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Overexpression of miRNA-410-3p protects hypoxia-induced cardiomyocyte injury *via* targeting TRAF5

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Abstract. – OBJECTIVE: This study aims to clarify the influence of microRNA-410-3p (miRNA-410-3p) on hypoxia-induced injury in cardiomyocytes.

MATERIALS AND METHODS: MiRNA-410-3p level, apoptotic rate, and cell viability in AC16 cells undergoing normoxia or hypoxia preconditioning were assessed. The regulatory effects of miRNA-410-3p and TRAF5 on the proliferative and apoptotic abilities of AC16 cells were evaluated. The binding relationship between miRNA-410-3p and TRAF5 was verified by Dual-Luciferase Reporter Gene Assay.

RESULTS: Hypoxia preconditioning triggered apoptosis and inhibited the viability in AC16 cells. MiRNA-410-3p was downregulated in cardiomyocytes under the hypoxic environment. The overexpression of miRNA-410-3p stimulated proliferation and inhibited apoptosis in hypoxia preconditioning AC16 cells. TRAF5 was proved to be the target of miRNA-410-3p. TRAF5 level was negatively regulated by miRNA-410-3p. The silence of TRAF5 could reverse viability and apoptosis changes in hypoxic AC16 cells overexpressing miRNA-410-3p.

CONCLUSIONS: MiRNA-410-3p protects hypoxia-induced proliferation suppression and apoptosis stimulation in cardiomyocytes *via* targeting TRAF5.

Key Words:
MiRNA-410-3p, TRAF5, Hypoxia, Cardiomyocytes.

Introduction

Acute myocardial infarction (AMI) is a clinical emergency with high mortality and severe complications resulted from myocardial ischemia and hypoxia. AMI is a major public health problem worldwide[1]. AMI leads to cell damage or apoptosis in cardiomyocytes because of persistent ischemia and hypoxia. Cardiomyocyte apoptosis at post-AMI is significant in cardiac remodeling and cardiac function improvement. The suppression of cardiomyocyte apoptosis and induction of cell survival are the key events in the treatment of AMI[2]. It is necessary to illustrate the mechanism underlying hypoxia-induced cardiomyocyte apoptosis.

MicroRNAs (miRNAs) are a group of highly conserved, non-coding, single-stranded molecules with 22-25 nucleotides long. They are capable of regulating the gene expressions and protein translation, thus participating in various life activities[3-5]. In recent years, miRNAs are discovered to be extensively involved in cardiovascular diseases, which are vital in mediating cardiomyocyte behaviors[6,7], angiogenesis[8], and function repair of progenitor cells and stem cells[9]. A plenty number of miRNAs, which could significantly suppress cardiomyocyte apoptosis, have been identified[10-16].

MiRNA-410-3p is differentially expressed in many types of tumors, suggesting the potential function during tumorigenesis and tumor progression[17-19]. Its specific function in AMI, however, remains unclear.

TRAF5 is a member of the recently discovered tumor necrosis factor receptor-associated factor (TRAF) family. The structure of the ring-type zinc finger gives TRAF5 the activity of E3 ubiquitin ligase. As a cytoplasmic adaptor protein, TRAF5 activates the nuclear factor-κB (NF-κB) pathway through its receptors, thus participating in neuroinflammation, neuronal apoptosis, and

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glia activation[20-22]. A recent study uncovered the protective effect of miR-29b-3p on cardiomyocytes from hypoxia-induced damage by targeting TRAF5[23]. Therefore, TRAF5 exerts a crucial role in maintaining the normal functions of cardiomyocytes.

In this paper, we mainly discussed the biological function of miRNA-410-3p in hypoxia-induced injury in AC16 cells, and the potential mechanism.

Materials and Methods

Cell Culture

Human-derived cardiomyocyte cell line AC16 was provided by American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Normoxia or hypoxia preconditioning in AC16 cells were applied for 24 h in an environment containing 21% O_2, 5% CO_2 and 74% N_2, or 1% O_2, 5% CO_2 and 94% N_2, respectively.

