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miR-138 protects cardiomyocytes from hypoxia-induced apoptosis via MLK3/JNK/c-jun pathway

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ABSTRACT

Cardiomyocytes experience a series of complex endogenous regulatory mechanisms against apoptosis induced by chronic hypoxia. MicroRNAs are a class of endogenous small non-coding RNAs that regulate cellular pathophysiological processes. Recently, microRNA-138 (miR-138) has been found related to hypoxia, and beneficial for cell proliferation. Therefore, we intend to study the role of miR-138 in hypoxic cardiomyocytes and the main mechanism. Myocardial samples of patients with congenital heart disease (CHD) were collected to test miR-138 expression. Agomir or antagomir of miR-138 was transfected into H9C2 cells to investigate its effect on cell apoptosis. Higher miR-138 expression was observed in patients with cyanotic CHD, and its expression gradually increased with prolonged hypoxia time in H9C2 cells. Using MTT and LDH assays, cell growth was significantly greater in the agomir group than in the negative control (NC) group, while antagomir decreased cell survival. Dual luciferase reporter gene and Western-blot results confirmed MLK3 was a direct target of miR-138. It was found that miR-138 attenuated hypoxia-induced apoptosis using TUNEL, Hoechst staining and Annexin V-PE/7-AAD flow cytometry analysis. We further detected expression of apoptosis-related proteins. In the agomir group, the level of pro-apoptotic proteins such as cleaved-caspase-3, cleaved-PARP and Bad significantly reduced, while Bcl-2 and Bcl-2/Bax ratio increased. Opposite changes were observed in the antagomir group. Downstream targets of MLK3, JNK and c-jun, were also suppressed by miR-138. Our study demonstrates that up-regulation of miR-138 plays a protective role in myocardial adaptation to chronic hypoxia, which is mediated mainly by MLK3/JNK/c-jun signaling pathway.

1. Introduction

Cyanotic congenital heart disease (CHD) is the most common innate malformation in infants and children, in which chronic hypoxia is the basic pathophysiological process. Hypoxia affects mitochondrial oxidative metabolism, leading to a heart remodeling process. However, adaptation to chronic hypoxia represents a well-defined and reproducible mean to improve cardiac hypoxic tolerance. Long-term clinical observations demonstrate that although patients with cyanotic CHD have been under the condition of low-oxygen blood perfusion, they can still survive in a certain period of time, and rarely progress to heart failure [1]. Until now, the existing data are not conclusive enough to explain this phenomenon and its detailed molecular mechanism remains unknown. Limited evidence exists for the involvement of reactive oxygen species (ROS), nitric oxide (NO) and some protein kinases [2]. Therefore, studying the protective adaptation of cardiomyocytes to chronic hypoxia and the underlying mechanism may provide the insight into treatments for limiting myocardial damage during cardiac surgery.

MicroRNAs (miRNAs) are a group of small, endogenous, non-coding single-stranded RNAs that regulate gene expression by controlling the stability and translation of protein-coding mRNAs [3], and recently, their role in cardiac protection and development has been increasingly valued [4]. MicroRNA-138 (miR-138) is previously proven to be an important regulator in heart development. Morton et al. discovered that it was expressed in specific domains in the zebrafish heart and was required to establish appropriate chamber-specific gene expression patterns [5]. Dysregulation of miR-138 may lead to disrupted heart morphogenesis and cardiac function [5,6]. Interestingly, in pulmonary artery smooth muscle cells (PASMCs), miR-138 expression was found to be up-regulated induced by hypoxia, and could directly inhibit the serine-threonine kinase (Mst1), an amplifier of cell apoptosis, thereby accelerating the proliferation of PASMCs [7]. Furthermore, miR-138 could also promote induced-pluripotent stem (iPS) cell colony formation via targeting p53 during the reprogramming process [8]. However, the possible effects of miR-138 on hypoxic adaptation in cardiomyocytes are less well-studied.

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In the present study, clinical myocardial samples of patients with cyanotic or acyanotic CHD were collected. Through preliminary literature retrieval, we screened several miRNAs related to hypoxia, and further investigated their changes. Our experiments revealed that miR-138 was significantly increased in hypoxic cardiomyocytes. By silencing and overexpressing of miR-138 *in vitro*, we aimed to explore its role in apoptosis of cardiomyocytes subjected to chronic hypoxia.

2. Materials and methods

2.1. Patients studied

All procedures were approved by the Human Ethical Committee of Xinqiao Hospital. A total of 21 patients, who underwent cardiac operations for congenital heart diseases, were enrolled in this study. 10 patients had cyanotic and 11 had acyanotic cardiac defects. The investigation confirmed with the principles outlined in the Declaration of Helsinki. Informed consents were obtained from all subjects before participation.

2.2. Collection of myocardial samples

Standardized anesthesia and operation were carried out routinely as previously described [9]. A biopsy was taken from the right ventricular outflow tract immediately after cardiac arrest. The myocardial samples were snap frozen in liquid nitrogen and stored at −70 °C for qRT-PCR analysis.

