MYBL2 protects against H9c2 injury induced by hypoxia via AKT and NF-κB pathways

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Received September 18, 2016; Accepted May 25, 2017

DOI: 10.3892/mmr.2018.8387

Abstract. Cardiovascular diseases have become one of the major public health problems in many countries. The downregulation of MYBL2 was found in H9c2 and native cardiomyocytes cells after hypoxia treatment. The present study aimed to investigate the effects of MYB proto-oncogene like 2 (MYBL2) on H9c2 injury induced by hypoxia. Reverse transcription-quantitative polymerase chain reaction and western blot were performed on H9c2 cells to determine the mRNA and protein levels of MYBL2, respectively. Small interfering RNA (siRNA) was employed to downregulate MYBL2 expression in H9c2 cells to investigate changes in cell proliferation and apoptosis. Cell proliferation was assessed by a Cell Counting kit-8 assay and the percentage of apoptotic cells was determined using an Annexin V-fluorescein isothiocyanate/propidium iodide apoptosis detection kit. The nuclear factor-κB (NF-κB) and AKT signaling pathways in H9c2 cells were investigated by western blot analysis. The results demonstrated that the overexpression of MYBL2 promoted cell proliferation and suppressed apoptosis. Furthermore, overexpression of MYBL2 suppressed the expression of phosphorylated (p)-AKT, p-NF-κB inhibitor α, p-p65 and B-cell CLL/lymphoma 3 (Bcl-3). The results indicated that MYBL2 may improve cell viability and inhibit H9c2 apoptosis via the inhibition of AKT and NF-κB pathways. Therefore, MYBL2 may be a potential therapeutic target for the treatment of myocardial infarction.

Introduction

Cardiovascular diseases are a major public health burden in numerous countries, and have high rates of morbidity and mortality. Coronary artery disease, such as acute myocardial infarction, has an important role in cardiovascular diseases (1).

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Key words: MYB proto-oncogene like 2, H9c2, hypoxia, proliferation, apoptosis, AKT, nuclear factor-κB

Acute myocardial infarction (AMI) is an acute coronary syndrome that is characterized by ischemic necrosis of the cardiac muscle, whose pathogeny is the absence of blood flow to the myocardium caused by the blocking of the coronary artery (2,3). At present, improved methods to prevent, diagnose and treat patients with AMI, which have lowered the mortality associated with AMI, have been developed. However, the morbidity of AMI remains high. The most effective method for treating AMI is myocardial reperfusion (4), however, myocardial reperfusion is associated with various side effects, including cardiomyocyte death and dysfunction, which are collectively termed myocardial reperfusion injury (5).

The MYB proto-oncogene like 2 (MYBL2) gene, also termed B-MYB, is a member of the MYB family that also includes MYB proto-oncogene like 1 (also termed A-MYB) and MYB proto-oncogene, transcription factor (also termed C-MYB). A-MYB is expressed in the testis and is rarely observed in the ovaries, spleen and brain. C-MYB, which was identified earlier than A-MYB, is primarily expressed in hematopoietic stem cells (6), the colon (7) and the brain (8). MYBL2, which is essential to the regulation of proliferation and differentiation, is vital to the generation of embryonic stem cells and the formation of inner cell mass (9). MYBL2 is predominantly expressed in proliferative cells and has an important role in guiding cell cycle progression (10). A previous study has reported that downregulation of MYBL2 leads to the inhibition of cell cycle progression (11). Other reports have demonstrated that MYBL2 is overexpressed in lung cancer (12), hepatocellular carcinoma (13) and breast cancer (14), and may promote cell proliferation and metastasis.

H9c2 embryonic rat cardiac cells are a subclone of the original clonal cell line that are derived from the embryonic hearts of rats and have previously been used as *in vitro* models for investigating the mechanisms of myocardial apoptosis induced by hypoxia (15). In the current study, H9c2 myocardial cells were subjected to hypoxia to simulate the process of myocardial ischemia *in vitro*. Downregulation of MYBL2 was observed in H9c2 cells following hypoxia, indicating that MYBL2 may participate in the hypoxia-induced apoptosis of H9c2 cells.