Cell Transfection

The cells were inoculated in a 6-well plate for overnight culture and the cell transfection was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 6 hours later, the complete medium was replaced.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted from the cells using the TRIzol kit (Invitrogen, Carlsbad, CA, USA), respectively, followed by the measurement of RNA concentration using an ultraviolet spectrophotometer (Hitachi, Tokyo, Japan). The complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to the instructions of the PrimeScript™ RT MasterMix kit (Invitrogen, Carlsbad, CA, USA). The QRT-PCR reaction conditions were as follows: 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s, for a total of 40 cycles. The relative expression level of the target gene was expressed by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: miRNA-410-3p: F: 5'-CGCAGAATATAACACAGATGGC-3' and R: 5'-AGGTCCAGTTTTTTTTTTTTTTA-CAG-3'; TRAF5: F: 5'-CCGAGCCCCACAATG-GCTTA-3' and R: 5'-CCGCTCCACAAACTGG-TACT-3'.

Western Blot

The cells were lysed using radioimmunoprecipitation assay (RIPA) and the isolated proteins were quantified by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein sample was loaded for electrophoresis and transferred on a polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking non-specific antibodies in 5% skim milk for 2 h, the membranes were subjected to incubation with primary and secondary antibodies. The bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Dual-Luciferase Reporter Gene Assay

The wild-type and mutant-type vectors were constructed based on the binding sites in the promoter regions of the two genes. The cells were co-transfected with miR-NC/miRNA-410-3p mimics and TRAF5-WT/TRAF5-MUT for 48 h. Afterwards, the cells were lysed to determine the Luciferase activity (Promega, Madison, WI, USA).

Cell Counting Kit-8 (CCK-8)

The cells were seeded in the 96-well plate with 3×10^3 cells per well and cultured overnight. Absorbance (A) at 450 nm was recorded at the appointed time points using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) to depict the viability curves.

5-Ethynyl-2'-Deoxyuridine (EdU) Assay

The cells inoculated into 96-well plates with 5×10^3 cells per well were labeled with 50 μM EdU reagent for 2 h (Thermo Fisher Scientific, Waltham, MA, USA). After washing with Phosphate-Buffered Saline (PBS), the cells were fixed in 50 μL of fixation buffer, decolored with 2 mg/mL glycine, and permeated with 100 μL of penetrant. After PBS washing, the cells were stained with 100 μL of 4',6-diamidino-2-phenylindole (DAPI) in the dark for 30 min. The EdU-positive cells and DAPI-labeled cells were observed under a fluorescent microscope.

Apoptosis Determination

The cells were washed with PBS twice and centrifuged at 3000 r/min for 5 min. The precipitant was resuspended in 500 μL of binding buffer, incubated with 5 μL of Annexin V in the dark for 15 min, and 5 μL of Propidium Iodide (PI) at

4°C, in the dark for other 15 min. After 5-min centrifugation at 3000 r/min, the precipitant was dissolved in 300 μL of binding buffer and subjected to flow cytometry (Partec AG, Arlesheim, Switzerland).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was used for data analyses. The data were expressed as mean ± standard deviation. The measurement data between the two groups were analyzed by the Student's *t*-test. The comparison between multiple groups was done using One-way ANOVA test followed by the post-hoc test (Least Significant Difference). $p<0.05$ was considered as statistically significant.

Results

Hypoxia Induced Cardiomyocyte Injury and MiRNA-410-3p Downregulation

AC16 cells were induced in normoxia and hypoxia environment, respectively. Compared with AC16 cells in normoxia preconditioning, miRNA-410-3p was downregulated in those with hypoxia preconditioning (Figure 1A). Hypoxia preconditioning stimulated apoptosis and attenuated viability in the AC16 cells (Figures 1B, 1C).