2.3. Cell culture

Embryonic rat heart-derived H9C2 cells and human renal epithelial-derived 293T cells (ATCC, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA). After serum-starved overnight, cells were placed in an Invivo200 cultivator (Ruskin Technology Ltd, UK) containing a gaseous mixture of 94% N_2, 5% CO_2 and 1% O_2 at 37 °C for durations of 12, 24, 48 and 72 h respectively. Cells in normoxia group were incubated under the same conditions except for 21% O_2 concentrations.

2.4. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's instructions. A PrimeScript RT reagent Kit (Takara, Japan) was used to create cDNA library. PCR analysis was performed on Applied Biosystems 7500 Sequence Detection system (ABI, USA) using SYBR Premix Ex Taq GC kit (Takara, Japan). The stem-loop primers used for the PCR amplification were synthesized by RiboBio (China). All reactions were run in triplicate. The relative expression level of miR-138 was normalized against U6 expression level. Fold changes relative to control samples were determined by the $2^{-\Delta\Delta Ct}$ methods.

2.5. MicroRNA agomir or antagomir transfection

For overexpression of miR-138, agomir (GenePharm, China), a kind of chemically modified microRNA simulants, was used to transfect cells, and agomir-negative control (agomir-NC), which was similar to agomir but with a scramble seeding sequence, acted as control. For inhibition of miR-138, a specific inhibitor antagomir was employed, and antagomir-negative control (antagomir-NC) was also used as control. Cells were transfected with 50 nM agomir/agomir-NC or 100 nM antagomir/antagomir-NC by using Lipofectamin 2000 (Invitrogen) following manufacturer's instructions. All reagents were labeled by FAM fluorescence to evaluate transfection efficacy, and qRT-PCR analysis was also used.

2.6. MTT assay

Cell survival was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Equal number of cells was seeded in 96-well plates. After incubation in hypoxic conditions for various hours, 20 μl MTT (5 mg/ml, Sigma) was added into each well. The plates were further cultured for 4 h before 150 μl dimethyl sulfoxide/well (DMSO, Sigma) was added. Absorbance was measured at 490 nm with a microplate spectrophotometer.

2.7. LDH assay

Cell death was assessed by the release of lactate dehydrogenase (LDH), a cytoplasmic enzyme, and a marker of membrane integrity. After hypoxic induction for 72 h, samples were centrifuged at 10,000g for 10 min, and supernatants were collected for analysis using a LDH assay kit (Beyotime, China). The results were divided by the value of maximal LDH release in each group, which were expressed as cell death rate.

2.8. Dual luciferase reporter gene assay

The 3′UTR of MLK3 was cloned into the psiCHECK-2 vectors (Promega, USA), immediately downstream of the stop codon of the luciferase gene to generate the psiCHECK-mlk3-3′UTR luciferase reporter plasmid. Mutagenesis was performed when the seed region was mutated to remove all complementarity to nucleotides of miR-138. Plasmid DNA and agomir/agomir-NC were co-transfected into 293T cells by using Lipofectamin 2000. 48 h later, luciferase activities were measured with a Dual-Glo Luciferase Assay System (Promega, USA). Firefly luciferase activity was normalized to Renilla luciferase activity.

2.9. Western blot

Polyclonal anti-MLK3 was purchased from Santa Cruz Biotechnology (SantaCruz, USA). Antibodies of caspase-3, PARP, Bad, Bcl-2, Bax, JNK, phospho-JNK, c-jun and phospho-c-jun were all from Cell Signaling Technology (CST, USA). Polyclonal anti-β-actin (Bioss, China) was used as an internal control antibody. Proteins of cells were extracted with SDS lysis buffer (Beyotime, China), and separated by SDS–PAGE gel. Subsequently, protein samples were transferred to polyvinylidene difluoride (PVDF; Roche). Membranes were probed with primary antibodies at 4 °C overnight, followed by incubation with horseradish peroxidase-conjugated secondary antibodies and detected by ECL.

2.10. Flow cytometry

Cell apoptosis was determined by annexin V assay. H9C2 cells were collected, washed, and suspended in annexin V binding buffer. Phycoerythrin (PE)-conjugated annexin V and 7-amino-actinomycin D (7-AAD; KeyGEN, China) were added to the cells successively. After incubation, annexin V binding buffer was added, and cells were analyzed by flow cytometry.

2.11. TUNEL assay

A TdT-mediated dUTP biotin nick-end labeling (TUNEL) kit (Beyotime, China) labeled by Cy3 fluorescence was also used to detect cell apoptosis according to manufacturer's instructions. The

Table 1
Clinical characteristics of included patients.

	Cyanotic	Acyanotic
n	10	11
Age at operation (month)	17.5 (8.2–33.7)	16.8 (6.9–28.0)
Weight at operation (kg)	10.3 (8.5–13.6)	9.8 (7.4–12.7)
Gender (male/female)	4/6	5/6
Oxygen saturation (%)	74.3 (65.0–85.0)*	98.0 (95.0–100)
Preoperative Hb (g/L)	171 (146–219)*	123 (108–150)
Preoperative Hct (%)	54.1 (46.0–65.7)*	40.3 (34.9–47.8)
Pathology		
TOF	9 (90)	
VSD & PA	1 (10)	
VSD & RVOS		11 (100)

Data are median (minimum-maximum) or number (%) of observations.
Hb, hemoglobin; Hct, hematocrit; TOF, tetralogy of fallot; VSD, ventricular septal defect; PA, pulmonary atresia; RVOS, right ventricular outflow tract obstruction.
* $p < 0.05$ *vs.* acyanotic group.

apoptotic cells in six random fields were photographed and counted for each group.

