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Original Article

MicroRNA-150 aggravates H_2O_2-induced cardiac myocyte injury by down-regulating *c-myb* gene

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MicroRNAs (miRNAs) are one class of non-coding RNAs that play an important role in post-transcriptional regulation *via* the degradation or translational inhibition of their target genes. MicroRNA-150 (miR-150) plays a vital role in regulating the development of B and T lymphocytes. Although the dysregulation of miR-150 was confirmed in human myocardial infarction, little is known regarding the biological functions of miR-150 in response to reactive oxygen species (ROS)-mediated gene regulation in cardiac myocytes. Using quantitative real-time reverse transcription-polymerase chain reaction, we demonstrated that the level of miR-150 was up-regulated in cardiac myocytes after treatment with hydrogen peroxide (H_2O_2). To identify the potential roles of miR-150 in H_2O_2-mediated gene regulation, we modulated expression of miR-150 using miR-150 inhibitor and miR-150 mimics. Results showed that silencing expression of miR-150 decreased H_2O_2-induced cardiac cell death and apoptosis. In lymphocytes, *c-myb* was a direct target of miR-150. In cardiac myocytes, we found that c-myb was also involved in miR-150-mediated H_2O_2-induced cardiac cell death. These results suggested that miR-150 participates in H_2O_2-mediated gene regulation and functional modulation in cardiac myocytes. MiR-150 may play an essential role in heart diseases related to ROS, such as cardiac hypertrophy, heart failure, myocardial infarction, and myocardial ischemia/reperfusion injury.

Keywords microRNA; cardiac myocyte apoptosis; cell death; reactive oxygen species

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Introduction

Reactive oxygen species (ROS)-induced cardiac cell injury plays a critical role in the pathogenesis of numerous heart diseases. Various genes, transcription factors, and the oxidation-sensitive signaling pathways are responsible for ROS-mediated cardiac cell injury [1–4]. The role of microRNAs (miRNAs) in the post-transcriptional control of gene expression, such as regulating translation, has been extensively studied [5,6]. MiRNAs negatively regulate gene expression by degrading or inhibiting the translation of target genes [7], and are involved in the regulation of almost all major cellular functions, including cell differentiation, growth, mobility, and death. Since the 1980s, about 700 miRNAs have been identified and sequenced in humans, and the actual number of miRNA genes may be larger [8–11]. Compared with the entire human genome, the number of miRNA genes is small; but an individual miRNA can regulate the expression of multiple target genes, and miRNAs may directly regulate at least 30% of the genes in a cell [12,13].

MicroRNA-150 (miR-150) is highly and specifically expressed in lymph nodes and the spleen, and its expression level is up-regulated during the development of mature and resting T and B lymphocytes [14–17]. Level of mRNA-150 remains low in pre-B and pro-B lymphocytes, which is important for B lymphocytes formation and function. During T-cell differentiation and leukemogenesis, miR-150 targets *NOTCH3*, a member of the Notch receptor family, and has inhibitory effects on cell proliferation and survival. In natural killer (NK)/T-cell lymphoma lines and samples of primary lymphoma, the expression level of miR-150 is significantly lower than that in normal NK cells, suggesting that miR-150 functions as a tumor suppressor [18–21].

Although the dysregulation of miR-150 was confirmed in human myocardial infarction (MI) [22], the biological functions of miR-150 in response to ROS-mediated gene regulation in cardiac myocytes are uncertain. Therefore, we assessed miR-150 expression in cultured cardiac myocytes treated with hydrogen peroxide (H_2O_2) to explore its potential role in gene regulation.

Materials and Methods

Cell culture

The hearts from 1- to 2-day-old Sprague–Dawley rats were exposed after being sterilized in 75% (v/v) ethanol for 15 s.

