a novel perspective on the molecular basis of hypoxia-induced cardiomyocyte apoptosis and suggests a possible drug target for the treatment of myocardial infarction.

Key words: apoptosis, cardiomyocyte, hypoxia, miR-130a, Smad4

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INTRODUCTION

Heart disease is a growing problem [1]. Despite significant progress in the treatment of heart disease with medication, a dismal general prognosis with high mortality still remains [2]. Ischaemia-reperfusion injury aggravates morbidity and increases mortality among a considerable variety of diseases, including myocardial infarction (MI) [3]. The heart is well known for its delicacy, having little capacity for regeneration and rehabilitation. The heart is also known to respond to both progressive and acute stressors.

The Smad family of proteins were initially identified as key downstream signal transducers in the transforming growth factor-β1 (TGF-β1) signalling pathway. Overexpression of a dominant negative Smad3 mutant or of Smad7, both of which im-

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pair Smad-mediated signal transduction, inhibits TGF-β1-dependent apoptosis [4]. TGF-β receptors activate Smad-independent pathways that do not only regulate Smad signalling but also allow Smad-independent TGF-β responses [5]. Smad4 is as an essential mediator of the TGF-β signalling pathway, and dysregulated TGF-β signalling is linked with thoracic aortic aneurysms [6]. Furthermore, miR-146b-5p has been shown to directly target Smad4 and thereby negatively regulate the TGF-β signalling pathway [7]. In a recent study, Smad4 was found to play an important role in heart development [8]. Smad4 is important in the TGF-β pathway, and it plays a key role as a transcription factor and inhibits cell growth after TGF-β1 stimulation [9].

Recently, microRNAs (miRNAs) have been shown to regulate complex molecular networks through feedback mechanisms [10]. miRNAs are a type of small, highly conserved non-coding RNA, and endogenous miRNAs typically have between 18 and 22 nucleotides. They participate in the regulation of cell proliferation, differentiation, and death by targeting the 3′-UTR of specific mRNA at the post-transcriptional level for degradation or translation repression [11]. Recent years have seen an increase in the interests of scholars in the pathological role that miRNAs may play in heart diseases [12].

A number of miRNA-based therapies have undergone pre-clinical and clinical trials [11]. miR-130a has previously been reported to be an apoptosis-associated miRNA [13]. Its expression was detected in embryo heart tissues and was actively involved in heart growth. In embryonic cardiac myocytes, upregulated miR-130a expression may lead to an atrophied ventricular muscle and an irregular heart structure [14]. In addition, it was recorded that miR-130a expression was decreased in various kinds of heart disease models [15]. However, whether miR-130a is associated with apoptosis of cardiac myocytes is still unclear. In addition, it has been shown that miR-130a expression is downregulated in various ischaemia/reperfusion (I/R) models. However, the exact role of miR-130a in the pathogenesis of I/R injury, particularly its association with autophagy, has not yet been fully elucidated.

In the present study, the expression of miR-130a, and the role of miR-130a in myocardial injury and apoptosis induced by hypoxia/reoxygenation (H/R) in cultured H9C2 rat cardiomyocytes were examined. The results show that miR-130a initiates H/R-induced apoptosis of cardiomyocytes by targeting Smad4, indicating that inhibition of miR-130a has therapeutic benefits.

METHODS

Cell culture

Rat myocardium-derived H9C2 and HEK293 cells were obtained from ATCC (VA, USA) and cultured in Dulbecco's modified Eagle's medium (Gibco BRL Inc., Waltham, MA, USA) with 10% foetal bovine serum (FBS) (Gibco BRL Inc., Waltham, MA, USA) and 1% penicillin (P/S; Invitrogen Inc., Waltham, MA, USA). The cells were incubated in a humidified CO_2 incubator (concentration: 5%) at a temperature of 37°C.

