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ORIGINAL ARTICLE

c-Jun promotes the survival of H9c2 cells under hypoxia via PTEN/Akt signaling pathway

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Abstract

Ischemia and hypoxia are common pathophysiological characteristics in cardiovascular diseases. c-Jun expression could be induced by extra- or intracellular stimuli and plays a pivotal role in regulating cell survival in response to the stress. However, previous studies of c-Jun in cell proliferation and apoptosis showed conflicting results. In the present study, we demonstrated that the expression of c-Jun was induced by hypoxia in H9c2 cells. Loss of function of c-Jun was investigated by CCK-8, LDH, and TUNEL assays in low oxygen (1% O_2) conditions. We revealed that c-Jun could promote cell survival and inhibit cell apoptosis under hypoxia. Knockdown of c-Jun also promoted the expression of apoptosis-related proteins under hypoxia, such as cleaved caspase-3, cleaved caspase-9, Bax, and Bim. Furthermore, we demonstrated that the knockdown of c-Jun inhibited the PTEN/Akt signaling pathway under hypoxia. Our findings suggested that c-Jun protected H9c2 cells from apoptosis and promoted the survival of H9c2 cells under hypoxia via PTEN/Akt signaling pathway.

Keywords c-Jun · Hypoxia · Apoptosis · Cardiomyocytes · H9c2 cells

Introduction

Cardiovascular diseases are the most common cause of death around the world and in China. In 2015, 17.9 million people died of cardiovascular disease, which accounted for nearly a third of global deaths [3]. Lists of cardiovascular diseases such as myocardial infarction, congenital heart disease, heart failure, and rheumatic heart disease are always accompanied by hypoxia [20]. Hypoxia is induced by maldistribution of blood flow or reductions in the partial pressure of oxygen. Under hypoxia, the oxygen concentration is unable to meet the physiological needs of heart tissue, which leads to the myocardial cytoskeleton damage and pathological heart remodeling [1]. At the cellular level, hypoxia induces cardiomyocyte injury by the changes of cell proliferation, necrosis, apoptosis, and cellular metabolism [23]. At the gene expression level, a series of genes are induced under low oxygen condition, which are referred to as hypoxia-sensitive genes [4]. Identification of new hypoxia-sensitive genes in cardiomyocytes could promote better understanding of the adaptation to hypoxia.

The c-Jun protein is a member of transcription factors, which is encoded by the gene JUN. In the form of dimeric complex, c-Jun constitutes the activator protein 1 (AP-1) early response transcription factor and regulates gene expression and cell function in response to diverse extra- or intracellular stimuli [28]. Under the environmental stress, such as pro-inflammatory cytokines, UV irradiation, ischemic stress, and hypoxic stress, c-Jun expression is elevated and plays a pivotal role in regulating cell proliferation and apoptosis to react to the stress [25].

So far, the exact role of c-Jun in cell proliferation and apoptosis still seems to be controversial. Previous studies demonstrated that c-Jun could promote apoptosis by increasing the expression of pro-apoptotic genes, such as Bim, Fas-L, and TNF-α [5]. However, c-Jun can also protect cells from apoptosis by counteracting the pro-apoptotic effects of p53 in cancer cells [14]. Moreover, c-Jun could regulate the cell cycle progression by promoting the transcriptional level of cyclin D1, which is required for cell cycle G_1/S transition [13]. In cardiomyocytes, c-Jun has been shown to be involved in the

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responses to hypoxia [24]. However, most of these studies are limited to describing the correlation between c-Jun and cell apoptosis in cardiomyocytes. The potential role and mechanism of c-Jun in cardiomyocytes under hypoxic condition need to be further investigated.

In the present study, we assessed the expression patterns of c-Jun in H9c2 cells under hypoxia. We subsequently focused on the role of c-Jun in responses to hypoxia by silencing of c-Jun in vitro. The mechanisms by which c-Jun participated in regulating cell survival and apoptosis under hypoxia were also revealed.

Materials and methods

Cell culture and treatment

The embryonic *rat* heart-derived H9c2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technology, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA). For hypoxia treatment, cells were cultured in serum-free medium overnight for starvation treatment and then exposed to a gaseous mixture of 94% N_2, 5% CO_2, and 1% O_2 at 37 °C for 6 h, 12 h, 24 h, 48 h, or 72 h in an Invivo2000 cultivator (Ruskin Technology Ltd., UK).