Then, the apexes were cut off and minced with scissors. The minced ventricular tissues were digested with collagenase type II (0.45 mg/ml; Sigma, St Louis, USA) and 0.1% trypsin (Life Technologies, Grand Island, USA). The released cells were pooled and separated on a discontinuous Percoll gradient procedure. The middle band at the interface of the two Percoll layers was collected into gelatin-coated dishes. Cardiomyocytes (3×10^5 cells/ml) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite, 2 g/l bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μg/ml

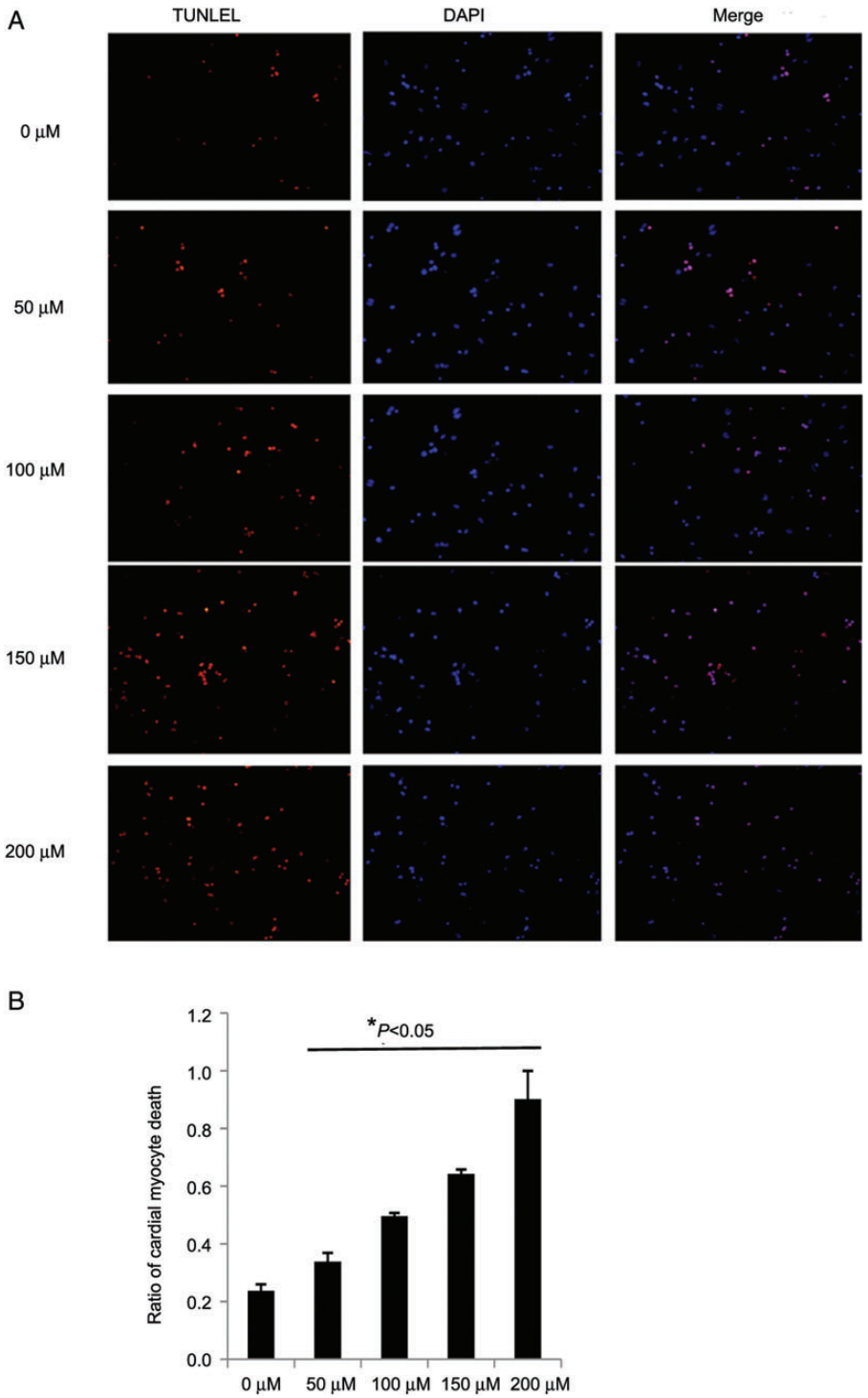

Figure 1 The effect of H_2O_2 on rat cardiac myocyte death Cultured neonatal rat cardiac myocytes were treated with vehicle (0 μM) or H_2O_2 (50–200 μM) for 24 h. Cell death was measured by TUNEL staining. (A) The effect of H_2O_2 on rat cardiac myocyte death. $n = 5$; $^*P < 0.05$ compared with the vehicle control. (B) Photomicrographs of TUNEL-stained cells. Magnification, ×200.

ampicillin, 5 μg/ml linoleic acid, 1% penicillin, and 1% streptomycin. To inhibit the proliferation of other cells, 5-bromo-2-deoxyuridine (100 μM) was added, and the cells were seeded into 24-well plates (pre-coated with gel).