MiRNA-410-3p Overexpression Protected Hypoxia-Induced Cardiomyocyte Injury

To fully elucidate the biological function of miRNA-410-3p in hypoxia-induced cell injury in cardiomyocytes, miRNA-410-3p mimics was constructed. The transfection of miRNA-410-3p mimics remarkably upregulated miRNA-410-3p level in AC16 cells (Figure 2A). The overexpression of miRNA-410-3p greatly enhanced viability and EdU-positive ratio in hypoxic AC16 cells (Figures 2B, 2D). Besides, the apoptotic rate decreased after transfection of miRNA-410-3p mimics in hypoxic AC16 cells (Figure 2C). Thus, it is proved that miRNA-410-3p overexpression protected hypoxia-induced cell injury in cardiomyocytes.

MiRNA-410-3p Bound to 3'UTR of TRAF5

Potential binding sites in the promoter regions of miRNA-410-3p and TRAF5 were identified through prediction in the miRanda (Figure 3A). The Luciferase activity decreased after co-transfection of miRNA-410-3p mimics and TRAF5-WT, verifying the binding relationship between miRNA-410-3p and TRAF5 (Figure 3B). In addition, hypoxia preconditioning greatly upregulated both mRNA and the protein levels of TRAF5. The overexpression of miRNA-410-3p downregulated the TRAF5 level in AC16 cells (Figures 3C, 3D). Collectively, TRAF5 was the direct target of miRNA-410-

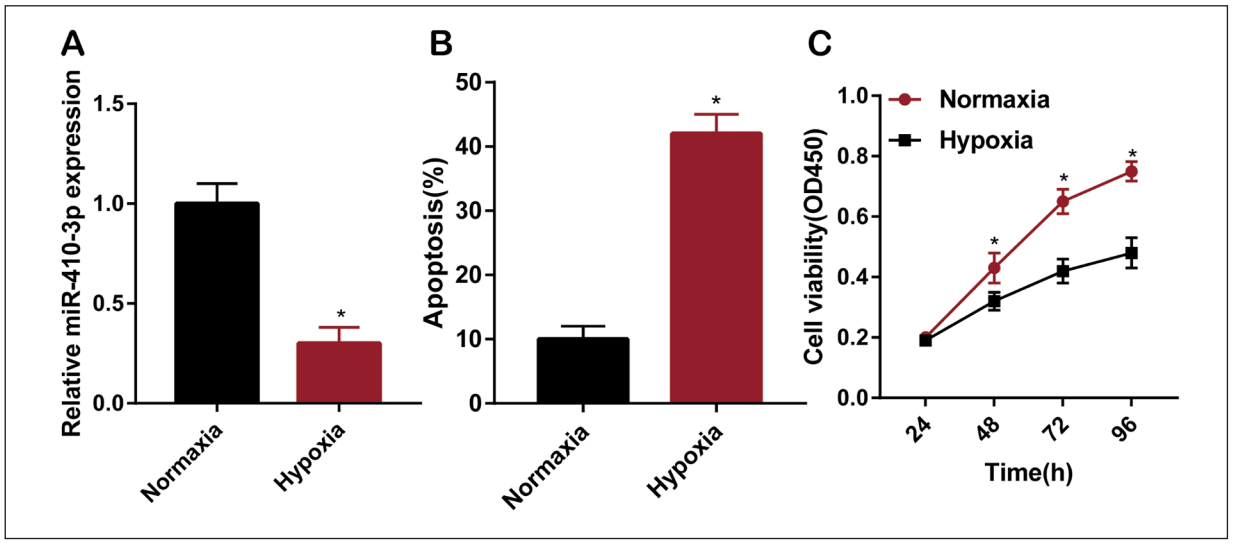

Figure 1. Hypoxia induced cardiomyocyte injury and miR-410-3p downregulation. **A,** MiR-410-3p level in AC16 cells with normoxia or hypoxia preconditioning. **B,** Apoptosis in AC16 cells with normoxia or hypoxia preconditioning. **C,** Cell viability at 24, 48, 72, and 96 h in AC16 cells with normoxia or hypoxia preconditioning.

