Epigenetic modification mechanism of histone demethylase KDM1A in regulating cardiomyocyte apoptosis after myocardial ischemia-reperfusion injury

Lin He [Corresp., 1], **Yanbo Wang** [1], **Jin Luo** [1]

[1] Department of Cardiology, The Center Hospital of Shaoyang, Shaoyang, China

Corresponding Author: Lin He
Email address: helinnn1022@163.com

Hypoxia and reoxygenation (H/R) plays a prevalent role in heart-related diseases. Histone demethylases are involved in myocardial injury. In this study, the mechanism of the lysine-specific histone demethylase 1A (KDM1A/LSD1) on cardiomyocyte apoptosis after myocardial ischemia-reperfusion injury (MIRI) was investigated. Firstly, HL-1 cells were treated with H/R to establish the MIRI models. The expressions of KDM1A and Sex Determining Region Y-Box Transcription Factor 9 (SOX9) in H/R-treated HL-1 cells were examined. The cell viability, markers of myocardial injury (LDH, AST, and CK-MB) and apoptosis (Bax and Bcl-2), and Caspase-3 and Caspase-9 protein activities were detected, respectively. We found that H/R treatment promoted cardiomyocyte apoptosis and downregulated KDM1A, and overexpressing KDM1A reduced apoptosis in H/R-treated cardiomyocytes. Subsequently, tri-methylation of lysine 4 on histone H3 (H3K4me3) level on the SOX9 promoter region was detected. We found that KDM1A repressed SOX9 transcription by reducing H3K4me3. Then, HL-1 cells were treated with CPI-455 and plasmid pcDNA3.1-SOX9 and had joint experiments with pcDNA3.1-KDM1A. We disclosed that upregulating H3K4me3 or overexpressing SOX9 reversed the inhibitory effect of overexpressing KDM1A on apoptosis of H/R-treated cardiomyocytes. In conclusion, KDM1A inhibited SOX9 transcription by reducing the H3K4me3 on the SOX9 promoter region and thus inhibited H/R-induced apoptosis of cardiomyocytes.

Epigenetic modification mechanism of histone demethylase KDM1A in regulating cardiomyocyte apoptosis after myocardial ischemia-reperfusion injury

Running title: KDM1A regulates cardiomyocyte apoptosis

Lin He*, Yanbo Wang, Jin Luo

Department of Cardiology, The Center Hospital of Shaoyang, Shaoyang City, 422000, Hunan Province, China

Corresponding Author:
Lin He
No.36 Qianyuan road, Daxiang district, Shaoyang City, 422000, Hunan Province, China.
Email address: helinnn1022@163.com

Abstract

Hypoxia and reoxygenation (H/R) plays a prevalent role in heart-related diseases. Histone demethylases are involved in myocardial injury. In this study, the mechanism of the lysine-specific histone demethylase 1A (KDM1A/LSD1) on cardiomyocyte apoptosis after myocardial ischemia-reperfusion injury (MIRI) was investigated. Firstly, HL-1 cells were treated with H/R to establish the MIRI models. The expressions of KDM1A and Sex Determining Region Y-Box Transcription Factor 9 (SOX9) in H/R-treated HL-1 cells were examined. The cell viability, markers of myocardial injury (LDH, AST, and CK-MB) and apoptosis (Bax and Bcl-2), and Caspase-3 and Caspase-9 protein activities were detected, respectively. We found that H/R treatment promoted cardiomyocyte apoptosis and downregulated KDM1A, and overexpressing KDM1A reduced apoptosis in H/R-treated cardiomyocytes. Subsequently, tri-methylation of lysine 4 on histone H3 (H3K4me3) level on the SOX9 promoter region was detected. We found that KDM1A repressed SOX9 transcription by reducing H3K4me3. Then, HL-1 cells were treated with CPI-455 and plasmid pcDNA3.1-SOX9 and had joint experiments with pcDNA3.1-KDM1A. We disclosed that upregulating H3K4me3 or overexpressing SOX9 reversed the inhibitory effect of overexpressing KDM1A on apoptosis of H/R-treated cardiomyocytes. In conclusion, KDM1A inhibited SOX9 transcription by reducing the H3K4me3 on the SOX9 promoter region and thus inhibited H/R-induced apoptosis of cardiomyocytes.

Introduction

Ischemia-reperfusion (I/R) injury, first described in the 1980s, is a severe pathological process that occurs in multiple diseases, such as myocardial infarction, cerebral stroke, and surgical interventions, and is frequently associated with severe cellular damage and death (Eltzschig & Eckle, 2011; Murry et al., 1986; Toldo et al., 2018). Hypoxia refers to the intracellular oxygen content and pressure lower than normal level, and would be triggered by factors such as hypoxemia, limited oxygen delivery, or impaired respiratory system (MacIntyre, 2014), while reoxygenation refers to the hypoxic cells regain oxygen and then the cells gradually recover to the normal state (Chen et al., 2020). Of note, the hypoxia–reoxygenation (H/R) process is known to cause neurological and cardiological damages and I/R injury (Leon et al., 2021). On a separate note, myocardial ischemia-reperfusion injury (MIRI) after cardiovascular disorders (CVDs) accompanied by myocardial systolic dysfunction and cardiomyocyte necrosis and apoptosis would lead to fatal cardiovascular outcomes (Lin et al., 2019) (Gao et al., 2020). Herein, we decided to probe the specific role of H/R in cardiomyocyte apoptosis after MIRI.

Histone methylation plays an important role in regulating gene expression and epigenetic inheritance of cells, serving as a part of the regulatory memory system of the epigenetic inheritance of cells (Klose et al., 2006). Furthermore, histone methylation is involved in myocardial injury induced by myocardial infarction (Wang et al., 2018a). Besides, the deletion of histone 3 lysine 4 methylation (H3K4) detriments the adaptive response to transverse aortic constriction and facilitate maladaptive cardiac remodeling (Stein et al., 2015). Additionally, the lysine-specific histone demethylase 1A (KDM1A/LSD1) is a demethylase first identified in 2004 that challenges the irreversibility of methylation signatures by demethylating H3K4 and H3K9 (Ismail et al., 2018; Shi et al., 2004). Prior studies demonstrated that KDM1A participates in cardiac development and diseases (Davis et al., 2021). And, KDM1A can increase the stability of hypoxia-inducible factor (HIF)-1α via demethylation of RACK1 and thus promotes cell adaptation to the hypoxic microenvironment (Kim et al., 2021; Yang et al., 2017). Moreover, LSD1 knockdown promotes apoptosis in multiple diseases (Han et al., 2021; Zou et al., 2017). Hence, we speculated that KDM1A may affect H/R-induced cardiomyocyte apoptosis through demethylating H3K4 methylation.

Sex Determining Region Y-Box Transcription Factors (SOXs) proteins are classified as a type of transcription factor possessing highly-conserved HMG box domain and could modulate developmental processes as well as homeostasis of adult tissues (Zhang & Hou, 2021). SOX9, belonging to the SOXs family, is a key transcription factor in the development and maturation of cartilage (Lefebvre et al., 2019). A study has shown that microRNA-30e mitigates myocardial I/R injury and promotes ventricular remodeling through repressing SOX9 (Cheng et al., 2021). In addition, SOX9 has been shown to regulate many fibrosis-related genes and is activated in the case of ischemic injury (Lacraz et al., 2017). Our study aimed to verify the hypothesis we proposed that whether KDM1A can function on H/R-induced cardiomyocyte apoptosis by regulating SOX9 through demethylation of H3K4 methylation. Accordingly, in the follow-up

experiments, the H/R cell models were established to induce cardiomyocyte apoptosis, and the expressions and roles of KDM1A and SOX9 in the models were detected.

Materials & Methods

Cell culture and H/R treatment

The mouse cardiomyocyte cell line HL-1 cells (GDC0606) were purchased from China Center for Typical Cultures Preservation (Wuhan, Hubei, China). HL-1 cells were cultured in RPMI-1064 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco) at 37℃ with 5% CO_2. Subsequently, HL-1 cells were first cultured under hypoxia conditions (95% N2 and 5% CO_2, 37℃) for 2 h and then under oxygen conditions (95% air and 5% CO_2, 37℃) for 12 h to establish the H/R cell models.

Cell transfection

PcDNA3.1-KDM1A (oe-KDM1A; RefSeq: NM_001347221.1), pcDNA3.1-SOX9 (oe-SOX9; RefSeq: NM_011448.4) and the corresponding control empty plasmids pcDNA3.1-NC (oe-NC) were synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). According to the manufacturer's instructions, 2 μg of pcDNA 3.1 plasmid was transfected into HL-1 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) 48 h prior to H/R treatment. HL-1 cells were treated with CPI-455 (10 mM, a specific KDM5 inhibitor) to increase H3K4me3 level 30 min before H/R treatment. CPI-455 (Cat. No. S8287) was purchased from Selleck Chemicals (Selleck Chemicals, Houston, TX, USA).

Enzyme-linked immunosorbent assay (ELISA)

HL-1 cells (2×10^5 cells) were transfected and then used for establishing H/R models. Afterwards, the cell culture medium was centrifuged at 850 g for 3 min to collect the cell supernatant. The concentrations of lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and creatine kinase MB (CK-MB) in cell supernatant were measured using ELISA kits according to the manufacturer's instructions. LDH kits (YX-E20034) were purchased from Wuhan Yipu Biological Technology Co., Ltd (Wuhan, Hubei, China), CK-MB kit (KE1544) from ImmunoWay (Plano, TX, USA), and AST kit (ab263882) from Abcam (Cambridge, MA, USA).