2.12. Hoechst staining

H9C2 cells were detected using Hoechst 33528 staining (Beyotime, China) following manufacturer's protocols. Cells with abnormal nuclei were considered apoptotic. A fluorescent microscope was used to image stained cells. The ratio of apoptotic nuclei/total nuclei was calculated averagely in six random fields.

2.13. Statistics

All results are expressed as mean ± SEM values. Data were analyzed using SPSS 18.0. Comparisons among various groups were determined by one-way analysis of variance (ANOVA) or *t*-test. Each experiment was repeated at least three times. All of the *p* values were two-tailed, and $p \leqslant 0.05$ was considered to be statistically significant.

3. Results

3.1. Clinical characteristics of included patients

A total of 21 patients (10 cyanotic and 11 acyanotic) were included in the present study. Clinical data were summarized in Table 1. Patients in the cyanotic group mainly underwent operation for tetralogy of Fallot (TOF), while ventricular septal defect (VSD) combined with right ventricular outflow tract obstruction (RVOS) was the only pathology in the acyanotic group. The two groups were well matched for gender, age and body weight. Oxygen saturation in the arterial blood was higher in the acyanotic group than in cyanotic group. In additon, preoperative hemoglobin (Hb) concentration and hematocrit (Hct) also had significant differences between the two groups.

3.2. Expression of miR-138 in hypoxic cardiomyocytes

According to previous reports, we detected expression of several miRNAs in hypoxic cardiomyocytes, of which miR-138

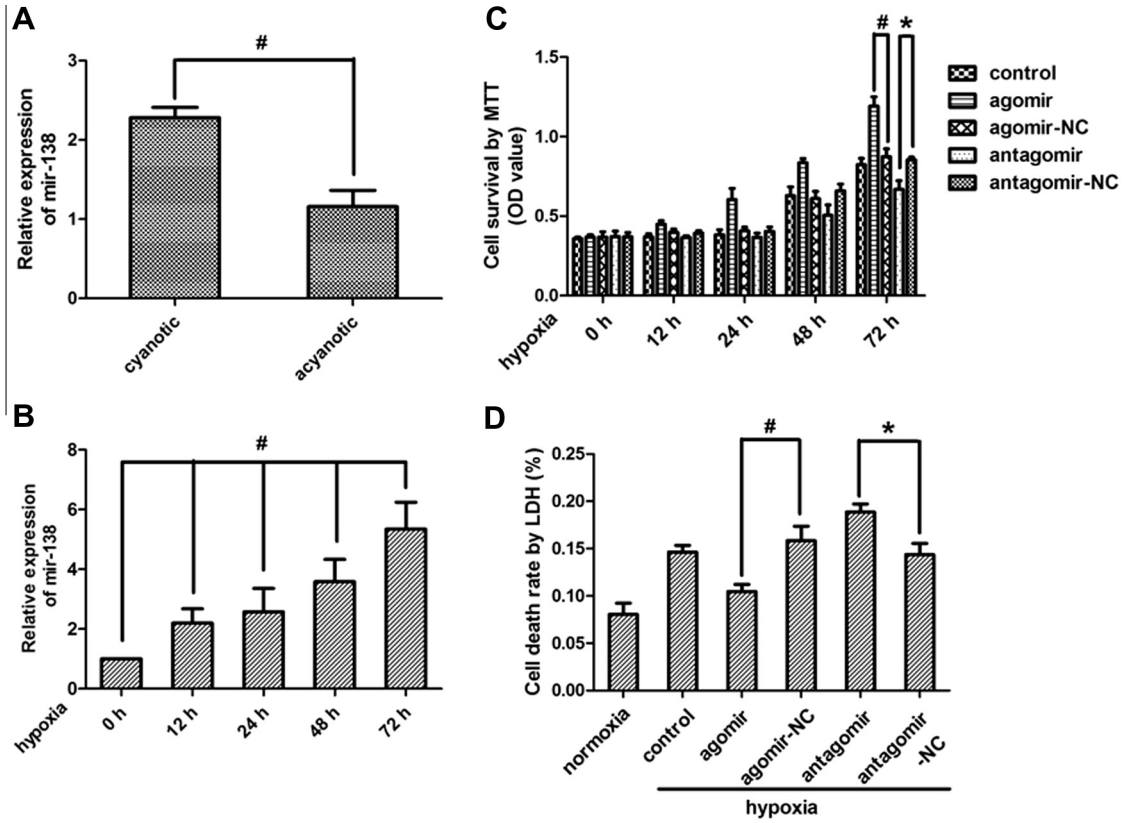

Fig. 1. Expression of miR-138 in hypoxic cardiomyocytes and its effect on cell growth. (A) qRT-PCR revealed that the expression of miR-138 was significantly increased in the myocardial samples of patients with cyanotic CHD ($^{\#}p < 0.05$). (B) H9C2 cells were cultured in 1% O_2 and 5% CO_2 for different duration, and expression of miR-138 increased in a time-dependent manner $^{\#}p < 0.05$. (C) After transfected with agomir or antagomir, cell survival curve was measured by MTT. (D) Cell death was determined by LDH release in normoxia or hypoxia at 72 h. Cell death rate = (LDH value − blank value)/(maximal release value − blank value). $^{\#}p < 0.05$ agomir group *vs.* agomir-NC group. $^{*}p < 0.05$ antagomir group *vs.* antagomir-NC group. Values are means ± SEM.