RNA extraction and qRT-PCR analysis

Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The quantity and quality of the total RNA were detected by spectrophotometry. One microgram of total RNA was reversely transcribed into complementary DNA (cDNA) using PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Real-time PCR was conducted using the SYBR Premix Ex Taq (TaKaRa) following the manufacturer's protocol. The qRT-PCR results were normalized to the expression levels of β-actin and were calculated using $2^{(-\triangle\triangle CT)}$ methods. The primer sequences of c-Jun and β-actin used in the study were as follows: c-Jun (*Rat*) forward primer, 5′-AGCGGATCAAGGCGGAGAGG-3′; c-Jun (*Rat*) reverse primer, 5′-CTGAGCATGTTGGCCGTGGAC-3′; β-actin (*Rat*) forward primer, 5′-CTTAGACTATAGGCATGGAC CT-3′; β-actin (*Rat*) reverse primer, 5′-GCTA ACGTTGCACGGTACGGAC-3′.

siRNA transfection

The c-Jun-specific small interfering RNAs (siRNAs) were designed and synthesized by Ribobio (Guangzhou, China). For c-Jun gene knockdown, 100 pmol siRNAs and 5 μl Lipofectamine 2000 (Invitrogen, USA) were added into 100 μl Opti-MEM medium, respectively, for 5 min. Then, the siRNA mixture and the Lipofectamine 2000 mixture were mixed together and incubated for 15 min at room temperature. Subsequently, the transfection mixture was added into 6-well plated cells and incubated at 37 °C in an Invivo2000 cultivator. The medium was replaced by normal medium after 5-h incubation.

Cell Counting Kit-8 assay

Cell survival was detected by Cell Counting Kit-8 (CCK-8) assay. H9c2 cells (5×10^3) were seeded in 96-well plates and transfected with c-Jun siRNAs (si-JUN) or negative control siRNAs (si-NC). After incubation in 1% O_2 at 37 °C for various hours, cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Jiangsu, China). Absorbance of each well was measured at 450 nm with a microplate spectrophotometer. All of the experiments were performed in quintuplicate.

Lactate dehydrogenase assay

The cell death was detected using lactate dehydrogenase (LDH) assay kit (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, H9c2 cells (5×10^3) were seeded in 96-well plates and treated with low oxygen (1% O_2) conditions for 24 h. Cells were centrifuged at 500*g* for 5 min and 120-μl supernatants were collected for analysis using the LDH assay kit. Absorbance of each well was measured at 450 nm with a microplate spectrophotometer. All of the experiments were performed in quadruplicate.

Cell cycle analysis by flow cytometry

H9c2 cells were harvested and fixed in 75% ethanol at 4 °C overnight. Then, the cells were stained with propidium iodide (Beyotime). 1.5×10^6 cells were collected and analyzed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ). The cell proportions of G_1 phase, S phase, and G_2 phase were calculated using ModFitLT software (Becton Dickinson, San Diego, CA). All of the experiments were performed in triplicate.

Cell apoptosis analysis by flow cytometry

Apoptosis was also detected by using Annexin V-APC/7-amino-actinomycin D (7-ADD) kit (KeyGEN, Nanjing, China). H9c2 cells were harvested and resuspended in 200-μl binding buffer. Then, 5 μl Annexin V-APC and 5 μl 7-ADD were both