Treatment with H_2O_2 for miR-150 expression

Neonatal rat cardiac ventricular myocytes were treated with H_2O_2 (50−200 μM) or vehicle (0 μM) for 6 h. Then, total RNA was isolated from the cultured cells using Trizol reagent (Life Technologies). Expression level of miR-150 was determined by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) using All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia, Rockville, USA). U6 and miR-150 primer were also purchased from GeneCopoeia.

Measuring cardiac myocyte death and apoptosis induced by H_2O_2

Cell death (apoptosis and necrosis) was assessed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. After exposure to H_2O_2 (0−200 μM) for 24 h, TUNEL staining was performed using an *in situ* cell death detection kit (Roche, Basel, Switzerland). According to the manufacturer's protocol, cardiac myocytes cultured in 24-well plates were fixed in 4% paraformaldehyde. The numbers of TUNEL-positive cells were counted under a fluorescence microscope.

Construction of the adenovirus expressing *c-myb* and the control virus expressing *GFP*

The adenovirus expressing *c-myb* (Ad-c-myb) or the control adenovirus expressing *GFP* (Ad-GFP) was generated using the Adeno-X™ expression systems 2 kit (Clontech, Mountain View, USA). Briefly, a fragment of the full length coding sequence amplified with primers 5′-aaatctagaatggccc-gagaccccggcacagc-3′ and 5′-aaagtcgactcgcttgtcatcgtcgtccttg-tagtccatgaccagcgtccgggctga-3′ using PCR was inserted into pDNR-CMV donor vector. Then *c-myb* fragment was excised from the pDNR-CMC, and inserted into the pLP-Adeno-X-CMV vector. The pLP-Adeno-X-CMV-c-myb plasmid was digested by *Pac*I and transfected to low-passage HEK293 cells with MegaTran1.0 (OriGene, Rockville, USA) to produce recombinant adenovirus. Ad-GFP was generated as described previously [23].

Modulating miR-150 in cultured cardiac myocytes

Oligo transfection was performed according to the manufacturer's instructions. Briefly, cells were transfected using a transfection reagent (MegaTran1.0) after 48 h of culture. To knockdown the miR-150, miR-150 inhibitor (RiboBio Co., Ltd, Guangzhou, China) was added to the culture media at final concentration of 160 nM. To up-regulate the miR-150, miR-150 mimics (RiboBio Co., Ltd) was directly added to the complexes at final concentration of 100 nM. The up-regulation of *c-myb* gene was performed by Ad-c-myb (30 multiplicity of infection). Before transfection, the medium was removed and the cells were gently washed twice with phosphate-buffered saline. The transfection mixture was directly added to the cells. After 4 h, the transfection mixture was replaced with DMEM containing 10% fetal bovine serum.

Determining RNA levels by qRT-PCR

RNA from cardiac myocytes was isolated using Trizol reagent. According to the manufacturer's protocol, qRT-PCR for miR-150 was performed using cDNA generated from 100 ng of total RNA. U6 was used as an internal control. The fluorescent signals were normalized to an internal reference, and the threshold cycle (C_t) was set within the exponential phase of the PCR. Relative gene expression

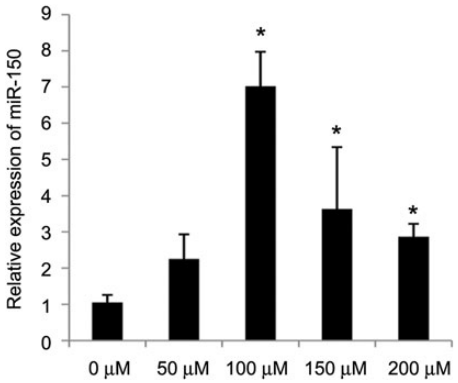

Figure 2 The effect of H_2O_2 on miR-150 expression in cultured rat cardiac myocytes Neonatal rat cardiac myocytes were treated with vehicle (0 μM) or H_2O_2 (50−200 μM) for 6 h. The miR-150 levels were determined by qRT-PCR. $n = 5$; $^*P < 0.05$ compared with the vehicle control.

