Downregulation of lncRNA ZFAS1 protects H9c2 cardiomyocytes from ischemia/reperfusion-induced apoptosis via the miR-590-3p/NF-κB signaling pathway

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Abstract. Long non-coding RNA (lncRNA) ZNFX1 antisense RNA 1 (ZFAS1) is upregulated in acute myocardial infarction; however, the role of ZFAS1 in myocardial ischemia/reperfusion (I/R) injury remains unknown. The present study aimed to detect microRNA (miR)-590-3p expression levels in cardiomyocytes subjected to I/R, and to investigate the effects of ZFAS1 on myocardial I/R injury. An *in vitro* model of I/R injury was established using rat H9c2 cardiomyocytes exposed to hypoxia/reoxygenation (H/R). It was demonstrated that ZFAS1 was upregulated and miR-590-3p was downregulated in the *in vitro* model of cardiac I/R injury. Western blotting results indicated that the protein expression levels of p50, tumor necrosis factor-α (TNF-α), interleukin (IL)-6, Bax and cleaved caspase-3 were upregulated, and the expression levels of Bcl-2 and pro-caspase-3 were downregulated. Flow cytometry results revealed that downregulation of ZFAS1 reduced H/R-induced apoptosis in H9c2 cells. In addition, downregulation of ZFAS1 significantly increased the expression of miR-590-3p, and p50 was identified as a target gene of miR-590-3p. Furthermore, with 12 h of hypoxia followed by 2 h of reoxygenation in H9c2 cells, ZFAS1 knockdown increased the expression levels of miR-590-3p, Bax and cleaved-caspase-3, and decreased the expression levels of Bcl-2 and pro-caspase-3. It was also found that the miR-590-3p-mimic transfection increased the expression levels of Bax and cleaved-caspase-3, and decreased the protein expression levels of p50, TNF-α, IL-6, Bcl-2 and pro-caspase-3. In addition, TNF-α treatment induced apoptosis of H9c2 cells, and the changes in Bax, Bcl-2, cleaved-caspase-3 and pro-caspase-3 expression levels in a dose-dependent manner. Collectively, the present results suggested that ZFAS1 was upregulated in H9c2 cells subjected to I/R injury, and that ZFAS1 knockdown protected against I/R-induced myocardial cell apoptosis by directly interacting with miR-590-3p, via the NF-κB pathway.

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Introduction

Coronary artery disease has one of the highest morbidity and mortality rates of any disease worldwide, and myocardial infarction is the most common coronary artery disease (1,2). Percutaneous coronary intervention (PCI) is the primary effective treatment for myocardial infarction in clinical practice; it can clear narrow lumens and occlude the coronary lumen, but ischemia-reperfusion (I/R) injury is the most important obstacle to PCI treatment (1,2). I/R injury is one of the main mechanisms of arrhythmia, myocardial contractile dysfunction and irreversible damage of cardiomyocytes (3). Increased inflammation induced by myocardial I/R is one of the main causes of myocardial cell apoptosis (4). However, the release of inflammatory mediators also initiates the repair of damaged tissues in the body (5,6). Moreover, inflammatory cytokines can induce cardiomyocyte apoptosis, which further promotes increases in inflammatory cytokine levels (7,8).

Long non-coding RNA (lncRNA) is a type of RNA that is >200 bp in length with no or little open reading frame, which does not encodes a protein (9,10). With the development of gene sequencing, gene chips and genomics, numerous lncRNAs have been revealed to be involved in the regulation of inflammation (9,11). Previous studies had reported that lncRNAs are not only involved in the development of cardiovascular diseases, such as cardiac hypertrophy, myocardial infarction, heart failure and myocardial fibrosis (12,13), but also in inflammation and inflammatory diseases through the regulation of numerous gene expression and signaling pathways (14).

lncRNA ZNFX1 antisense RNA 1 (ZFAS1) is abnormally expressed in patients with acute myocardial infarction (15) and in atherosclerotic model rats (16). Furthermore, lncRNA

ZFAS1 was reported to contribute to the impairment of cardiac contractile function in myocardial infarction (17,18). The aim of the present study was to investigate the relationship between lncRNA ZFAS1 and I/R injury using hypoxia/reoxygenation (H/R)-induced H9c2 cardiomyocytes as an *in vitro* model to examine apoptosis and inflammation. It was found that H/R increased the expression level of ZFAS1 in H9c2 cells, and that ZFAS1 knockdown reduced inflammation and apoptosis by targeting the microRNA (miR)-590-3p/NF-κB pathway.