Cell treatment

Cells were subjected to hypoxia for 24 h at 37°C in 1% O_2, 94% N_2, and 5% CO_2 using a modular incubator (Model 3131; Forma; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, reoxygenation (5% CO_2; 37°C, 95% air [79% N_2/21% O_2]) was performed for 24 h. Cells cultured under normoxic conditions served as a control. Ischaemia and reperfusion were simulated by culturing the cells in DMEM containing 1% or 10% FBS, respectively.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cardiac myocytes using TRIzol Reagent (Invitrogen) following the manufacturer's manuals. We carried out reverse transcription (RT) to synthesise cDNA with 20 mL ddH_2O, 4 mL 5 × Mix, and 500 ng/μL RNA. The temperature conditions for RT were 37°C, 50°C, and 98°C for 15 min, 5 min, and 5 min, respectively. qPCR was carried out using the QuantiNova SYBR Green kit (Qiagen, Hilden, Germany). We used Primer Premier 5 to design the primers. The PCR primer sequences were: miR-130a, 5′-CAGUGCAAUGUUAAAAG-3′; GAPDH forward, 5′-CCTCAAGATTGTCAGCAAT-3′ and reverse, 5′-CCATCCACAGTCTTCTGAGT-3′. The reaction involved denaturation at a temperature of 95°C for 1 min and 15 s, followed by 40 cycles of incubation at 72°C and 60°C for 30 s and 15 s, respectively, followed by another 1 min reaction at 72°C. A melting curve analysis was performed in the range 65°C to 95°C at 0.2°C intervals. The $2^{-\Delta\Delta}$Cq method was adopted for the calculation of the target gene's expression level. Negative controls were prepared with ddH_2O. All experiments were done in triplicate.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

Cells were plated in 96-well plates at a density of 1 × 10^3 cells/well and incubated overnight. They were subsequently treated with 20 μL of MTT (Sigma-Aldrich, St. Louis, MO, USA; 5 mg/mL). The supernatants were discarded after 4 h, and 200 μL of dimethyl sulfoxide (SigmaAldrich, St. Louis, MO, USA) was added to each well and incubated for 15 min to dissolve the formazan crystals in the living cells. The absorbance at 490 nm was measured with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Lactate dehydrogenase (LDH) assay

Cells were lysed using 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA). Supernatants were harvested after centrifugation and then incubated with pyruvate and nicotinamide adenine dinucleotide hydrogen (Roche Applied Science, Indi-

anapolis, IN, USA) for 30 min. The absorbance at 530 nm was detected by a microplate reader (Bio-Rad, Berkeley, CA, USA).

Caspase-3 activity assay

Cells were lysed, and protein concentration was measured. Protein samples (100 μg) were treated with 10 μL of Ac-DEVD-pNA (Roche Applied Science) for 4 h at 37°C. The absorbance at 405 nm was measured with a microplate reader (Bio-Tek Instruments, Burlington, VT, USA).

Western blot assay

Myocardial cells were suspended in 1% NP-40 buffer for 30 min. Protein concentration was measured via the detergent compatible DC Protein Assay (Bio-Rad; Hercules, CA, USA) method after the cells were centrifuged at 12,000 × g for 20 min at 4°C. Thirty micrograms of protein lysate was loaded onto 10% SDS-PAGE gels, and electrophoresis was run at 140 mV for 1 h. Afterwards, the proteins on the gels were transferred onto nitrocellulose membranes at 220 mV and 300 mA for 3 h. The nitrocellulose membranes were then soaked in 5% skimmed milk for 1 h before being incubated with rat anti-Smad4 primary antibody (1:1000; Santa Cruz Biotechnology, sc-73040) and anti-β-actin primary antibody (1:2000; Santa Cruz Biotechnology) separately for 14 h at 4°C. Next, the membrane was incubated in goat anti-mouse secondary antibody (1:5000 Santa Cruz Biotechnology) for 1 h in normal temperature conditions. The Western blot was mapped by the enhanced chemiluminescence (ECL) method. Protein bands were analysed via Image J software (National Institutes of Health, Bethesda, MD, USA).

Luciferase reporter assessment

We used TargetScan software (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) to screen for potential target sequences of miR-130a in the 3′-UTR of Smad4. The wild type and mutated sequences are as follows: wild type Smad4-3′-UTR: 5′-AUUUUGCACUG-3′ and mutant Smad4 3′-UTR: 5′-AGUCUACGCAG-3′. The sequences were amplified and cloned into Promega's psiCHECK™ (Promega Corporation, Madison, WI, USA) reporter plasmids to obtain the plasmids wt-Smad4-3′-UTR and mut-Smad4-3′-UTR. The plasmids were then co-transfected with miR-130a/negative control (NC) mimics into HEK-293 cells. The results of the experiments were analysed following the manufacturer's operation manual. Each experiment was carried out in triplicate.

Bioinformatics

The potential target genes of miR-130a were predicted using TargetScan software.

Statistical analysis

Data were recorded as mean ± standard deviation. GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was employed for statistical analysis. The t test was used for pair-wise comparison, and variance was calculated for multiple comparisons. $P < 0.05$ (indicated by an asterisk) was considered to suggest a significant difference.

RESULTS

miR-130a was considerably increased in hypoxic cardiomyocytes

To determine whether hypoxia affects miR-130a expression in cardiomyocytes, we first examined the level of miR-130a expression in response to hypoxia via qRT-PCR. Data showed that the miR-130a expression in hypoxic cardiomyocytes was greatly increased compared to that in control normoxic cardiomyocytes (Fig. 1A), indicating the important role of miR-130a in hypoxic cardiomyocytes.