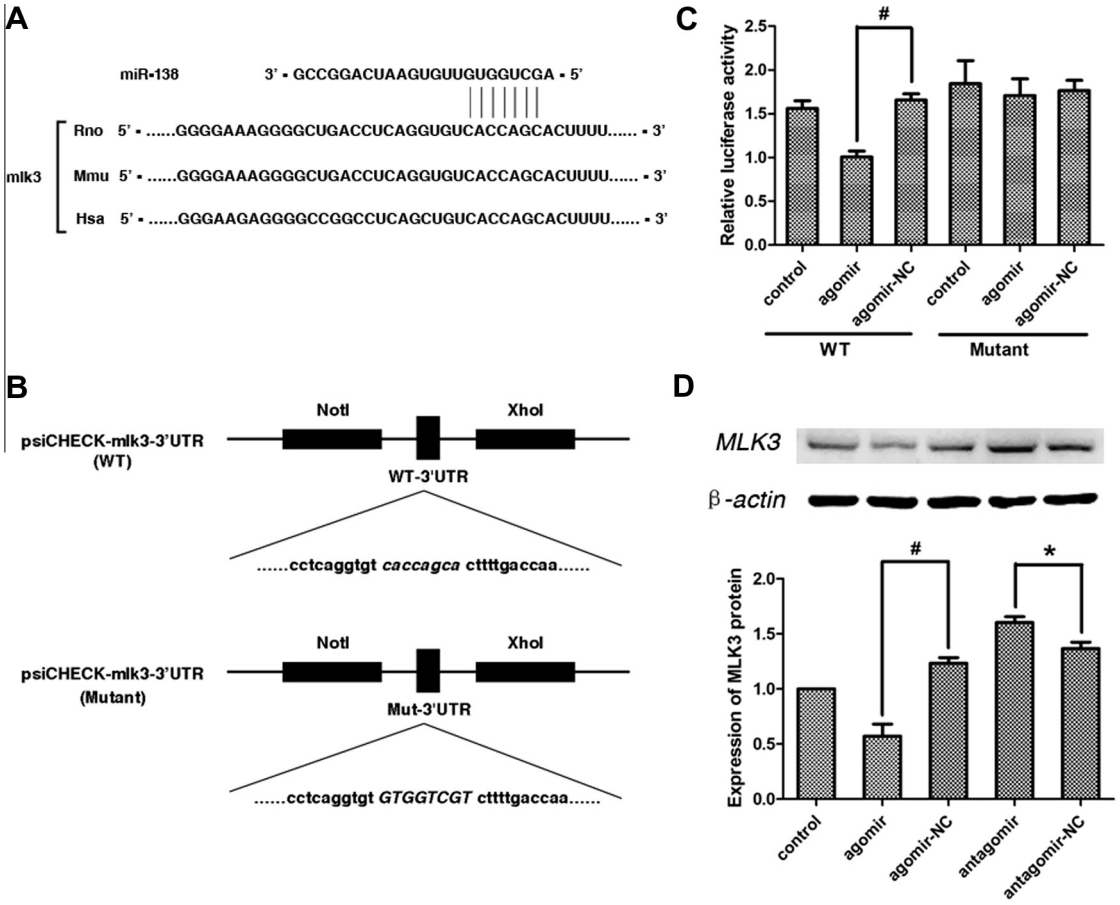

Fig. 2. MLK3 is a target of miR-138. (A) Sequence alignment of miR-138 and 3′UTR of MLK3 using TagetScan algorithm. (B) Diagram of plasmid construction. 3′UTR of MLK3 or a mutated sequence was cloned into psi-CHECK-2 vector. (C) Luciferase reporter assay with co-transfection of wild-type or mutant 3′UTR and agomir or agomir-NC or blank control in 293T cells $^{\#}p < 0.05$. (D) Western blot analyses were performed to examine the effects of miR-138 on expression of endogenous MLK3 protein levels. β-Actin was used as an internal control. $^{\#}p < 0.05$ agomir group *vs.* agomir-NC group. $^{*}p < 0.05$ antagomir group *vs.* antagomir-NC group. Values are means ± SEM.

was the one most sensitive to hypoxia. qRT-PCR analysis revealed that miR-138 expression in the cyanotic patients was 211% of that in acyanotic group ($^{\#}p < 0.05$) (Fig. 1A). H9C2 cells were exposed to hypoxia for 0, 12, 24, 48 and 72 h at 1% oxygen concentration. The expression of miR-138 was found to be gradually increased in a time-dependent manner ($^{\#}p < 0.05$) (Fig. 1B), and peaked by 5.34-fold at 72 h after the onset of hypoxia. These results suggested that miR-138 was up-regulated by hypoxia in cardiomyocytes.