Materials and methods

Cell culture and H/R stress. Rat H9c2 cardiomyocyte cells (cat. no. CRL-1446; American Type Culture Collection) were cultured with DMEM (cat. no. 12491-15; Thermo Fisher Scientific, Inc.) with 10% of FBS (cat. no. 10100-147; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (cat. no. 15640055; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. H/R treatment was used to establish an I/R injury model in H9c2 cells. H9c2 cells were sequentially exposed to hypoxia for 2, 4, 8, 12, 18 and 24 h (95:5 CO_2:N_2 ratio) at 37˚C and re-oxygenated for (95:5 O_2:CO_2 ratio) 2 h at 37˚C (19).

Cell transfection. Small interfering (si)RNA for ZFAS1 knockdown (si-ZFAS1 forward, 5'-UGGAUUUGUACC AUUCUUCUG-3' and reverse, 5'-GAAGAAUGGUAC AAAUCCAAG-3'), negative control knockdown (si-NC forward, 5'-AGUUUCAACCGUCUUAAUCAG-3' and reverse, 5'-GAUUAAGACGGUUGAAACUAG-3'), hsa-miR-590-3p-inhibitor (5'-AAUUUUCAUAUUCGA UCA-3'), hsa-miR-590-3p-mimic (5'-UUAAAAGUAUAA GCUAGU-3') and hsa-miR-590-5p-NC (5'-GGAUGGCCA AUCUUCGCGGGCU-3') were designed and synthesized by Shenggong Bioengineering Co., Ltd. Cells ($1x10^6$) were directly transfected with 25 nmol si-RNA, si-NC, miR-inhibitor, miR-mimic or miR-NC using Lipofectamine® 2000 transfection reagent (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 72 h. Subsequent experiments were performed at 72 h post-transfection.

Dual-luciferase reporter assay. The StarBase database (starbase.sysu.edu.cn/index.php) was used to identify the binding sites between miR-590-3p and ZFAS1. The wild-type (WT) or mutant (MUT) mRNA 3'-untranslated regions (UTRs) of ZFAS1 and p50 were cloned into the psiCHECK2 vector (Promega Corporation). Cells ($5x10^6$) were transfected with psiCHECK2 vectors using Lipofectamine® 2000. The Dual-Lucy Assay kit (cat. no. D00100; Beijing Solarbio Science & Technology Co., Ltd.) was used to detect luciferase activities according to the manufacturer's protocol. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Reverse transcription-quantitative PCR (RT-qPCR). RT-qPCR was used to detect the mRNA expression levels of U6, miR-590-3p and lncRNA ZFAS1 in cells. TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract the total RNA from H9c2 cells. The extracted RNA was reverse transcribed into cDNA using PrimeScript RT Master mix RT kit (cat. no. RR036B; Takara Bio, Inc.) at 37˚C for 15 min and 85˚C for 15 sec. qPCR was set up and conducted according to the SYBR Green qPCR Master Mix kit instructions (cat. no. 638320; Takara Bio, Inc.) and amplified using an ABI 7500 fluorescence qPCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation at 94˚C for 30 sec; 40 cycles of 93˚C for 2 min, 93˚C for 1 min and 55˚C for 2 min; followed by final extension at 72˚C for 1.5 min. PCR primers were as follows: U6, forward 5'-AUA AAUCCCUUUACACCUCTT-3', reverse 5'-AAUAAAUCC CUUUACACCUCTT-3'; GAPDH, forward 5'-AGGTCGGTG TGAACGGATTTG-3', reverse, 5'-GGGGTCGTTGATGGC AACA-3'; miR-590-3p, forward 5'-ACACTCCAGCTGGGT GATCGAATATGTAT-3', reverse 5-TGGTGTCGTGGAGTC G-3; and ZFAS1, forward 5'-ACGTGCAGACATCTACAA CCT-3', reverse 5'-TACTTCCAACACCCGCAT-3'. miRNA and mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (20) and normalized to the internal reference genes U6 and GAPDH, respectively.