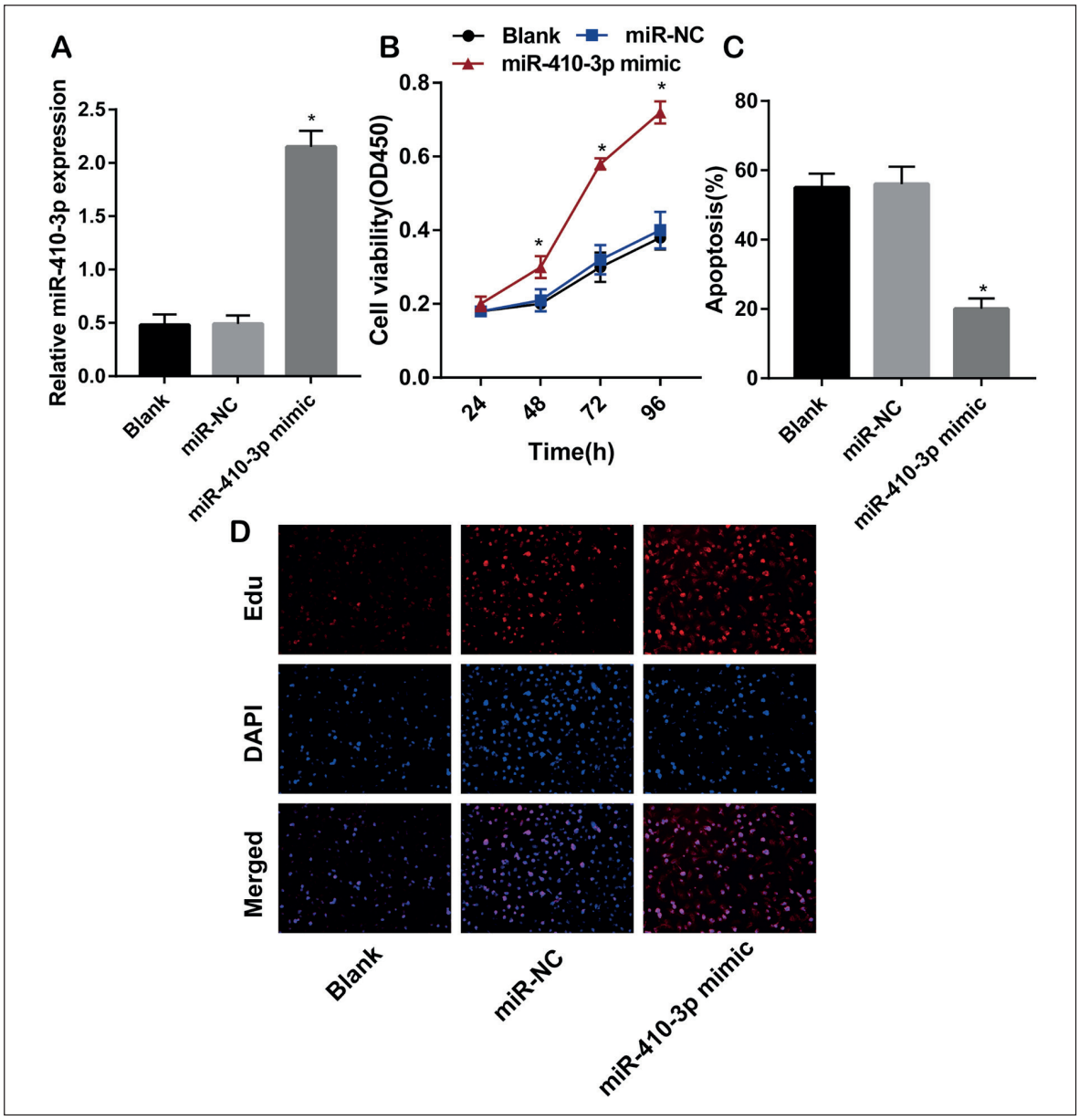

Figure 2. MiR-410-3p overexpression protected hypoxia-induced cardiomyocyte injury. **A,** Transfection efficacy of miR-410-3p mimics in AC16 cells. **B,** Cell viability at 24, 48, 72, and 96 h in hypoxic AC16 cells with blank control, transfection of miR-NC, or miR-410-3p mimics. **C,** Apoptosis in hypoxic AC16 cells with blank control, transfection of miR-NC or miR-410-3p mimics. **D,** EdU-positive ratio in hypoxic AC16 cells with blank control, transfection of miR-NC, or miR-410-3p mimics (magnification ´ 40).