Caspase activity detection

The activity of Caspase-3 and Caspase-9 was determined using a colorimetric assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, HL-1 cells were collected and treated with 100 μL lysis buffer for 15 min. Then, the protein concentration was determined using a bicinchoninic acid (BCA) protein detection reagent (Beyotime). Subsequently, 150 μg of the lysates were incubated with 10 μL of Caspase-3 and Caspase-9 substrates (2 mM) at 37℃ for 4 h. The absorbance was measured at 405 nm using a microplate spectrophotometer (FLx800; BioTek, Winooski, Vermont, USA).

Cell counting kit-8 (CCK-8) assay

The cell viability of HL-1 cells was detected using the CCK-8 assay kit (Beyotime) according to the manufacturer's instructions. HL-1 cells were loaded on 96-well plates at 2×10^4 cells/well and incubated for 24 h, 48 h, and 72 h, after which 10 μL CCK-8 reagent was added into each well for continuous incubation in the dark for 2 h. The absorbance of the reagent at a wavelength of 450 nm was calculated using a microplate reader (Hercules, CA, USA).

Flow cytometry

Flow cytometry combined with the Annexin V/PI kit (BD Bioscience, San Jose, CA, USA) was used to detect HL-1 cell apoptosis. In short, HL-1 cells were collected, washed with PBS, and then re-suspended in 1 × binding buffer. Next, HL-1 cells were stained with Annexin V and PI in a dark room for 30 min. Flow cytometry (BD Bioscience) was performed through exciting lasers at 488 nm for flow cytometric analysis, and PI-stained HL-1 cells were collected using 575 nm optical filter configurations.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from HL-1 cells using Trizol (TaKaRa, Dalian, China) reagent and reversely transcribed into cDNA using PrimeScript RT kit (Takara). qRT-PCR amplification was performed using SYBR Green Real-time kit (Takara) on a CFX96 RT-PCR system (BioRad, Hemel Hempstead, Hertfordshire, UK) according to the producer's instructions. GAPDH was used as the endogenous control. The primers are shown in Table 1. The relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

Total protein of histone was extracted using the histone extraction kit (#ab113476; Abcam). And, total protein of HL-1 cells was extracted using the cell lysis buffer RIPA and was quantified by BCA kits (Pierce, Rockford, IL, USA). The protein was isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to poly (vinylidene fluoride) (PVDF) membrane (Invitrogen, Carlsbad, CA, USA). The membrane was blocked with 5% skim milk and incubated with the primary antibodies anti-KDM1A (1:10000; ab129195; Abcam), anti-H3K4me3 (1:1000; ab213224; Abcam), anti-H3 (1: 1000; ab1791; Abcam), and anti-GAPDH (1: 2500; ab9485; Abcam) and secondary antibody (1:2000; ab6728; Abcam). H3 was used as the internal reference for H3K4me3, and GAPDH was used as the internal reference for other proteins. The protein bands were observed using an enhanced chemiluminescence kit (Pierce).

Chromatin immunoprecipitation (ChIP) assay

ChIP analysis was performed using EZ-ChIP (Millipore, Billerica, MA). Briefly, HL-1 cells were crosslinked in 1% formaldehyde for 10 min and lysed in SDS lysis buffer, and DNA was sheared by ultrasound using Covaris at 4℃ (5 min, 20 s on, 20 s off). After dilution of the lysates

with ChIP buffer, immunoprecipitation was conducted with mouse IgG (1:100; Ab6789, Abcam) and anti-H3K4me3 (1:20, ab213224, Abcam). The antibody-chromatin complex was precipitated with ChIP blocking protein G agarose at 4℃ for 1 h, followed by washing and elution. After reverse cross-linking of the protein-DNA complex, DNA was purified on a rotating column and analyzed by RT-PCR. SOX9 promoter PCR primers were as follows: forward 5' AAGAGAGCATCATAAGGAGAC 3' and reverse 3' GGAGTATTTATTAGAGACCCT 5'.

Statistical analysis

Statistical analysis of the data was performed using GraphPad Prism 8.0 statistical software (GraphPad Software Inc., San Diego, CA, USA). The measurement data were expressed in the form of mean ± standard deviation. The data between two groups were compared using the *t* test. One-way ANOVA or two-way ANOVA was used for comparisons among multiple groups, and Tukey's multiple comparisons test was used for the post-hoc test. $p < 0.05$ indicated the difference was significant.

Results

H/R treatment promotes cardiomyocyte apoptosis and downregulates KDM1A

In order to investigate the role of KDM1A in MIRI, we constructed *in vitro* MIRI cell models through treating HL-1 cells with H/R. After H/R treatment, the level of KDM1A was significantly reduced ($p < 0.05$, Figure 1A-B), HL-1 cell viability was reduced ($p < 0.05$, Figure 1C), the concentrations of myocardial injury markers (LDH, AST, CK-MB) were increased ($p < 0.05$, Figure 1D), the level of pro-apoptotic protein (Bax) was increased, the level of anti-apoptotic protein (Bcl-2) was decreased ($p < 0.05$, Figure 1E), the apoptosis rate was notably increased ($p < 0.05$, Figure 1F), and the activities of Caspase-3 and Caspase-9 proteins were significantly increased ($p < 0.05$, Figure 1G). The above results indicated that H/R treatment promoted the apoptosis of cardiomyocytes treated by H/R and downregulated the expression of KDM1A.

Overexpressing KDM1A reduces apoptosis of H/R-treated cardiomyocytes

To further study the role of KDM1A in H/R-induced cardiomyocyte apoptosis, HL-1 cells were transfected with pcDNA3.1-KDM1A (oe-KDM1A) ($p < 0.05$, Figure 2A). In HL-1 cells overexpressing KDM1A, KDM1A protein level was elevated ($p < 0.05$, Figure 2B), cell viability was increased ($p < 0.05$, Figure 2C), the levels of LDH, AST, and CK-MB were decreased ($p < 0.05$, Figure 2D), Bax mRNA expression was decreased, Bcl-2 mRNA expression was increased ($p < 0.05$, Figure 2E), the apoptosis rate was decreased ($p < 0.05$, Figure 2F), and the activities of Caspase-3 and Caspase-9 were significantly decreased ($p < 0.05$, Figure 2G). The above results indicated that overexpression of KDM1A reduced apoptosis of H/R-treated cardiomyocytes

KDM1A inhibits the transcription of SOX9 by reducing H3K4me3

It has been reported that KDM1A could inhibit the expressions of its downstream genes by removing the H3K4me3 markers from the gene promoters (Zhao et al., 2020). In order to explore the downstream mechanism of KDM1A in MIRI, we performed Western blot to test the level of H3K4me3. The results showed that the level of H3K4me3 was increased in H/R-treated HL-1 cells and was decreased after overexpressing KDM1A ($p < 0.05$, Figure 3A). Additionally, H3K4me3 reduction can inhibit the transcription of SOX9 (Wang et al., 2018b), and SOX9 is upregulated in MIRI (Cheng et al., 2021). Therefore, we speculated that KDM1A in H/R cells could inhibit the transcription of SOX9 by reducing H3K4me3, thereby affecting apoptosis of cardiomyocytes after MIRI. The above speculation was subsequently supported by ChIP analysis, which showed that H3K4me3 was enriched on the SOX9 promoter but not on the SOX9 exon, and overexpressing KDM1A reduced the level of H3K4me3 on the SOX9 promoter ($p < 0.05$, Figure 3B). The level of SOX9 mRNA was increased in H/R-treated HL-1 cells and was decreased after overexpressing KDM1A ($p < 0.05$, Figure 3C). The above results indicated that KDM1A could inhibit the transcription of SOX9 by reducing H3Kme3 on the SOX9 promoter.

Upregulating H3K4me3 reverses the effect of overexpressing KDM1A on alleviating H/R-induced cardiomyocyte apoptosis

To prove that KDM1A limited apoptosis of H/R-treated HL-1 cells by reducing H3K4me3, we used CPI-455 to promote H3K4me3 in HL-1 cells transfected with oe-KDM1A. The results showed that, compared with HL-1 cells overexpressing KDM1A alone, in the H/R + oe-KDM1A + CPI group, the level of H3K4me3 was increased ($p < 0.05$, Figure 4A), the mRNA expression of SOX9 was elevated ($p < 0.05$, Figure 4B), HL-1 cell viability was decreased ($p < 0.05$, Figure 4C), the levels of LDH, AST, and CK-MB were increased ($p < 0.05$, Figure 4D), Bax mRNA level was increased, Bcl-2 mRNA level was decreased ($p < 0.05$, Figure 4E), the apoptosis rate was increased ($p < 0.05$, Figure 4F), and the activities of Caspase-3 and Caspase-9 were increased ($p < 0.05$, Figure 4G). Briefly, upregulating H3K4me3 could reverse the inhibition of overexpression of KDM1A on H/R-induced cardiomyocyte apoptosis.