3.3. Effect of miR-138 on cell survival in hypoxic conditions

To analyze the potential role of up-regulated miR-138 in cardiomyocytes, we induced silence and overexpression of miR-138 in H9C2 cells by transfection with agomir or antagomir. MTT assay was used to test cell growth. With prolonged time of hypoxia, overexpression of miR-138 efficiently promoted H9C2 cells proliferation, and at 72 h, the amount of cells reached 136% of that in agomir-NC group ($^{\#}p < 0.05$) (Fig. 1C). Inhibition of miR-138 with its complementary RNA analogs (antagomir) resulted in slower cell growth, which was 0.9-fold changes compared with antagomir-NC group in hypoxic conditions at 72 h ($^{*}p < 0.05$) (Fig. 1C). Cell death rate was measured by LDH release. After 72 h exposure to hypoxia, the agomir group achieved the lowest cell death rate ($^{\#}p < 0.05$ *vs.* agomir-NC group), whereas the antagomir group had the highest ($^{*}p < 0.05$ *vs.* antagomir-NC group) (Fig. 1D). Together, these data indicated that hypoxia-induced upregulation of miR-138 was beneficial for cardiomyoctes survival.

3.4. MLK3 is the target of miR-138

In order to elucidate the underlying molecular mechanism, we performed a bioinformatic analysis using TargetScan algorithm to predict the possible target gene of miR-138. We found that mixed-lineage kinase 3 (MLK3) contained theoretical miR-138 binding site in its 3′UTR (Fig. 2A). MLK3 is a well-known pro-apoptotic enzyme, and its inhibitor exhibits anti-inflammatory and cytoprotective effects. Given that miR-138 promoted cell growth, it was strongly supported that MLK3 may be regulated by miR-138. To confirm this hypothesis, we cloned the 3′UTR of MLK3 into psi-CHECK-2 vector and co-transfected miR-138 agomir with the vector in 293T cells. The normalized luciferase activity was significantly reduced compared with that of the negative control ($^{\#}p < 0.05$) (Fig. 2C). We also constructed a vector in which miR-138 binding sites were all mutated. miR-138 agomir failed to decrease the activity of luciferase gene with mutant 3′UTR (Fig. 2C). Furthermore, transfection of H9C2 cells with miR-138 agomir obviously reduced MLK3 protein level ($^{\#}p < 0.05$ *vs.* agomir-NC group), whereas transfection of miR-138 antagomir significantly increased the protein amount of MLK3 ($^{*}p < 0.05$ *vs.* antagomir-NC group) (Fig. 2D). All these findings suggested that miR-138 inhibited MLK3 protein expression by directly binding to its 3′UTR.

3.5. miR-138 attenuated hypoxia-induced apoptosis

MLK3 activates multiple MAPK pathways and can initiate apoptosis in different cell types [10]. In cardiomyocytes, hypoxia

induced increased expression of miR-138, and subsequently inhibited MLK3 protein, which would suppress cell apoptosis theoretically. To verify this assumption, we used flow cytometry, TUNEL and Hoechst staining to analyze cell apoptosis under hypoxic conditions. Because transfection agents (agomir and antagomir) were labeled by FAM, which emitted green light, we employed annexin V-PE/7-AAD with red light. As shown in Fig. 3A, flow cytometry revealed that the amount of apoptotic cells in all hypoxic groups greatly increased after exposure for 72 h. When exogenous miR-138 was added into H9C2 cells (the agomir group), the pro-apoptotic actions were weakened almost close to the normoxia group. The antagomir group had the highest number of apoptotic cells up to 56.06% of the total (Fig. 3A). TUNEL was also labeled by Cy3 launching red fluorescence. In contrast to negative control cells, a smaller proportion of cells treated with agomir exhibited pyknotic, TUNEL-positive nuclei ($^{\#}p < 0.05$), while the number significantly increased in the antagomir group compared with its negative control ($^{*}p < 0.05$) (Fig. 3B and D). As similar to TUNEL results, the ratio of cells with abnormal nuclei stained by Hoechst was low in the agomir group ($^{\#}p < 0.05$ *vs.* agomir-NC group), and high in the antagomir group ($^{*}p < 0.05$ *vs.* antagomir-NC group) (Fig. 3C and E).

Next, we monitored the key changes of apoptosis-related proteins by using Western blotting. The caspase cascade is crucial for cell apoptosis, and caspase-3 and its substrate PARP are main effectors. Hypoxia induced high expression of cleaved caspase-3 and PARP, which could be eased up by agomir ($^{\#}p < 0.05$ *vs.* agomir-NC group) and enhanced by antagomir ($^{*}p < 0.05$ *vs.* antagomir-NC group) (Fig. 4B and D). Members of B-cell lymphoma-2 (Bcl-2) family proteins are thought to play regulatory roles in the apoptotic execution of cells. In hypoxic conditions, overexpression of miR-138 by agomir displayed increased levels of anti-apoptotic protein Bcl-2 (Fig. 4C), together with reduced pro-apoptotic protein Bcl-xl/Bcl-2 associated death promoter (Bad) (Fig. 4E). Although expression of Bcl-2 associated X protein (Bax) showed no obvious changes in the agomir or antagomir group, the ratio of Bcl-2/Bax varied significantly in an opposite trend towards expression of miR-138 (Fig. 4F).

