

KLF7 promotes LPS induced apoptosis, inflammation, and oxidative stress in H9c2 cells by activating NF- κ B pathway

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ABSTRACT: This study aimed to investigate and explore the molecular mechanisms of Krüppel-like factor 7 (KLF7) on apoptosis, inflammation, and oxidative stress in H9c2 cells. In the study, the protein expression of KLF7 in H9c2 cells were ascertained by western blot after lipopolysaccharide (LPS) treatment and small interfering RNA (siRNA) transfection. The results confirmed that the expression levels of the protein KLF7 were dramatically increased after LPS treatment and decreased after siRNA transfection. Then, the viability of the LPS treated and siRNA transfected H9c2 cells was tested by cell counting kit-8 (CCK8) assay and enzyme-linked immunosorbent assay (ELISA). The results indicated that down-regulation of KLF7 inhibited LPS-induced injury of H9c2 cells. Flow cytometry assays were applied to test the apoptosis of H9c2 cells, and cell apoptosis-associated proteins in H9c2 cells were also examined by western blot. The results showed that down-regulation of KLF7 inhibited LPS-induced apoptosis of H9c2 cells. The inflammation and oxidative stress related factors, subsequently tested by quantitative polymerase chain reaction (q-PCR) and ELISA, supported the inhibition of LPS-induced oxidative stress in H9c2 cells by the down-regulation of KLF7. Finally, the western blot assays were employed to determine the expression of NF- κ B p65 and I κ B α after LPS treatment and siRNA transfection. These findings proved that KLF7 promotes LPS-induced apoptosis, inflammation, and oxidative stress in H9c2 cells via activating NF- κ B pathway, which hinted that KLF7 knockdown could be a potential therapeutic approach for the treatment of sepsis.

KEYWORDS: KLF7, H9c2, inflammation, oxidative stress, apoptosis, NF- κ B

INTRODUCTION

Sepsis, usually induced by a host response disorder caused by infection, can lead to organ dysfunction [1]. Despite the considerable attention given by researchers to advance the disease monitoring and support technologies, sepsis remains one of the top ten causes of death in non-coronary critical patients [2, 3]. It is predicted that sepsis remains to be a major clinical challenge and a serious global health burden which will affect at least 20 million patients worldwide [4]. Sepsis-induced cardiomyopathy (SIC), a typical complication of sepsis, can induce abnormal inflammatory response, disturbances in calcium regulation, and autonomic nervous system dysregulation [5–7]. Although many aggressive measures have been taken to limit the advancement of SIC; these measures have often been depressing, and the mechanisms underlying SIC are currently unknown.

The inflammatory response is the marker and initial process of SIC advancement. TNF- α , IL-1, and IL-6 are the main inflammatory factors of SIC [8]. TNF- α /IL-1 and inducible nitric oxide synthase were considered to have negative inotropic effects on SIC [9]. Apoptosis has been widely used as an important indicator of SIC, and upregulation of caspase activity and cellular damage were observed in SIC; whereas

caspase inhibition improved the function of the septic rat heart [10]. Oxidative stress indicates the imbalance between oxidation and antioxidation, including the massive production of ROS and malondialdehyde (MDA) *in vivo*, which is mainly cleared by antioxidant enzymes such as catalase and superoxide dismutase (SOD) [11, 12]. It has been reported that salidroside inhibits LPS-induced myocardial lipid peroxidation, inflammatory cytokines, and myocardial injury via inhibition of the ROS-mediated PI3K/Akt/mTOR signalling [13].

Krüppel-like factors (KLFs) are transcription factors with zinc finger structures that are extensively involved in the regulation of many biological events, such as tumor cell fate, stress response, and embryonic development. *Klf7* is a highly conserved gene and plays a role in transcriptional activation [14]. It has been reported that miR-19b-3p protects against inflammatory injury in mouse sepsis models and *in vitro* LPS-induced lung microvascular endothelial cells through inhibition of the NF- κ B signaling pathway where KLF7 serves as a potential target [15]. The above studies suggest that KLF7 may be involved in acute lung injury in sepsis, but the exact mechanism of action is unclear. Moreover, there are only few studies investigating the effects of KLF7 on myocardial injury in sepsis, so this study aimed to investigate and explore the molecular

mechanisms of KLF7 in LPS-induced apoptosis, inflammation, and oxidative stress levels in H9c2 cells and their related mechanisms, which may conceivably pave the path for advanced therapeutic targets in sepsis.

