

# CTRP12 alleviates cardiomyocyte ischemia-reperfusion injury via regulation of KLF15

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**Abstract.** Myocardial ischemia-reperfusion (I/R) serves a crucial role in myocardial infarction. C1q/TNF-related protein 12 (CTRP12) is a secretory protein involved in metabolism. It has been reported that CTRP12 participates in the regulation of numerous cardiovascular diseases. However, its role in myocardial I/R injury remains unclear. In the present study, the left anterior descending coronary artery in mice was ligated to establish a mouse I/R model. A myocardial hypoxia-reoxygenation (H/R) cell model was also established. Cardiomyocyte injury was evaluated using hematoxylin and eosin staining, Cell Counting Kit-8 and a lactate dehydrogenase (LDH) kit. The expression levels of CTRP12 and Krueppel-like factor 15 (KLF15) in murine myocardial tissues and H9c2 cells were determined using reverse transcription-quantitative PCR and western blotting, as KLF15 was previously reported to protect against I/R-induced cardiomyocyte damage. Furthermore, inflammatory factors TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were analyzed using ELISA while apoptosis was assessed using TUNEL assays and western blotting. Moreover, the activity of the CTRP12 promoter was determined using a dual-luciferase reporter assay. The results demonstrated that I/R surgery markedly exacerbated myocardial tissue damage, whereas H/R treatment significantly reduced cell viability and significantly increased LDH activity as well as the release of inflammatory factors and apoptosis. I/R and H/R induction significantly reduced the expression levels of CTRP12 and KLF15. CTRP12 overexpression significantly alleviated H/R-induced cell injury and significantly inhibited inflammation and apoptosis. Further analysis demonstrated that KLF15 could significantly promote the activity of the CTRP12 promoter. However, following CTRP12 knockdown,

KLF15 overexpression exacerbated cell injury, inflammation and apoptosis. In conclusion, the present study demonstrated that CTRP12 may mitigate inflammation and apoptosis in H/R-induced cardiomyocytes, possibly via the regulation of KLF15, which provided a theoretical basis for the potential treatment of I/R-induced myocardial infarction.

## Introduction

Myocardial infarction (MI) is an ischemic heart condition that poses a significant burden to society and may lead to death or disability (1-3). Myocardial ischemia-reperfusion (I/R) injury is considered as the main pathophysiology of ischemic heart diseases (4). It has been reported that acute and chronic cell death following MI affects cardiac function and patient prognosis (5,6). A previous study has reported that the apoptotic cascade in myocardial I/R is initiated either by mitochondrial damage and activation of caspase-9 or by death receptor ligation and activation of caspase-8 (7). Myocardial ischemia may induce inflammatory responses, while reperfusion may promote cell apoptosis, thus resulting in impaired myocardial structure and function (8-10). Therefore, inhibiting inflammatory responses and apoptosis following I/R could be considered as an effective strategy for preventing myocardial I/R injury.

C1q/TNF-related proteins (CTRPs) are a family of adipokines, similar to adiponectin, which exert numerous biological activities, including anti-atherosclerotic, insulin sensitivity, anti-inflammatory and vascular functions (11). CTRP12, also known as adipolin, is a member of the CTRP family, which is abundantly expressed in the fat tissue (12). It has previously been reported that CTRP12 is involved in the protection against metabolic disorders such as type 2 diabetes, atherosclerosis and obesity (13-15). Furthermore, it has been suggested that CTRP12 may serve a critical role in cardiovascular injury (16). Another study has demonstrated that CTRP12 could alleviate lipopolysaccharide (LPS)-induced cardiomyocyte injury by preventing inflammation and apoptosis (17), which suggests that CTRP12 may be tightly linked with the cardiovascular system. However, its role in myocardial I/R injury has not been previously reported.

