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Original Article

Inhibition of microRNA-182 reduces hypoxia/re-oxygenation-induced HL-1 cardiomyocyte apoptosis by targeting the nuclear respiratory factor-1/mitochondrial transcription factor A (NRF-1/mtTFA) pathway

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Abstract: Apoptosis is a component of cardiac remodeling following myocardial ischemia and infarction. MicroRNAs (miRNAs) are involved in regulating a range of cellular biological processes, including apoptosis, by regulating gene expression at the post-transcriptional level. Nuclear respiratory factor-1 (*NRF-1*) is a transcriptional activator of nuclear genes that encode a range of mitochondrial proteins that sensitize cells to apoptosis. The HL-1 mouse cardiomyocyte hypoxia/re-oxygenation (H/R) *in vitro* model included a 24 h period of hypoxia followed by a 48 h period of re-oxygenation. qRT-PCR was used to study gene expression at the mRNA level. MicroRNA target identification was done using bioinformatics data with analysis using a luciferase dual reporter assay. SiRNA-*NRF-1* was used to inhibit *NRF1* gene expression. Functional studies included the assessment of cardiomyocyte apoptosis and lactate dehydrogenase (LDH) levels. The protein levels of mitochondrial transcription factor A (*mtTFA*) and *NRF-1* were measured using western blots. The results of this study showed that increasing the duration of HL-1 cardiomyocyte H/R resulted in increased expression of miRNA-182 and a reduction in the expression of *NRF-1*. Inhibition of miRNA-182 reduced HL-1 cardiomyocyte apoptosis and cell death, increased *NRF-1* gene expression and protein levels. These effects were reduced with co-transfection of HL-1 cardiomyocytes using siRNA-*NRF-1*. A role for miRNA-182 in H/R-induced cardiomyocyte apoptosis involves regulation of the NRF-1/mtTFA pathway. Future studies may determine whether miRNA-182 knocked down can reduce reperfusion injury associated with myocardial ischemia and infarction.

Keywords: Apoptosis, microRNA-182, reperfusion injury, cardiomyocyte, NRF-1, mtTFA

Introduction

Adaptation to endogenous and exogenous stress is an important survival mechanism for cells, tissues and organisms [1]. One of the most common external stresses for cells is the lack of oxygen, which may lead to cell death. This form of ischemic cell death is termed 'infarction' when it involves organs such as the heart [2-4]. Ischemic heart disease and myocardial infarction are leading causes of morbidity and mortality, worldwide [5].

Following myocardial ischemia, due to lack of oxygen to the cardiac myocytes, reperfusion and re-supply of oxygen results in cell damage, termed 'reperfusion injury' [5-7]. Apoptosis is the hallmark of reperfusion injury at the cellular level.

A number of *in vitro* models of hypoxia/re-oxygenation (H/R)-induced injury have been developed recently to allow studies to be conducted on the cellular mechanisms of reperfusion injury and its control. These *in vitro* studies have shown that reactive oxygen species play a critical role in cardiac reperfusion injury [6, 7]. HL-1 cells are derived from an AT-1 mouse atrial cardiomyocyte tumor lineage and have characteristics typical of embryonic atrial cardiac muscle cells [8]. The advantage of using this *in vitro* cardiomyocyte cell line is that it can be suc-

cessfully maintained and passaged [8]. Molecular studies using *in vitro* and *in vivo* models and clinical studies have revealed that multiple genetic factors are associated with ischemic heart disease in individuals and patient populations [9]. MiRNAs are small non-coding RNAs (between 18-25 nucleotides in length) that regulate gene expression at the post-transcriptional level and control cell proliferation, cell differentiation and apoptosis [10, 11]. These miRNAs play an important role in a number of disease processes, including ischemic cardiac disease [12]. Recently, miRNAs have been shown to have different expression levels in ischemic and normal cardiac myocytes in rat and mouse *in vivo* and *in vitro* models [13, 14].

There has been recent supporting evidence for the role of miRNA-182 in cardiac diseases including acute myocardial infarction, heart failure and stroke [15]. In a recent study, cultured rat H9C2 cardiomyocytes transfected with a miR-92a inhibitor showed reduced cardiomyocyte damage and apoptosis induced by H/R [14].