MATERIALS AND METHODS

Cell culture

H9C2 cells, the rat cardiomyoblast cell line, were obtained from ATCC (CRL-1446™). The cells were cultured in an incubator with DMEM (Gibco, Carlsbad, CA, USA) containing 10% FBS (Hyclone, Logan, Utah, USA) with 100 IU/ml streptomycin and penicillin (Gibco).

Cell transfection

For KLF7 knockdown, small interfering RNA (siRNA) was cloned into the pSuper-retro-puro vector. The syntheses of si-Klf7 and si-Negative control (NC) were accessed from GenePharma (Shanghai, China). After 24 h cell culture, si-Klf7 and si-NC were separately transfected into cells by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the protocol [16]. The sequences of si-Klf7 and si-NC were shown in Table S1.

Lipopolysaccharide (LPS) exposure

H9C2 cells in LPS-treated group were administrated with different concentrations of LPS (0, 1, 2.5, 5, and 10 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 24 h to induce cellular inflammation, oxidative stress, and apoptosis according to the protocol [17].

Western blot

H9C2 cells were washed 3 times with PBS and lysed by RIPA buffer (Solarbio, China). The BCA protein assay kit (Tiangen, Beijing, China) was used to measure the protein concentration. The protein sample was electrophoresed on SDS-PAGE and transferred to PVDF membrane (Millipore), which was blocked with 5% skim milk in Tris buffer and then incubated with the primary antibodies (all were from Abcam, Cambridge, MA, USA) against KLF7 (ab197690), Cleaved Caspase-3 (ab214430), Bax (ab53154), Bcl-2 (ab182858), p-IkBα (ab133462), IkBα (ab32518), p-NF-κB p65 (ab86299), NF-κB p65 (ab16502), β-actin (ab8227), and GAPDH (ab9485). After the incubation with the primary antibodies, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (ab205718, Abcam), and the blots were visualized by the ECL chemiluminescence reagent (Beyotime, Shanghai, China).

CCK-8 cell viability assay

After the treatment, 2.5×10^3 H9C2 cells were seeded into plates for 24 h and treated with CCK-8 solution (Solarbio, Beijing, China) under different conditions

for 1.5 h according to the manufacturer's instructions. Then, the optical density values were measured at 450 nm.

ELISA

After the treatment, the protein levels of LDH, TNF-α, IL-6, IL-1β, SOD, MDA, and GSH-Px in H9C2 cells were detected by ELISA with corresponding ELISA kits (Abcam) according to the manufacturer's instructions.

Cell apoptosis

The H9C2 cells were re-suspended in Annexin V and PI (Invitrogen) incubation solution and stained for 25 min without light; then, the cell apoptosis was analyzed by flow cytometer (CytoFLEX, Beckman, Brea, CA, USA) according to the manufacturer's instructions.

qPCR

Trizol (Invitrogen) was used to extract total RNA in the H9c2 cells. Nano Drop (Thermo Fisher, Waltham, MA, USA) was used to measure the quantity and the integrity of total RNA. The expressions of TNF-α, IL-6, and IL-1β were tested by the SYBR Premix EX Taq (Takara, Kyoto, Japan) and analyzed by the $2^{-\Delta\Delta CT}$ method. Primer sequences are shown in Table S2.

Statistical analysis

All data were shown as mean ± SD from 3 independent experiments. Results were analyzed by one-way ANOVA with Tukey's multiple comparison test. *p*-values < 0.05 (two-tailed) were considered a significant difference.