Materials and methods

Cell culture and hypoxia treatment. The myoblast cell line H9c2 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany)

was cultured in Dulbecco's modified Eagle's Medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (100 U/ml and 100 mg/ml, respectively) and 1% GlutaMAX (200 mM) (All purchased from Thermo Fisher Scientific, Inc.) in a 5% CO_2 containing atmosphere at a relative humidity of 95% at 37˚C. Metabolic ischemia was induced by a buffer exchange to an ischemia-mimetic solution (in mM: 125 NaCl, 8 KCl, 1.2 KH2PO4, 1.25 MgSO4, 1.2 CaCl2, 6.25 NaHCO3, 5 sodium lactate, 20 HEPES, pH 6.6) and by placing the dishes in hypoxic pouches with an indicator strip in a GasPak EZ Anaerobe Gas Generating Pouch System (BD Biosciences, Franklin Lakes, NJ, USA) for 2 h at 37˚C. As certified by the manufacturer, the Anaerobe Gas Generating Pouch System produces an atmosphere containing 10% CO_2 and 1% O_2. Non-hypoxia cells exposed to normoxic conditions (95% O_2, 5% CO_2) were included as the control for hypoxia.

Plasmids and small interfering RNA (siRNA) transfection. An MYBL2 expression vector (pc-MYBL2) was constructed by subcloning the full-length wild-type MYBL2 coding sequence into pcDNA3.1 (+) vector (Thermo Fisher Scientific, Inc.), and confirmed by Sanger sequencing. The empty construct pcDNA3.1 was transfected as a control. The target sequence for MYBL2-specific siRNA was 5'GCAGAGGACAGUAUC AACA(dT)(dT)'3, and both MYBL2 siNRA and control siRNA were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells were seeded into 6-well plates at a concentration of 10^5 cells/well and incubated at 37˚C in a humidified atmosphere containing 5% CO_2 for 24 h. Then plated cells were transfected with MYBL2 siRNA or siNC at a final concentration of 50 nM using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Approximately 4 weeks later, stable MYBL2 transfection was generated under G418 (Gibco; Thermo Fisher Scientific, Inc.) selection, as previously described (16).

Cell proliferation assay. H9c2 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well. Cell proliferation was assessed by a Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Briefly, following stimulation of hypoxia, CCK-8 solution was added to the culture medium and the cultures were incubated for 1 h at 37˚C in humidified 95% air and 5% CO_2. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Apoptosis analysis. Apoptosis analysis was performed to identify and quantify the apoptotic cells by using an Annexin V-Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) Apoptosis Detection kit (Beijing Biosea Biotechnology, Beijing, China). The cells ($1x10^5$ cells/well) were seeded into 6 well-plates. Treated cells were washed twice with cold PBS and resuspended into single cell solution of $1x10^6$ cell/ml in 500 μl binding buffer of Annexin V-Fluorescein Isothiocyanate/Propidium Iodide (FITC/PI) Apoptosis Detection kit (Beyotime Institute of Biotechnology, Haimen, China). Then, cells were stained with 5 μl Annexin V-FITC and 5 μl PI for 30 min at room temperature in the dark. Samples were measured with a flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) to differentiate apoptotic cells (Annexin V positive and PI-negative) from necrotic cells (Annexin V positive and PI-positive). Flow cytometry results were analyzed using FlowJo Software (version 7.6, TreeStar, Inc., Ashland, OR, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from transfected cells ($1x10^6$) by using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), which was followed by treatment with DNase I (Promega Corporation, Madison, WI, USA). RT was performed by using the TaqMan™ Reverse Transcription Reagents kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RT conditions were 10 min at 25˚C, 30 min at 48˚C and a final step of 5 min at 95˚C. qRT-PCR was performed using a SYBR Premix Ex Taq kit (TaKaRa Biotech, Kyoto, Japan) in a 7500 Real-Time PCR System (ABI; Applied Biosystems, Carlsbad, CA, USA). The PCR primers were obtained from Invitrogen, and the reaction conditions included the following steps: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The PCR primers were obtained from Invitrogen (Thermo Fisher Scientific, Inc.), and the sequences of the primers used for PCR were as follows: MYBL2, 5'AAAACA GTGAGGAGGAAC'3 (forward) and 5'CAGGGAGGTCAA ATTTAC'3 (reverse); GAPDH, 5'GCACCGTCAAGGCTG AGAAC'3 (forward) and 5'TGGTGAAGACGCCAGTGGA3' (reverse). mRNA expression of MYBL2 was normalized to GAPDH expression which was used as an internal control and relative expression changes of MYBL2 were calculated using the $2^{-\Delta\Delta Cq}$ method as previous described (17). The experiments were repeated three times.