Overexpressing SOX9 reverses the alleviative effect of overexpressing KDM1A on H/R-induced cardiomyocyte apoptosis

To prove that SOX9 may be involved in the regulation of KDM1A on H/R-induced HL-1 cell apoptosis, pcDNA3.1-SOX9 (oe-SOX9) was transfected into HL-1 cells and the transfection efficiency was verified ($p < 0.05$, Figure 5A). And, the transfected HL-1 cells underwent a joint experiment with oe-KDM1A. The results showed that compared with the group transfected with oe-KDM1A alone, in the H/R + oe-KDM1A + SOX9 group, HL-1 cell viability was decreased ($p < 0.05$, Figure 5B), the concentrations of LDH, AST, and CK-MB were increased ($p < 0.05$, Figure 5C), Bax mRNA level was increased, Bcl-2 mRNA level was decreased ($p < 0.05$, Figure 5D), the apoptosis rate was increased ($p < 0.05$, Figure 5E), and Caspase-3 and Caspase-9 activities were increased ($p < 0.05$, Figure 5E). The above results indicated that overexpressing

SOX9 could reverse the effect of overexpression of KDM1A on apoptosis of H/R-treated HL-1 cells.

Discussion

MIRI is regarded as the primary manifestation of CVDs (Hausenloy & Yellon, 2013). H/R process is considered a pivotal trigger factor for CVDs and exerts an irreversible impairment to homeostasis, causing cardiomyocyte apoptosis and injury, heart failure, or other detrimental outcomes (Chen et al., 2021). In addition, histone demethylases KDMs are proven to modulate the cellular processes of cardiomyocytes and be implicated with CVD progression (Akerberg et al., 2017; Mokou et al., 2019). Herein, our investigation in this work highlighted the regulatory functions of KDM1A on cardiomyocyte apoptosis after MIRI.

It has been reported that H/R induces cardiomyocyte apoptosis and inflammation (Chen et al., 2021; Fornes et al., 2020). Notably, a previous study illustrated that changes in KDM1A expression in neonatal mice could affect cardiomyocyte proliferation as well as myocardial regeneration through demethylation (Fei et al., 2021). In our study, HL-1 cells were subjected to H/R to establish MIRI cell models. Afterwards, the expression of KDM1A in H/R-induced HL-1 cells was decreased. Besides, HL-1 cell viability was decreased, LDH, AST, and CK-MB concentrations were increased, and HL-1 cell apoptosis was promoted. Through literature review, the decreased expressions of cardiomyocyte injury markers, LDH, AST, and CK-MB indicates the improvement of injury (Chen et al., 2018; Yu et al., 2020). And, many previous studies have proved the promotion of H/R on cardiomyocyte apoptosis and injury (Lu et al., 2019; Wang et al., 2021). The above indicated that H/R treatment promoted apoptosis of H/R-treated cardiomyocytes and downregulated KDM1A. Subsequently, we overexpressed KDM1A in HL-1 cells, after which, HL-1 cell viability was increased, myocardial injury was mitigated, and apoptosis was decreased. A relevant study has shown that KDM1A upregulation permits autophagy within the appropriate range, thereby reducing apoptosis of H/R-treated cardiomyocytes (Song et al., 2018), which supported our results that overexpression of KDM1A reduced apoptosis of H/R cardiomyocytes.

H3K4me3 is closely related to several heart-related pathologies, such as hypertrophic/dilated cardiomyopathy (Raveendran et al., 2020; Tran et al., 2022), and is associated with the conversion of the induced cardiomyocytes (Liu et al., 2016). Additionally, dexmedetomidine precondition in lung tissues alleviated I/R-induced lung injury via increasing H3K4me3 modification on the KGF-2 promoter (Hong et al., 2021). KDM1A may function on MIRI through demethylating H3K4me3 in MIRI. Accordingly, we found that the level of H3K4me3 was increased in H/R-treated HL-1 cells and was decreased after overexpressing KDM1A. On top of that, a prior study stated that KDM6A knockdown elevates H3K27me3 level but decreases H3K4me3 level on SOX9 promoter (Wang et al., 2018b) and SOX9 inhibition could reduce

apoptosis of hypoxia-treated cardiomyocytes (Rui et al., 2022). Hence, we speculated that KDM1A might inhibit the transcription of SOX9 and thus affect apoptosis of H/R-treated cardiomyocytes by reducing H3K4me3. As expected, overexpressing KDM1A reduced the level of H3K4me3 on the SOX9 promoter, and decreased the mRNA level of SOX9. Besides, a previous study has found that KDM1A bound to Hotair could inhibit the molecule transcription by reducing the H3K4me3 level located on the Hotair promoter (Zhao et al., 2020). Overall, the above results indicated that KDM1A could inhibit the transcription of SOX9 by reducing H3K4me3 on the SOX9 promoter region.

CPI-455 could be used as a KDMs inhibitor, and CPI-455-mediated KDM inhibition elevates the global levels of H3K4me3 (Liu et al., 2019; Vinogradova et al., 2016). Thereafter, CPI-455 was used to promote H3K4me3 in H/R-treated HL-1 cells overexpressing KDM1A. After H3K4me3 promotion, HL-1 cell viability was significantly reduced, myocardial injury was aggravated, and cell apoptosis was increased. It has been verified that H3K4me3 inhibition could alleviate I/R-induced renal and limit fibrosis and inflammation (Shimoda et al., 2019). The above result demonstrated that H3K4me3 upregulation could reverse the effect of overexpressing KDM1A on cardiomyocyte apoptosis after MIRI.

In addition, previous studies have proved that H/R process could modulate SOX9 expression in rat chondrocytes and the interaction between KDM1A and SOX9 in endochondral ossification (Li et al., 2018; Sun et al., 2020). Subsequently, HL-1 cells were transfected with oe-SOX9 and received a joint experiment with oe-KDM1A to probe the involvement of SOX9 in the regulation of KDM1A on H/R-treated cardiomyocyte apoptosis. Afterwards, we found that overexpressing SOX9 reduced HL-1 cell viability, promoted myocardial injury, and facilitated cell apoptosis. Previous literature showed that SOX9 suppression encourages cell activity and limits inflammation apoptosis in H/R-treated cardiomyocytes and further alleviated MIRI (Cheng et al., 2021), while overexpression of SOX9 limits cell activity and thus promotes hepatic I/R (Fan et al., 2018). These findings made it plausible that overexpressing SOX9 could reverse the mitigating effect of overexpressing KDM1A on cardiomyocyte apoptosis in MIRI.

Conclusions

In conclusion, we revealed that KDM1A inhibited the transcription of SOX9 by reducing H3K4me3 on the SOX9 promoter region, and thereby inhibited H/R-induced cardiomyocyte apoptosis. And we hope our study can provide a promising direction for the research and treatment related to MIRI. However, our study still has its limitations. We only investigated the demethylation of KDM1A on H3K4me3 located on the SOX9 promoter region and failed to study other histone demethylases. Besides, we verified the KDM1A/H3K4me3/SOX9 mechanism only in the *in vitro* models established by a single cell type. Going forward, we will conduct further studies to probe other sites of KDM1A for histone demethylation, to explore the

roles of other histone demethylases in cardiomyocyte apoptosis after MIRI. Accordingly, we will use different types of cardiomyocytes, such as H9C2 cells, to establish MIRI cell models and will validate our study in the *in vivo* animal models.

Acknowledgements

Not applicable.

References

Akerberg AA, Henner A, Stewart S, Stankunas K. 2017. Histone demethylases Kdm6ba and Kdm6bb redundantly promote cardiomyocyte proliferation during zebrafish heart ventricle maturation. *Developmental Biology* **426(1)**:84-96. doi:10.1016/j.ydbio.2017.03.030

Chen M, Guo Y, Sun Z, Meng X. 2021. Long non-coding RNA SENCR alleviates hypoxia/reoxygenation-induced cardiomyocyte apoptosis and inflammatory response by sponging miR-1. *Cardiovasc Diagn Ther* **11(3)**:707-715. doi:10.21037/cdt-20-1037

Chen X, Guo H, Li Q, Zhang Y, Liu H, Zhang X, Xie K, Zhu Z, Miao Q, Su S. 2018. Protective effect of berberine on aconiteinduced myocardial injury and the associated mechanisms. *Mol Med Rep* **18(5)**:4468-4476. doi:10.3892/mmr.2018.9476

Chen Z, Guo W, Wu Q, Tan L, Ma T, Fu C, Yu J, Ren X, Wang J, Liang P et al. . 2020. Tumor reoxygenation for enhanced combination of radiation therapy and microwave thermal therapy using oxygen generation in situ by CuO nanosuperparticles under microwave irradiation. *Theranostics* **10(10)**:4659-4675. doi:10.7150/thno.42818

Cheng N, Li L, Wu Y, Wang M, Yang M, Wei S, Wang R. 2021. microRNA-30e up-regulation alleviates myocardial ischemia-reperfusion injury and promotes ventricular remodeling via SOX9 repression. *Molecular Immunology* **130(**96-103. doi:10.1016/j.molimm.2020.11.009

Davis K, Azarcon P, Hickenlooper S, Bia R, Horiuchi E, Szulik MW, Franklin S. 2021. The role of demethylases in cardiac development and disease. *Journal of Molecular and Cellular Cardiology* **158(**89-100. doi:10.1016/j.yjmcc.2021.05.018