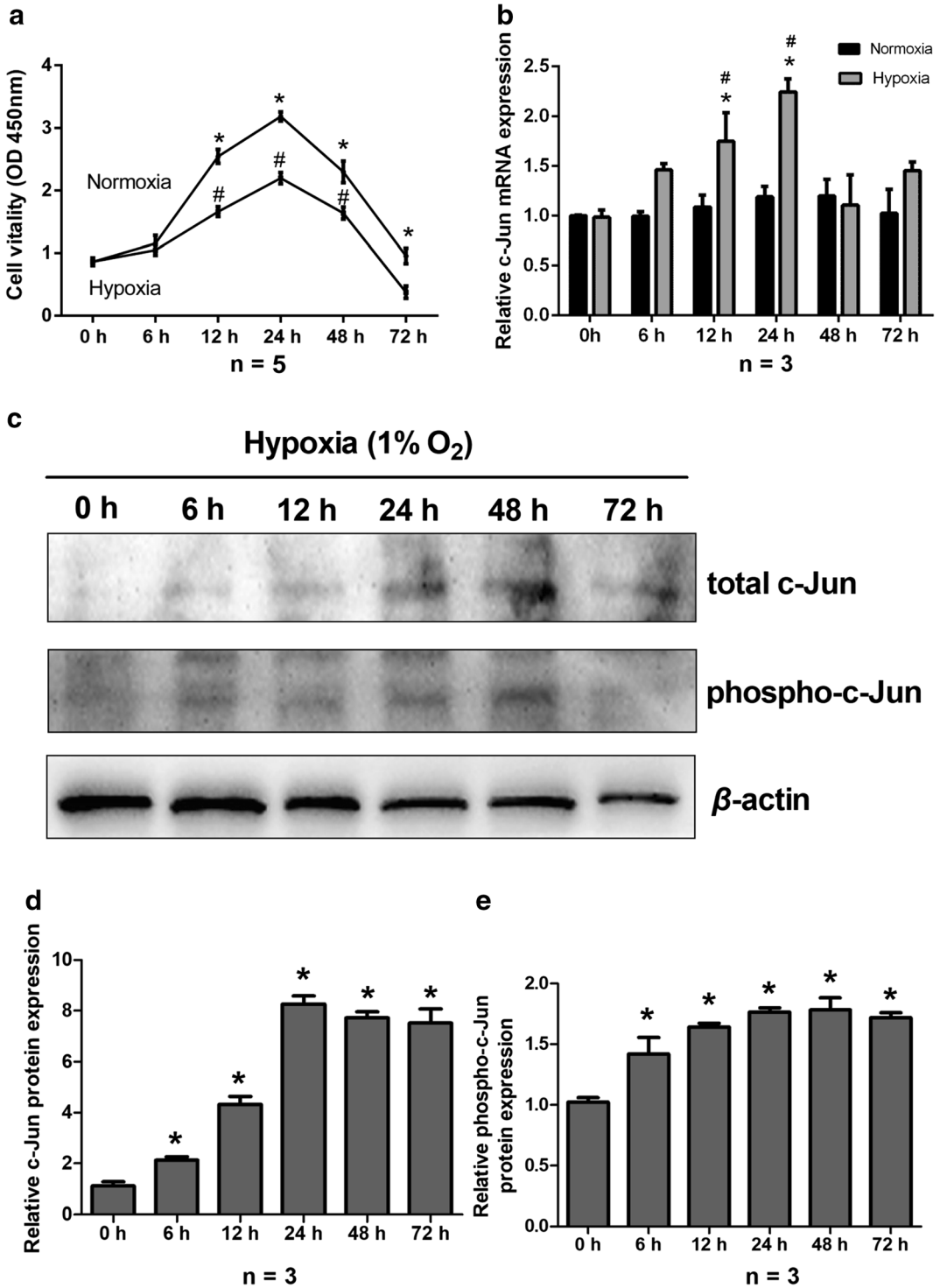
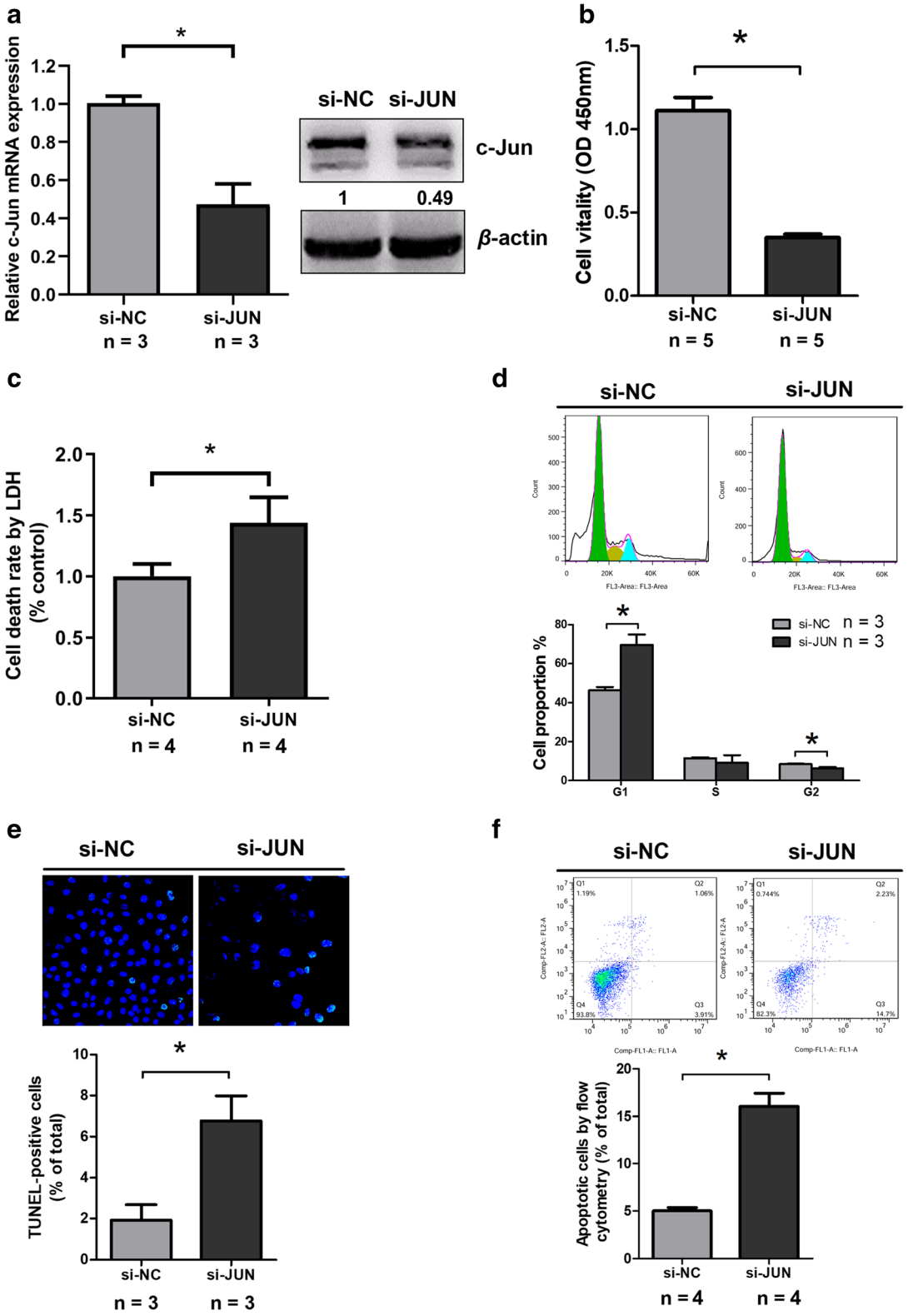