RESULTS

Down-regulation of KLF7 inhibited LPS-induced injury of H9c2 cells

To evaluate the correlation between the expression of KLF7 and LPS treatment in H9c2 cells, the protein expression level of KLF7 in the H9c2 cells was measured by western blot. The result confirmed that the protein expression level of KLF7 was dramatically higher in the LPS treatment group than that in the control group (*p* < 0.01) (Fig. 1A). The protein expression of KLF7 was increased in a dose-dependent manner with increasing concentration of LPS (*p* < 0.001). Then, the cell viability of H9c2 cells was analyzed after administration with different concentrations of LPS via CCK8 assay. The result showed that LPS treatment obviously decreased the cell viability of H9c2 cells, compared with the control group. in a dose-dependent manner, indicating that LPS administration caused injury to H9c2 cells in a dose-dependent manner as well (*p* < 0.001) (Fig. 1B). So, 10 µg/ml LPS was used in subsequent experiments. To assess the effects of KLF7 on cell injury, H9c2 cells were transfected with si-NC and si-Klf7, respectively. After transfection,

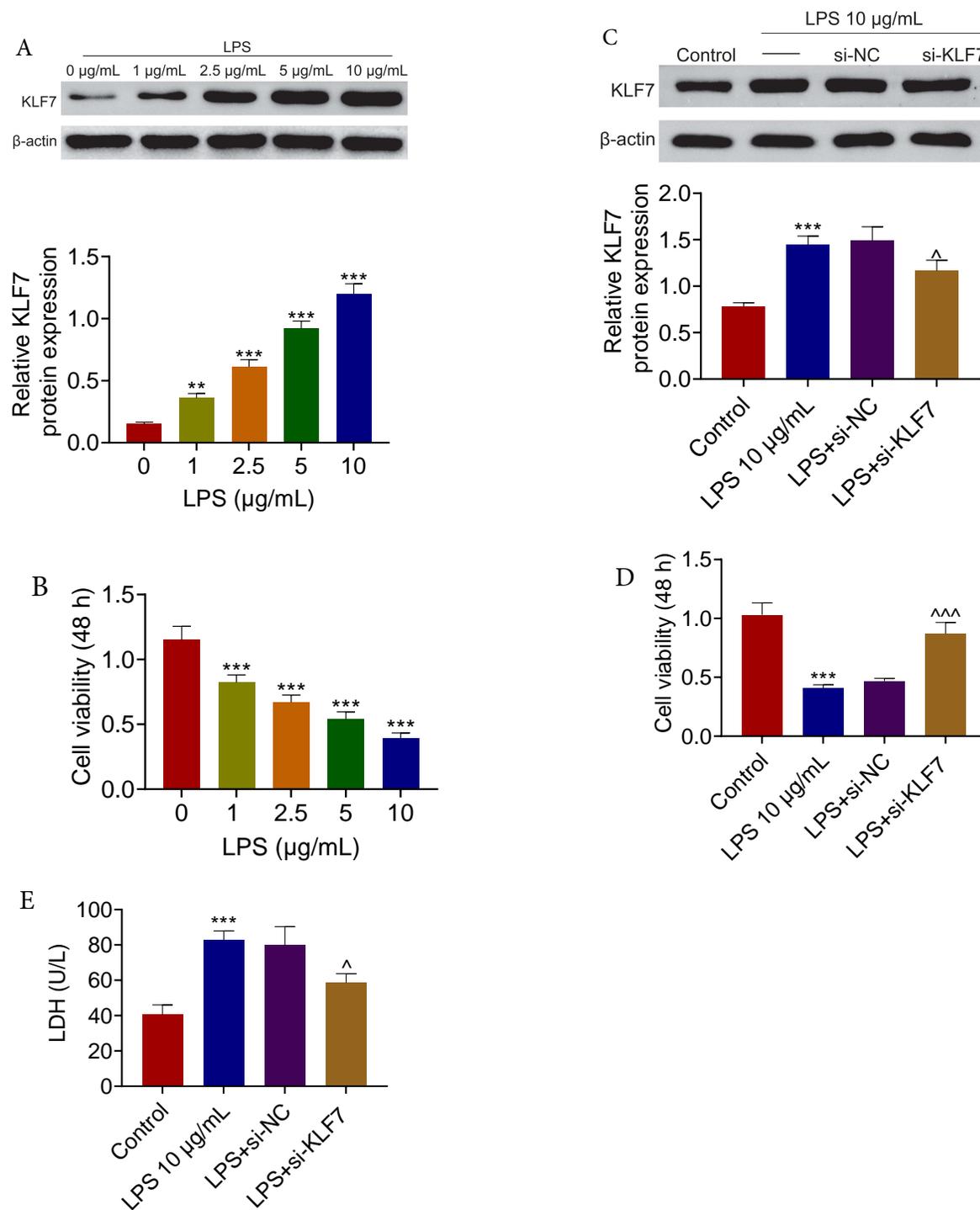


Fig. 1 Down-regulation of KLF7 inhibited LPS-induced injury of H9c2 cells. (A), protein expression levels of KLF7 in H9c2 cells treated with LPS of different concentrations; (B), cell viability of H9c2 cells treated with LPS of different concentrations; (C), protein expression levels of KLF7 in H9c2 cells transfected with siRNA; (D), cell viability of H9c2 cells transfected with siRNA; (E), protein expression levels of LDH in H9c2 cells transfected with siRNA. Data were presented as the mean \pm SD of three independent experiments. (^ $p < 0.05$ versus LPS + NC group) (** $p < 0.01$ and *** $p < 0.001$ versus control group).

the protein expression of KLF7 in H9c2 cells was examined. Cells transfected with si-Klf7 showed strikingly lower KLF7 protein expression levels than the control, confirming the transfection efficiency of KLF7 ($p < 0.05$) (Fig. 1C). Then, the cell viability of H9c2 cells were assessed after administration with different concentrations of LPS through CCK8 assay again. The result showed that down-regulation of KLF7 obviously increased the cell viability of H9c2 cells ($p < 0.001$) (Fig. 1D). Furthermore, the expression of LDH was also determined by ELISA, and the result revealed that LPS administration could increase the expression levels of LDH, while down-regulation of KLF7 could decrease the expression levels of LDH (Fig. 1E). These results showed that down-regulation of KLF7 inhibited LPS-induced injury of H9c2 cells.