Western blotting. Total protein was extracted from H9c2 cells using RIPA lysis buffer (cat. no. P0013K; Beyotime Institute of Biotechnology) and quantified using the BCA Protein Assay kit (cat. no. P0010S; Beyotime Institute of Biotechnology). Proteins (50 μg) were separated via 12% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk powder at room temperature for 2 h. Membranes were incubated overnight at 4˚C with the following primary antibodies: Anti-p50 (1:1,000; cat. no. ab32360; Abcam), anti-tumor necrosis factor-α (TNF-α; 1:2,000; cat. no. ab6671; Abcam), anti-interleukin (IL)-6 (1:1,000; cat. no. 12912; Cell Signaling Technology, Inc.), anti-Bax (1:3,000; cat. no. ab32503; Abcam), anti-Bcl-2 (1:500; cat. no. ab692; Abcam), anti-cleaved-caspase 3 (1:5,000; cat. no. ab2302; Abcam), anti-pro-caspase-3 (1:10,000; cat. no. ab32499; Abcam) and anti-GAPDH (1:3,000; cat. no. ab9484; Abcam). The membranes were subsequently incubated with the following horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature: Goat anti-mouse (cat. no. ab6789; 1:3,000; Abcam) or goat anti-rabbit (cat. no. ab6721; 1:3,000; Abcam). Protein bands were visualized using the BeyoECL Plus kit (cat. no. P0018S; Beyotime Institute of Biotechnology). Protein expression levels were quantified using ImageJ software (version 1.8.0; National Institutes of Health) with GAPDH as the loading control.

MTT assay. H9c2 cells ($2x10^4$ cells/well) were seeded into a 96-well plate and cultured for 12 h in 5% CO_2 at 37˚C. Cells were subjected to H/R exposure and then washed twice with PBS. Cell viability was measured using an MTT assay kit (cat. no. C0009; Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The absorbance was measured in a Bio-Rad 680 microplate reader at 490 nm (Bio-Rad Laboratories, Inc.).

Flow cytometry. H9c2 cells ($2x10^6$) were treated with 0, 1, 2 and 4 μg/ml TNF-α (cat. no. P6231; Beyotime Institute of Biotechnology) at 37˚C for 12 h. H9c2 cells ($1x10^6$) were collected and an Annexin V FITC/PI kit (Invitrogen; Thermo

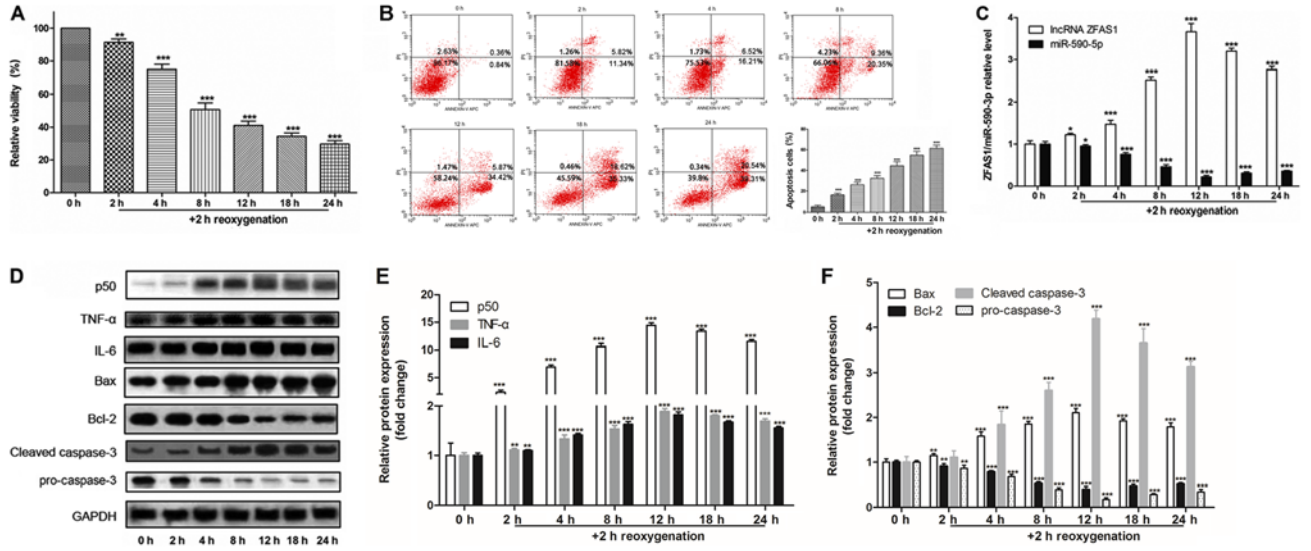

Figure 1. H/R induces cell apoptosis and gene expression changes in H9c2 cells. (A) MTT assay for cell viability following H/R exposure. (B) Apoptotic rate of cells was detected by flow cytometry. (C) Reverse transcription-quantitative PCR was used to detect the expression level of ZFAS1, which was normalized by GAPDH, and of miR-590-5p, which was normalized by U6. (D) Representative western blotting images used to determine protein expression levels of (E) p50, TNF-α and IL-6, and (F) Bax, Bcl-2, pro-caspase-3 and cleaved caspase-3. *P<0.05, **P<0.01, ***P<0.001 vs. 0 h. H/R, hypoxia/reoxygenation; IL, interleukin; miR, microRNA; TNF-α, tumor necrosis factor-α; ZFAS1, ZNFX1 antisense RNA 1.