Fig. 1 Expression of c-Jun in H9c2 cells under hypoxia. **a** H9c2 cells viability in normal (21% O_2) or low (1% O_2) oxygen conditions for 0, 6, 12, 24, 48, and 72 h. **b** Expression of c-Jun mRNA in H9c2 cells in normal (21% O_2) or low (1% O_2) oxygen conditions for 0, 6, 12, 24, 48, and 72 h. **c** c-Jun and phosphorylated c-Jun protein levels in H9c2 cells in low oxygen (1% O_2) conditions for 0, 6, 12, 24, 48, and 72 h. **d** The quantitative analysis results of c-Jun protein levels in three independent Western blots. **e** The quantitative analysis results of phosphorylated c-Jun protein levels in three independent Western blots. Asterisk symbols in panels **a** and **b** compared between both groups, $P < 0.05$; number symbols in panels **a** and **b** compared with 0 h, $P < 0.05$; asterisk symbols in panels **d** and **e** compared with 0 h, $P < 0.05$

added to the cell suspension. After dual staining for 15 min, cells were analyzed by FACSCalibur System (BD Biosciences). All of the experiments were performed in quadruplicate.



TUNEL assay

The cell apoptosis was detected using a TUNEL kit (Beyotime). H9c2 cells in 96-well plates were washed by PBS, fixed in 4% paraformaldehyde for 30 min, permeabilized by 0.5% Triton X-100 for 5 min, and then incubated with TUNEL solution in dark environment at 37 °C for 60 min. The cells were further counterstained with Hoechst 33258 (Beyotime) for 5 min at room

◀ **Fig. 2** Effects of c-Jun knockdown by siRNAs on cell survival and apoptosis under hypoxia for 24 h. **a** The knockdown of c-Jun in H9c2 cells was identified by qRT-PCR (left) and WB (right). The numbers in the WB result indicate the quantitative analysis results. **b** CCK-8 assays were used to examine the effects of c-Jun knockdown on cell viability in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. **c** LDH assays were used to examine the effects of c-Jun knockdown on cell death rate in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. **d** Cell cycle analysis results from H9c2 cells in si-NC and si-JUN groups in low oxygen (1% O_2) conditions for 24 h. Upper chart: representative images of cell cycle. Lower chart: the quantitative analysis of cell cycle. **e** TUNEL staining analysis was used to examine the effects of c-Jun knockdown on cell apoptosis in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. TUNEL-positive cells were labeled by FITC in green fluorescence and total cells were stained by Hoechst in blue fluorescence. **f** Cell apoptosis analysis results from H9c2 cells in si-NC and si-JUN groups in low oxygen (1% O_2) conditions for 24 h. Upper chart: representative images of cell apoptosis. Lower chart: the quantitative analysis of cell apoptosis. Asterisk symbols indicate Mann-Whitney test, $P < 0.05$

temperature. The cells were examined by a fluorescent microscope and 5 fields of vision were captured in each group. Apoptotic percentage was calculated as the ratio of TUNEL-positive nuclei to the total cell nuclei counterstained by Hoechst. All of the experiments were performed in quintuplicate.

Western blot

Western blot (WB) was performed as described in our previous study [8]. The antibodies targeting c-Jun, phosphorylated c-Jun, cleaved caspase-3, cleaved caspase-9, Bim, and Bax were purchased from Cell Signaling Technology (CST, USA). The antibodies targeting PTEN, phosphorylated PTEN, Akt, phosphorylated Akt, and β-actin were purchased from Abcam (Cambridge, USA). All of the experiments were performed in triplicate.