KLF7 promoted LPS-induced apoptosis in H9c2 cells.

To assess the effects of KLF7 on H9c2 cell apoptosis, flow cytometry experiment was employed in each group. As evidenced by enhanced apoptotic cells with LPS treatment ($p < 0.001$), it was proved that LPS administration dramatically facilitates apoptosis of H9c2 cells. Besides, down-regulation of KLF7 substantially inhibited H9c2 cell apoptosis, as evidenced by reduced apoptotic cells with si-Klf7 administration ($p < 0.001$) (Fig. 2A). Then, cell apoptosis-related proteins were checked, and the results proved that LPS administration markedly elevated the expression of Bax and cleaved caspase-3 while suppressed the expression of Bcl-2 ($p < 0.001$). However, the expressions of Bax and cleaved caspase-3 were strikingly decreased, and the expression of Bcl-2 was significantly increased in si-Klf7 administration group ($p < 0.05$) (Fig. 2B). These results showed that knockdown of KLF7 inhibited LPS-induced apoptosis in H9c2 cells.

KLF7 promoted LPS-induced inflammation in H9c2 cells

Then, qPCR and ELISA were employed to identify the mRNA and protein expressions of TNF- α , IL-6, and IL-1 β to confirm that down-regulation of KLF7 could inhibit LPS-induced H9c2 cell inflammation. Both the qPCR and ELISA results hinted that the mRNA and protein expressions of TNF- α , IL-6, and IL-1 β were drastically enhanced in the LPS administration group, suggesting that LPS administration could increase inflammatory response ($p < 0.001$). However, si-Klf7 treatment markedly decreased the mRNA and protein expressions of TNF- α , IL-6, and IL-1 β ($p < 0.05$) (Fig. 3A and 3B). These results revealed that down-regulation of KLF7 inhibited LPS-induced H9c2 cell inflammation.

KLF7 promoted LPS-induced oxidative stress in H9c2 cells

In addition, ELISA was employed to detect the changes in protein expressions of MDA, SOD, and GSH-Px to find out if down-regulation of KLF7 could inhibit LPS-induced H9c2 cell oxidative stress. The ELISA results suggested that the protein expression of MDA was considerably elevated, while the protein expressions of SOD and GSH-Px were attenuated in the LPS administration group, indicating that LPS treatment could enhance the state of oxidative stress ($p < 0.001$). Furthermore, the oxidative stress induced by LPS administration gradually abated with si-Klf7 treatment ($p < 0.05$) (Fig. 4). These results supported that down-regulation of KLF7 inhibited LPS-induced oxidative stress in H9c2 cells.