Western blot analysis. Protein from $2x10^6$ cells was extracted using radio immune precipitation assay lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors (Roche Applied Science, Penzberg, Germany). Proteins were quantified using the BCA Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein samples (30 μg) were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Subsequently, membranes were blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. Primary antibodies were prepared in 5% BSA at a dilution of 1:1,000.

The western blotting system was established using a Bis-Tris Gel system (Bio-Rad Laboratories, Inc.), according to the manufacturer's protocol. MYBL2 antibody (cat. no. ab76009) was purchased from Abcam (Cambridge, UK) and GAPDH antibody (cat. no. G9545) was purchased from Sigma-Aldrich (Merck KGaA). Antibodies: t-AKT (cat. no. 4685), p-AKT (p308; cat. no. 13038), p-AKT (p473; cat. no. 4060), t-IκBα (cat. no. 4812), p-IκBα (cat. no. 2859), t-p65 (cat. no. 8242) and p-p65 (cat. no. 3033) were purchased from Cell Signaling Technology (Beverly, MA, USA). Bcl-3 antibody (cat. no. ab27780) was purchased from Abcam (Cambridge, UK). Primary antibodies were incubated with the corresponding membranes at 4˚C overnight, followed by washing and incubation with a goat anti-rabbit IgG secondary antibody horseradish peroxidase

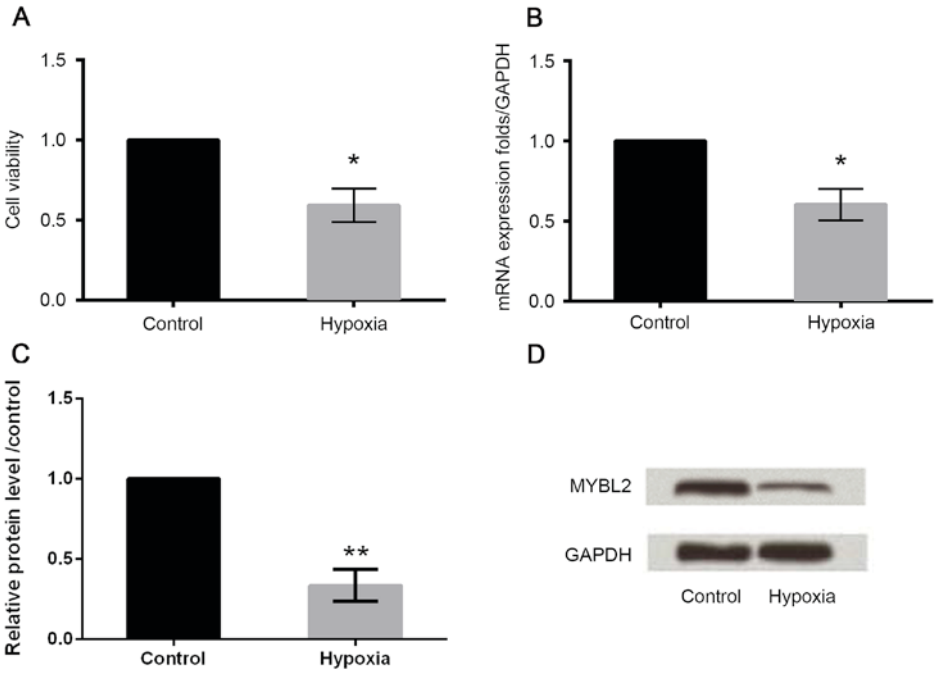

Figure 1. (A) H9c2 cells were treated with hypoxia and cell viability was analyzed by a Cell Counting kit-8 assay. (B) Hypoxia downregulated the mRNA expression of MYBL2 in H9c2 cells. (C and D) Hypoxia downregulated the protein expression of MYBL2 in H9c2 cells. Results are presented as the mean ± standard deviation, n=3. *P<0.05 vs. control group. MYBL2, MYB proto-oncogene like 2.

(HRP) conjugated (cat. no. sc-2004; 1:5,000 in 5% BSA) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. After rinsing, membranes were transferred into a ChemiDoc XRS system (Bio-Rad Laboratories, Inc.) and 200 μl Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software version 4.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were repeated three times. Data are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P-values were calculated using one-way analysis of variance followed by Tukey's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Hypoxia induces H9c2 cell injury and downregulates the expression of MYBL2. As demonstrated in Fig. 1A, the treatment of H9c2 cells with hypoxia reduced cell viability compared with the control group. In addition, following hypoxia treatment, the mRNA and protein expression of MYBL2 in H9c2 cells was downregulated compared with the control group (Fig. 1B-D), which indicated that hypoxia may induce injury in H9c2 cells.