Eltzschig HK, Eckle T. 2011. Ischemia and reperfusion--from mechanism to translation. *Nature Medicine* **17(11)**:1391-1401. doi:10.1038/nm.2507

Fan XD, Zheng HB, Fan XS, Lu S. 2018. Increase of SOX9 promotes hepatic ischemia/reperfusion (IR) injury by activating TGF-beta1. *Biochemical and Biophysical Research Communications* **503(1)**:215-221. doi:10.1016/j.bbrc.2018.06.005

Fei Q, Qiu M, Fan G, Zhang B, Wang Q, Zhang S, Wang S, Yang B, Zhang L. 2021. Downregulation of Hotair or LSD1 Impaired Heart Regeneration in the Neonatal Mouse. *DNA and Cell Biology* **40(9)**:1177-1184. doi:10.1089/dna.2021.0095

Fornes O, Castro-Mondragon JA, Khan A, van der Lee R, Zhang X, Richmond PA, Modi BP, Correard S, Gheorghe M, Baranasic D et al. . 2020. JASPAR 2020: update of the

399 open-access database of transcription factor binding profiles. *Nucleic Acids Res*
400 **48(D1)**:D87-D92. doi:10.1093/nar/gkz1001
401 **Gao G, Chen W, Yan M, Liu J, Luo H, Wang C, Yang P. 2020.** Rapamycin regulates the
402 balance between cardiomyocyte apoptosis and autophagy in chronic heart failure by
403 inhibiting mTOR signaling. *International Journal of Molecular Medicine* **45(1)**:195-209.
404 doi:10.3892/ijmm.2019.4407
405 **Han B, Yang Y, Tang L, Yang Q, Xie R. 2021.** Roles of SET7/9 and LSD1 in the Pathogenesis
406 of Arsenic-induced Hepatocyte Apoptosis. *J Clin Transl Hepatol* **9(3)**:364-372.
407 doi:10.14218/JCTH.2020.00185
408 **Hausenloy DJ, Yellon DM. 2013.** Myocardial ischemia-reperfusion injury: a neglected
409 therapeutic target. *Journal of Clinical Investigation* **123(1)**:92-100.
410 doi:10.1172/JCI62874
411 **Hong H, Huang Q, Cai Y, Lin T, Xia F, Jin Z. 2021.** Dexmedetomidine preconditioning
412 ameliorates lung injury induced by pulmonary ischemia/reperfusion by upregulating
413 promoter histone H3K4me3 modification of KGF-2. *Experimental Cell Research*
414 **406(2)**:112762. doi:10.1016/j.yexcr.2021.112762
415 **Ismail T, Lee HK, Kim C, Kwon T, Park TJ, Lee HS. 2018.** KDM1A microenvironment, its
416 oncogenic potential, and therapeutic significance. *Epigenetics Chromatin* **11(1)**:33.
417 doi:10.1186/s13072-018-0203-3
418 **Kim D, Kim KI, Baek SH. 2021.** Roles of lysine-specific demethylase 1 (LSD1) in homeostasis
419 and diseases. *Journal of Biomedical Science* **28(1)**:41. doi:10.1186/s12929-021-00737-3
420 **Klose RJ, Kallin EM, Zhang Y. 2006.** JmjC-domain-containing proteins and histone
421 demethylation. *Nature Reviews: Genetics* **7(9)**:715-727. doi:10.1038/nrg1945
422 **Lacraz GPA, Junker JP, Gladka MM, Molenaar B, Scholman KT, Vigil-Garcia M,**
423 **Versteeg D, de Ruiter H, Vermunt MW, Creyghton MP et al. . 2017.** Tomo-Seq
424 Identifies SOX9 as a Key Regulator of Cardiac Fibrosis During Ischemic Injury.
425 *Circulation* **136(15)**:1396-1409. doi:10.1161/CIRCULATIONAHA.117.027832
426 **Lefebvre V, Angelozzi M, Haseeb A. 2019.** SOX9 in cartilage development and disease.
427 *Current Opinion in Cell Biology* **61(**39-47. doi:10.1016/j.ceb.2019.07.008
428 **Leon J, Castillo MC, Gayubas B. 2021.** The hypoxia-reoxygenation stress in plants. *Journal of*
429 *Experimental Botany* **72(16)**:5841-5856. doi:10.1093/jxb/eraa591
430 **Li H, Li X, Jing X, Li M, Ren Y, Chen J, Yang C, Wu H, Guo F. 2018.** Hypoxia promotes
431 maintenance of the chondrogenic phenotype in rat growth plate chondrocytes through the
432 HIF-1alpha/YAP signaling pathway. *International Journal of Molecular Medicine*
433 **42(6)**:3181-3192. doi:10.3892/ijmm.2018.3921
434 **Lin J, Lin H, Ma C, Dong F, Hu Y, Li H. 2019.** MiR-149 Aggravates Pyroptosis in Myocardial
435 Ischemia-Reperfusion Damage via Silencing FoxO3. *Medical Science Monitor* **25(**8733-
436 8743. doi:10.12659/MSM.918410

Liu Y, Yu Y, Zhang J, Wang C. 2019. The therapeutic effect of dexmedetomidine on protection from renal failure via inhibiting KDM5A in lipopolysaccharide-induced sepsis of mice. *Life Sciences* **239(**116868. doi:10.1016/j.lfs.2019.116868

Liu Z, Chen O, Zheng M, Wang L, Zhou Y, Yin C, Liu J, Qian L. 2016. Re-patterning of H3K27me3, H3K4me3 and DNA methylation during fibroblast conversion into induced cardiomyocytes. *Stem Cell Res* **16(2)**:507-518. doi:10.1016/j.scr.2016.02.037

Lu Y, Bu M, Yun H. 2019. Sevoflurane prevents hypoxia/reoxygenation-induced cardiomyocyte apoptosis by inhibiting PI3KC3-mediated autophagy. *Human Cell* **32(2)**:150-159. doi:10.1007/s13577-018-00230-4

MacIntyre NR. 2014. Tissue hypoxia: implications for the respiratory clinician. *Respiratory Care* **59(10)**:1590-1596. doi:10.4187/respcare.03357

Mokou M, Klein J, Makridakis M, Bitsika V, Bascands JL, Saulnier-Blache JS, Mullen W, Sacherer M, Zoidakis J, Pieske B et al. . 2019. Proteomics based identification of KDM5 histone demethylases associated with cardiovascular disease. *EBioMedicine* **41(**91-104. doi:10.1016/j.ebiom.2019.02.040

Murry CE, Jennings RB, Reimer KA. 1986. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74(5)**:1124-1136. doi:10.1161/01.cir.74.5.1124

Raveendran VV, Al-Haffar K, Kunhi M, Belhaj K, Al-Habeeb W, Al-Buraiki J, Eyjolsson A, Poizat C. 2020. Protein arginine methyltransferase 6 mediates cardiac hypertrophy by differential regulation of histone H3 arginine methylation. *Heliyon* **6(5)**:e03864. doi:10.1016/j.heliyon.2020.e03864

Rui L, Liu R, Jiang H, Liu K. 2022. Sox9 Promotes Cardiomyocyte Apoptosis After Acute Myocardial Infarction by Promoting miR-223-3p and Inhibiting MEF2C. *Molecular Biotechnology*. doi:10.1007/s12033-022-00471-7

Shimoda H, Doi S, Nakashima A, Sasaki K, Doi T, Masaki T. 2019. Inhibition of the H3K4 methyltransferase MLL1/WDR5 complex attenuates renal senescence in ischemia reperfusion mice by reduction of p16(INK4a). *Kidney International* **96(5)**:1162-1175. doi:10.1016/j.kint.2019.06.021

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y. 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119(7)**:941-953. doi:10.1016/j.cell.2004.12.012

Song H, Feng X, Zhang M, Jin X, Xu X, Wang L, Ding X, Luo Y, Lin F, Wu Q et al. . 2018. Crosstalk between lysine methylation and phosphorylation of ATG16L1 dictates the apoptosis of hypoxia/reoxygenation-induced cardiomyocytes. *Autophagy* **14(5)**:825-844. doi:10.1080/15548627.2017.1389357

Stein AB, Goonewardena SN, Jones TA, Prusick PJ, Bazzi AA, Belyavskaya JM, McCoskey MM, Dandar RA. 2015. The PTIP-Associated Histone Methyltransferase Complex Prevents Stress-Induced Maladaptive Cardiac Remodeling. *PloS One* **10(5)**:e0127839. doi:10.1371/journal.pone.0127839

Sun J, Feng H, Xing W, Han Y, Suo J, Yallowitz AR, Qian N, Shi Y, Greenblatt MB, Zou W. 2020. Histone demethylase LSD1 is critical for endochondral ossification during bone fracture healing. *Sci Adv* **6(45)**. doi:10.1126/sciadv.aaz1410

Toldo S, Mauro AG, Cutter Z, Abbate A. 2018. Inflammasome, pyroptosis, and cytokines in myocardial ischemia-reperfusion injury. *American Journal of Physiology: Heart and Circulatory Physiology* **315(6)**:H1553-H1568. doi:10.1152/ajpheart.00158.2018