KLF7 activated NF- κ B signaling pathway

To assess the mechanism of KLF7 promoting LPS induced apoptosis, inflammation, and oxidative stress in H9c2 cells, western blot was employed to detect the expressions of NF- κ B p65 and I κ B- α in H9c2 cells. The result hinted that the phosphorylation levels of NF- κ B p65 and I κ B- α were notably up-regulated in H9c2 cells after LPS administration ($p < 0.001$). However, the phosphorylation of NF- κ B p65 and I κ B- α were reversed after si-Klf7 treatment ($p < 0.001$) (Fig. 5). These results suggested that KLF7 could activate NF- κ B signaling.

DISCUSSION

In this study, the results suggested that down-regulation of KLF7 had beneficial effects on LPS induced apoptosis, inflammation, and oxidative stress in H9c2 cells. The protein expression level of KLF7 was dramatically increased after LPS treatment and decreased after siRNA transfection. Down-regulation of KLF7 inhibited LPS-induced injury of H9c2 cells and attenuated the expression of LDH in H9c2 cells. Flow cytometry assays and western blot also confirmed that down-regulation of KLF7 inhibited LPS-induced apoptosis of H9c2 cells. Besides, down-regulation of KLF7 inhibited the expressions of cell apoptosis-related proteins, inflammation related factors, and oxidative stress related factors, which confirmed the therapeutic effect of KLF7 knockdown. Additionally, western blot suggested that knockdown of KLF7 in H9c2 cells would promote phosphorylation of NF- κ B p65 and I κ B α proteins, suggesting that KLF7 promotes LPS induced apoptosis, inflammation, and oxidative stress in H9c2 cells by activating NF- κ B pathway.

LPS is a major component of the membrane of Gram-negative bacteria, and it is also an endotoxin that causes immune response. In the human body or in cell line *in vitro* study, LPS binds to the lipopolysaccharide receptor complex (CD14/TLR4/MD-2) on the

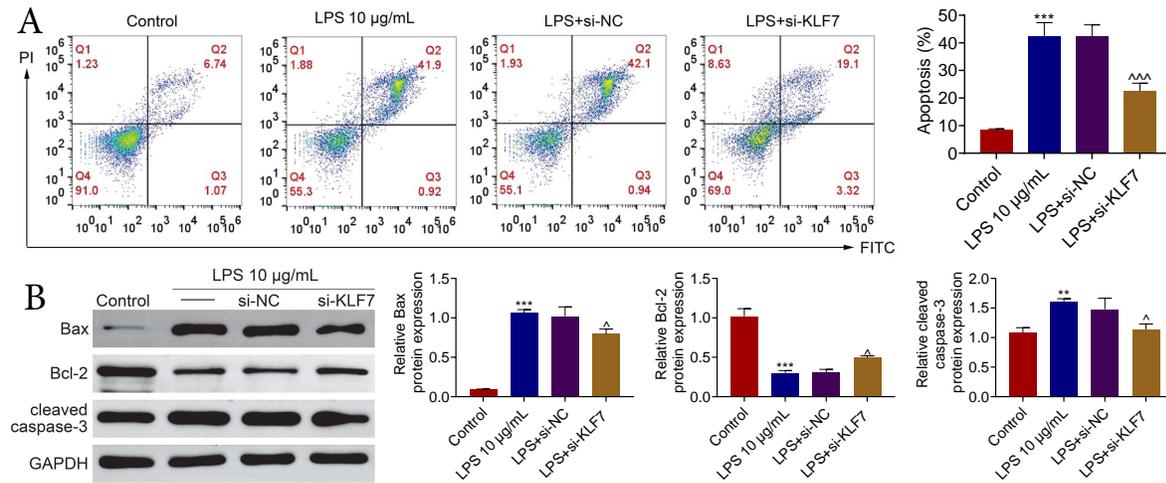


Fig. 2 KLF7 promoted LPS-induced apoptosis in H9c2 cells. (A), serial flow cytometry assays detected apoptosis of H9c2 cells treated with LPS of different concentrations; (B), cell apoptosis-related proteins in H9c2 cells. Data were presented as the mean \pm SD of three independent experiments. ($\Delta p < 0.05$ versus LPS + NC group) (** $p < 0.01$ and *** $p < 0.001$ versus control group).