Upregulation and downregulation of MYBL2 in H9c2 cells. To investigate the function of MYBL2, myocardial cells were transfected with MYBL2-specific siRNA or an overexpression plasmid. Overexpression of MYBL2 upregulated MYBL2 mRNA and protein expression in these cells (Fig. 2A, C and D, respectively), while knockdown of MYBL2 downregulated MYBL2 mRNA and protein expression, compared with control cells (Fig. 2B, E and F, respectively).

MYBL2 gene enhances cell proliferation of H9c2 cells. The results indicated that following hypoxia treatment, the cell viability of H9c2 cells increased over time. The growth of the MYBL2-specific siRNA-transfected cells was slower compared with the negative control cells, which indicated that downregulation of MYBL2 may suppress cell proliferation. Furthermore. overexpression of MYBL2 alleviated cellular damage caused by hypoxia, as cell viability was increased compared with hypoxia-treated cells that were transfected with pcDNA3.1 vector-only (Fig. 3).

Overexpression of MYBL2 inhibits H9c2 apoptosis and downregulation of MYBL2 promotes H9c2 apoptosis. To determine whether MYBL2 has an association with apoptosis, the percentage of apoptotic cells was assessed using Annexin V-FITC/PI staining. Overexpression of MYBL2 resulted in a significant decline in the percentage of apoptotic cells compared with cells transfected with pcDNA3.1 vector-only, which indicates that overexpression of MYBL2 inhibited H9c2 apoptosis. However, the transfection of H9c2 cells with MYBL2-specific siRNA resulted in a significant increase in apoptotic cells compared with cells transfected with negative control siRNA (Fig. 4), which indicates that downregulation of MYBL2 induced apoptosis in H9c2 cells.

Overexpression of MYBL2 suppresses AKT and NF-κB pathways, while downregulation of MYBL2 activates AKT and