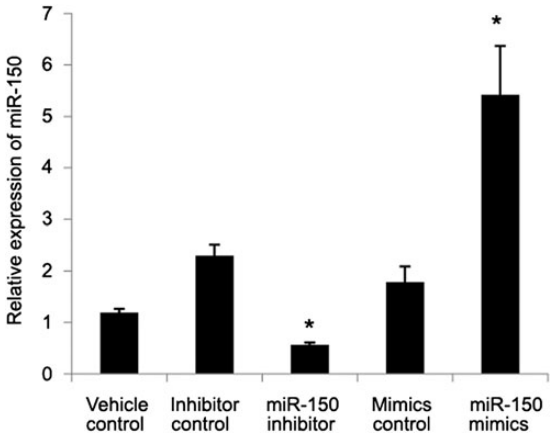

Figure 3 Modulating miR-150 expression in cultured cardiac myocytes Cultured cardiac myocytes were treated with vehicle, miR-150 inhibitor (160 nM), miR-150 mimics (100 nM), and control oligo for 4 h. The miR-150 levels were determined by qRT-PCR. $n = 5$; $^*P < 0.05$ compared with the vehicle control.

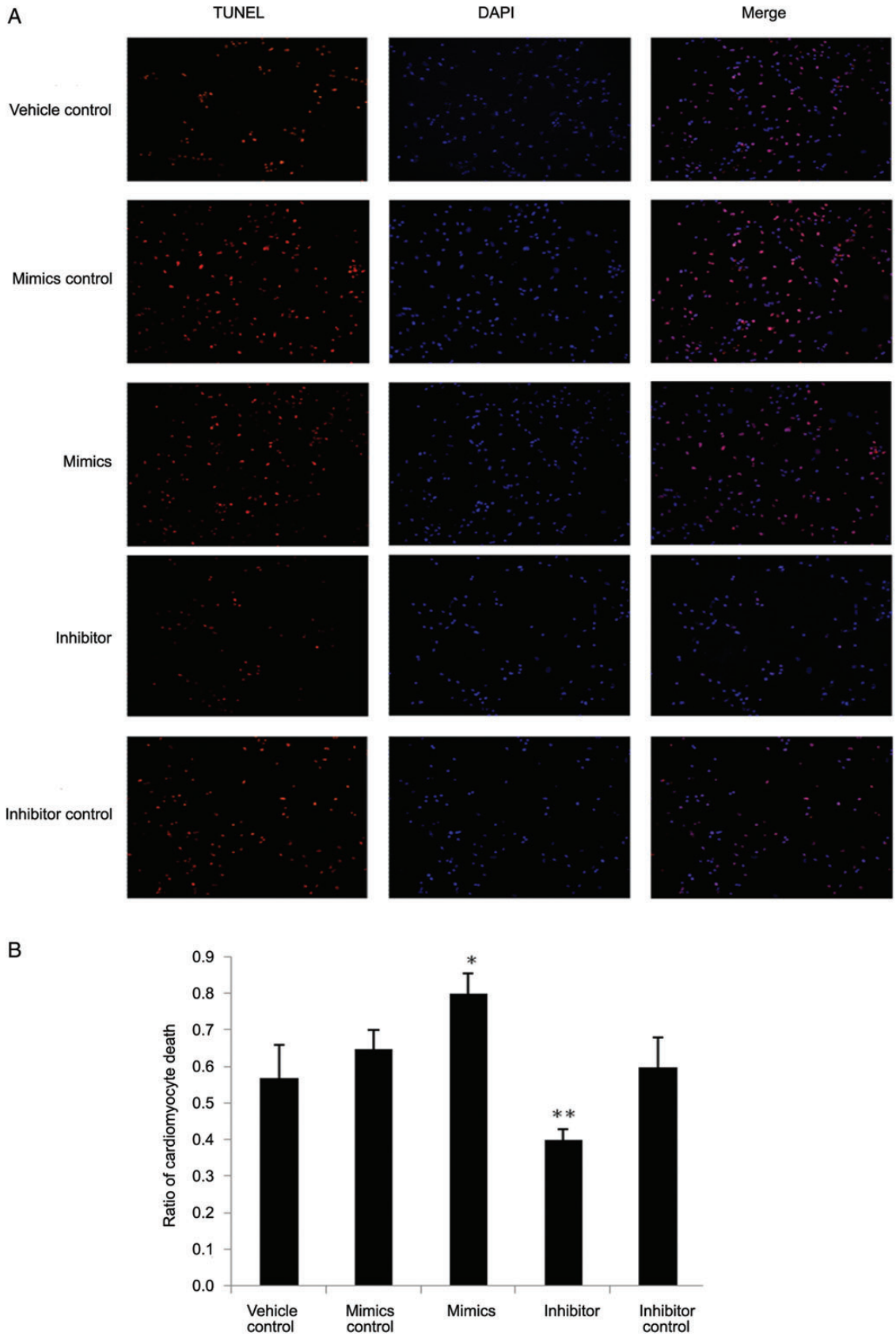

Figure 4 The effect of miR-150 on H_2O_2-induced cardiac myocytes apoptosis Cultured rat cardiac myocytes pre-treated with vehicle, miR-150 inhibitor (160 nM), miR-150 mimics (100 nM), and oligo control were treated with H_2O_2 (100 μM) for 24 h. (A) Representative TUNEL-stained cell photomicrographs from different groups. Magnification, ×200. (B) The effect of miR-150 on H_2O_2-induced cardiac myocyte death. $n = 6$; $^*P < 0.05$ compared with the vehicle control; $^{**}P < 0.01$ compared with the vehicle control.

was calculated by comparing cycle times for each target PCR. The relative expression between treatments was then calculated using the following equation: relative gene expression $= 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ control})}$.

Western blot analysis

Proteins were isolated from cultured cardiac myocytes and 60–100 μg of protein was run on 10% sodium dodecyl sulfate–polyacrylamide gels, and visualized using anti-Bcl-2 (1 : 1000; SC-492; Santa Cruz, Santa Cruz, USA), anti-actived-caspase-3 (1 : 1000; SC-7148, Santa Cruz), anti-c-myb (1 : 1000; SC-517; Santa Cruz), and anti-β-actin (1 : 2000; Santa Cruz) antibodies.

Statistical analysis

All data are presented as the mean ± SD from, experiment repeated at least three. Two-tailed unpaired Student's *t*-tests and one-way analysis of variance were used to evaluate the data. SPSS version 16.0 (IBM) was used for the data analysis. A P value <0.05 was considered significant.

Results

Effect of H_2O_2 on cardiac myocyte injury

We confirmed that high concentrations of H_2O_2 (50–200 μM) increased cardiac myocyte death in a dose-dependent manner after 24 h treatment under our experimental conditions (**Fig. 1**). To better understand the effects of H_2O_2 on cardiac myocyte death, expression levels of the apoptosis-associated Bcl-2 protein and actived-caspase-3 protein were evaluated after treatment with H_2O_2 for 24 h. The expression level of anti-apoptosis protein Bcl-2 was reduced in parallel with the H_2O_2 concentration, while the level of actived-caspase-3 protein was concomitantly activated and up-regulated. These results suggested that H_2O_2 triggers the caspase-3-mediated apoptosis signaling pathway.

Effect of H_2O_2 on miR-150 expression in cultured cardiac myocytes

As shown in **Fig. 2**, 6 h exposure of cardiac myocytes to H_2O_2 resulted in a dose-dependent increase of miR-150 expression with a peak at ~100 μM.