Tran TA, Zhang QJ, Wang L, Gonzales C, Girard L, May H, Gillette T, Liu ZP, Martinez ED. 2022. Inhibition of Jumonji demethylases reprograms severe dilated cardiomyopathy and prolongs survival. *Journal of Biological Chemistry* **298(2)**:101515. doi:10.1016/j.jbc.2021.101515

Vinogradova M, Gehling VS, Gustafson A, Arora S, Tindell CA, Wilson C, Williamson KE, Guler GD, Gangurde P, Manieri W et al. . 2016. An inhibitor of KDM5 demethylases reduces survival of drug-tolerant cancer cells. *Nature Chemical Biology* **12(7)**:531-538. doi:10.1038/nchembio.2085

Wang F, Min X, Hu SY, You DL, Jiang TT, Wang L, Wu X. 2021. Hypoxia/reoxygenation-induced upregulation of miRNA-542-5p aggravated cardiomyocyte injury by repressing autophagy. *Human Cell* **34(2)**:349-359. doi:10.1007/s13577-020-00466-z

Wang P, Fan F, Li X, Sun X, Ma L, Wu J, Shen C, Zhu H, Dong Z, Wang C et al. . 2018a. Riboflavin attenuates myocardial injury via LSD1-mediated crosstalk between phospholipid metabolism and histone methylation in mice with experimental myocardial infarction. *Journal of Molecular and Cellular Cardiology* **115(**115-129. doi:10.1016/j.yjmcc.2018.01.006

Wang P, Li Y, Meng T, Zhang J, Wei Y, Meng Z, Lin Y, Liu D, Sui L. 2018b. KDM6A promotes chondrogenic differentiation of periodontal ligament stem cells by demethylation of SOX9. *Cell Proliferation* **51(3)**:e12413. doi:10.1111/cpr.12413

Yang SJ, Park YS, Cho JH, Moon B, An HJ, Lee JY, Xie Z, Wang Y, Pocalyko D, Lee DC et al. . 2017. Regulation of hypoxia responses by flavin adenine dinucleotide-dependent modulation of HIF-1alpha protein stability. *EMBO Journal* **36(8)**:1011-1028. doi:10.15252/embj.201694408

Yu W, Qin X, Zhang Y, Qiu P, Wang L, Zha W, Ren J. 2020. Curcumin suppresses doxorubicin-induced cardiomyocyte pyroptosis via a PI3K/Akt/mTOR-dependent manner. *Cardiovasc Diagn Ther* **10(4)**:752-769. doi:10.21037/cdt-19-707

Zhao D, Zhao Y, Wang J, Wu L, Liu Y, Zhao S, Guo F, Ma X, Zhang H, Li Z et al. . 2020. Long noncoding RNA Hotair facilitates retinal endothelial cell dysfunction in diabetic retinopathy. *Clinical Science (London, England: 1979)* **134(17)**:2419-2434. doi:10.1042/CS20200694

Zhang Y, Hou L. 2021. Alternate Roles of Sox Transcription Factors beyond Transcription Initiation. *International Journal of Molecular Sciences* **22(11)**. doi:10.3390/ijms22115949

Zou ZK, Huang YQ, Zou Y, Zheng XK, Ma XD. 2017. Silencing of LSD1 gene modulates

histone methylation and acetylation and induces the apoptosis of JeKo-1 and MOLT-4 cells. *International Journal of Molecular Medicine* **40(2)**:319-328. doi:10.3892/ijmm.2017.3032

Legends

Figure 1 H/R treatment promotes cardiomyocyte apoptosis and downregulates KDM1A. HL-1 cells were subjected to hypoxia-reoxygenation treatment (the H/R group), with HL-1 cells untreated as the control (the Blank group). A-B: qRT-PCR and Western blot were adopted to detect the levels of KDM1A in HL-1 cells; C: CCK-8 was used to verify HL-1 cell viability; D: ELISA was performed to detect the concentrations of myocardial injury markers in HL-1 cells; E: qRT-PCR was used to detect the mRNA levels of apoptotic proteins in HL-1 cells; F: Flow cytometry was conducted to detect the apoptosis rate of HL-1 cells; G: Colorimetric method was used to examine the activities of Caspase-3 and Caspase-9. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panels A-B and D-G were tested by independent *t* test; data in panel C were analyzed by two-way ANOVA, followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

Figure 2 Overexpressing KDM1A reduces apoptosis of H/R-treated cardiomyocytes. Plasmid pcDNA3.1-KDM1A (oe-KDM1A) was transfected into HL-1 cells, with pcDNA3.1-NC (oe-NC) as the control. A: qRT-PCR was used to measure the transfection efficiency; B: Western blot was performed to examine the protein level of KDM1A in HL-1 cells; C: CCK-8 assay was adopt to detect cell viability; D: ELISA was conducted to the concentrations of myocardial injury markers in HL-1 cells; E: qRT-PCR was performed to verify the mRNA levels of apoptotic proteins in HL-1 cells; F: Flow cytometry was used to detect the apoptosis rate; G: Colorimetric method was conducted to measure the activities of Caspase-3 and Caspase-9. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panel C were analyzed using two-way ANOVA and data in panels A-B and D-G were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

Figure 3 KDM1A inhibits the transcription of SOX9 by reducing H3K4me3. A: Western blot was used to detect the level of H3K4me3 in HL-1 cells. B: ChIP analysis was used to detect the

changes of H3K4me3 on the SOX9 promoter and exon; C: qRT-PCR to detect the level of SOX9 mRNA in HL-1 cells; The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panel B were analyzed by two-way ANOVA and data in panels A and C were analyzed by one-way ANOVA, and followed by Tukey's post-hoc test.

Figure 4 Upregulating H3K4me3 reverses the effect of overexpressing KDM1A on alleviating H/R-induced cardiomyocyte apoptosis. H3K4me3 in H/R cells was promoted using CPI-455 (solvent control: DMSO) and then the cells were combined with oe-KDM1A. A: Western blot was used to detect the levels of H3K4me3 in HL-1 cells; B: qRT-PCR was performed to examine the mRNA expression of SOX9 in HL-1 cells; C: CCK-8 was adopted to detect cell viability; D: ELISA was performed to verify the concentrations of myocardial injury markers in HL-1 cells; E: qRT-PCR was conducted to examine the mRNA levels of apoptotic proteins in HL-1 cells; F: Flow cytometry was used to detect the apoptotic rate; G: Colorimetric method was to conducted to test the activities of Caspase-3 and Caspase-9 in the cells. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panel C were analyzed by two-way ANOVA and data in panels A-B and D-G were analyzed by one-way ANOVA, and followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

Figure 5 Overexpressing SOX9 reverses the alleviative effect of overexpressing KDM1A on H/R-induced cardiomyocyte apoptosis. pcDNA3.1-SOX9 (oe-SOX9) was transfected into HL-1 cells using oe-NC as the blank. A: qRT-PCR was adopted to detect the transfection efficiency. Then, the H/R-treated HL-1 cells overexpressing SOX9 were combined with oe-KDM1A. B: CCK-8 was used to detect cell viability; C: ELISA was used to detect the concentrations of myocardial injury markers in HL-1 cells; D: qRT-PCR was conducted to verify the mRNA levels of apoptotic proteins in HL-1 cells; E: Flow cytometry was used to examine the apoptotic rate; F: Colorimetric method was adopted to test that activities of Caspase-3 and Caspase-9 proteins in HL-1 cell. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panels B were analyzed by two-way ANOVA and data in panels A and C-F were analyzed by one-way ANOVA, followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

Table 1(on next page)

PCR primers

Table 1 PCR primers

	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
KDM1A	CGGCCCGAGATGTTATCT	AGGCCCCCGGGGGGCGAG
SOX9	GGCTCGCGTATGAATCTCCTG	TTCTTCAGATCCGGCTCG
Bax	GCGGCAGTGATGGACGGGTCC	AGCTCCATATTGCTGTCCAG
Bcl-2	CGGGGAAGGATGGCGCAAGCC	GCAGCCATGTCCCGGTGC
GAPDH	AGAGGGATGCTGCCCTTACCC	AGTTGAGGTCAATGAAGG

Figure 1

H/R treatment promotes cardiomyocyte apoptosis and downregulates KDM1A.