Inhibition of miR-130a suppresses hypoxia-induced apoptosis

The biological role of miR-130a in hypoxic cardiomyocytes was determined. The expression of miR-130a was suppressed in hypoxic cardiomyocytes by transfecting an miR-130a inhibitor (Fig. 1B). The effect of the miR-130a inhibitor on cell survival was determined by the MTT assay. The results indicated that inhibition of miR-130a expression considerably improved the survival rate of impaired, hypoxic cardiomyocytes (Fig. 1C). The LDH assay showed that inhibition of miR-130a markedly inhibited hypoxia-induced cell injury in cardiomyocytes (Fig. 1D).

We then evaluated the impact of miR-130a inhibition on apoptosis via caspase-3 analysis. The results indicated that inhibition of miR-130a could lead to a considerable decline in caspase-3 activity in hypoxic cardiomyocytes (Fig. 2A) and to an increase in the expression of the anti-apoptotic protein Bcl-2 (Fig. 2B). In general, the results suggested that inhibition of miR-130a inhibited hypoxia-induced apoptosis of cardiac myocytes.

Smad4 as a target gene of miR-130a

To further explore the mechanism that enables miR-130a inhibition to suppress hypoxia-induced apoptosis, we predicted the target genes of miR-130a via a bioinformatic analysis. Among the identified putative target genes, Smad4, a gene important for cell survival in cardiomyocytes, showed promising characteristics. The binding site for miR-130a at the 3′-UTR of Smad4 is shown in Figure 3A. To determine whether miR-130a directly targets the 3′-UTR of Smad4, we performed a dual-luciferase reporter assay. We found that overexpression of miR-130a considerably inhibited the activity of the luciferase reporter from the vector containing the 3′-UTR of Smad4 (Fig. 3B). However, as shown in Figure 3B, miR-130a overexpression had little influence on the activity of the reporter from the vector bearing the mutated 3′-UTR of Smad4, implying that miR-130a directly targeted the 3′-UTR