Effect of silencing miR-150 on H_2O_2-induced cardiac myocyte death and apoptosis

Next, we modulated the miR-150 expression to determine its role in regulating the cardiomyocyte response to ROS. Compared with the vehicle control, miR-150 mimics treatment increased the ratio of cardiac myocyte death and apoptosis to 78% ± 1.8%, but the ratio of cardiac myocyte death and apoptosis was decreased to 39% ± 1.2% when treated with miR-150 inhibitor, and the protective effect of the miR-150 inhibitor was dose-dependent. Using concentrations from 50–200 nM, the maximum effect occurred at 160 nM (**Fig. 3**). TUNEL-stained photomicrographs of cardiac myocytes treated with vehicle (control oligo), miR-150 mimics, and miR-150 inhibitor are shown in **Fig. 4**. These results suggested that miR-150 aggravates

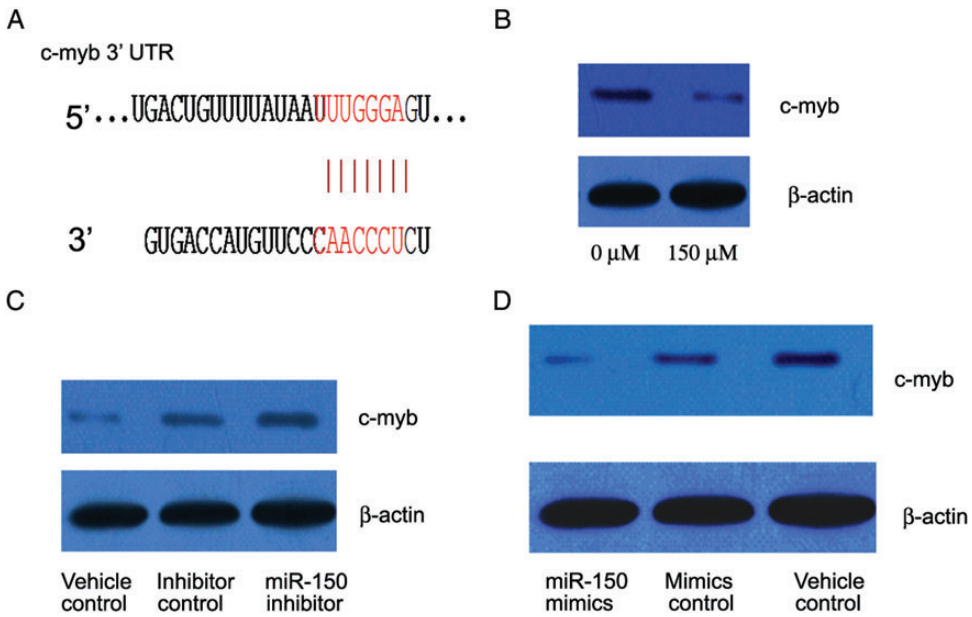

Figure 5 ***c-Myb*** **gene is involved in miR-150-mediated H_2O_2-induced cardiomyocytes injury** (A) Conserved miR-150 binding site in the 3′-untranslated region (3′-UTR) of *c-myb*. (B) H_2O_2 decreased c-myb expression in cultured cardiac myocytes. (C) miR-150 inhibitor (160 nM) increased c-myb expression in cardiac myocytes compared with vehicle. (D) miR-150 (100 nM) decreased c-myb expression in cardiac myocytes.