Nuclear respiratory factor-1 (*NRF-1*) is a transcriptional activator of nuclear genes encoding a range of mitochondrial proteins that sensitize cells to apoptosis; *NRF-1* is a target for miR-182. Mitochondrial transcription factor A (*mtTFA*) plays a key role in the regulation of mitochondrial DNA (mtDNA) replication, with the measured levels of *mtTFA* being proportional to mtDNA. Data from a recent study using cardiomyocyte-specific *NRF-1* knockout mice has elucidated a role for cardiac *NRF-1* in cardiac ischemia [16]. This recent *in vivo* mouse study showed that the loss of *NRF-1* resulted in cardiomyocyte apoptosis and reduced left ventricular function [16].

In view of these recent findings, this study was conducted to investigate the role of miRNA-182 in H/R-induced cardiomyocyte apoptosis *in vitro* by regulating *NRF-1* gene expression.

Materials and methods

Cell culture - HL-1 cardiomyocytes

HL-1 cardiomyocytes derived from mouse ventricle were purchased from Cell Bank, Chinese Academy of Sciences, Shanghai, China. HL-1 cells were seeded at a density of 10^4 cells/cm^2 in 6-well culture plates at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone, USA).

The HL-1 cardiomyocyte H/R model

Cell hypoxia was induced by incubation of the HL-1 cells in a modular incubator (Thermo Forma Scientific, USA) with 1% O_2, 94% N_2, and 5% CO_2 for 24 h. Re-oxygenation was performed for different time periods. Control HL-1 cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Oligonucleotide transfection

HL-1 cardiomyocytes were cultured in six-well plates at a density of 10^4 cells/ well for 24 h, and then transfected with oligonucleotides mixed with 5.0 mL Lipofectamine 2000 (Invitrogen, USA). MiR-182 mimic, non-targeting negative control (NC) mimic, miR-182 inhibitor and NC inhibitor were synthesized by Shangai GenePharma (GenePharma, China), either the short interfering RNA (siRNA) for Nuclear respiratory factor-1 (siR-*NRF-1*) and NC siRNA (siR-NC). All transfections were performed following a 12 h period of serum starvation prior to the H/R experiments.

FACS analysis of HL-1 cardiomyocyte apoptosis

The Annexin-V fluorescein isothiocyanate (FITC) apoptosis KGA107 detection kit (KeyGene, China) for flow cytometry fluorescence-activated cell sorting (FACS) (FACS Calibur, BD Biosciences, USA) was used. In this protocol, 5 × 10^5 HL-1 cardiomyocytes were harvested and washed twice with. phosphate-buffered saline (PBS), then mixed with 500 µL binding buffer to form a cell suspension. Annexin-V-FITC 5 µL was mixed with 5 µL of propidium iodide (PI). HL-1 cardiomyocytes were incubated for 10 minutes at room temperature in the dark and analyzed immediately using a FACS. The experiments were performed in triplicate.

Lactate dehydrogenase (LDH) assay

HL-1 cardiomyocytes were harvested, and LDH concentrations were measured using a commercial kit (Jiancheng Bioengineering Co. Ltd., Nanjing, China), according to the manufacturer's instructions.

Table 1. The primers used for polymerase chain reaction (PCR) analysis

Primer	Forward	Reverse
miR-182	5'-ACACTCCAGCTGGGAAGTGCCCCCA-3'	5'-TGGTGTCGTGGAGTCG-3'
NRF1	5'-GAGCACGGAGTGACCCAAAC-3'	5'-GTCCGAGTCATCATAAGAAGTG-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-GGGAAGCCCATCACCATCTT-3'	5'-CCTTTTGGCTCCACCCTTCA-3'

Luciferase reporter assay

Luciferase activity was measured using a dual luciferase reporter assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions. The 3'UTR of the *NRF-1* gene (*NRF-1* 3'UTR) and a mutated targeting fragment in the 3'-UTR of *NRF-1* lacking the putative miR-182 binding sequence (*NRF-1* 3'UTR-Mut) were cloned into psiCheck-2 reporter plasmid (Promega, Madison, WI, USA) between the *Not*1 and *Sgf*1 sites, repectively. 293T cells were co-transfected with 50 nM of miR-182 mimic, NC mimic, miR-182 inhibitor, NC inhibitor and 0.5 μg of psiCheck-2 reporter plasmid, containing the *NRF-1* 3'-UTR or *NRF-1* 3'UTR-Mut, respectively. The cells were harvested 24 h following transfection. The ratio of renilla luciferase luminescence to firefly luciferase luminescence was calculated. The experiments were performed in triplicate.