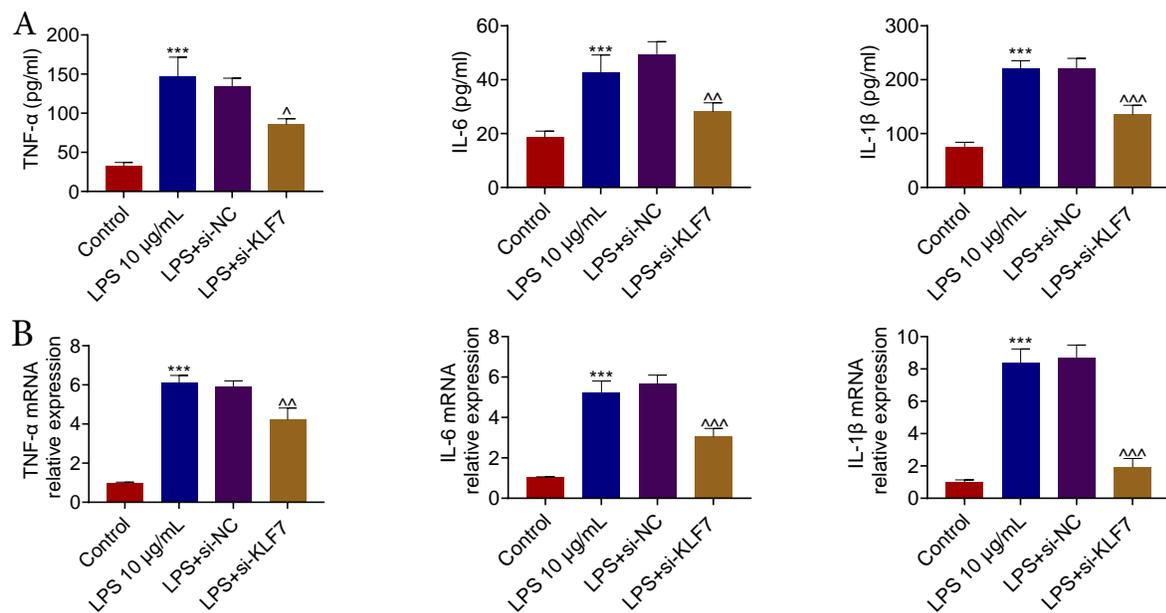


Fig. 3 KLF7 promoted LPS-induced inflammation in H9c2 cells. (A), protein expression levels of TNF- α , IL-6, and IL-1 β in H9c2 cells of each group; (B), mRNA expression levels of TNF- α , IL-6, and IL-1 β in H9c2 cells. Data were presented as the mean \pm SD of three independent experiments. ($\Delta p < 0.05$, $\Delta\Delta p < 0.01$, and $\Delta\Delta\Delta p < 0.001$ versus LPS + NC group) (***) $p < 0.001$ versus control group).

cell membrane and promotes the secretion of a variety of cytokines in inflammatory cells [18]. Inflammation and cell viability are closely related to many coronary artery diseases, such as acute myocardial infarction, ischemic heart disease, and microvascular reperfusion injury [19]. Several studies have shown that LPS exposure can induce apoptosis, leading to decreased

cell viability [20]. In our study, the result showed that LPS administration obviously abated the cell viability of H9c2 cells in a dose-dependent manner, indicating that LPS treatment caused injury to H9c2 cells.

KLF7 has been shown to be associated with mediating inflammatory response and cell apoptosis helping maintain normal homeostasis and tissue functions, and