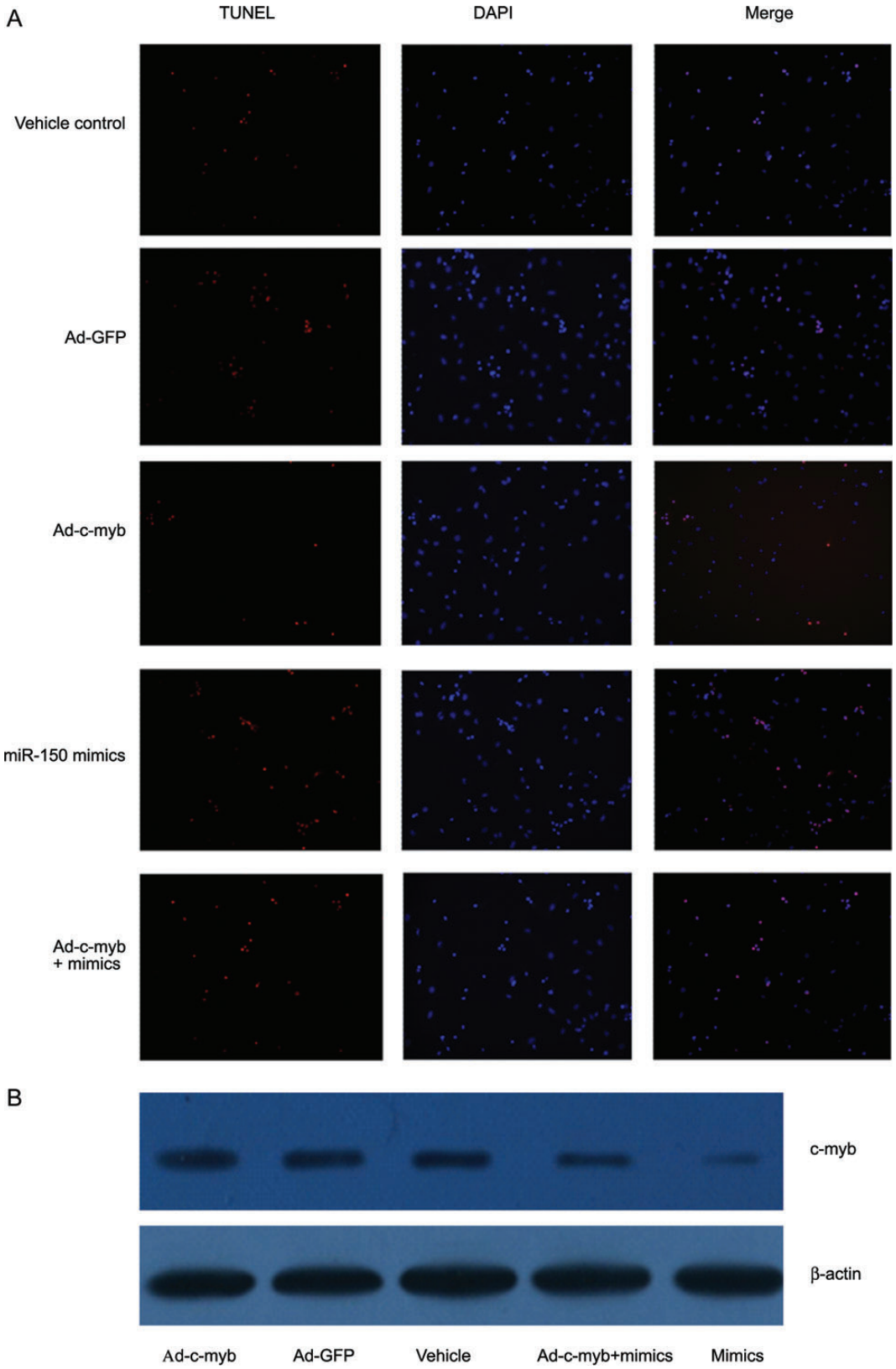

Figure 6 ***c-Myb*** **is a functional target gene that is involved in miR-150-mediated H_2O_2-induced cardiac cell apoptosis** (A) H_2O_2 (100 μM)-induced cardiac myocytes apoptosis was inhibited by miR-150 inhibitor (160 nM). Overexpression of c-myb via Ad-c-myb decreased the H_2O_2-cardiac myocyte apoptosis. The miR-150 mimics-mediated cardiac myocyte apoptosis was inhibited via adenovirus-mediated overexpression of c-myb. (B) Representative western blots of c-myb from each group.

H_2O_2-induced cardiac myocytes injury, while inhibiting the expression of miR-150 may protect cardiac myocyte from H_2O_2-induced death and apoptosis.