Real-time quantitative reverse transcription polymerase chain reaction PCR (qRT-PCR)

Total RNA in the HL-1 cells was extracted using TRIzol reagent (Invitrogen, USA). RNA was quantified by measuring the absorption at 260 nm. RNA purity was assessed by the absorbance ratio at 260 nm and 280 nm. A fraction was electrophoresed in 1% w/v agarose to verify RNA integrity. The mRNA level of miR-182, *NRF-1* was measured using the SYBR® Green qPCR Master mix (Applied Biosystems, USA). Gene expression was normalized to the level of GAPDH (*NRF-1*) or U6 (miR-182) within each sample using the relative $_{\Delta\Delta}$CT method. The samples were analyzed in triplicate. The primers used are detailed in **Table 1**.

Western blot assays

HL-1 cells were harvested in lysis buffer (Beijing Biotech Co. Ltd, China) and centrifuged at 12,000 rpm for 15 min at 4°C. Supernatants were analyzed for protein content. Samples were separated by 12% SDS-polyacrylamide gel (SDS-PAGE) and electro-transferred to nitrocellulose membranes. Nitrocellulose membranes were incubated with 5% non-fat skimmed milk powder in Tris-buffered saline (TBS) (100 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 0.1% Tween 20 (TBST) for 1 h. Overnight incubation was performed with primary antibodies to *NRF-1* (diluted 1:1000) and *mtTFA* protein (diluted 1:1000), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. After rinsing in TBST, the nitrocellulose membranes were exposed to an X-ray film using western blot detection reagents (Thermo Scientific, Pierce ECL, USA). Gel band density was scanned using the Gel Doc 2000® system and analyzed by Quantity One® image software (Bio-Rad, USA). All antibodies were purchased from Cell Signaling Technology, USA.

Statistical analysis

Data were expressed as a mean ± SEM. The statistical analysis was performed using one-way analysis of variance (ANOVA) or two-tailed student's t-test for multiple comparisons. The differences between comparisons were considered to be statistically significant at $P < 0.05$. SPSS software version 18.0 (IBM, USA) was used for data analysis. All experiments were performed in triplicate.

Results

The HL-1 cardiomyocyte H/R model

The results of LDH concentrations associated with the HL-1 cells after 24 h of hypoxia followed by 6 h of re-oxygenation was significantly increased compared with control HL-1 cells (16.37 ± 0.23 *vs*. 9.52 ± 0.86; $P < 0.05$). LDH concentrations were 30.56 ng/mL when hypoxia of 24 h was followed by re-oxygenation of 72 h (30.56 ± 2.62 *vs*. 12.56 ± 1.37; $P < 0.05$) (**Figure 1A**). The HL-1 cardiomyocyte apoptosis rate, measured after 24 h of hypoxia followed by 72 h of re-oxygenation, was significantly