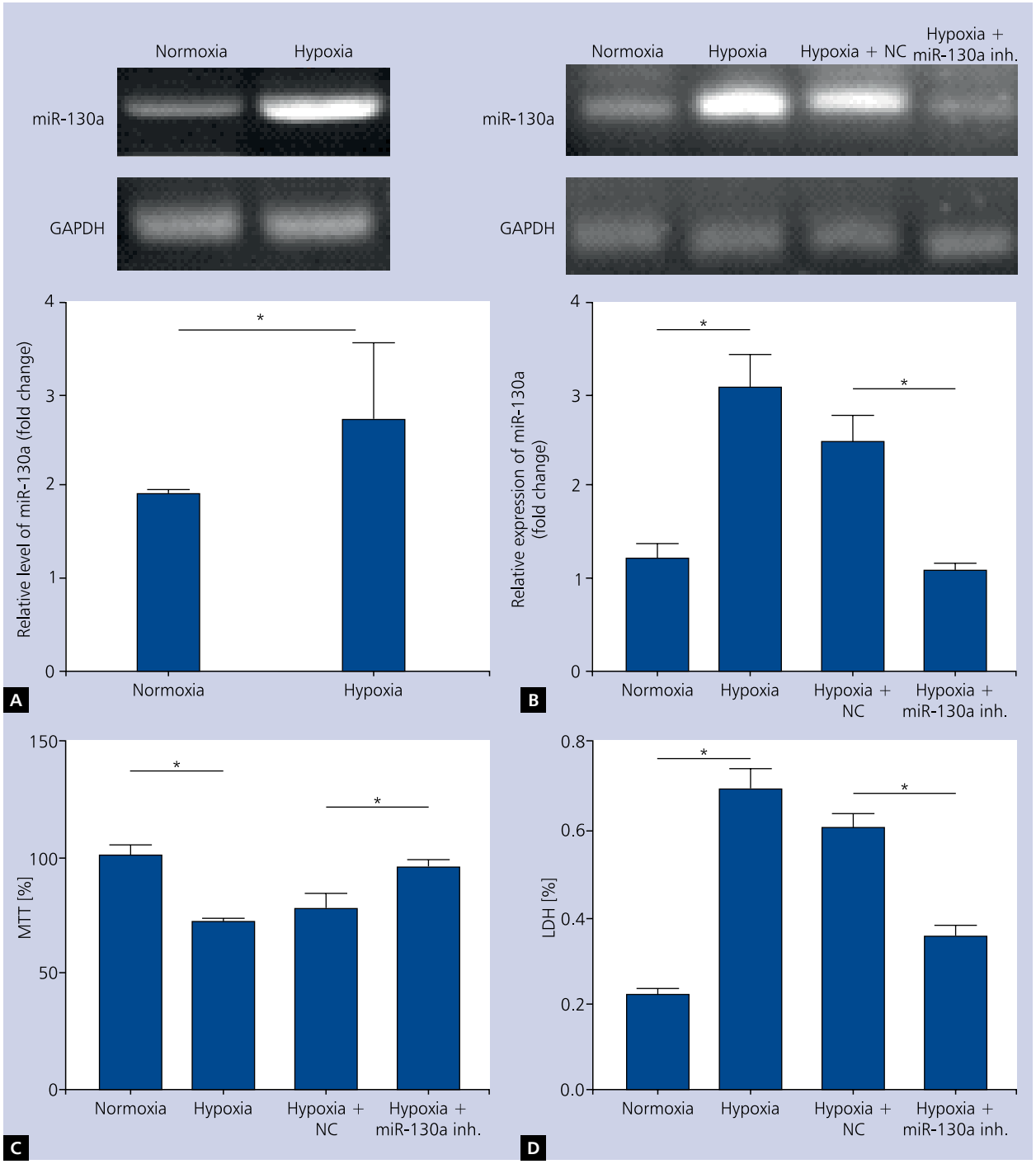

Figure 1. MiR-130a expression in hypoxic cardiomyocytes. H9C2 cells were exposed to hypoxic conditions for 24 h and then harvested for quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis; $p < 0.05$ as compared with cells cultured in normoxic conditions. **A.** Inhibition of miR-130a increased the survival rate of hypoxic cardiomyocytes. H9C2 cells were transfected with either the miR-130a inhibitor or negative control (NC) for 24 h and then exposed to hypoxia for another 24 h. **B.** miR-130a expression was measured through qRT-PCR analysis. Cell survival of cardiomyocytes was determined by MTT (**C**) and lactate dehydrogenase (LDH) (**D**) analysis. Data are presented as mean ± standard deviation from three independent experiments; *$p < 0.05$ as compared with the control group; GAPDH — glyceraldehyde-3-phosphate dehydrogenase; inh. — inhibitor

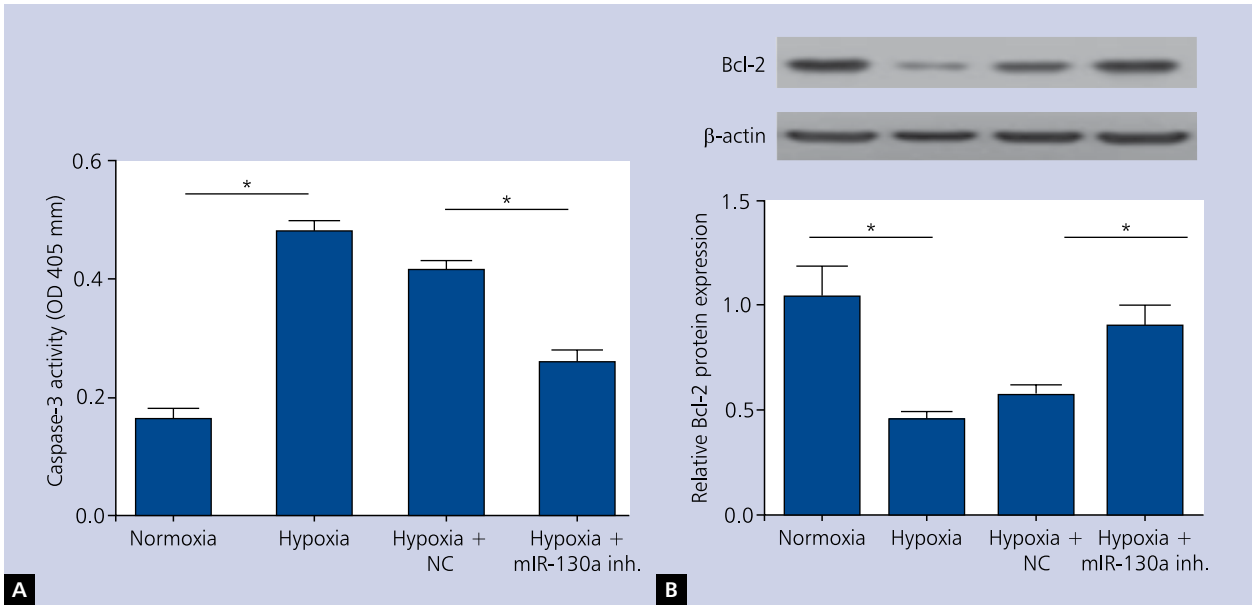

Figure 2. Inhibition of miR-130a reduces apoptosis triggered by hypoxia in myocardial cells. H9C2 cells were transfected with miR-130a inhibitor/negative control (NC) for 24 h and then exposed to hypoxia for another 24 h; **A.** Apoptosis was assessed using the caspase-3 activity assay; **B.** Protein expression level was measured through a Western blot assay. Data are presented as mean ± standard deviation from three independent experiments; *p < 0.05 as compared with the control group; inh. — inhibitor; OD — optical density