The *c-myb* gene is involved in miR-150-mediated H_2O_2-induced cardiac myocyte death

Bioinformatics analysis indicates that *c-myb* is a potential target gene of miR-150 [**Fig. 5(A)**]. To confirm this, we incubated cardiac myocytes with either vehicle or H_2O_2 (50 μM) for 24 h and the protein level of c-myb was determined by western blot analysis. As shown in **Fig. 5(B)**, H_2O_2 decreased the protein expression of c-myb. To further verify *c-myb* as a target gene of miR-150 in cardiac myocytes, both gain-of-function and loss-of-function approaches were applied. As shown in **Fig. 5(C,D)**, miR-150 inhibitor increased, whereas miR-150 mimics decreased c-myb expression in cultured cardiac myocytes. These results suggested that *c-myb* may be a target gene of miR-150 in cardiac myocytes. To verify the functional involvement of c-myb in miR-150-mediated cellular effect, we determined the role of c-myb in H_2O_2-induced cardiac myocyte apoptosis. Overexpression of c-myb *via* Ad-c-myb decreased H_2O_2-mediated cell apoptosis. Furthermore, the miR150-mediated effect on cardiac myocytes apoptosis was inhibited *via* adenovirus-mediated overexpression of c-myb. Representative TUNEL-stained photomicrographs from cardiac myocytes treated with vehicle, Ad-GFP, Ad-c-myb, miR-150 mimics, and miR-150 mimics plus Ad-c-myb were displayed in **Fig. 6(A)**, and representative western blots of c-myb for each group were shown in **Fig. 6(B)**.

Discussion

Genes, transcription factors, and ROS modulate the oxidation-sensitive signaling pathways that may be critical in ROS-mediated cardiac ischemic disease. In this study, we found that the expression level of miR-150 was up-regulated in a dose-dependent manner in cardiac myocytes after treatment with H_2O_2 for 6 h.

To determine the potential role of miR-150 in H_2O_2-mediated cardiac myocyte injury, the expression of miR-150 was modulated by administering miR-150 inhibitor and mimics. Up-regulated expression of miR-150 exacerbated H_2O_2-mediated neonatal rat cardiac myocytes apoptosis and death. In contrast, H_2O_2-mediated cardiac myocyte apoptosis and death were attenuated after inhibiting miR-150 expression. These results suggested that miR-150 is a new target for the treatment of ROS-induced cardiac cell injury.

MiRNAs play vital roles *via* the degradation or translational inhibition of their target mRNAs. Although their potential gene targets can be predicted by bioinformatics analysis, these targets must be experimentally verified in specific cells because the miRNA-mediated effects on gene expression and cellular functions are cell-specific, and a single miRNA might target various genes. In this study, bioinformatics analysis suggested that *c-myb* was an miR-150 target gene. Moreover, c-myb regulation by miR-150 has been reported in lymphocytes and cancer cells. The transcription factor c-myb regulates self-renewal and lineage specification of adult hematopoietic stem cells. c-Myb regulates cell cycle-dependent intracellular Ca^{2+} concentrations and proliferation of vascular smooth muscle cells (SMCs), and therapies targeting c-myb limit neointima formation after carotid artery injury. Embryonic stem cells lacking c-myb fail to form contractile SMCs in embryoid bodies and have limited ability to contribute to the SMC compartments of chimeric embryos and adults. The potential role of c-myb in cardiomyocyte is uncertain [24,25]. To determine whether c-myb is involved in miR-150-mediated H_2O_2-induced cardiomyocytes death and injury, we performed western blot analysis to evaluate the expression of c-myb protein. We found that the level of c-myb was down-regulated in cardiomyocytes treated with H_2O_2. Modulating the expression of c-myb via Ad-c-myb decreased H_2O_2-mediated cell apoptosis. Furthermore, the miR150-mediated effect on cardiac myocyte apoptosis was inhibited *via* adenovirus-mediated overexpression of c-myb. These results suggested that *c-myb* gene might be miR-150 target in cardiomyocytes.

In summary, our results suggested that the expression of miR-150 in cardiac myocytes is up-regulated after H_2O_2 treatment. MiR-150 aggravates H_2O_2-induced injury of cardiac myocytes by down-regulating c-myb. These novel findings may have potential implications for the therapy of ROS-related diseases, such as MI, cardiac hypertrophy, and myocardial ischemia/reperfusion injury.

Funding

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