Statistical analysis

The differences of c-Jun expression, cell viability, cell death rate, and cell apoptosis were tested using a non-parametric test (Mann-Whitney test for two groups or Kruskal-Wallis for more than two groups). A two-sided P value less than 0.05 was taken as statistically significant. Statistical analyses were performed using the SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The graphs were drawn using GraphPad Prism 5.0 and CorelDRAW 2018 software.

Results

Expression patterns of c-Jun under hypoxia

To explore the expression patterns of c-Jun under hypoxia, H9c2 cells were cultured in normal or low oxygen (1% O_2) conditions for 0, 6, 12, 24, 48, and 72 h respectively. First, we examined the effects of hypoxic condition on cell proliferation. CCK-8 assays showed that cell viability was markedly higher at 24 h after hypoxic treatment compared with other time points (Fig. 1a). Next, we detected the expression levels of c-Jun mRNA under hypoxia. The c-Jun mRNA exhibited the highest expression level at 24 h after hypoxic treatment, which was in accordance with cell viability under hypoxia (Fig. 1b). Moreover, Western blot analysis showed that the expression of c-Jun protein and phosphorylated c-Jun protein were both significantly increased after exposure to hypoxia for 6, 12, 24, 48, and 72 h (Fig. 1c–e).

Knockdown of c-Jun inhibited cell survival under hypoxia

To explore the potential role of c-Jun under hypoxia, we established a loss-of-function cell model by transfecting siRNAs into the H9c2 cells. The expression level of c-Jun was significantly reduced after siRNA transfection as shown by qRT-PCR and WB assays (Fig. 2a). The cell viability was detected using CCK-8 assay after 24-h exposure to hypoxia. We observed that the knockdown of c-Jun expression significantly inhibited the viability of H9c2 cells under hypoxia (Fig. 2b). In addition, cell death rate was detected by using LDH-release assay. At the time point of 24 h under hypoxia, the knockdown of c-Jun expression significantly increased the cell death rate of H9c2 cells compared with the negative control (Fig. 2c).

Knockdown of c-Jun inhibited cell cycle and promoted cell apoptosis under hypoxia

To dissect the mechanisms of the cell survival promotion effects of c-Jun in cardiomyocytes under hypoxia, we further examined the effects of c-Jun knockdown on cell cycle and apoptosis in H9c2 cells. By using a flow cytometry analysis, we found that the knockdown of c-Jun in H9c2 cells significantly inhibited the cell cycle transition from G_1 phase to the S phase and G_2 phase under hypoxia (Fig. 2d). TUNEL staining analysis showed that the knockdown of c-Jun in H9c2 cells resulted in an increase of apoptotic cells after 24-h exposure to hypoxia (Fig. 2e). The suppressive effect of c-Jun on cell apoptosis was confirmed by flow cytometry, which revealed that the amounts of apoptotic cells were significantly increased after the knockdown of c-Jun in H9c2 cells under hypoxia (Fig. 2f).

Effects of c-Jun on expression of apoptosis-related proteins

To elucidate the molecular basis of apoptosis, we further assessed the expression of apoptosis-related proteins by

Western blot. Cleaved caspase-3 and cleaved caspase-9 were pro-apoptotic mediators for cell apoptosis. We measured cleaved caspase-3 and cleaved caspase-9 expression in H9c2 cells after the knockdown of c-Jun by siRNAs under hypoxia. The knockdown of c-Jun markedly promoted the expression of cleaved caspase-3 and cleaved caspase-9 compared with the control under hypoxia (Fig. 3a–c). We also detected the expression of pro-apoptotic proteins Bim and Bax. Consistent with the results of cleaved caspase-3 and cleaved caspase-9, Bim and Bax protein levels were also markedly increased after the knockdown of c-Jun under hypoxia (Fig. 3d–f). Collectively, our findings suggested that c-Jun negatively regulated cardiomyocyte apoptosis under hypoxia.

Effects of c-Jun on PTEN/Akt signaling pathway under hypoxia

We next explored the potential molecular pathways by which c-Jun regulated cell survival and cell apoptosis under hypoxia. We examined the changes of PTEN/Akt signaling pathway activity after the knockdown of c-Jun under hypoxia. We found that the knockdown of c-Jun did not markedly change the expression of PTEN protein, while significantly increased the expression of phosphorylated PTEN after 24-h exposure to hypoxia (Fig. 4a–c). Meanwhile, accompanied by changes of phosphorylated PTEN expression, the knockdown of c-Jun inhibited the protein expression of phosphorylated Akt under hypoxia (Fig. 4d–f). Taken together, these findings indicated that c-Jun might promote cell survival and inhibit cell apoptosis under hypoxia via the PTEN/Akt signaling pathway.