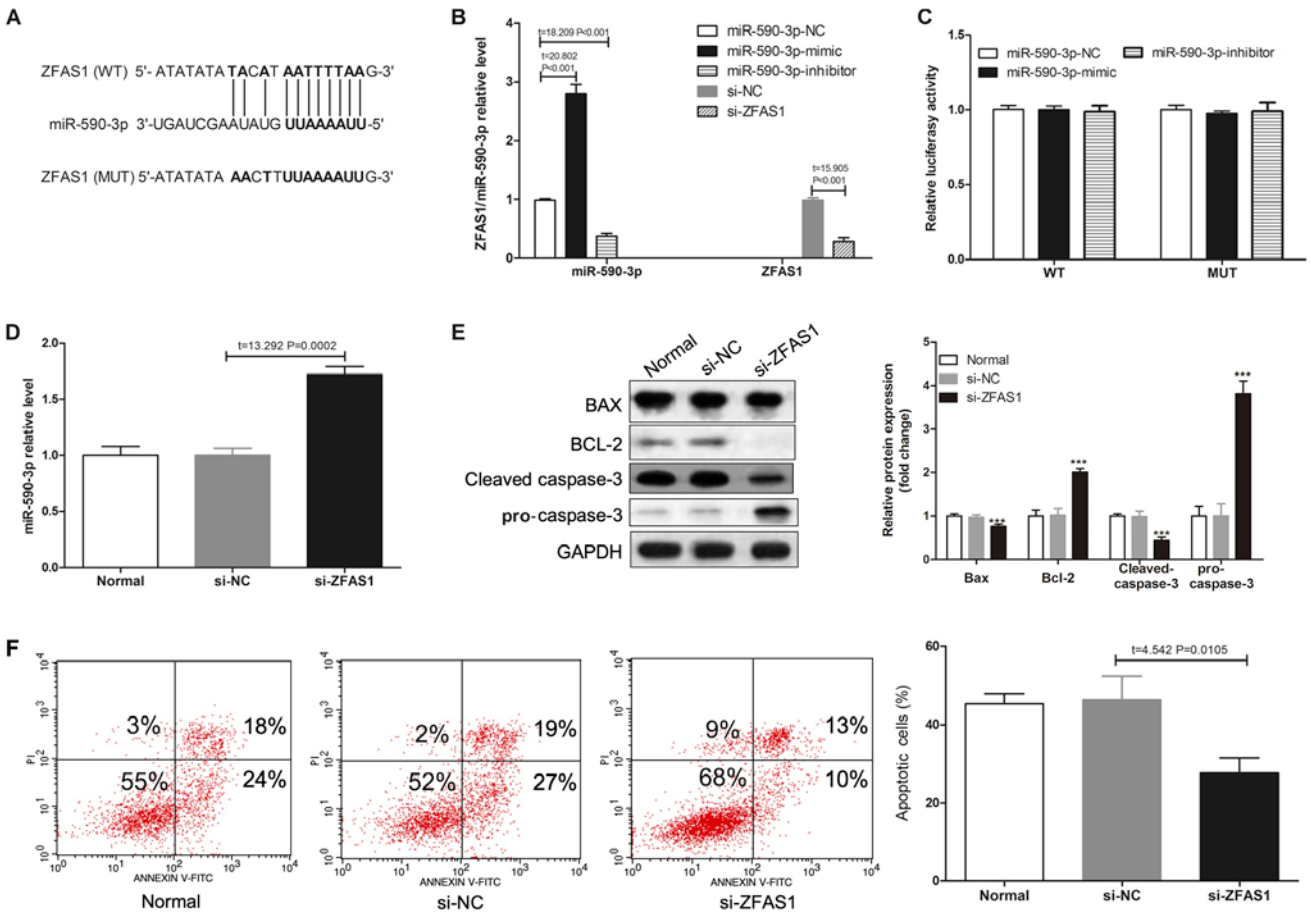

Figure 2. ZAFS1 knockdown reduces H/R-induced H9c2 apoptosis. (A) A WT-ZFAS1 3'UTR luciferase reporter vector and a MUT-ZSAF1 3'UTR luciferase reporter vector with mutations on the miR-590-3p binding sites of the ZFAS1 3'UTR were constructed. (B) RT-qPCR was used to detect the expression levels of ZAFS1 or miR-590-3p in H9c2 cells without H/R induction. (C) miR-590-3p-NC, miR-590-3p-mimic and miR-590-3p-inhibitor were transected into H9c2 cells, and luciferase activity was determined. (D) RT-qPCR results of miR-590-3p expression level in H9c2 cells transfected with si-ZFAS1. (E) Western blotting was used to detect the protein expression levels of Bax, Bcl-2, pro-caspase-3 and cleaved caspase-3 in H9c2 cells after si-ZFAS1 transfection and H/R injury. (F) Flow cytometry results of the percentage of apoptotic cells of different transfected groups following H/R induction. ***P<0.001 vs. si-NC group. 3'UTR, 3'untranslated regions; H/R, hypoxia/reoxygenation; miR, microRNA; MUT, mutation; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR; WT, wild-type; ZFAS1, ZNFX1 antisense RNA 1.