HL-1 cells were subjected to hypoxia-reoxygenation treatment (the H/R group), with HL-1 cells untreated as the control (the Blank group). A-B: qRT-PCR and Western blot were adopted to detect the levels of KDM1A in HL-1 cells; C: CCK-8 was used to verify HL-1 cell viability; D: ELISA was performed to detect the concentrations of myocardial injury markers in HL-1 cells; E: qRT-PCR was used to detect the mRNA levels of apoptotic proteins in HL-1 cells; F: Flow cytometry was conducted to detect the apoptosis rate of HL-1 cells; G: Colorimetric method was used to examine the activities of Caspase-3 and Caspase-9. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panels A-B and D-G were tested by independent *t* test; data in panel C were analyzed by two-way ANOVA, followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

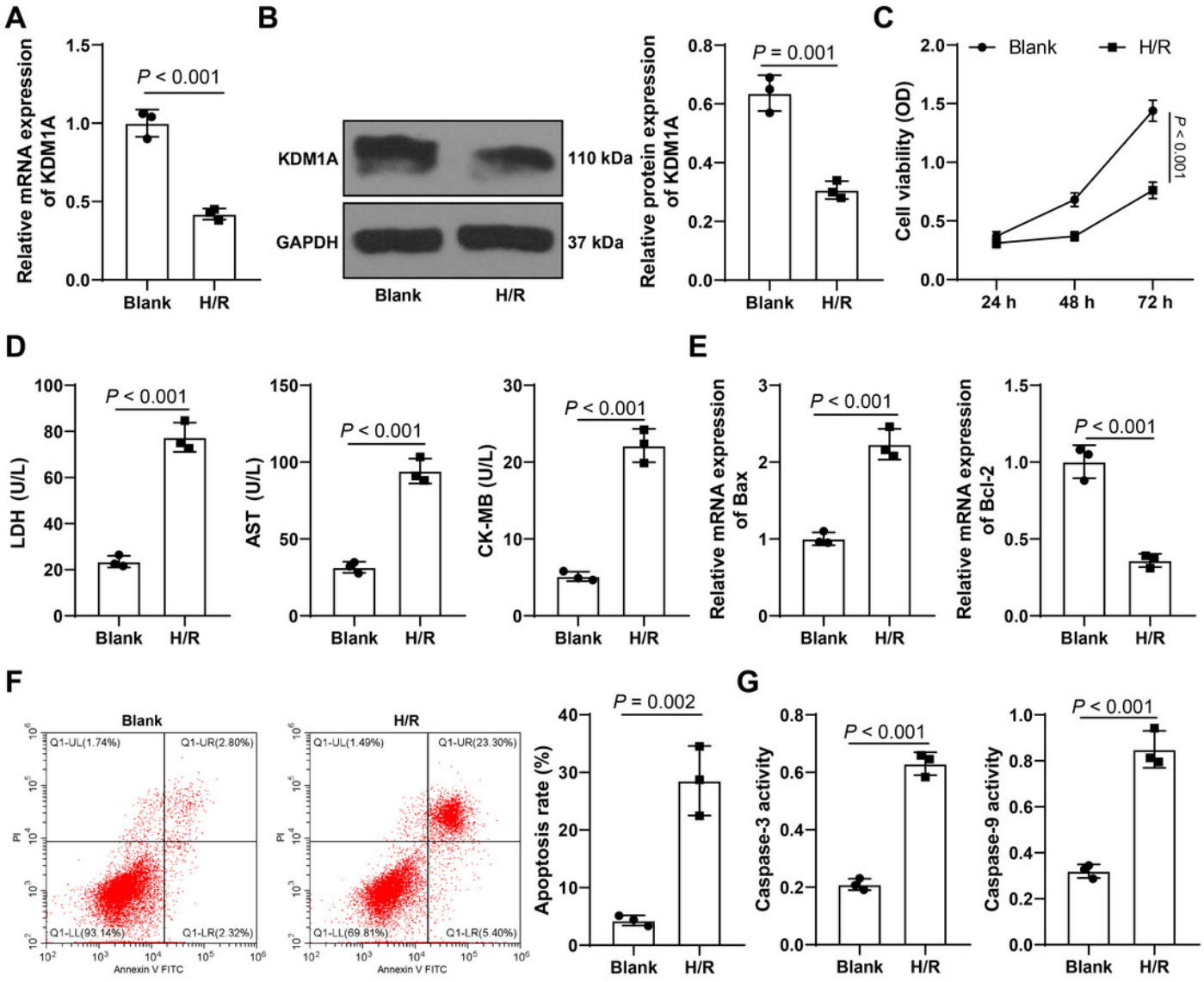

A
Relative mRNA expression of KDM1A
P < 0.001
Blank
H/R
B
KDM1A
110 kDa
GAPDH
37 kDa
Blank
H/R
Relative protein expression of KDM1A
P = 0.001
C
Blank
H/R
Cell viability (OD)
P < 0.001
24 h
48 h
72 h
D
LDH (U/L)
AST (U/L)
CK-MB (U/L)
E
Relative mRNA expression of Bax
Relative mRNA expression of Bcl-2
F
Blank
H/R
Q1-UL(1.74%)
Q1-UR(2.80%)
Q1-LL(93.14%)
Q1-LR(2.32%)
Q1-UL(1.49%)
Q1-UR(23.30%)
Q1-LL(69.81%)
Q1-LR(5.40%)
PI
Annexin V FITC
Apoptosis rate (%)
P = 0.002
G
Caspase-3 activity
Caspase-9 activity

Figure 2

Overexpressing KDM1A reduces apoptosis of H/R-treated cardiomyocytes.

Plasmid pcDNA3.1-KDM1A (oe-KDM1A) was transfected into HL-1 cells, with pcDNA3.1-NC (oe-NC) as the control. A: qRT-PCR was used to measure the transfection efficiency; B: Western blot was performed to examine the protein level of KDM1A in HL-1 cells; C: CCK-8 assay was adopt to detect cell viability; D: ELISA was conducted to the concentrations of myocardial injury markers in HL-1 cells; E: qRT-PCR was performed to verify the mRNA levels of apoptotic proteins in HL-1 cells; F: Flow cytometry was used to detect the apoptosis rate; G: Colorimetric method was conducted to measure the activities of Caspase-3 and Caspase-9. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panel C were analyzed using two-way ANOVA and data in panels A-B and D-G were analyzed using one-way ANOVA, followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

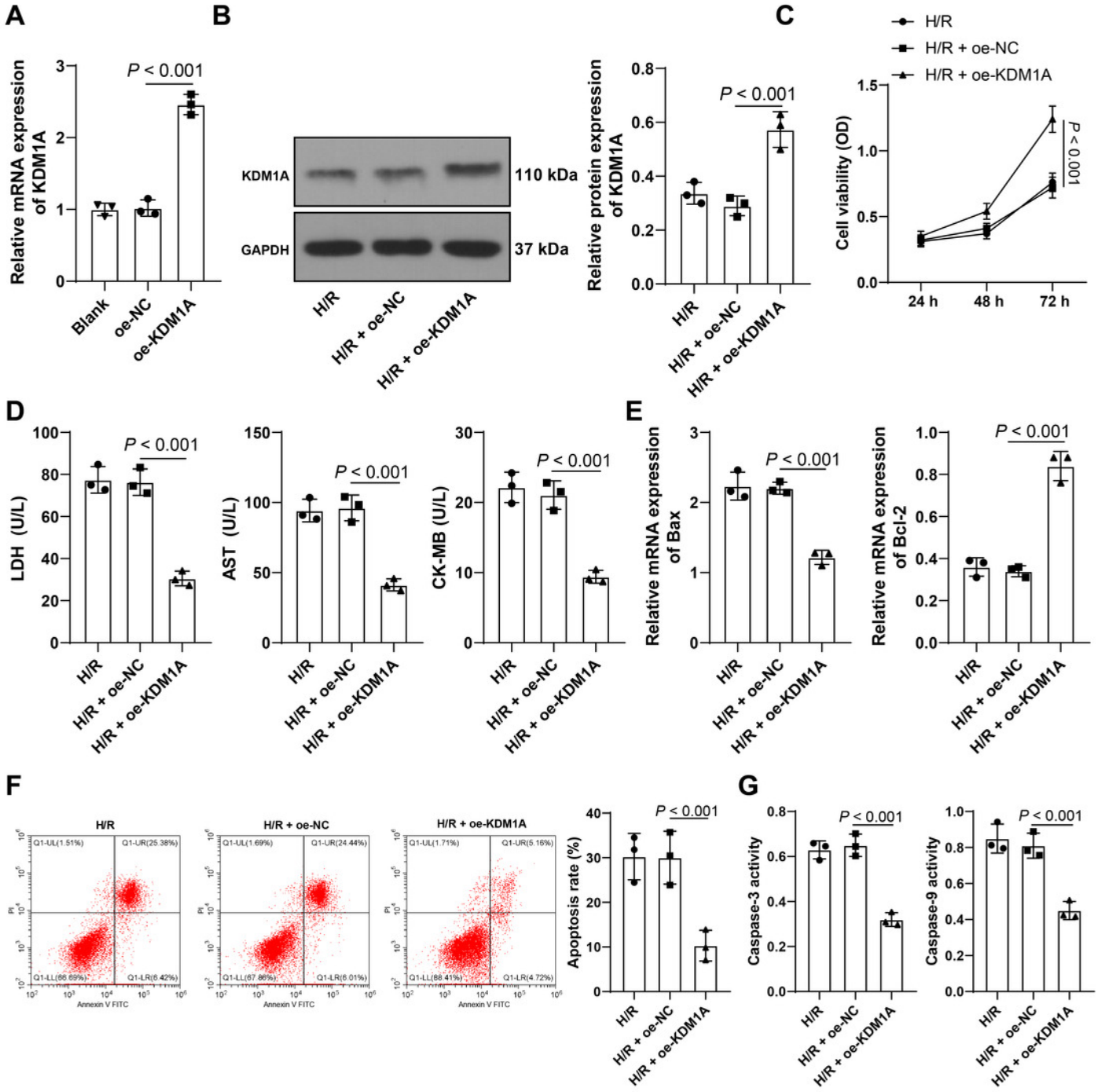

A
Relative mRNA expression of KDM1A
P < 0.001
Blank
oe-NC
oe-KDM1A
B
KDM1A
110 kDa
GAPDH
37 kDa
H/R
H/R + oe-NC
H/R + oe-KDM1A
Relative protein expression of KDM1A
P < 0.001
C
H/R
H/R + oe-NC
H/R + oe-KDM1A
Cell viability (OD)
P < 0.001
24 h
48 h
72 h
D
LDH (U/L)
P < 0.001
AST (U/L)
P < 0.001
CK-MB (U/L)
P < 0.001
E
Relative mRNA expression of Bax
P < 0.001
Relative mRNA expression of Bcl-2
P < 0.001
F
H/R
H/R + oe-NC
H/R + oe-KDM1A
Q1-UL(1.51%)
Q1-UR(25.38%)
Q1-LL(66.69%)
Q1-LR(6.42%)
Q1-UL(1.69%)
Q1-UR(24.44%)
Q1-LL(67.86%)
Q1-LR(6.01%)
Q1-UL(1.71%)
Q1-UR(5.16%)
Q1-LL(88.41%)
Q1-LR(4.72%)
PI
Annexin V FITC
Apoptosis rate (%)
P < 0.001
G
Caspase-3 activity
P < 0.001
Caspase-9 activity
P < 0.001

Figure 3

KDM1A inhibits the transcription of SOX9 by reducing H3K4me3.