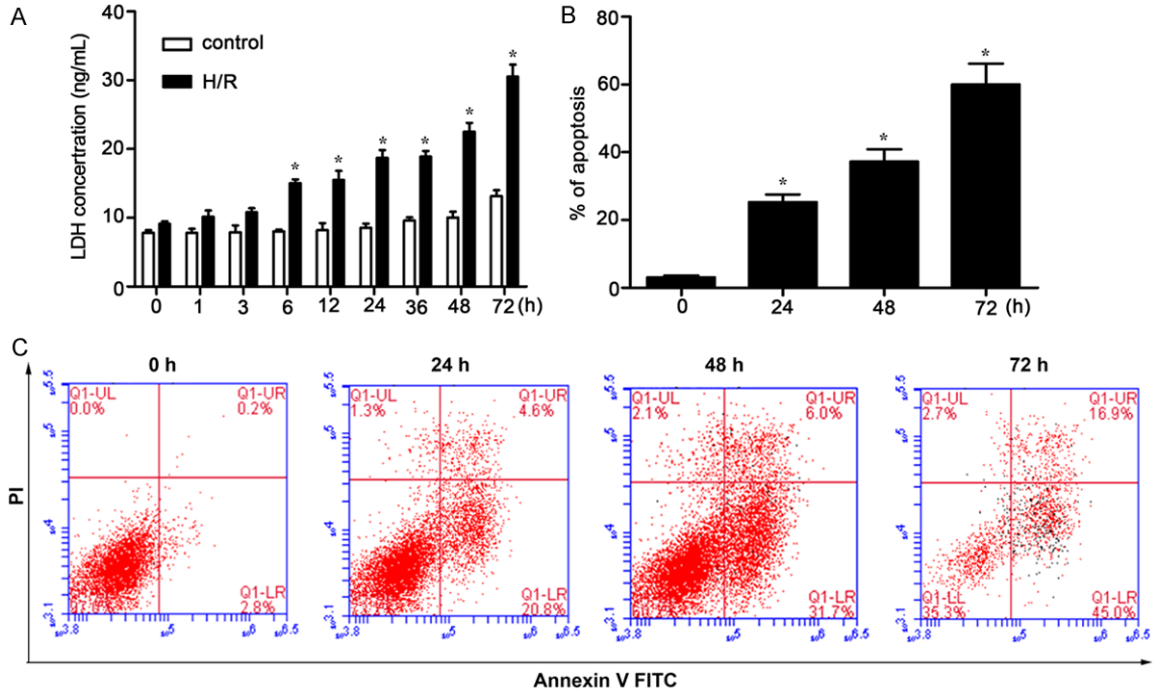

Figure 1. LDH concentrations (ng/mL) and HL-1 cardiomyocyte apoptosis in the H/R model. A. The LDH concentrations from the cultured HL-1 cardiomyocytes after 24 h of hypoxia followed by different time periods (0 to 72 h) of re-oxygenation stress. $^{*}P < 0.05$ *vs.* control. B. The apoptosis rate of the cultured HL-1 cardiomyocytes after 24 h of hypoxia followed by 0, 24, 48 and 72 h of re-oxygenation. $^{*}P < 0.05$ *vs.* H/R of 24/0 h. C. H/R-induced HL-1 cardiomyocyte apoptosis was detected by flow cytometry after 24 h of hypoxia followed by 0, 24, 48 and 72 h of re-oxygenation.

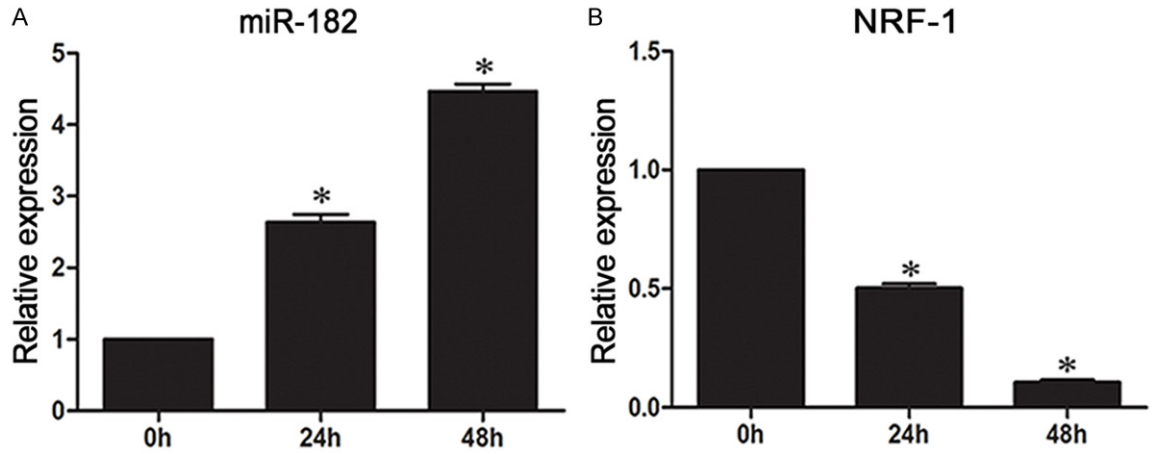

Figure 2. Expression levels of miR-182 and *NRF-1* in HL-1 cardiomyocytes by qRT-PCR. A. The expression level of miR-182 after 24 h of hypoxia followed by 0, 24 and 48 h of re-oxygenation. B. The expression levels of *NRF1* after 24 h of hypoxia followed by 0, 24 and 48 h of re-oxygenation $^{*}P < 0.05$ *vs.* H/R of 24/0 h.

increased by up to 60% (60.25 ± 6.02 *vs.* 3.1 ± 0.56; $P < 0.05$) (**Figure 1B**, **1C**). On the basis of these findings, an *in vitro* HL-1 model was used that included a 24 h period of hypoxia followed by a 48 h period of re-oxygenation, or H/R of 24/48 h.