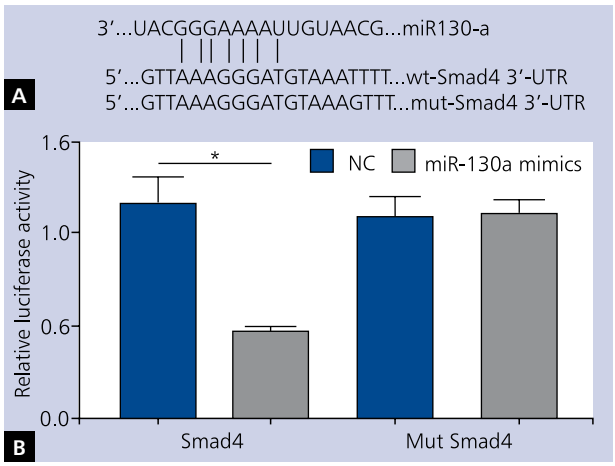

Figure 3. miR-130a targets the 3'-UTR of Smad4. **A.** Sequence alignment of miR-130a and the 3'-UTR of Smad4; **B.** Dual-luciferase reporter gene assay. HEK293 cells were transfected with miR-130a mimics as well as a luciferase reporter gene carrying either the wild type or a mutated form of the Smad4 3'-UTR and incubated for 48 h. Relative luciferase activities were measured through the dual luciferase reporter assay system. Data are presented as mean ± standard deviation from three independent experiments; *p < 0.05 as compared with the control group; NC — negative control

of Smad4. To further validate whether Smad4 is a direct target of miR-130a, we analysed the effect of inhibition of miR-130a on the expression of Smad4. The results showed that inhibition of miR-130a significantly promoted the expression of both Smad4 mRNA (Fig. 4A) and Smad4 protein (Fig. 4B). Overall, these results indicate that Smad4 is a direct target gene of miR-130a.

Inhibition of miR-130a upregulates the TGF-β signalling targeting Smad4

To further investigate the molecular mechanisms underlying the regulation of hypoxia-induced apoptosis by miR-130a, we determined the effect of miR-130a inhibition on TGF-β signalling. The results showed that TGF-β protein levels increased greatly upon miR-130a inhibition (Fig. 4C). Moreover, the expression levels of downstream target genes of the TGF-β signalling pathway, including Smad4, also significantly increased upon miR-130a inhibition. These results suggest that inhibition of miR-130a promotes the activation of TGF-β signalling.

Knockdown of miR-130a abolishes its protective impact

To confirm that TGF-β is involved in the induction of protective mechanisms against hypoxia, upon miR-130a inhibition, we silenced the expression of TGF-β gene while miR-130a was inhibited (Fig. 5A). Similarly, the increased protein levels of Smad4 induced by miR-130a inhibition were markedly decreased upon TGF-β silencing (Fig. 5B). As predicted, the protective effects of miR-130a inhibition against hypoxia-induced apoptosis (Fig. 5C, D) were markedly reversed by