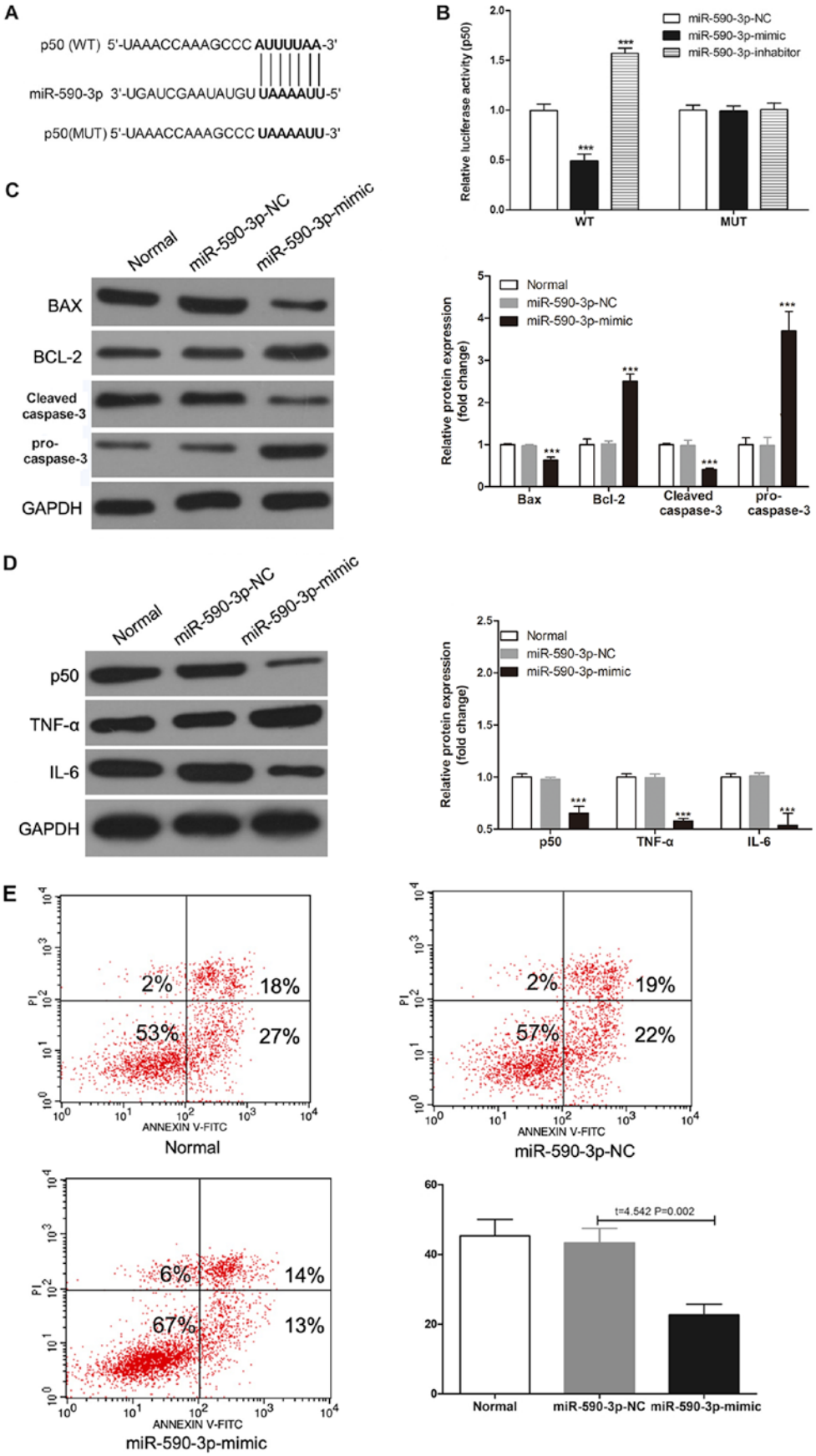

Figure 3. miR-590-3p targets p50 and reduces H/R-induced apoptosis and inflammation. (A) A WT-p50 3'UTR and a MUT-p50 3'UTR luciferase reporter vector, with mutations on miR-590-3p binding sites of the p50 3'UTR were constructed. (B) miR-590-3p-NC, miR-590-3p-mimic or miR-590-3p-inhibitor were transected into H9c2 cells, and luciferase activity was detected. (C) Protein expression levels of Bax, Bcl-2, pro-caspase-3 and cleaved-caspase-3, and (D) p50, TNF-α and IL-6 were detected by western blotting in H/R-induced H9c2 cells. (E) Flow cytometry results of the percentage of apoptotic cells in different transfected groups with H/R. ***P<0.001 