Discussion

In the present study, we demonstrated that the expression of c-Jun was induced by hypoxia in H9c2 cells. Loss-of-function studies confirmed that c-Jun promoted cell survival and repressed cell apoptosis under hypoxia. Furthermore, c-Jun could regulate PTEN/Akt signaling pathway, which may mediate its protective effects in H9c2 cells under hypoxia.

Ischemia and hypoxia in the heart are common pathophysiological characteristics in cardiovascular diseases [11]. In the

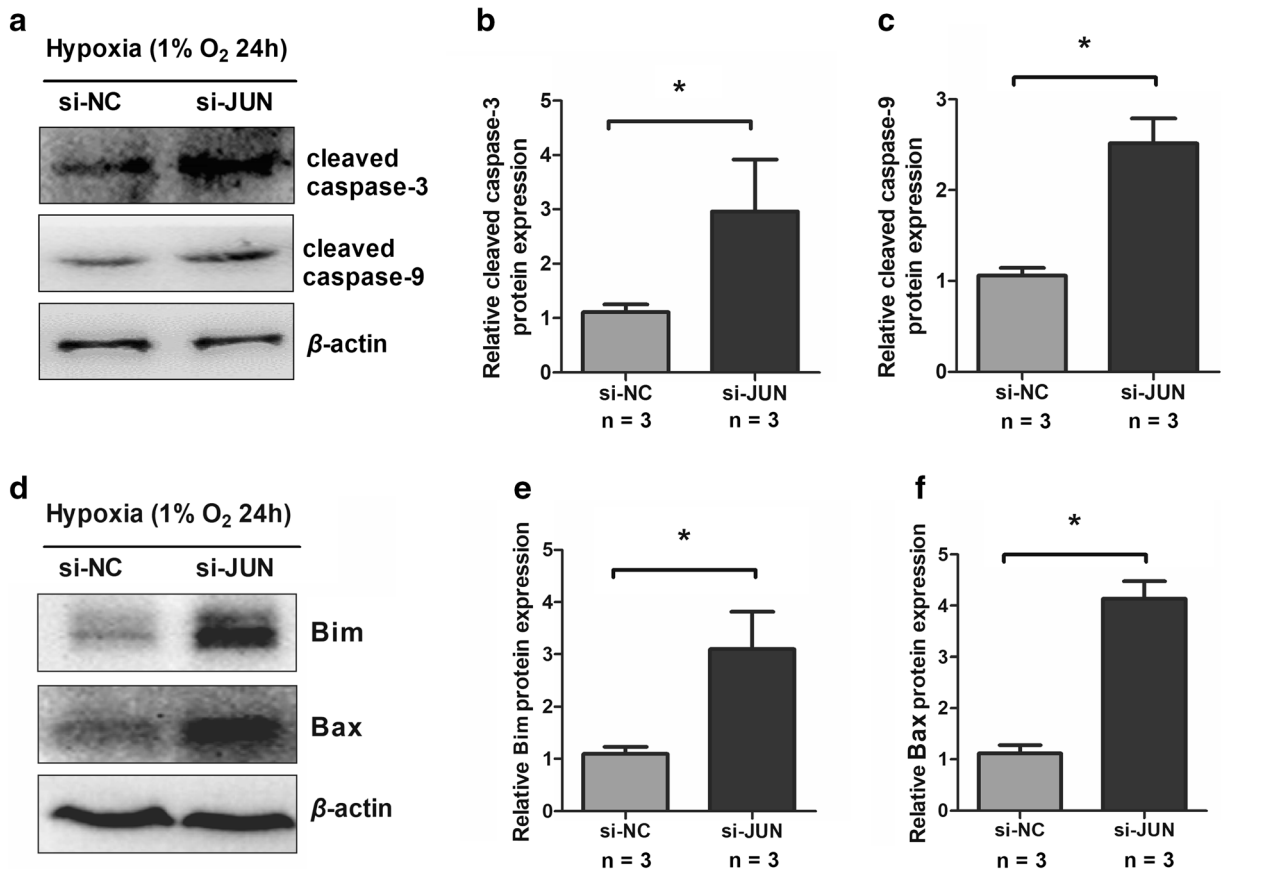


Fig. 3 Effects of c-Jun knockdown on the expression of apoptosis-related proteins under hypoxia for 24 h. **a** Cleaved caspase-3 and cleaved caspase-9 protein levels in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. **b** The quantitative analysis results of cleaved caspase-3 protein levels in three independent Western blots. **c** The quantitative analysis results of cleaved caspase-9 protein levels in three independent Western blots. **d** Bim and Bax protein levels in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. **e** The quantitative analysis results of Bim protein levels in three independent Western blots. **f** The quantitative analysis results of Bax protein levels in three independent Western blots. Asterisk symbols indicate Mann-Whitney test, $P < 0.05$