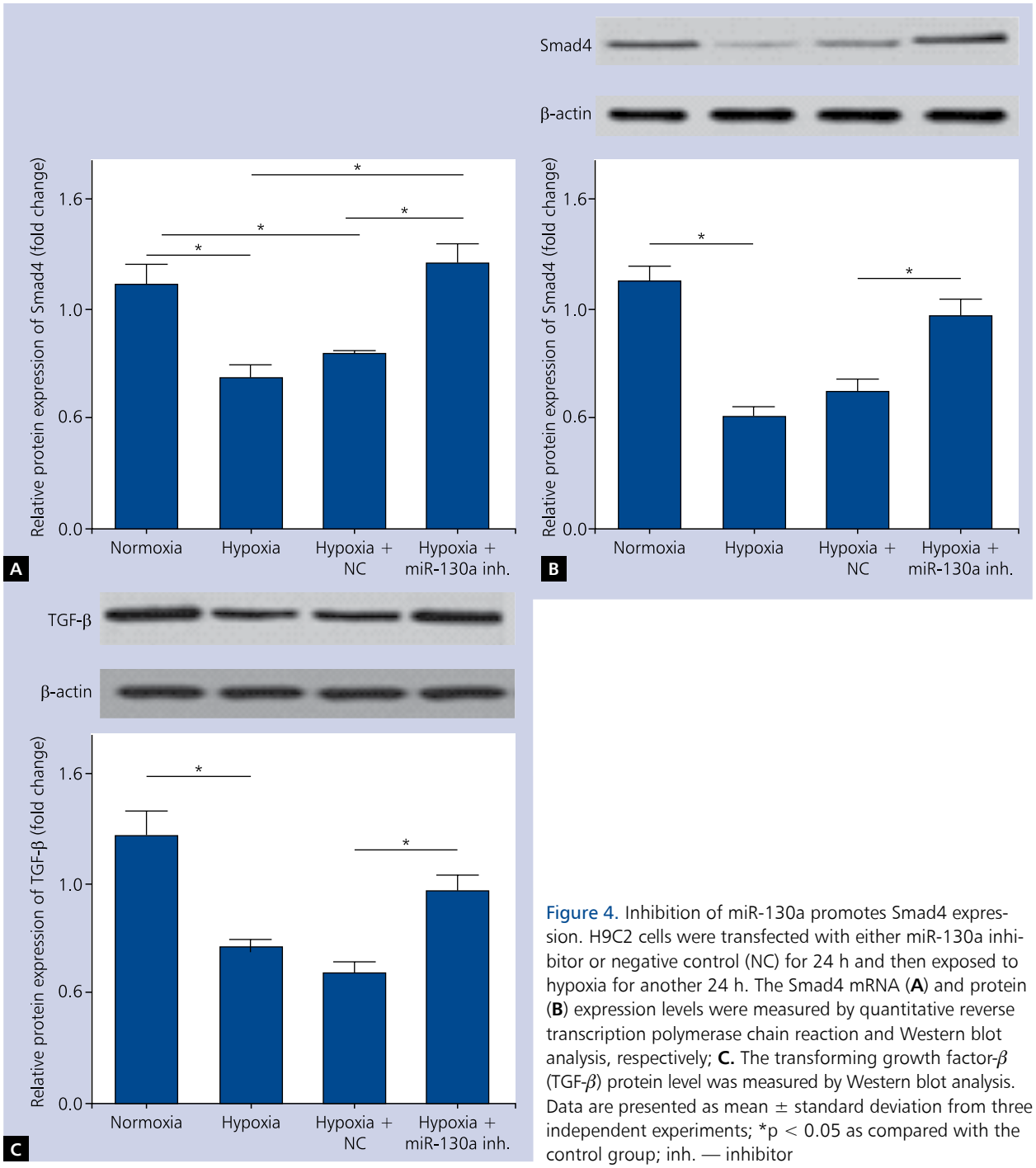

Figure 4. Inhibition of miR-130a promotes Smad4 expression. H9C2 cells were transfected with either miR-130a inhibitor or negative control (NC) for 24 h and then exposed to hypoxia for another 24 h. The Smad4 mRNA (**A**) and protein (**B**) expression levels were measured by quantitative reverse transcription polymerase chain reaction and Western blot analysis, respectively; **C.** The transforming growth factor-β (TGF-β) protein level was measured by Western blot analysis. Data are presented as mean ± standard deviation from three independent experiments; *$p < 0.05$ as compared with the control group; inh. — inhibitor

TGF-β silencing. Overall, these results suggest that miR-130a inhibition protects cardiomyocytes against hypoxia-induced apoptosis by promoting Smad4 expression.

To further verify whether the Smad4 signalling pathway is involved in miR-130a inhibition-induced protective effects against hypoxia, we blocked Smad4 signalling using RepSox and then assessed its effect on miR-130a inhibition-induced protective effects. The results showed that blocking Smad4 signalling significantly decreased the protective effects of miR-130a inhibition against hypoxia-induced apoptosis (Fig. 6A, B), implying that this protection is conferred by miR-130a inhibition through the regulation of the Smad4 signalling pathway.