vs. miR-590-3p-NC. 3'UTR, 3'untranslated region; H/R, hypoxia/reoxygenation; IL, interleukin; miR, microRNA; MUT, mutation; NC, negative control; TNF-α, tumor necrosis factor-α; WT, wild-type.

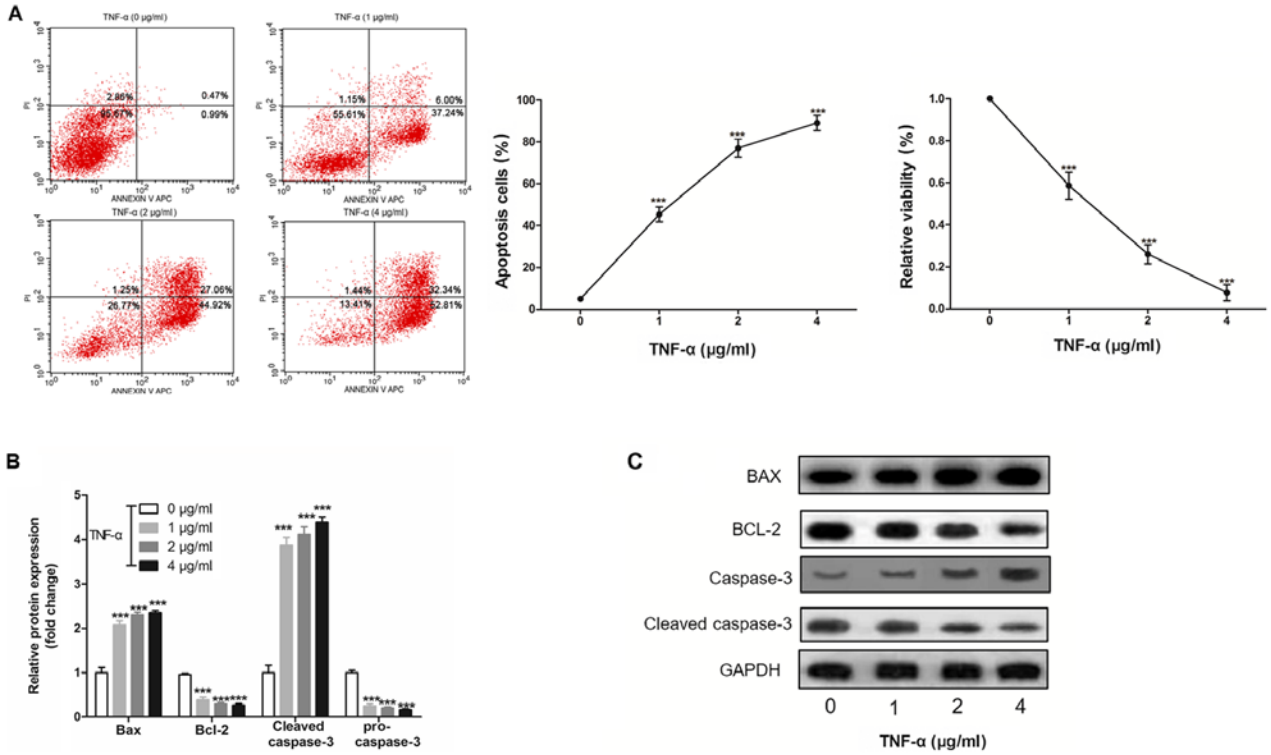

Figure 4. TNF-α induces H9c2 cell apoptosis in a dose-dependent manner. (A) Apoptotic rate of H9c2 cell was detected by flow cytometry in cells treated with 0, 1, 2 and 4 μg/ml TNF-α for 12 h. (B) MTT was used to detect the viability of H9c2 cells exposed to TNF-α. (C) Western blotting was used to detect the protein expression levels of Bax, Bcl-2, pro-caspase-3 and cleaved-caspase-3. ***P<0.001 vs. 0 μg/ml group, TNF-α, tumor necrosis factor-α.