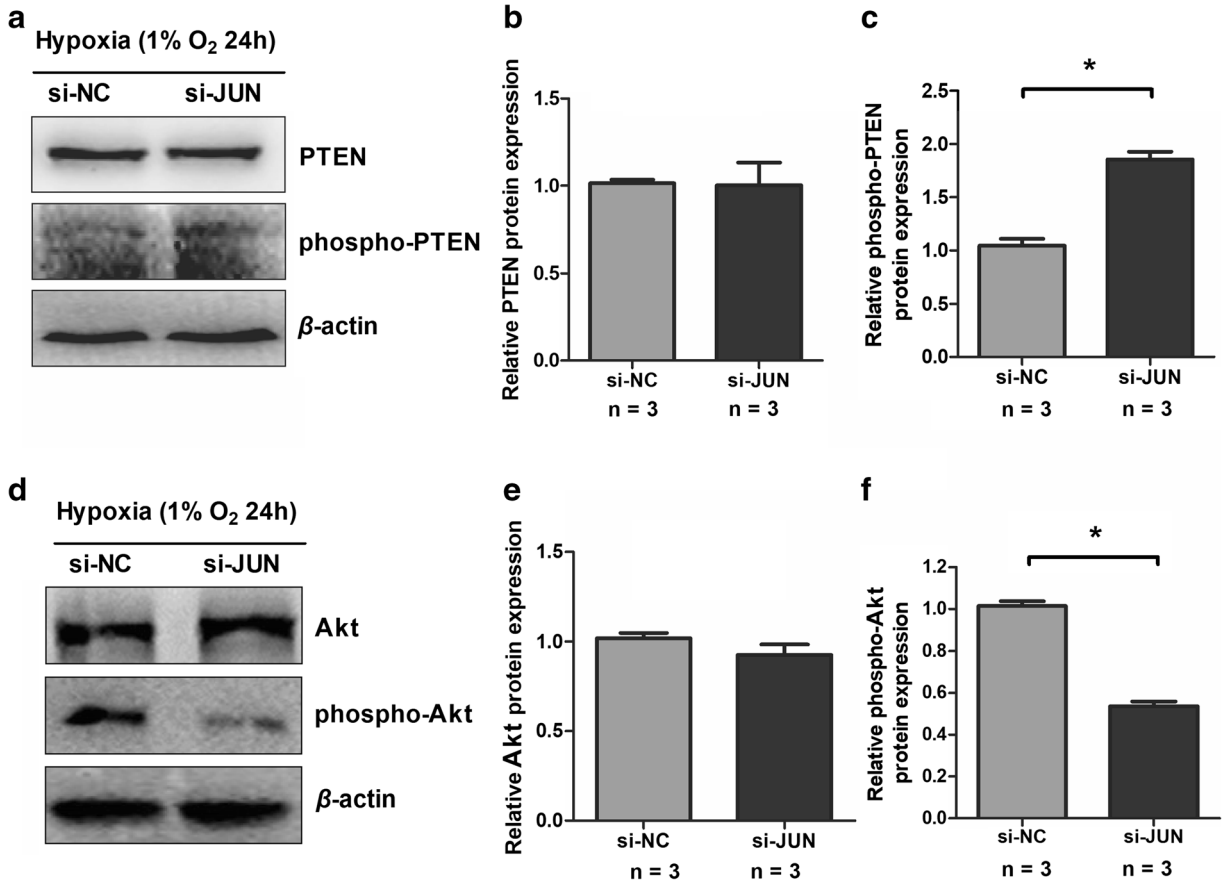


Fig. 4 Effects of c-Jun knockdown on PTEN/Akt signaling pathway under hypoxia for 24 h. **a** PTEN and phosphorylated PTEN protein levels in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. **b** The quantitative analysis results of PTEN protein levels in three independent Western blots. **c** The quantitative analysis results of phosphorylated PTEN protein levels in three independent Western blots. **d** Akt and phosphorylated Akt protein levels in H9c2 cells in low oxygen (1% O_2) conditions for 24 h. **e** The quantitative analysis results of Akt protein levels in three independent Western blots. **f** The quantitative analysis results of phosphorylated Akt protein levels in three independent Western blots. Asterisk symbols indicate Mann-Whitney test, $P < 0.05$