A: Western blot was used to detect the level of H3K4me3 in HL-1 cells. B: ChIP analysis was used to detect the changes of H3K4me3 on the SOX9 promoter and exon; C: qRT-PCR to detect the level of SOX9 mRNA in HL-1 cells; The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panel B were analyzed by two-way ANOVA and data in panels A and C were analyzed by one-way ANOVA, and followed by Tukey's post-hoc test.

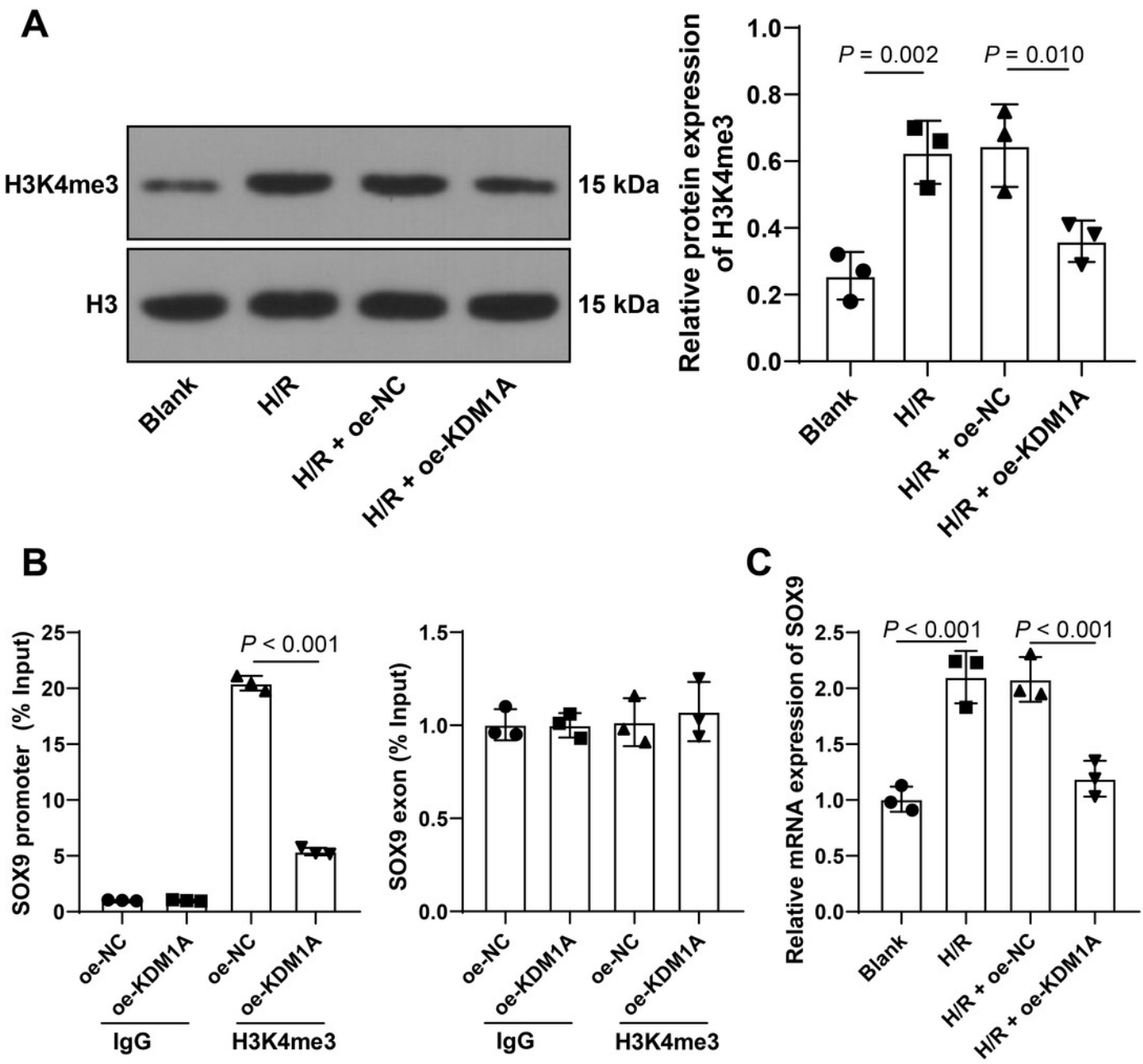

A
H3K4me3
15 kDa
H3
15 kDa
Blank
H/R
H/R + oe-NC
H/R + oe-KDM1A
Relative protein expression of H3K4me3
P = 0.002
P = 0.010
B
SOX9 promoter (% Input)
P < 0.001
oe-NC
oe-KDM1A
oe-NC
oe-KDM1A
IgG
H3K4me3
SOX9 exon (% Input)
C
Relative mRNA expression of SOX9
P < 0.001
P < 0.001

Figure 4

Upregulating H3K4me3 reverses the effect of overexpressing KDM1A on alleviating H/R-induced cardiomyocyte apoptosis.

H3K4me3 in H/R cells was promoted using CPI-455 (solvent control: DMSO) and then the cells were combined with oe-KDM1A. A: Western blot was used to detect the levels of H3K4me3 in HL-1 cells; B: qRT-PCR was performed to examine the mRNA expression of SOX9 in HL-1 cells; C: CCK-8 was adopted to detect cell viability; D: ELISA was performed to verify the concentrations of myocardial injury markers in HL-1 cells; E: qRT-PCR was conducted to examine the mRNA levels of apoptotic proteins in HL-1 cells; F: Flow cytometry was used to detect the apoptotic rate; G: Colorimetric method was to conducted to test the activities of Caspase-3 and Caspase-9 in the cells. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panel C were analyzed by two-way ANOVA and data in panels A-B and D-G were analyzed by one-way ANOVA, and followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.