Fisher Scientific, Inc.) was used for flow cytometric analysis to detect apoptosis, according to the manufacturer's protocol. A Beckman CytoFLEX flow cytometer (Beckman Coulter, Inc.) and FlowJo software (version 10.0.7; FlowJo LLC) were used to analyze the rate of apoptosis.

Statistical analysis. Data are presented as the mean ± SD, and were analyzed using SPSS 20.0 (IBM Corp.). Student's t-test was used to compare differences between two groups, and one-way ANOVA with Tukey's test was used to compare the difference between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

H/R induces cell apoptosis and gene expression changes in H9c2 cells. H9c2 cells were cultured under anoxic conditions for 2, 4, 8, 12, 18 and 24 h, and then re-oxygenated for 2 h to establish an *in vitro* I/R model. MTT assay (Fig. 1A) and flow cytometry (Fig. 1B) results suggested that H/R-induced cell injury decreased cell viability and increased apoptotic rates in a time-dependent manner.

To investigate the underlying molecular mechanisms, the expression levels of genes of interest were assessed. It was demonstrated that, compared with control cells (0 h), H/R increased the expression levels of ZFAS1 (Fig. 1C), as well as the protein expression levels of p50, TNF-α, IL-6, Bax and cleaved-caspase-3 (Fig. 1D-F). Furthermore, H/R decreased the expression levels of miR-590-3p (Fig. 1C), as well as the protein expression levels of Bcl-2 and pro-caspase-3 (Fig. 1D and F). Therefore, the present results suggested that lncRNA ZFAS1 and miR-590-3p may be related to H/R-induced apoptosis of H9c2 cells.

lncRNA ZFAS1 regulates apoptosis in H9c2 cells. The StarBase database was used to identify the binding sites between miR-590-3p and ZFAS1 (Fig. 2A). miR-590-3p-mimic significantly increased the expression of miR-590-3p, whereas miR-590-3p-mimic significantly decreased the expression of miR-590-3p compared with miR-590-3p-NC. Moreover, si-ZFAS1 significantly decreased the expression of ZFAS1 compared with si-NC (Fig. 2B). Results from the luciferase gene reporter assay indicated that infection with miR-590-3p mimic or miR-590-3p inhibitor did not change the expression level of ZFAS1 (Fig. 2C). However, ZFAS1 knockdown increased the expression level of miR-590-3p (Fig. 2D). Furthermore, flow cytometry results suggested that lncRNA ZFAS1 knockdown could affect the protein expression levels of Bax, Bcl-2, cleaved-caspase-3 and pro-caspase-3 (Fig. 2E), as well as decrease H/R-induced apoptosis in H9c2 cells (Fig. 2F). Collectively, the results indicated that ZFAS1 knockdown reduced H/R-induced H9c2 apoptosis, and ZFAS1 knockdown increased miR-590-3p expression.

miR-590-3p regulates inflammation and apoptosis in H9c2 cells. The StarBase was also used to searched for target sites of miR-590-3p in p50 3'UTR (Fig. 3A). To assess whether miR-590-3p can regulate p50 expression level, a luciferase gene reporter assay was performed. It was demonstrated that transfection of miR-590-3p mimic significantly decreased WT type 3'UTR luciferase activity in H9c2 cells (P<0.001; Fig. 3B); however, no effect was observed with the MUT in

any group. Furthermore, the miR-590-3p mimic transfection could decrease the protein expression levels of Bax and cleaved-caspase-3, and increase the protein expression levels of Bcl2 and pro-caspase-3 (Fig. 3C). Furthermore, miR-590-3p mimic decreased the expression levels of p50, TNF-α, IL-6 in H9c2 cells following H/R injury (Fig. 3D). In addition, transfection with the miR-590-3p mimic decreased H/R-induced apoptosis (Fig. 3E). Therefore, these results suggested that miR-590-3p may have a protective effect in reducing H/R-induced apoptosis by targeting p50.

H9c2 cell apoptosis is induced by TNF-α in a dose-dependent manner. To investigate the effect of inflammation on apoptosis, H9c2 cells were treated with TNF-α, and it was revealed that TNF-α induced H9c2 cell apoptosis and cell viability in a dose-dependent manner (Fig. 4A and B). Furthermore, it was demonstrated that TNF-α altered the protein expression levels of Bax, Bcl-2, cleaved-caspase-3 and pro-caspase-3 in a dose-dependent manner (Fig. 4C).