hypoxic environment, multiple physiological and pathological changes occur in the cardiovascular system. For example, the heart rate and stroke volume are increased and left ventricular end-diastolic volume is reduced after acute hypoxia [12]. During chronic exposure to hypoxia, cardiac remodeling, sterile inflammation, metabolic disorders, and contractile dysfunction may occur [7]. In this study, H9c2 cells showed higher viability at 24 h under hypoxia. After hypoxia treatment for more than 24 h, the cell viability was decreased, indicating that hypoxia treatment may have a time-dependent effect on H9c2 cell viability. At the genetic level, different mechanisms have been studied to elucidate the cell responses to hypoxia. Hypoxia-inducible factor-1α (HIF-1α) and HIF-2α are representative hypoxia-inducible genes, which mediate a number of cellular responses and gene expression under hypoxia [21]. In addition to HIF-1α and HIF-2α, several mRNAs, miRNAs, and lncRNAs are also altered by hypoxia in cardiomyocytes, such as VEGFA [19], miR-210 [6], and lncRNA-TUG1 [26]. These hypoxia-sensitive mRNAs, miRNAs, and lncRNAs could play important roles in regulating cell survival and apoptosis under hypoxia. However, these hypoxia-sensitive genes could not fully elucidate the mechanisms of cardiomyocytes' responses to hypoxia. Thus, better understanding of new hypoxia-sensitive genes in cardiomyocytes is going to be very important.

In the previous studies, miR-138 was found to be upregulated in cardiomyocytes under hypoxia [8]. The upregulated miR-138 could promote cell survival and attenuate cell apoptosis in hypoxic conditions. Mechanistically, miR-138 exerted protective functions by targeting MLK3 [8]. MLK3 is a member of the serine/threonine kinase family and functions as a positive regulator of Jun N-terminal kinase (JNK) signaling pathway [16]. The subsequent study found that the expression of MLK3 was decreased in hypoxic conditions [9]. The low expression of MLK3 could promote cell survival and decrease cell death under hypoxia. Interestingly, MLK3 could also regulate the expression of c-Jun under hypoxia [9]. These previous studies suggested that c-Jun might also participate in the responses to hypoxia in cardiomyocytes.

c-Jun is a well-known early response transcription factor and exerts its functions by forming a dimer with other AP-1 family members [28]. Studies have shown that c-Jun participated in stress-adaptive responses and regulated cellular proliferation, apoptosis, differentiation, and transformation. c-Jun is also a famous oncogene in cancer and is upregulated in non-small-cell lung cancer [17], prostate cancer [22], and breast cancer [10]. The activities of c-Jun protein rely on the N-terminal phosphorylation of c-Jun, which could be regulated by the JNKs [2]. In cardiovascular diseases, JNK-1 plays a pivotal role in protecting cardiomyocytes against ischemia and hypoxia, and c-Jun is one of the targets of JNK-1 [24]. In the present study, both c-Jun mRNA and protein levels were upregulated after hypoxic treatment and were concordant with the cardiomyocyte viability under hypoxia. Phosphorylated c-Jun protein was also upregulated after exposure to hypoxia in our study, which could reflect c-Jun protein activity. Besides, the phosphorylated c-Jun can move into or outside of the nucleus, so the changes of nuclear phosphorylated c-Jun under hypoxia need further study. In addition, cell function studies demonstrated that the knockdown of c-Jun inhibited cell proliferation and increased cell apoptosis under hypoxia. Caspase-3 could be activated by caspase-9 and plays a central role in the final execution phase of apoptosis [15]. Bcl-2 family proteins were most notable for their regulation of apoptosis, which consisted of members that either promote or inhibit apoptosis [27]. Bim and Bax belonged to pro-apoptotic proteins [27]. Here, we demonstrated that c-Jun negatively regulated the expression of cleaved caspase-3, cleaved caspase-9, Bim, and Bax under hypoxia. These genes were involved in key steps of cell apoptosis, underlining the protective effects of c-Jun in myocardial adaptation to hypoxia.

We further explored the molecular basis of c-Jun as a protector in cardiomyocytes under hypoxia. The Akt signaling pathway is involved in promoting cell survival by inhibiting apoptotic processes [18]. PTEN acts as a natural inhibitor of the Akt signaling pathway [29]. PTEN could specifically dephosphorylate PIP3 to PIP2 and inhibit the activities of the Akt signaling pathway [29]. We found that c-Jun negatively regulated the phosphorylation of PTEN protein and positively regulated the phosphorylation of Akt protein. Our data suggested that c-Jun might promote cardiomyocyte survival via the PTEN/Akt signaling pathway under hypoxia.

In conclusion, this study demonstrated that c-Jun played a protective role in myocardial responses to hypoxia by promoting cell survival and inhibiting cell apoptosis. Our results suggested that c-Jun could exert its protective function via PTEN/Akt signaling pathway and may be a promising therapeutic target for cardioprotection.

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Compliance with ethical standards

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflicts of interest.

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