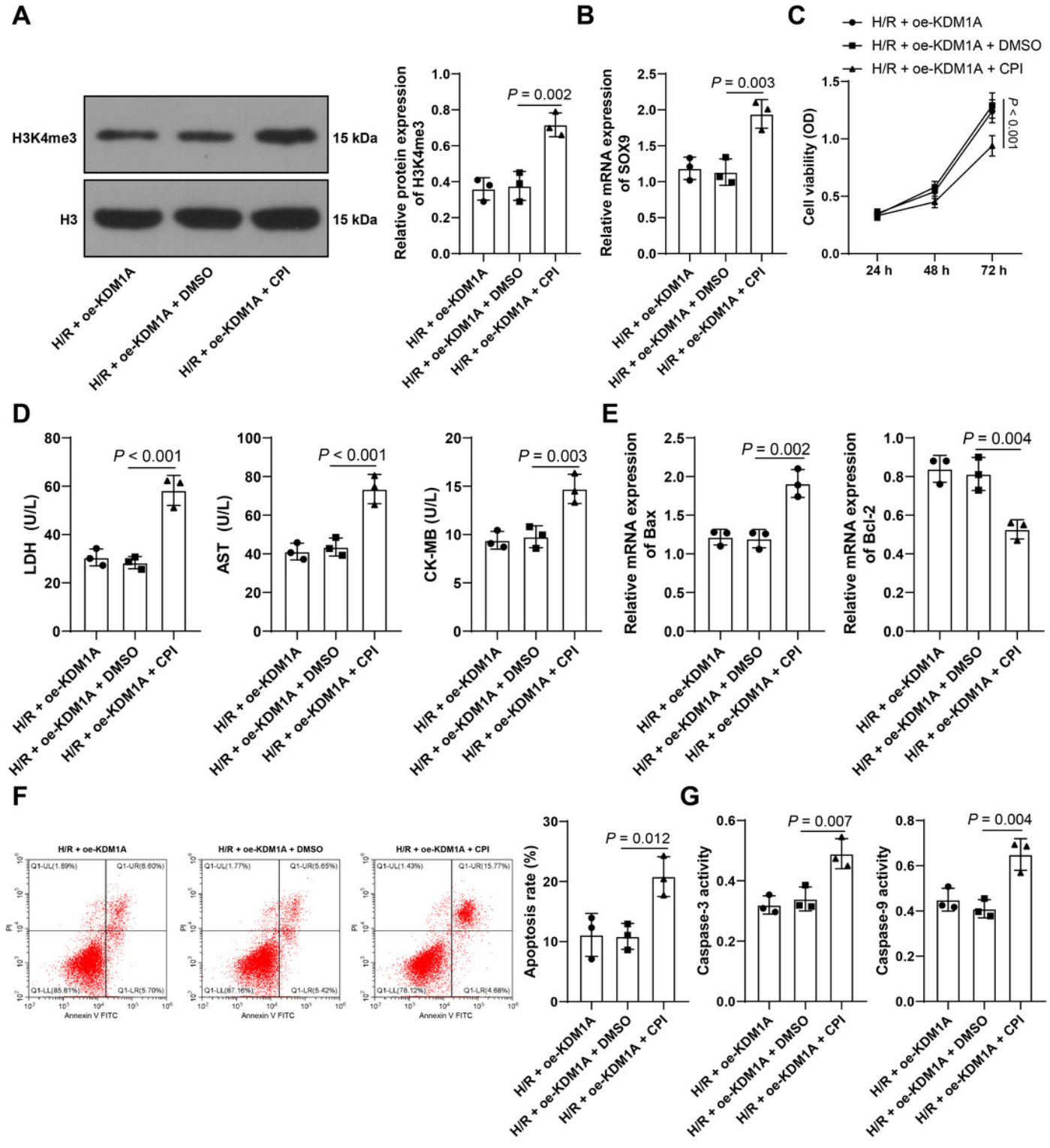
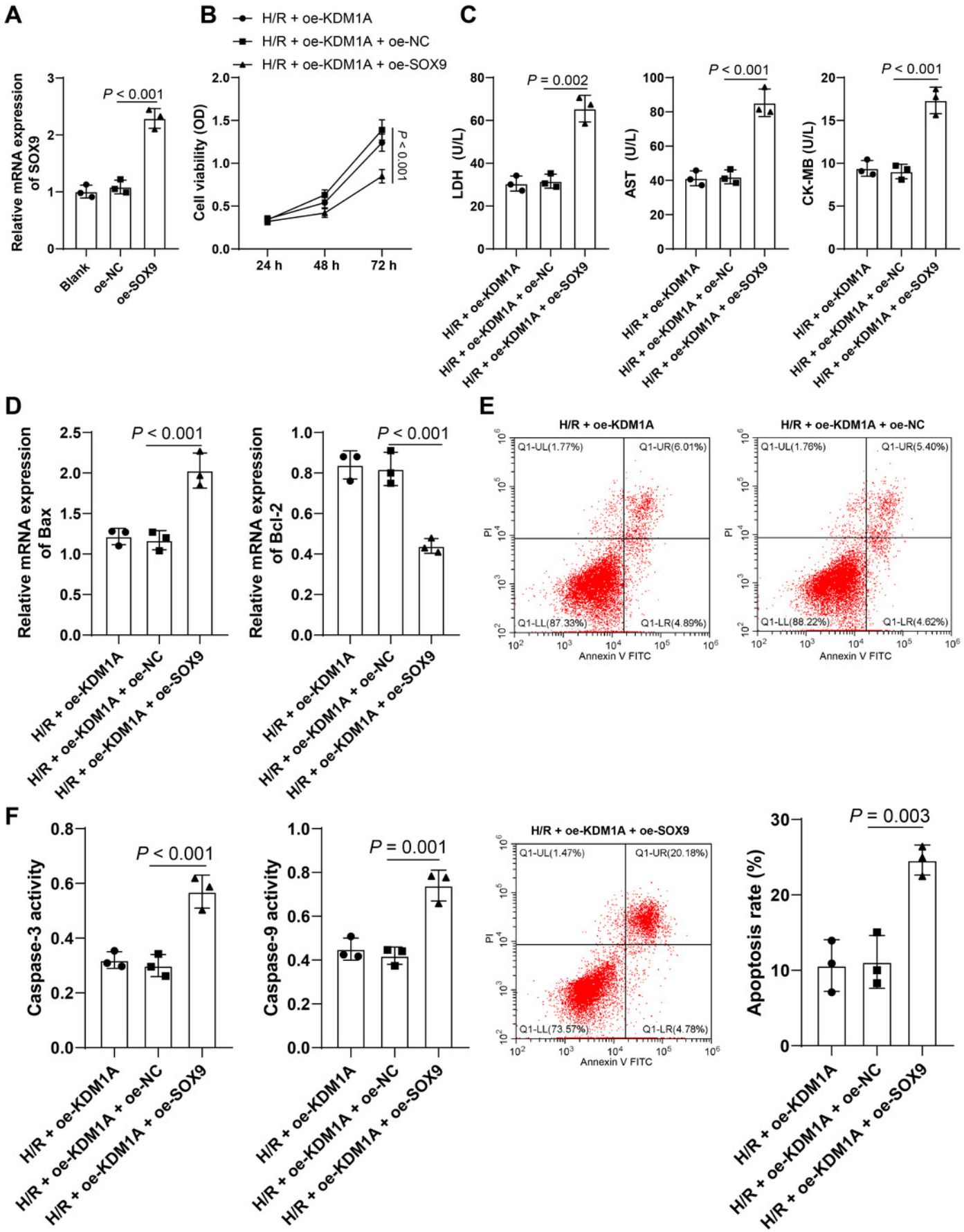

A
H3K4me3
H3
15 kDa
15 kDa
H/R + oe-KDM1A
H/R + oe-KDM1A + DMSO
H/R + oe-KDM1A + CPI
Relative protein expression of H3K4me3
P = 0.002
B
Relative mRNA expression of SOX9
P = 0.003
C
H/R + oe-KDM1A
H/R + oe-KDM1A + DMSO
H/R + oe-KDM1A + CPI
Cell viability (OD)
P < 0.001
24 h
48 h
72 h
D
LDH (U/L)
P < 0.001
AST (U/L)
P < 0.001
CK-MB (U/L)
P = 0.003
E
Relative mRNA expression of Bax
P = 0.002
Relative mRNA expression of Bcl-2
P = 0.004
F
H/R + oe-KDM1A
H/R + oe-KDM1A + DMSO
H/R + oe-KDM1A + CPI
Q1-UL(1.89%)
Q1-UR(6.60%)
Q1-LL(85.81%)
Q1-LR(5.70%)
Q1-UL(1.77%)
Q1-UR(5.65%)
Q1-LL(87.16%)
Q1-LR(5.42%)
Q1-UL(1.43%)
Q1-UR(15.77%)
Q1-LL(78.12%)
Q1-LR(4.68%)
PI
Annexin V FITC
G
Apoptosis rate (%)
P = 0.012
Caspase-3 activity
P = 0.007
Caspase-9 activity
P = 0.004

Figure 5

Overexpressing SOX9 reverses the alleviative effect of overexpressing KDM1A on H/R-induced cardiomyocyte apoptosis.

pcDNA3.1-SOX9 (oe-SOX9) was transfected into HL-1 cells using oe-NC as the blank. A: qRT-PCR was adopted to detect the transfection efficiency. Then, the H/R-treated HL-1 cells overexpressing SOX9 were combined with oe-KDM1A. B: CCK-8 was used to detect cell viability; C: ELISA was used to detect the concentrations of myocardial injury markers in HL-1 cells; D: qRT-PCR was conducted to verify the mRNA levels of apoptotic proteins in HL-1 cells; E: Flow cytometry was used to examine the apoptotic rate; F: Colorimetric method was adopted to test that activities of Caspase-3 and Caspase-9 proteins in HL-1 cell. The cell experiment was repeated 3 times independently; data were expressed as mean ± standard deviation. Data in panels B were analyzed by two-way ANOVA and data in panels A and C-F were analyzed by one-way ANOVA, followed by Tukey's post-hoc test. LDH: Lactate Dehydrogenase; AST: Aspartate Aminotransferase; CK-MB: Creatine Kinase-MB.


A
Relative mRNA expression of SOX9
P < 0.001
Blank
oe-NC
oe-SOX9
B
H/R + oe-KDM1A
H/R + oe-KDM1A + oe-NC
H/R + oe-KDM1A + oe-SOX9
Cell viability (OD)
P < 0.001
24 h
48 h
72 h
C
LDH (U/L)
P = 0.002
AST (U/L)
P < 0.001
CK-MB (U/L)
P < 0.001
H/R + oe-KDM1A
H/R + oe-KDM1A + oe-NC
H/R + oe-KDM1A + oe-SOX9
D
Relative mRNA expression of Bax
P < 0.001
Relative mRNA expression of Bcl-2
P < 0.001
E
H/R + oe-KDM1A
Q1-UL(1.77%)
Q1-UR(6.01%)
Q1-LL(87.33%)
Q1-LR(4.89%)
H/R + oe-KDM1A + oe-NC
Q1-UL(1.76%)
Q1-UR(5.40%)
Q1-LL(88.22%)
Q1-LR(4.62%)
PI
Annexin V FITC
F
Caspase-3 activity
P < 0.001
Caspase-9 activity
P = 0.001
H/R + oe-KDM1A + oe-SOX9
Q1-UL(1.47%)
Q1-UR(20.18%)
Q1-LL(73.57%)
Q1-LR(4.78%)
Apoptosis rate (%)
P = 0.003