Discussion

The present study established an I/R injury *in vitro* model by H/R exposure in H9c2 rat cardiomyocytes, and found that H/R increased the expression levels of ZFAS1, p50, TNF-α and IL-6, decreased miR-590-3p expression, and induced apoptosis of H9c2 cells in a time-dependent manner. There have been an increasing number of studies investigating the potential role of lncRNA in heart disease, which have reported that lncRNAs serve a key role in the regulation of heart disease, such as cardiac hypertrophy, cardiac graft rejection and ischemic heart failure (21,22). Moreover, lncRNA ZFAS1 is abnormally expressed in patients with acute myocardial infarction (15) and atherosclerotic model rats (16), and contributes to the impairment of cardiac contractile function in myocardial infarction (17,18).

lncRNAs are non-coding RNAs that exert biological functions by regulating the expression levels of other genes (9,10). Previous studies have shown that there are several mechanisms by which lncRNAs can regulate gene expression (23,24). The interaction mechanism between lncRNAs and miRNAs is an important way that lncRNAs regulate gene expression (25). However, lncRNA not only acts as a target for miRNAs to inhibit their binding to target genes, but also an endogenous miRNA sponge that can inhibit the expression of miRNAs and indirectly inhibits the negative control of miRNAs to target genes (9,10). Moreover, RNA competes with miRNAs to bind to the 3'UTR of target gene mRNA (9,10). In addition, miRNAs can target a large number of protein-coding genes and lncRNAs. The present results suggested that ZFAS1 could directly bind to miR-590-3p and inhibit its expression. There had been a number of previous studies investigating the relationship between miR-590-3p and the NF-κB signaling pathway. For example, Zhao *et al* (26) found that miR-590-3p is a novel miRNA in myocarditis by targeting NF-κB *in vivo*. In addition, Bao *et al* (27) showed that miR-590 protects against oxidized low-density lipoprotein-induced endothelial cell apoptosis via the p53/NF-κB pathway. The present study results indicated that p50 was a target gene of miR-590-3p, and that the miR-590-3p-mimic could downregulate the protein expression levels of p50, TNF-α and IL-6. Moreover, it was found that ZFAS1 knockdown or miR-590-3p overexpression attenuated H/R-induced apoptosis in H9c2 cardiomyocytes.

The important role of lncRNA regulation of the NF-κB signaling pathway has been a focus of research into inflammatory diseases. The lncRNA Lethe binds directly to the NF-κB heterodimeric subunit RelA and inhibits the DNA-binding activity of NF-κB (28). Thus, Lethe acts as a negative feedback regulator of the TNF-α pathway and regulates the inflammatory response (29). lncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) inhibits DNA binding activity of NF-κB, reduces inflammatory cytokine production and downregulates the autoimmune inflammatory response (30). Furthermore, knockdown of MALAT1 upregulates lipopolysaccharide-induced TNF-α and IL-6 expression (30). In I/R injury, the inflammatory response plays a key role in myocardial I/R injury and occurs during the whole process of myocardial cell injury (31). Moreover, adhesion molecules and cytokines involved in the inflammatory reaction have the same NF-κB gene initiation site (32), and NF-κB is activated to mediate overexpression of these factors, thus aggravating the inflammatory response after myocardial I/R (33).

p50 protein is an important part of the NF-κB signaling pathway, and the dimeric complex consisting of p50-p65 is called the standard NF-κB protein complex (34). A previous study found that deletion of the NF-κB subunit p50 reduces I/R injury *in vivo* (35). The present results suggested that the miR-590-3p-mimic decreased the protein expression level of p50, thus miR-590-3p may inhibit the activation of the NF-κB signaling pathway. In addition, it was found that H9c2 cardiomyocyte apoptosis was induced by TNF-α in a dose-dependent manner. Therefore, downregulation of lncRNA ZFAS1 may protect H9c2 cardiomyocytes from I/R-induced apoptosis via the miR-590-3p/NF-κB pathway. However, there are some limitations to the present study, including the lack of *in vivo* experiments and the absence of clinical data.

In conclusion, it was demonstrated that ZFAS1 was upregulated and miR-590-3p was downregulated in H9c2 cells subjected to I/R injury. Furthermore, the present results suggested that downregulation of ZFAS1 protected against I/R-induced myocardial cell apoptosis by increasing miR-590-3p expression via the NF-κB signaling pathway.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY and YS conceived and designed the present study. PH and DY performed the experiments and analyzed the data.

LY substantially contributed to drafting the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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