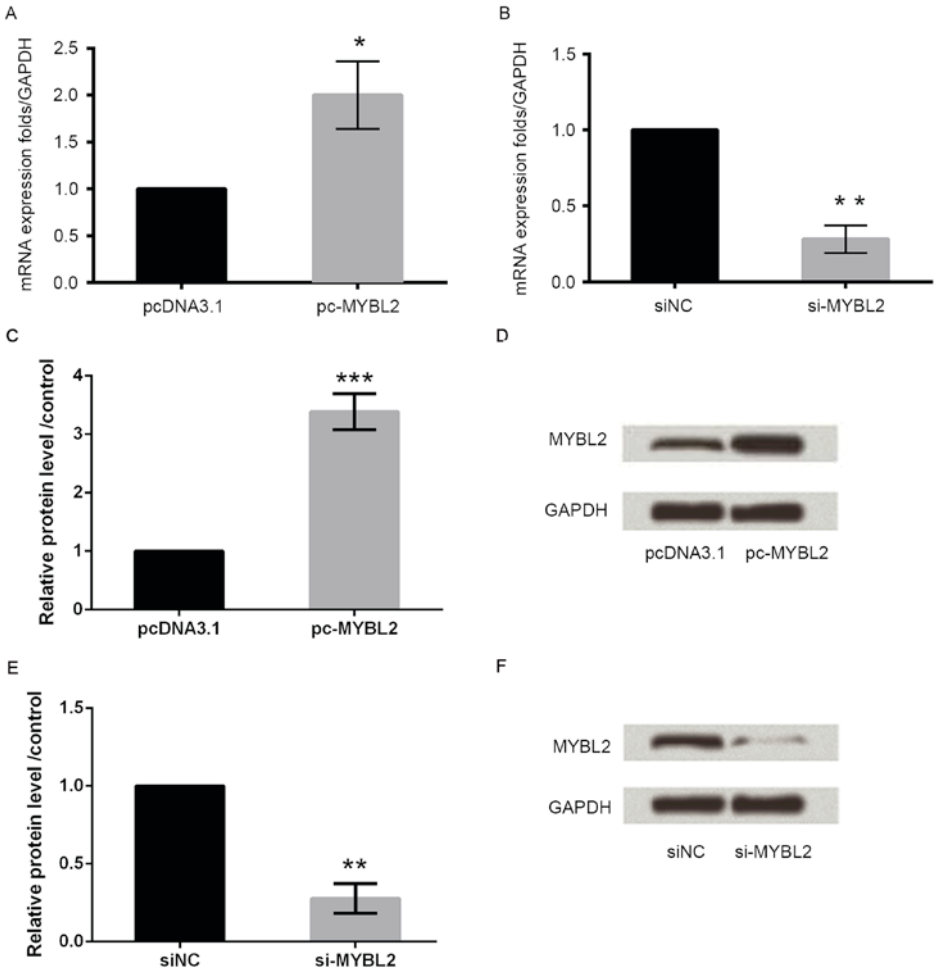

Figure 2. The effect of overexpression and knockdown of MYBL2 on the expression of MYBL2 in H9c2 cells. (A) mRNA expression of MYBL2 in H9c2 cells transfected with pcDNA3.1 vector or pc-MYBL2 overexpression plasmid. (B) mRNA expression of MYBL2 in H9c2 cells transfected with siNC or si-MYBL2. (C and D) Protein expression of MYBL2 in H9c2 cells transfected with pcDNA3.1 vector or pc-MYBL2 overexpression plasmid. (E and F) Protein expression of MYBL2 in H9c2 cells transfected with siNC or si-MYBL2. Results are presented as the mean ± standard deviation, n=3. *P<0.05 and **P<0.01 vs. control group. MYBL2, MYB proto-oncogene like 2; si, small interfering RNA; NC, negative control.

NF-κB pathways. To investigate the protection mechanism of MYBL2 on H9c2 injury induced by hypoxia, the present study analyzed the expression of AKT and NF-κB by western blotting. The protein levels of p-AKT, p-IκBα, p-p65 and Bcl-3 were markedly increased in the hypoxia-treated group compared with the control group, while overexpression of MYBL2 suppressed the expression of p-AKT, p-IκBα, p-p65 and Bcl-3, which indicates that overexpression of MYBL2 suppressed AKT and NF-κB pathways (Fig. 5). By contrast, the expression of p-AKT, p-IκBα, p-p65 and Bcl-3 in the siRNA-MYBL2 group was enhanced to a certain extent compared with the group transfected with negative control siRNA, indicating that downregulation of MYBL2 may activate AKT and NF-κB pathways.

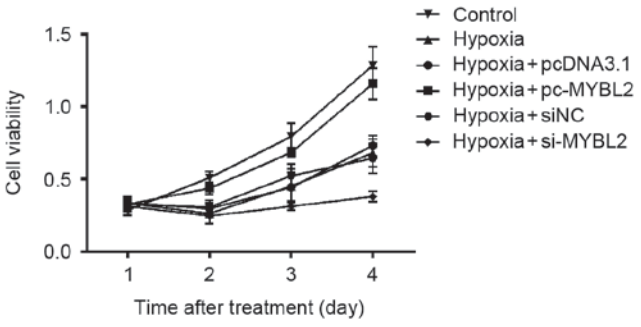

Figure 3. Cell viability of H9c2 cells in different treatment groups. Results are presented as the mean ± standard deviation, n=3. MYBL2, MYB proto-oncogene like 2; pcDNA3.1, control vector; pc-MYBL2, MYBL2 overexpression plasmid; si, small interfering RNA; NC, negative control.

Discussion

AMI is associated with various suppressor genes, such as MYBL2. MYBL2 is a positive growth control gene, which participates in cell apoptosis and cell cycle progression. Previous studies have reported that MYBL2 is overexpressed in various types of cancer, including hepatocellular carcinoma (18) and colorectal cancer, amongst others (19). Furthermore, overexpression of MYBL2 indicates poor prognosis in patients with acute myeloid leukemia (20) and breast cancer (21). The present study, to the best of our knowledge, was the first to investigate the effect of MYBL2 on H9c2 injury induced by hypoxia. The current study investigated the effect of hypoxia