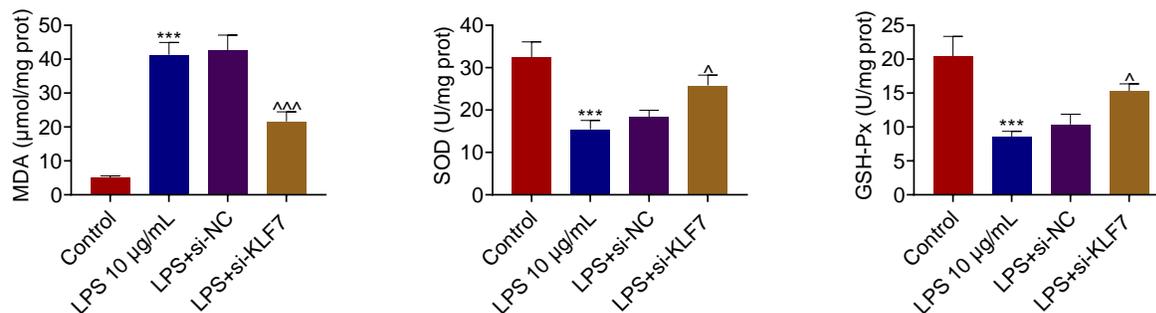


Fig. 4 KLF7 promoted LPS-induced oxidative stress in H9c2 cells: protein expression levels of MDA, SOD, and GSH-Px in H9c2 cells. Data were presented as the mean ± SD of three independent experiments. (^ $p < 0.05$ and ^^ $p < 0.001$ versus LPS + NC group) (***) $p < 0.001$ versus control group).

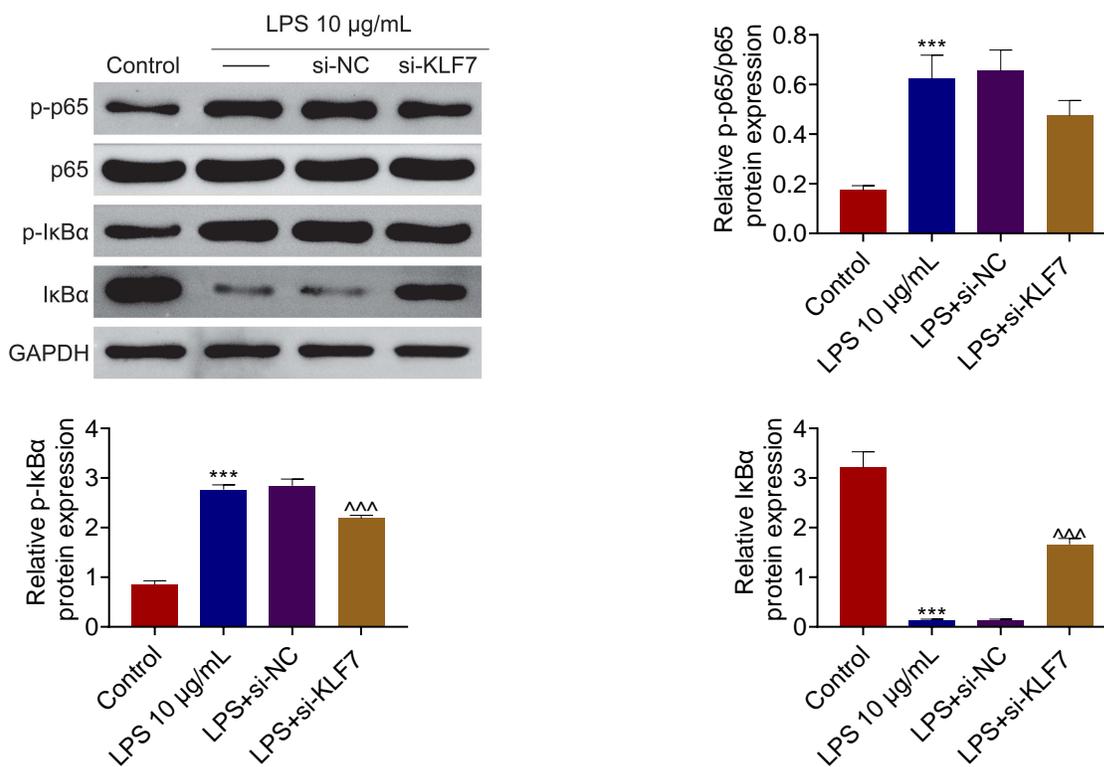


Fig. 5 KLF7 activated NF-κB signaling pathway: protein expression levels of IκB-α and NF-κB p65 in H9c2 cells. Data were presented as the mean ± SD of three independent experiments. (^^ $p < 0.001$ versus LPS + NC group) (***) $p < 0.001$ versus control group).