Taken together, these findings strongly supported that hypoxia-induced cell damage would be alleviated by miR-138.

3.6. Effect of miR-138 on MLK3/JNK/c-jun pathway in hypoxic cardiomyocytes

MLK3 is traditionally known to generate effects via activation of c-jun N-terminal kinase (JNK). C-jun protein is a well-known substrate of JNK. We found miR-138 agomir or antagomir did not influence total JNK and c-jun protein levels after exposure to hypoxia for 72 h in H9C2 cells. As MLK3 was inhibited by miR-138 agomir, the expression of phospho-JNK and phospho-c-jun increased significantly in the agomir group compared with the agomir-NC group ($^{\#}p < 0.05$). miR-138 antagomir promoted the phosphorylation of JNK and c-jun ($^{*}p < 0.05$ *vs.* antagomir-NC group) (Fig. 4H and I). Thus, the above results indicated that miR-138 could block activation of JNK and c-jun, followed by directly targeting MLK3 in hypoxic cardiomyocytes.

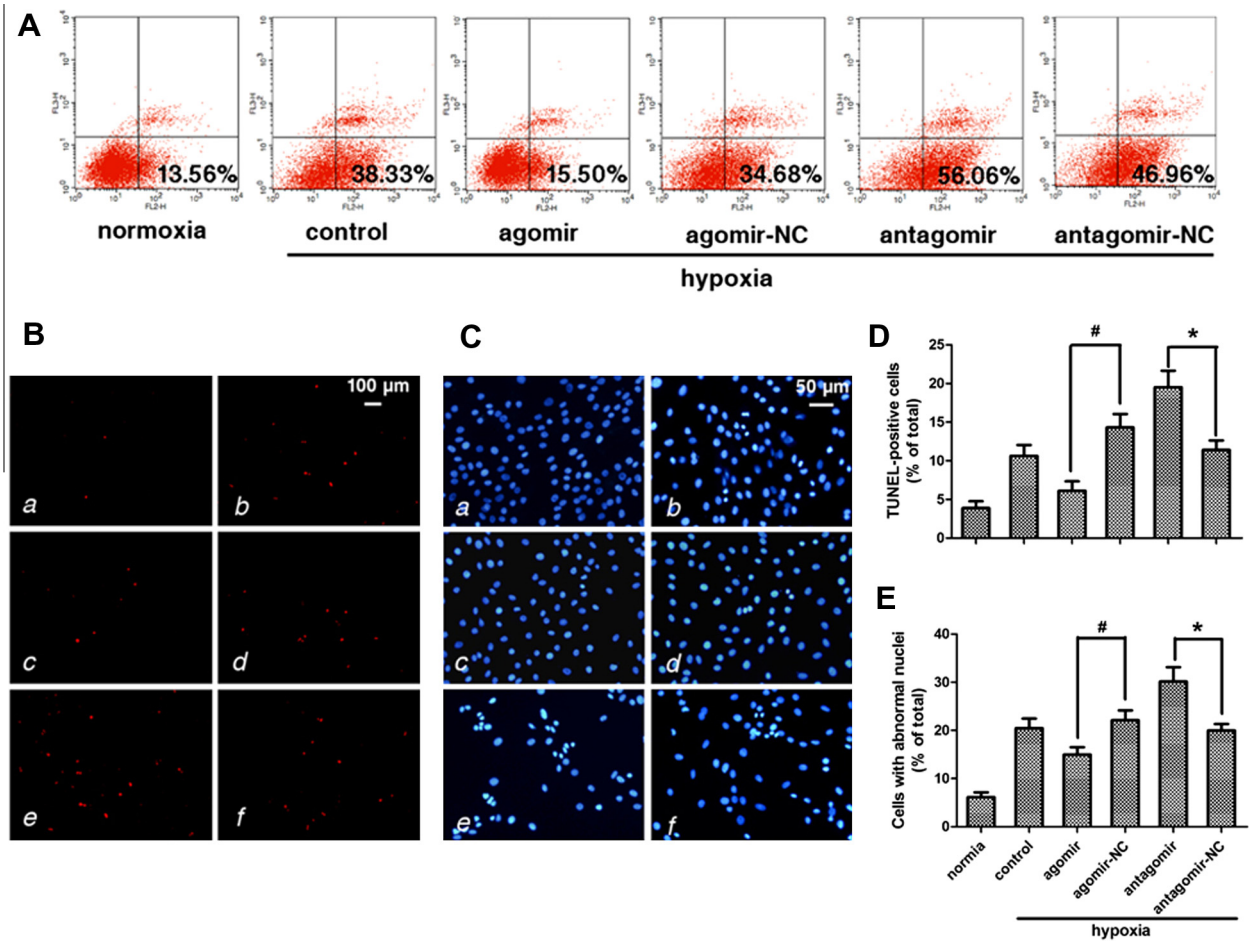

Fig. 3. miR-138 attenuates hypoxia-induced apoptosis in cardiomyocytes. Exposed to hypoxia for 72 h, cell apoptosis was tested by Annexin V-PE/7-AAD flow cytometry (A), TUNEL