MiR-182 and NRF-1 expression in HL-1 cardiomyocytes following H/R

In order to determine the potential involvement of miR-182 and *NRF-1* in H/R injury, qRT-PCR was used to determine miR-182 and *NRF-1*

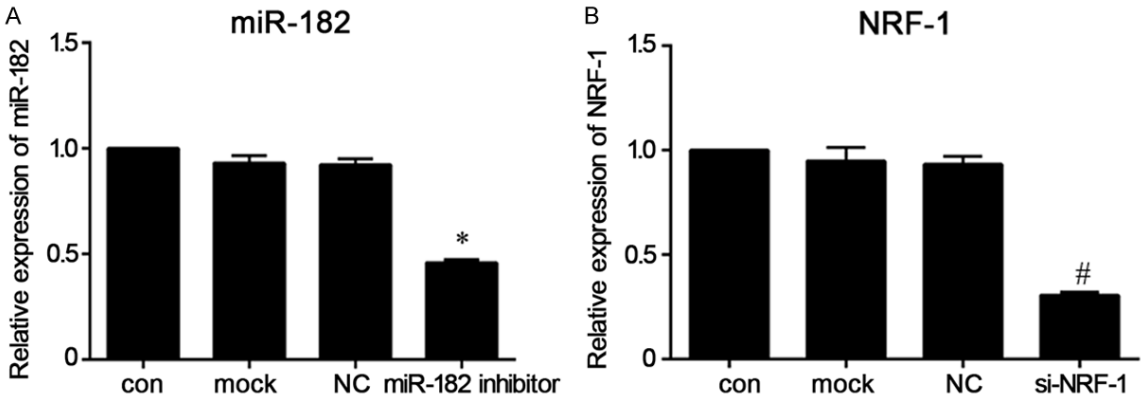

Figure 3. Relative gene expression levels in HL-1 cardiomyocytes transfected with miR-182 inhibitor or siRNA-*NRF-1*. HL-1 cardiomyocytes were transfected with miR-182 inhibitor or siRNA-NR-1. A. The expression level of miR-182. $^{*}P < 0.05$ *vs.* NC inhibitor. B. The expression level of *NRF-1*. $^{\#}P < 0.05$ *vs.* siR-NC.

expression levels. The expression of miR-182 following H/R of 24/48 h was about 4-fold of the control HL-1 cells (4.52 ± 0.46 *vs*. 1.02 ± 0.00; $P < 0.05$) (**Figure 2A**). *NRF-1* mRNA expression following 24 h of hypoxia and 48 h of re-oxygenation was significantly down-regulated compared to expression at 0 h (0.13 ± 0.02 *vs*. 1.0 ± 0.00; $P < 0.05$) (**Figure 2B**).

The effects of RNA interference

In cultured HL-1 cells under normal control conditions, When compared with the NC inhibitor group, the level of miR-182 was significantly decreased when transfected with miR-182 inhibitor (0.45 ± 0.01 *vs*. 1.00 ± 0.00; $P < 0.05$) (**Figure 3A**). However, transfection with siRNA-*NRF-1* significantly decreased the level of *NRF-1* in normal HL-1 cardiomyocytes (0.30 ± 0.01 *vs*. 1.00 ± 0.00; $P < 0.05$) (**Figure 3B**).

Inhibition of miR-182 protects against H/R-induced injury and apoptosis

Flow cytometry analysis (**Figure 4A**) revealed increased apoptosis in the HL-1 cardiomyocytes following the H/R schedule (35.2 ± 3.5% *vs*. 9.8 ± 1.3%; $P < 0.01$). Transfection with miR-182 inhibitor significantly decreased the percentage of apoptotic cells induced by the H/R schedule (17.8 ± 2.5). When co-transfected with siRNA-*NRF-1* the apoptotic rate increased, equating with an attenuating effect of the miR-182 inhibitor (30.2 ± 2.3) (**Figure 4B**).