3p. MiRNA-410-3p could negatively regulate TRAF5 level in cardiomyocytes under hypoxia preconditioning.

MiRNA-410-3p Protected Hypoxia-Induced Cardiomyocyte Injury Via TRAF5

Transfection of si-TRAF5 effectively down-regulated TRAF5 level in AC16 cells (Figure 4A). The silence of TRAF5 enhanced the viability and inhibited apoptosis in hypoxic AC16 cells. Notably, the silence of TRAF5 could reverse the regulatory effects of the overexpressed miRNA-410-3p on hypoxia-induced changes in viability and apoptosis (Figures 4B, 4C). Hence, it is confirmed that miRNA-410-3p protected cardiomyocytes from hypoxia injury *via* TRAF5.

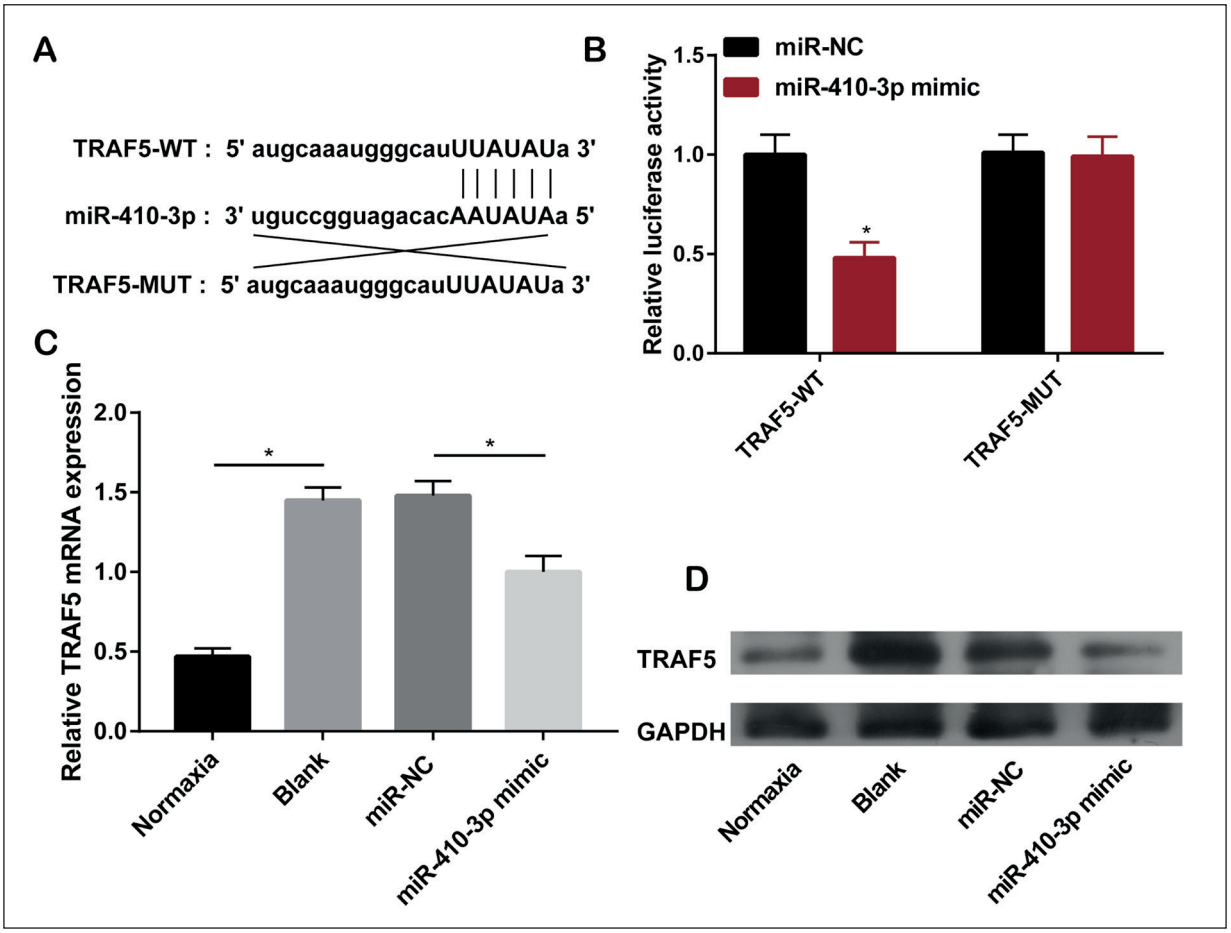

Figure 3. MiR-410-3p bound to 3'UTR of TRAF5. **A,** The binding sites in the promoter regions of miR-410-3p and TRAF5. **B,** The Luciferase activity in AC16 cells co-transfected with miR-NC/miR-410-3p mimics and TRAF5-WT/TRAF5-MUT, respectively. **C,** The mRNA level of TRAF5 in AC16 cells with normoxia or hypoxia preconditioning, and in hypoxic AC16 cells transfected with miR-NC or miR-410-3p mimics. **D,** The protein level of TRAF5 in AC16 cells with normoxia or hypoxia preconditioning, and in hypoxic AC16 cells transfected with miR-NC or miR-410-3p mimics.

Discussion