assay (labeled by Cy3) (B) and Hoechst staining (C). (D) Proportion of TUNEL-positive cells. (E) Percentage of cells with abnormal nuclei. Each value was counted in six random fields. $^{\#}p < 0.05$ agomir group *vs.* agomir-NC group. $^{*}p < 0.05$ antagomir group *vs.* antagomir-NC group. Values are means ± SEM.

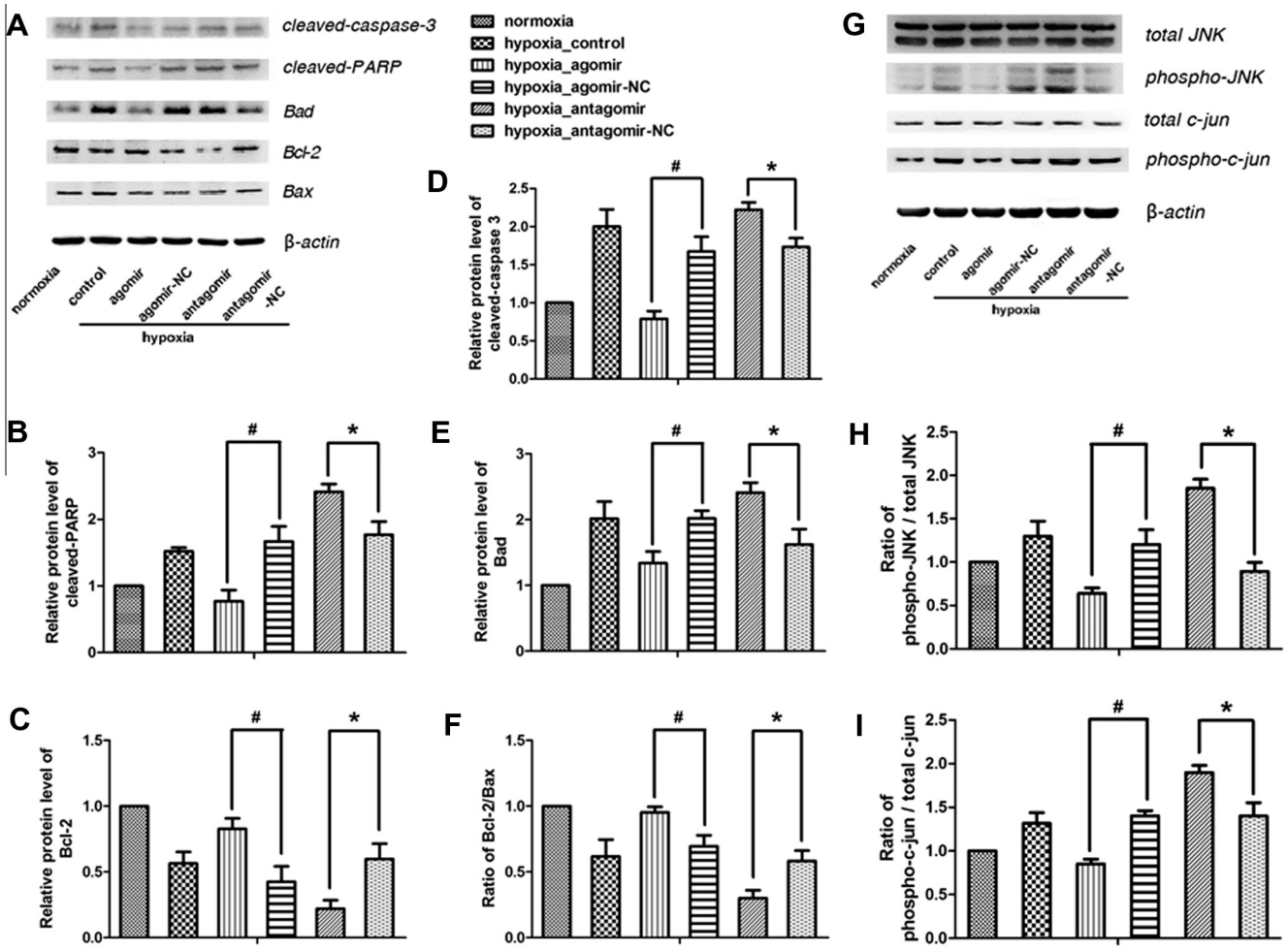

Fig. 4. Role of miR-138 in expression of apoptosis-related proteins and regulation of MLK3/JNK/c-jun signaling pathway. (A and G) Western-blot results showed relative expression of proteins in both normoxia and hypoxia at 72 h. miR-138 agomir significantly reduced the expression of cleaved-PARP (B), cleaved-caspase-3 (D) and Bad (E), together with increased expression of Bcl-2 (C) and ration of Bcl-2/Bax (F). Expression of Bax had no significant changes. miR-138 agomir significantly inhibited ratio of phospho-JNK/total JNK (H) and ratio of phospho-c-jun/total c-jun (I), whereas its antagomir exerted opposite effects. $^{\#}p < 0.05$ agomir group *vs.* agomir-NC group. $^{*}p < 0.05$ antagomir group *vs.* antagomir-NC group. β-Actin was used as an internal control. Values are means ± SEM.