The H/R schedule of a 24 h period of hypoxia followed by a 48 h period of re-oxygenation of the HL-1 cardiomyocytes up-regulated LDH concentrations (27.03 ± 1.06 *vs*. 9.86 ± 0.79; $P < 0.05$). The miR-182 inhibitor significantly decreased LDH release in the HL-1 cardiomyocytes during the H/R schedule (19.03 ± 2.57). Co-transfection with siRNA-*NRF-1* reduced the effects of the miR-182 inhibitor (25.54 ± 2.06) (**Figure 4C**).

NRF-1 as a target of miR-182

In order to investigate the mechanism of miR-182, an approach was made to target the genes for miR-182 using TargetScanMouse online software (Whitehead Institute for Biomedical Research, USA) and miRBase (Manchester University, UK). *NRF-1* was predicted to be a putative target gene of miR-182. Specifically, the 3'-UTR of the *NRF-1* mRNA contains one binding site for miR-182 (**Figure 5A**).

This miR-182 mimic reduced the activity of the luciferase reporter when fused with the *NRF-1*-UTR by 35% compared with the NC mimic (2.61 ± 0.26 *vs*. 4.01 ± 0.14; $P < 0.05$). Conversely, the miR-182 inhibitor increased the luciferase activity compared with the NC inhibitor (6.01 ± 0.32; $P < 0.05$) (**Figure 5B**).

Activation of the NRF-1/mtTFA signaling pathway

The miR-182 inhibitor promoted *NRF-1* expression and activated the NRF-1/mtTFA signaling pathway. The protein expression levels of *mtTFA* and *NRF-1* are shown in **Figure 6A**. Following the H/R schedule, the protein levels of *NRF-1* in