KLF7 disorders play a vital role in the advancement and occurrence of diseases [21]. Specifically, studies have also showed that KLF7 promotes cell migration and epithelial-mesenchymal transition [22]. Recently, the interrelationship between MAP7 and TLR4 was also showed in inflammatory response [23]. Moreover, MAP7 was found to be associated with cell migration and adhesion in tongue cancer [24]. In our study, the result confirmed that the protein expression of

KLF7 was markedly enhanced after LPS treatment and reduced after si-Klf7 transfection. Down-regulation of KLF7 obviously increased the cell viability of H9c2 cells, indicating that KLF7 knockdown inhibited LPS-induced injury of H9c2 cells. It is important to note that the result cannot prove that KLF7 expression is directly affected by LPS treatment. In order to further verify the relationship between KLF7 expression and LPS treatment, more classical signaling pathways need

to be systematically studied to determine whether they are involved.

LDH is a specific enzyme in the myocardial cytoplasm that enters the blood during myocardial ischemia. Therefore, this enzyme is recognized as the diagnostic standard of myocardial injury [25]. In our study, the result showed that the expression levels of LDH could be increased by LPS treatment and decreased by down-regulation of KLF2, which also suggested that KLF7 knockdown inhibited LPS-induced injury of H9c2 cells.

Cleaved caspase-3, Bcl-2, and Bax are among the most important oncogenes in apoptosis research [26]. Cleaved caspase-3 is the activated form of caspase-3, a vital mediator of programmed cell death or apoptosis. Bcl-2 has been reported to significantly inhibit apoptosis. Bax can form a heterodimer with Bcl-2 and suppress Bcl-2. The ratio of Bax/Bcl-2 protein is the vital factor to determine the inhibitory effect of apoptosis. Therefore, Bax is considered to be one of the critical pro-apoptotic genes. In our study, the results showed that down-regulation of KLF7 inhibited LPS-induced apoptosis of H9c2 cells. Toll-like receptors (TLRs) on cell surfaces and endosomal membranes detect extracellular foreign bodies and activate multiple host defense signaling pathways that induce the expression of pro-inflammatory cytokines [27]. In our study, the results revealed that down-regulation of KLF7 inhibited LPS-induced H9c2 cell inflammation. In patients with sepsis, antioxidant consumption leads to the accumulation of reactive oxygen species, which assault polyunsaturated fatty acids and lead to lipid peroxidation. MDA is one of the many aldehydes produced by lipid peroxidation, and it is considered as an indirect marker of oxidative stress due to its stable properties and convenient detection [28]. SOD is an antioxidant enzyme that specifically scavenges free radicals, and GSH-Px is a major intracellular antioxidant and cellular signaling regulator. Our results supported that down-regulation of KLF7 inhibited LPS-induced H9c2 cell oxidative stress.

In conclusion, this study demonstrated that down-regulation of KLF7 alleviated LPS induced apoptosis, inflammation, and oxidative stress in H9c2 cells. Knockdown of KLF7 suppressed these activities by mediating NF- κ B pathway. Therefore, KLF7 knockdown could be a potential therapeutic approach for the treatment of sepsis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at <http://dx.doi.org/10.2306/scienceasia1513-1874.2022.092>.

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Appendix A. Supplementary data**Table S1** Sequences of si-Klf7 and si-NC.

	Sequence (5' → 3')
si-KLF7	GCCAACCAGCUCUUCUCUATT UAGAGAAGAGCUGGUUGGCTT
si-NC	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT

Table S2 Primers for TNF- α , IL-6, IL-1 β , and β -actin (reference) genes.

Gene	Primer	Sequence (5' → 3')
IL-6	Forward	CACATGTTCTCTGGGAAATCG
	Reverse	TTGTATCTCTGGAAGTTCAGATTGTT
IL-1 β	Forward	ACCTTCCAGGATGAGGACATGA
	Reverse	CTAATGGGAACGTCACACACCA
TNF- α	Forward	GCCACCACGCTCTTCTGTCTAC
	Reverse	GGGTCTGGGCCATAGAACTGAT
β -actin	Forward	GTGACGTTGACATCCGTAAAGA
	Reverse	GCCGGACTCATCGTACTCC