AMI is one of the most severe cardiovascular events with the characteristics of acute onset, serious illness, multiple complications, and high mortality[24]. AMI generally results from necrotic myocardium following ischemia and hypoxia. During the process of AMI, many miRNAs are abnormally expressed in the heart, which may be utilized as novel hallmarks and therapeutic targets.

MiRNA-410-3p is abnormally expressed in many diseases, including cancers, inflammation, and autoimmune diseases. It is widely involved in the regulation of cellular behaviors, differentiation, and drug resistance[25-28]. A previous study[29] illustrated that miRNA-410-3p mediates the proliferative and invasive capacities of glioma by regulating EMT. In rheumatoid arthritis, miRNA-410-3p acts as an inflammatory inhibitor by suppressing the NF-κB pathway[30]. In this paper, we first uncovered the inhibited viability and stimulated apoptosis in hypoxic AC16 cells. Under the hypoxia preconditioning, miRNA-410-3p was markedly downregulated in cardiomyocytes. Moreover, the overexpression of miRNA-410-3p protected hypoxia-induced proliferation suppression and apoptosis stimulation in cardiomyocytes, exerting a protective role in AMI.

Cytokines of the tumor necrosis factor (TNF) family trigger a variety of cellular responses, including inflammatory response and immune defense[31]. TRAFs are present in mammalian and multicellular organisms (e.g., fly, nematode, dictyostelium), involving in multiple signaling pathways[32]. TRAF5 is a key molecule in mediating