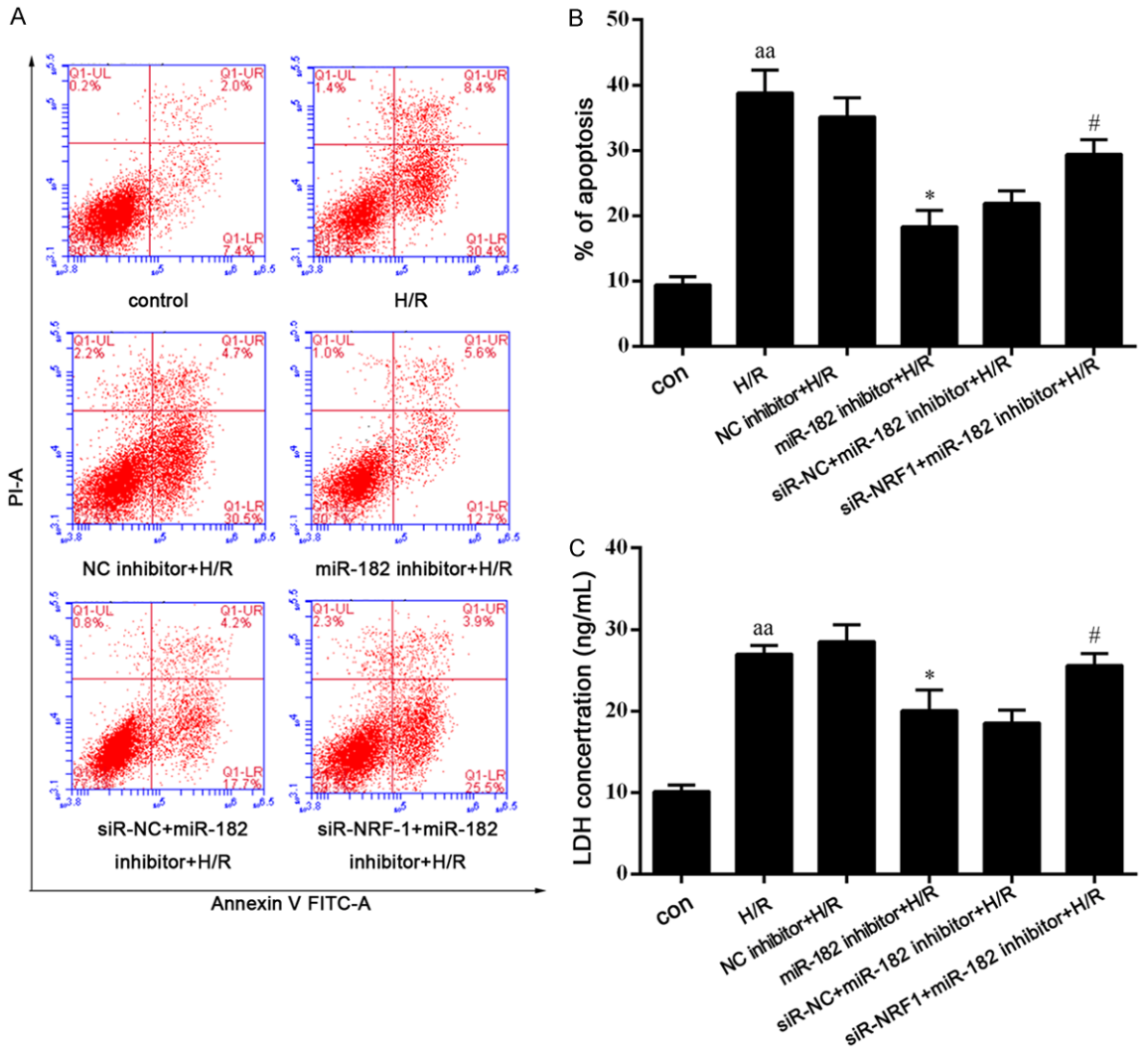

Figure 4. HL-1 cardiomyocyte apoptosis and LDH concentration. A. Representative dot-plot diagrams of Annexin-V/propidium iodide (AV/PI) flow cytometry. B. Percentage of apoptotic cardiomyocytes. C. LDH concentration of HL-1 cardiomyocytes. $^{aa}P < 0.01$ control; $^{*}P < 0.05$ *vs.* NC inhibitor + H/R; $^{\#}P < 0.05$ *vs.* siR-NC+miR-182 inhibitor + H/R.

the HL-1 cardiomyocytes were significantly increased by the miR-182 inhibitor. The effects of miR-182 inhibitor were decreased by co-transfection with *siRNA-NRF-1* (**Figure 6B**). Following the *in vitro* H/R schedule, the protein levels of *mtTFA* in the HL-1 cardiomyocytes were significantly increased by the miR-182 inhibitor. The effects of the miR-182 inhibitor on *mtTFA* were decreased by co-transfection with siRNA-*NRF-1* (**Figure 6B**).

Discussion

Cardiac myocyte apoptosis is an important mechanism for cardiac damage in myocardial ischemia [5-7, 17]. Recent studies have shown that several genes are abnormally expressed in ischemic heart disease, but as yet the function of the majority of these genes remains unclear [18]. A number of miRNAs regulate the expression of apoptosis-related genes and have been implicated in reperfusion and H/R injury [19]. The miRNAs down-regulate gene expression by degrading target mRNAs through binding to 3'-UTR [20, 21].

In this study, cultured HL-1 mouse cardiomyocytes were subjected to a regime of 24 h of hypoxia followed by 48 h of re-oxygenation. In this *in vitro* model, the findings showed that miR-182 expression in HL-1 cardiomyocytes supported the role for miR-182 as a H/R-related cardiomyocyte miRNA. Further findings of this study were that cardiomyocyte apoptosis and

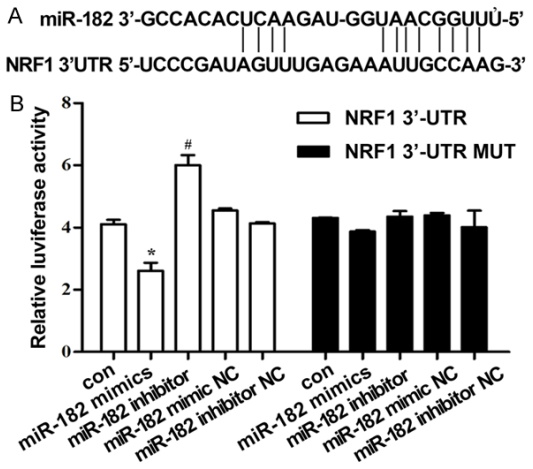

Figure 5. MiR-182 directly regulates *NRF1* expression by 3'-UTR site. A. The protential binding site for miR-182 in the 3'-UTR of *NRF1*. B. Luciferase reporter assay was performed by co-transfection of 293T cells with psiCheck2 containing the *NRF1* 3'-UTR or *NRF-1* 3'UTR-Mut of mouse *NRF-1* with miR-182 mimic, NC mimic, miR-182 inhibitor, NC inhibitor, respectively. $^{*}P < 0.05$ miR-182 mimic *vs.* NC mimic; $^{\#}P < 0.05$ miR-182 inhibitor *vs.* NC inhibitor.