DISCUSSION

In recent studies, miRNAs have been detected in body fluids, including urine, saliva, and plasma [16]. Numerous miRNAs are dysregulated in MI, serving as potential targets for the de-

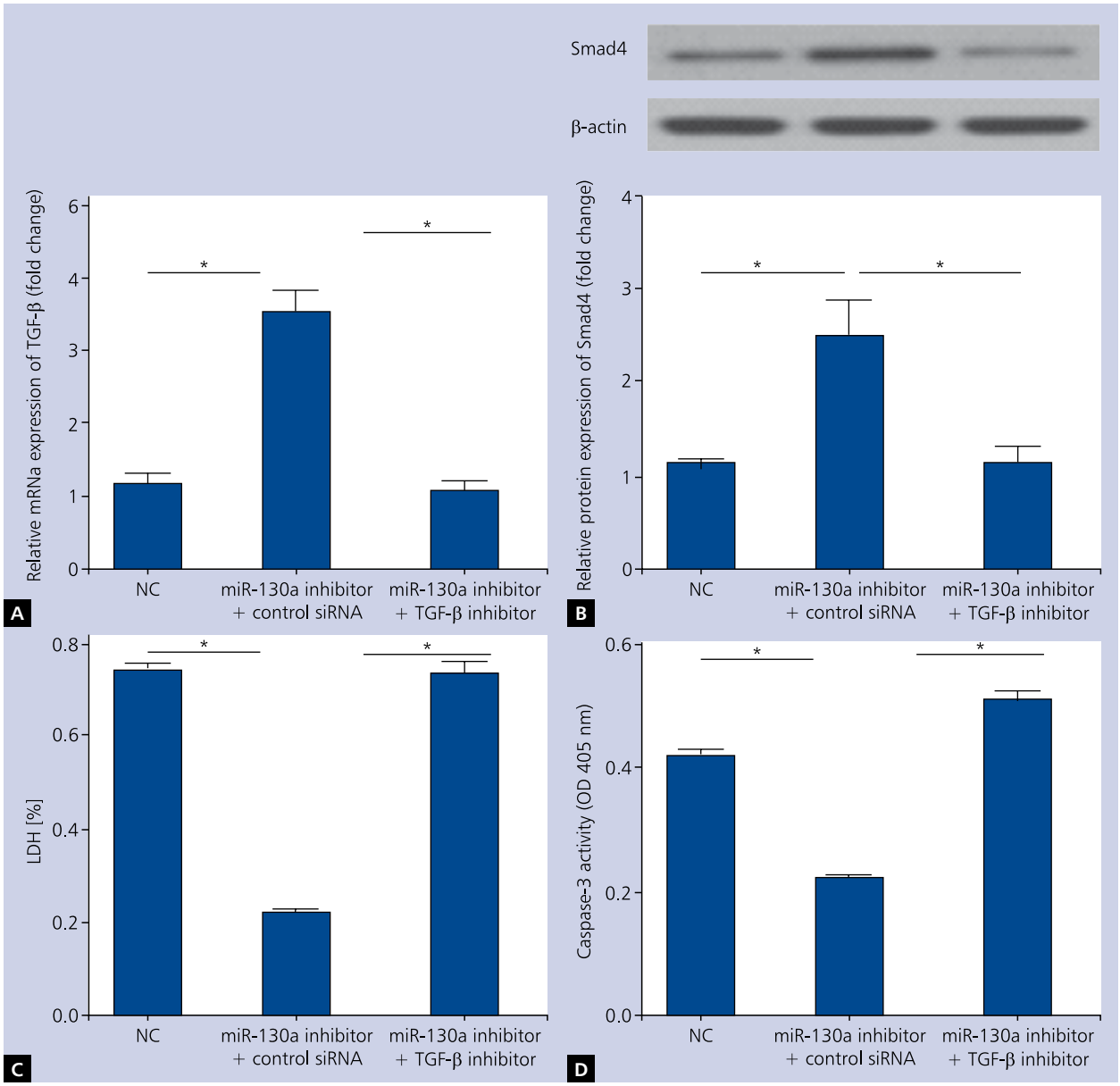

Figure 5. Knockdown of transforming growth factor-β (TGF-β) abolishes the protective effects of miR-130a inhibition. H9C2 cells were co-transfected with an miR-130a inhibitor and a TGF-β siRNA for 24 h and then exposed to hypoxia for another 24 h. Protein expression levels of TGF-β (**A**) and Smad4 (**B**) were measured by Western blot analysis; **C.** Cell injury was assessed by lactate dehydrogenase (LDH) assay; **D.** Apoptosis was evaluated using the caspase-3 activity assay; *$p < 0.05$; NC — negative control; OD — optical density

velopment of miRNA-based therapy. A good understanding of the role of miRNAs in regulating cardiomyocyte apoptosis will help the development of novel therapies for MI. Overall, the limited amount of evidence supporting the use of miRNAs in clinical settings calls for more epidemiological investigation to translate their molecular implications into clinical applications as biomarkers of disease severity, diagnosis, and ultimately prognosis. In the present study, we found that miR-130a expression was greatly increased in hypoxic cardiac myocytes, and the downregulation of miR-130a expression effectively shielded cardiac myocytes from hypoxia-triggered apoptosis. We also showed that the Smad4 gene was the target of miR-130a. The inhibition of miR-130a significantly promoted the activation of Smad4 in hypoxic cardiomyocytes. Blocking of Smad4 significantly abrogated the protective effects of miR-130a inhibition. Our findings indicate that inhibition of miR-130a shields cardiac myocytes from hypoxic apoptosis via the target gene Smad4.