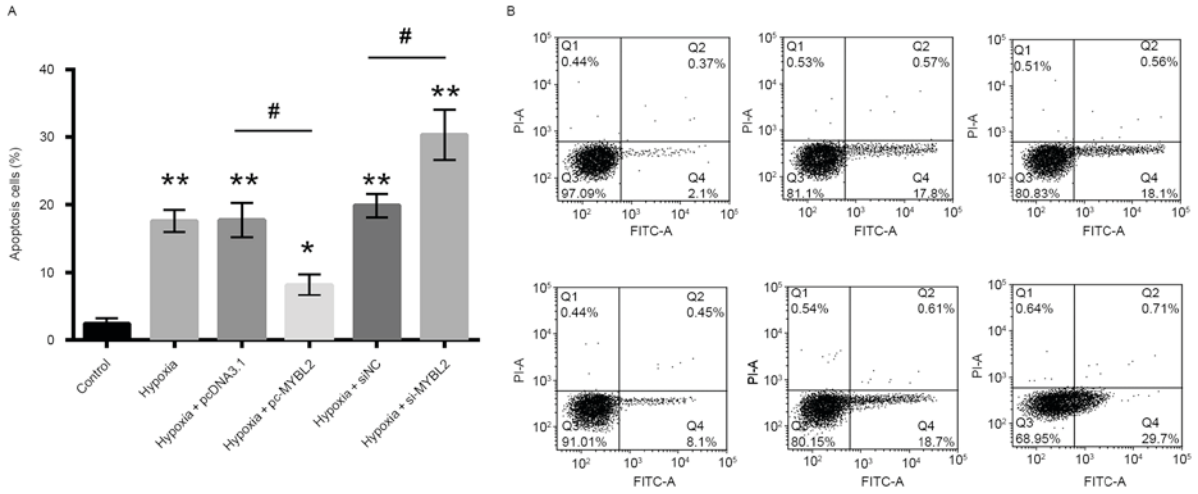

Figure 4. The percentage of apoptotic cells was determined by Annexin V-FITC/PI staining and flow cytometry. (A) Percentage of apoptotic cells as determined by flow cytometry. (B) Representative flow cytometry scatter plots for each treatment group. The percentage of apoptotic cells was reduced following transfection with pc-MYBL2, and was increased following transfection with si-MYBL2. Q4 was considered to indicate apoptotic cells. *P<0.05 and **P<0.01 vs. control group; $^{\#}$P<0.05, as indicated FITC, fluorescein isothiocyanate; PI, propidium iodide; MYBL2, MYB proto-oncogene like 2; pc-MYBL2, MYBL2 overexpression plasmid; si, small interfering RNA; pcDNA3.1, control vector; NC, negative control; Q, quadrant.

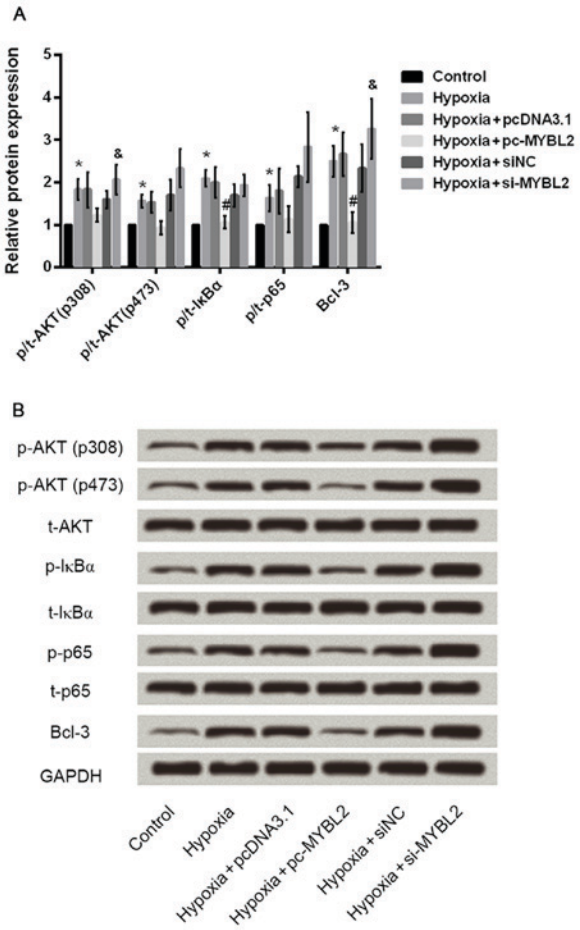

Figure 5. AKT and NF-κB pathways were investigated by RT-qPCR and western blotting. (A) The quantified results for protein expression of AKT and NF-κB pathways-associated factors. (B) Representative western blots of proteins associated with AKT and NF-κB pathways. GAPDH was used as the loading control. *P<0.05 vs. control group, $^{\#}$P<0.05 vs. hypoxia + pcDNA3.1, $^{\&}$P<0.05 vs. hypoxia + siNC. NF-κB, nuclear factor-κB; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p-, phosphorylated-; t-, total-; IκBα, NF-κB inhibitor α; Bcl-3, B-cell CLL/lymphoma 3; pcDNA3.1, control vector; MYBL2, MYB proto-oncogene like 2; pc-MYBL2, MYBL2 overexpression plasmid; si, small interfering RNA.

on H9c2 cells and discovered that hypoxia induced H9c2 cell injury and downregulated the expression of MYBL2.