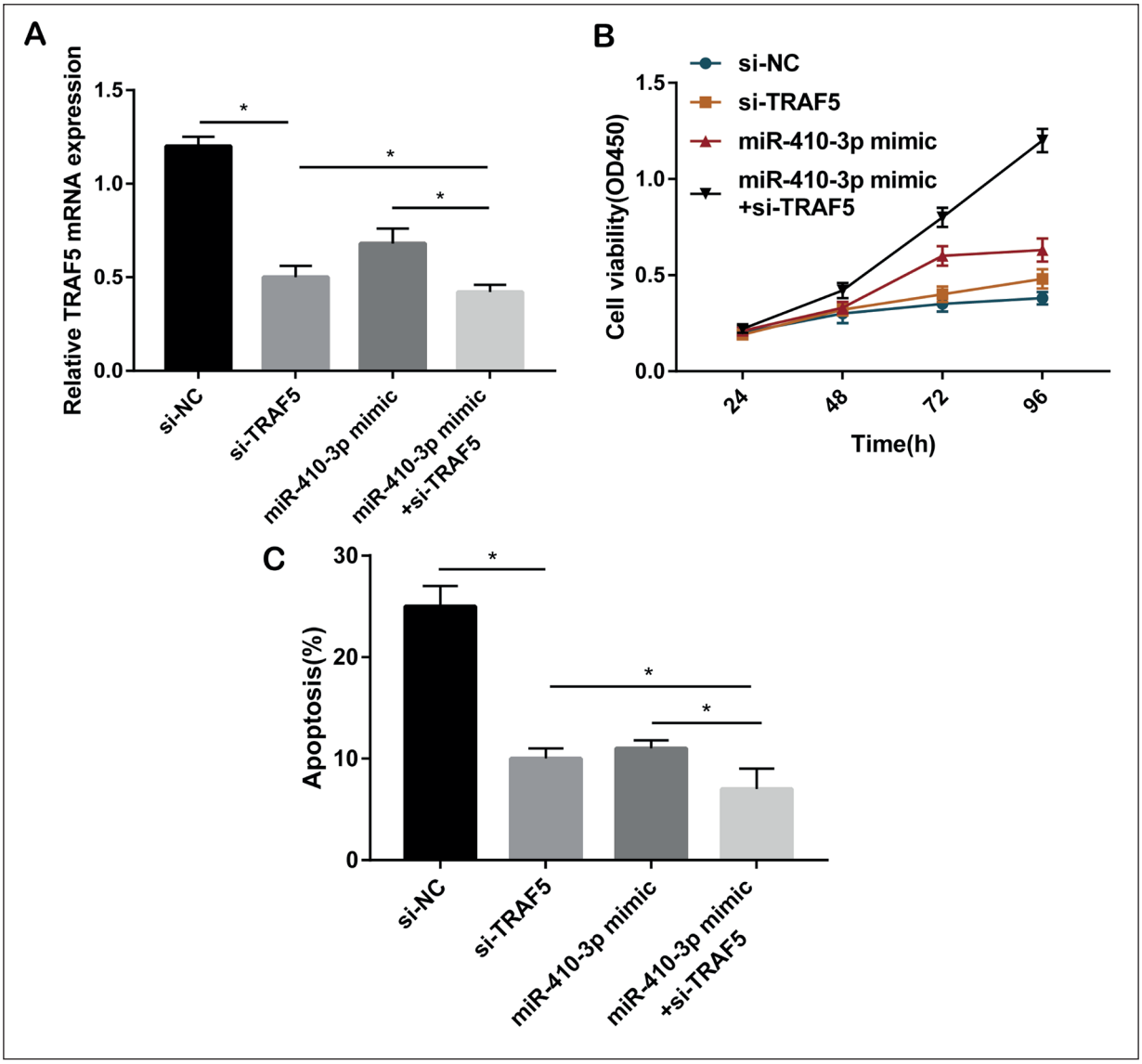

Figure 4. MiR-410-3p protected hypoxia-induced cardiomyocyte injury via TRAF5. AC16 cells were transfected with si-NC, si-TRAF5, miR-410-3p mimics, or miR-410-3p mimics + si-TRAF5. The mRNA level of TRAF5 (**A**) cell viability at 24, 48, 72 and 96 h (**B**) and apoptosis (**C**).

cell differentiation, survival, and other behaviors[33,34]. As a mediator to activate NF-κB, TRAF5 protects cell injury from TNF-α induction[35]. Missiou et al[36] found that the severity of atherosclerosis in TRAF5 knockout mice was worse than that in the controls after high-fat feeding. The mRNA level of TRAF5 in blood monocytes of patients with the acute coronary syndrome is remarkably downregulated, further confirming the protective role of TRAF5 in atherosclerosis. Our findings verified that TRAF5 was the direct target of miRNA-410-3p. A negative correlation was discovered between them under the hypoxia preconditioning. Moreover, the silence of TRAF5 protected hypoxia-induced cardiomyocyte injury. Importantly, the protective effect of miRNA-410-3p on hypoxia-induced AMI was achieved by negatively regulating TRAF5.

Conclusions

MiRNA-410-3p protects hypoxia-induced proliferation suppression and apoptosis stimulation in cardiomyocytes *via* targeting TRAF5.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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