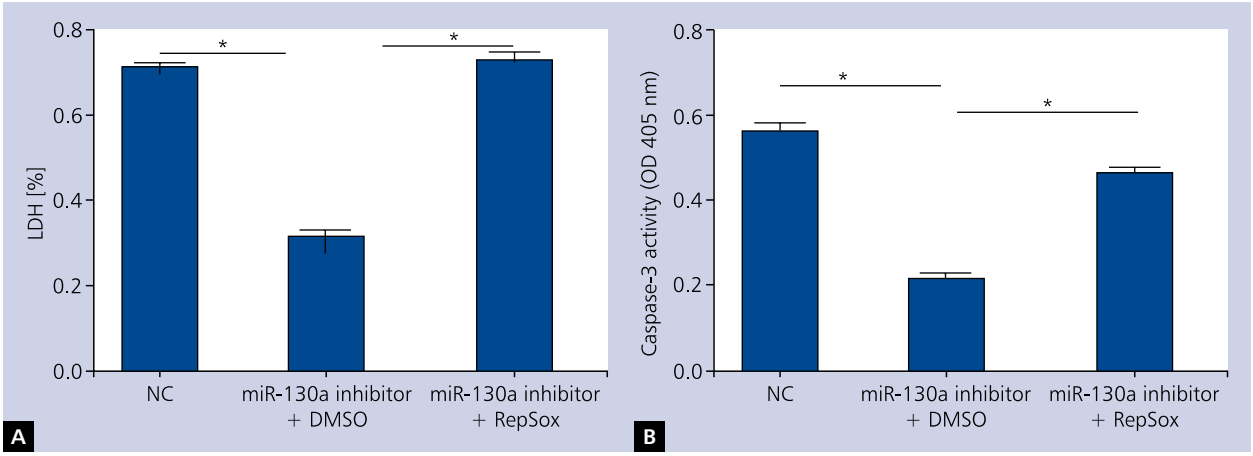

Figure 6. Blocking Smad4 signalling abolishes the protective effects of miR-130a inhibition. H9C2 cells were transfected with an miR-130a inhibitor in the presence of 100 nM RepSox, incubated for 24 h, and then exposed to hypoxia for another 24 h. **A.** Cell injury was assessed by lactate dehydrogenase (LDH) assay; **B.** Apoptosis was evaluated by a caspase-3 activity assay. Data are presented as mean ± standard deviation from three independent experiments; *$p < 0.05$ as compared with the control group; DMSO — dimethyl sulfoxide; NC — negative control; OD — optical density

Through bioinformatic analysis and the dual-luciferase reporter assay, we identified Smad4 as a potential target gene of miR-130a. Unlike other Smads, Smad4, which is active in signalling, influences the interaction between bone morphogenetic proteins (BMPs) and TGF-β. Patients with hereditary disorders that arise due to changes in TGF-β or in the BMP family of genes seldom demonstrate cardiovascular disorders that correspond to the affected pathway [17]. Smad4 mutations separate family members into various disease groups such as aortopathy, juvenile polyposis syndrome, and mitral valve disorder [18]. Smad4 is a tumour suppressor that transduces TGF-β signalling and regulates genomic stability [19]. The expression of SMADs in the ischaemic cardiac muscles changes significantly following MI, suggesting a close relationship between Smad4 proteins and heart remodelling. Moreover, it is known that the expression of Smad4 is regulated by TGF-β and BMPs [20]. Smad4 is also involved in the regulation of MI. Based on these findings, regulating the expression of Smad4 proteins by targeting TGF-β and BMP in the ischaemic myocardium may be considered as a potential treatment for MI. In this study it has been revealed that miR-130a inhibition causes de-repression of Smad4 and therefore attenuation of cardiomyocyte apoptosis (Smad4 is a protective agent for myocardial cells).

Overall, our research implied that miR-130a regulates cardiomyocyte apoptosis. We found that miR-130a inhibition shielded cardiac myocytes from apoptosis triggered by hypoxia through the upregulation of Smad4 expression and activation of Smad4 signalling. Our study provides a novel understanding of the molecular mechanism of cardiomyocyte death triggered by hypoxia and offers a possible target in the treatment of MI. However, the precise effect of Smad4 inhibition on the treatment of MI needs to be further investigated in animal models.

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Conflict of interest: *none declared*

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