4. Discussion

Chronic hypoxia is commonly observed in patients with cyanotic CHD, and its main clinical manifestations are low blood oxygen saturation combined with a high Hb concentration and Hct [11]. Infants with cyanotic CHD would experience stresses induced by chronic and systemic hypoxia as well as increased overload caused by cardiac defects, contributing to high mortality and morbidity. However, endogenous cellular protection against hypoxia-induced injury may be conferred by several intracellular components such as protein kinases [1] and heat stress proteins [12]. In the present study, we discovered the up-regulation of miR-138 in hypoxic H9C2 cells and myocardial biopsy of patients with cyanotic CHD, and it was confirmed to be beneficial for cardiomyocyte survival (Fig. 1). Based on the available evidence that miR-138 regulates heart development [5] and promotes cell proliferation in hypoxic PASMCs [7], we inferred that the up-regulation of miR-138 was probably a compensatory mechanism for cardioprotection against chronic hypoxia. The major objective of our study was to examine the role of miR-138 in adaptation of hypoxic cardiomyocytes.

MLK3, a member of mitogen-activated protein kinase (MAPK) family, is characterized by the presence of signature sequences of serine/threonine kinases within its catalytic domain in mammalian cells [13]. It is involved in many inflammatory signaling cascades. Upon activation, MLK3 phosphorylates MAP2K members, subsequently activating JNK and p38 signaling pathway [13]. MLK3 serves as a pro-apoptotic kinase, leading to cell death after trophic factor withdrawal [14] or in response to exogenous stimuli [15]. When MLK3 activity was blocked using RNAi methods or some specific inhibitors, its pro-apoptotic role would be abrogated [16]. Using dual luciferase reporter gene assay and Western blot analysis, we found that miR-138 could directly bind 3′UTR of MLK3, and decrease its protein level in H9C2 cells (Fig. 2D). Once hypoxia occurs, the oxygen demands cannot be met and a set of apoptotic responses will be evoked. As expected, when the agomir or antagomir of miR-138 was added into H9C2 cells, apoptosis induced by hypoxia was attenuated or aggravated respectively. This strongly implies that miR-138-induced suppression of apoptosis is mediated by targeting MLK3.

Caspase-3 is an essential member of the caspase family known to be involved in the final execution phase of apoptosis [17]. Bcl-2 family is also a critical regulator in cell apoptosis, among which Bcl-2 plays an anti-apoptotic role while Bad and Bax have pro-apoptotic effects [18]. As the most important interactions during apoptosis are considered to lie in Bcl-2/Bax dimerization, the ratio of Bcl-2/Bax serves as a rheostat that determines the survival or death of cells following an apoptotic stimulus [19]. Previous data suggest that overexpression of the dominant-negative MLK3 results in the inhibition of cytochrome c release and caspase-3

activation [20]. When MLK3 signaling pathway was inhibited, members of Bcl-2 family experienced corresponding changes to protect cells from apoptosis [21]. In the present study, miR-138 significantly regulated the expression of cleaved-caspase-3, cleaved-PARP, Bcl-2 and Bad via targeting MLK3, except for Bax expression. This can probably be interpreted as that the regulation of cell apoptosis by Bax does not rely on its expression, but chiefly on its oligomerization in the mitochondrial outer membrane [22] and intracellular movement from cytosol to mitochondria [23]. Further studies are needed to investigate the expression of Bax dimers. It was pleased to find that significant changes still occurred in the ratio of Bcl-2/Bax.

JNK signaling is a well-known target of MLK3. Activated JNK is translocated from cytoplasm to nucleus, and subsequently phosphorylates ser63 and ser73 of transcription factor c-jun, thereby regulating expression of apoptosis-related proteins [24]. Our study demonstrates that miR-138 efficiently inhibits MLK3 protein and phosphorylation of downstream JNK and c-jun under hypoxic conditions. Myocardial activation of JNK signaling was previously found in adult rats exposed to hypoxia for varying periods of time [25]. Blockade of JNK phosphorylation protects cardiomyocytes from hypoxia-induced apoptosis [26]. Therefore, we suppose that suppression of MLK3/JNK/c-jun signaling pathway activation is probably a main mechanism by which miR-138 is involved in hypoxic adaptation of cardiomyocytes.

In summary, hypoxia induces up-regulation of miR-138, which decreases the protein level of its target MLK3. via inhibiting MLK3/JNK/c-jun signaling pathway, miR-138 obviously attenuates hypoxia-induced apoptosis in cardiomyocytes. Our study provides a valuable clue for studying the adaptation of cardiomyocytes to chronic hypoxia as well as a significant target for clinical treatment of patients with CHD.

Acknowledgments

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