The MYBL2 gene participates in cell growth, the cell cycle and cell apoptosis. To understand the function of MYBL2 in H9c2 injury, MYBL2-specific siRNA was used to downregulate MYBL2 expression in H9c2 cells and cell proliferation was analyzed using the CCK-8 assay. The growth of cells transfected with siRNA was slower compared with the negative control cells, which indicates that the MYBL2 gene may enhance cell proliferation. Similar results have previously been reported in neuroblastoma cell lines (22) and hepatocellular carcinoma (23). We hypothesized that the inhibition of apoptosis by MYBL2 may explain the cell proliferation results in H9c2 cells. In the present study, Annexin V-FITC/PI apoptosis staining was used to assess apoptotic cells. The results demonstrated that upregulation of MYBL2 resulted in a significant decline in the percentage of apoptotic cells, indicating that the MYBL2 gene may exhibit anti-apoptotic functions. A previous study reported similar results for hepatocellular carcinoma (13), which demonstrated that overexpression of MYBL2 led to the downregulation of proapoptotic genes.

Previous studies have reported that MYBL2 was contained in a segment of the Bcl-2 promoter (24), and stimulated the activity of the promoter at the 5' end of Bcl-2 (25). NF-κB is a transcription factor that has important roles in inflammation (26). Activation of NF-κB due to inflammatory agents leads to the induction of a large number of genes, including inflammatory cytokines and adhesion molecules (27). The activation of NF-κB requires the phosphorylation of NF-κB proteins, such as p65 (28). To further understand the mechanism responsible for the induction of proliferation, the current study analyzed the expression of p-AKT, p-IκBα, p-p65 and Bcl-3 by western blotting. The results demonstrated that upregulation of MYBL2 suppressed the expression of p-p65 and p-IκBα, indicating that the protective effect of MYBL2 on H9c2 cell injury may be mediated by the inhibition of the phosphorylation of p65 and IκBα. In terms of Bcl-3, it has

been reported that Bcl3 is an IκB-related protein with ankyrin repeat motifs (29). Bcl-3 tightly associates with the NF-kB p50 or p52 subunits and strongly enhances cell proliferation and oncogenesis in various cancers (30-32). However, the role of Bcl-3 in cardiomyocytes is rarely reported. The present results indicate that MYBL2 might have a regulatory effect on BCL-3 in hypoxia-injured H9c2 cells.

The phosphoinositide 3-kinase (PI3K)/AKT pathway has a key role in various biological responses, which include cellular proliferation and survival. Previous studies have reported that the PI3K/AKT pathway may be involved in NF-κB activation (33,34). In the present study, upregulation of MYBL2 suppressed the expression of p-AKT, and the down-regulation of MYBL2 promoted the expression of p-AKT, indicating that MYBL2 may inhibit NF-κB activation via the PI3K/AKT pathway. Based on the results of the present study, MYBL2 may inhibit apoptosis and promote cell proliferation by suppressing AKT and NF-κB pathways.

The present study investigated the effect of MYBL2 on H9c2 injury induced by hypoxia. The expression of MYBL2 was demonstrated to be reduced in H9c2 cells exposed to hypoxia. The results indicated that MYBL2 may inhibit apoptosis and promote cell proliferation by suppressing AKT and NF-κB pathways, and that MYBL2 may protect against H9c2 injury induced by hypoxia via AKT and NF-κB pathways. Myocardial infarction is determined by numerous mediators and signaling pathways, leading to the irreparable loss of cardiomyocytes due to oxidative stress, along with insufficient oxygen and blood supply to the heart (35). It is critical to maintain efficient cardiomyocytes for the preservation of cardiac structural integrity and function. Therefore, MYBL2 is a potential therapeutic target for the treatment of myocardial infarction.

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