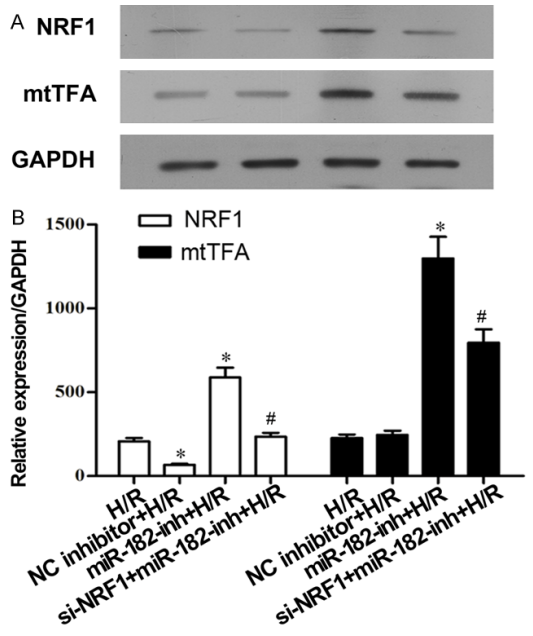

Figure 6. Inhibition of miR-182 promotes *NRF1* expression and activates the NRF1/mtTFA signaling pathway. A. Western blots for *NRF1* and mtTFA protein level in the HL-1 cardiomyocytes transfected with miR-182 inhibitor, or co-transfected with miR-182 inhibitor and siRNA-*NRF1*, following by H/R of 24/48 h. B. 'Grey value' image analysis of western blot data. $^{*}P < 0.05$ *vs.* NC inhibitor + H/R; $^{\#}P < 0.05$ *vs.* miR-182 inhibitor + H/R.

the secretion of LDH were reduced when miR-182 was knocked down.

NRF-1 drives the expression of genes involved in transcription, oxidative phosphorylation, replication of the mitochondrial genome and the expression of antioxidant genes [22]. In human cardiac myocytes, *NRF-1* has been shown to play an important role in defects in *mtTFA* DNA [23]. A recent study has shown that *NRF-1* and *mtTFA* regulate mtDNA transcription in many tissues during embryogenesis [24].

Bioinformatics analysis (TargetScan and miRBase) was used in this study to identify *NRF-1* as a target of miR-182. This prediction was confirmed by the data from a dual luciferase reporter assay. Inhibition of miR-182 increased *NRF-1* protein levels. This study has shown that in the HL-1 *in vitro* mouse cardiomyocyte model, miR-182 inhibition reduced H/R-induced cardiomyocyte apoptosis by an NRF-1/mtTFA signaling pathway. Recently studies have shown the involvement of other regulatory factors in H/R-induced cardiomyocyte apoptosis, including *Smad-7* [14, 20], *Hsp-20* [25], *IRAK-1* and *TRAF-6* [26].

There are, of course, limitations on the extrapolation of *in vitro* data to the *in vivo* situation and clinical cardiac disease. Studies on cell lines may not reflect responses found in whole-organ studies [8]. For example, in a recent review of the role of TGFβ in cardiac apoptosis following ischemia, the protective effects of transforming growth-factor β (TGFβ) were found in HL-1 cells, which are neonatal cells, whereas apoptosis promoting effects were described for TGFβ in adult cardiac myocytes [27]. It would, therefore, be of interest if future studies could be done to investigate the NRF-1/mtTFA signaling pathway using *in vivo* models of cardiac ischemic reperfusion injury.

In conclusion, the findings of this study have shown that miR-182 increased apoptosis induced by H/R in cultured HL-1 mouse cardiomyocytes and that miR-182 inhibition inhibited apoptosis. These findings support the view that future *in vivo* studies should be done to investigate a role for a miRNA-182 knockdown in reducing apoptosis and reperfusion injury associated with myocardial ischemia and infarction. These further studies would be

important as they may form the basis for the future therapeutic modulation of the NRF-1/mtTFA signaling pathway by administration of miR-182 as cardio-protective therapy in myocardial ischemia.

Disclosure of conflict of interest

None.

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