Krueppel-like factor 15 (KLF15), a member of the zinc-finger family of transcription factors, is tightly associated with several disorders, including inflammation, obesity

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and metabolic dysfunction (18). Emerging evidence has indicated that KLF15 enhances the activity of the CTRP12 promoter and regulates CTRP12 expression in adipocytes (19). Furthermore, another study reported that KLF15 attenuates hypoxia-induced apoptosis and oxidative stress in myocardial cells (20). Therefore, the aim of the present study was to evaluate the functional role of CTRP12 in myocardial I/R injury and explore the possible underlying molecular mechanisms of CTRP12 and KLF15.

## Materials and methods

**Animals.** The present study was approved by the Animal Care and Use Committee of the Shenzhen Peking University, The Hong Kong University of Science and Technology Medical Center (Shenzhen, China) and conducted in accordance with Chinese legislation regarding animal experiments (21). A total of 30 c57BL/6J male mice (age, 8 weeks; weight, 18–22 g) were purchased from the Comparative Medicine Centre of Yangzhou University and housed under standard conditions of 25°C, with a relative humidity of 60%, in a 12-h light/dark cycle with free access to standard laboratory food and water.

**Establishment of the myocardial I/R model.** To establish the I/R mouse model, the left anterior descending coronary artery (LAD) was ligated (22). Mice were anesthetized using isoflurane (4% for induction and 2% for maintenance). Subsequently, a 6–0 silk suture slipknot was placed around the LAD and was released after 30 min. During the operation, mice were subcutaneously injected with 0.05 mg/kg buprenorphine hydrochloride as an intraoperative analgesic. Mice in the sham group underwent the same surgical procedure but without LAD ligation. Following the operation, 0.1 ml 5% glucose was subcutaneously injected into the mice to prevent dehydration. Following recovery from anesthesia, mice received buprenorphine hydrochloride (0.05 mg/kg subcutaneously) for post-operative analgesia (23–26). Animals were sacrificed by cervical dislocation following anesthesia at 24 h following reperfusion and myocardial tissue samples were subsequently collected and stored at -80°C for further analysis.

**Histopathological examination.** Myocardial tissue specimens derived from mice who underwent myocardial I/R and sham surgery were collected and fixed using 4% paraformaldehyde for 24 h at room temperature. Subsequently, the tissues were dehydrated in different concentrations of ethanol, embedded in paraffin and sliced into 4- $\mu$ m-thick sections. The tissue sections were then stained with hematoxylin (0.4%) and eosin (0.1%) (H&E) solution at 37°C for 5 min and the pathological changes after myocardial I/R injury were observed using a light microscope.

**Cell culture and hypoxia-reoxygenation (H/R) treatment.** The rat myoblast H9c2 cell line was obtained from the American Type Culture Collection. Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin solution (Gibco; Thermo Fisher Scientific,

Inc.) in a humidified atmosphere with 5% CO<sub>2</sub> at 37°C. To establish an *in vitro* cardiomyocyte I/R model, H9c2 cells were cultured in glucose-free/serum-free DMEM and maintained in an incubator containing a gaseous mixture of 94% N<sub>2</sub>, 5% CO<sub>2</sub> and 1% O<sub>2</sub> at 37°C for 4 h. Following incubation, cells were transferred to 30 mM high-glucose DMEM, containing 10% FBS, was cultured under normoxic conditions for 4 h at 37°C for reoxygenation. H9c2 cells were not previously cultured under hypoxic conditions and these cells were cultured in high glucose DMEM containing 10% FBS under normoxic conditions and served as the control group.

**Cell transfection.** CTRP12- or KLF15-specific pcDNA overexpression plasmids [overexpressed (Ov)-CTRP12 or Ov-KLF15] and the corresponding negative control (NC; Ov-NC), CTRP12-specific small interfering (si)RNA-CTRP12 (siRNA-CTRP12) and the corresponding NC (siRNA-NC), were synthesized by Shanghai GenePharma Co., Ltd. The following siRNA sequences were used: CTRP12 forward (F), 5'-CUGUGACGGUAGACAAGAAGA-3' and reverse (R), 5'-UUCUUGUCUACCGUCACAGGG-3'; and siRNA-NC F, 5'-GAUCAUACGUGCGAUCAGA-3' and R, 5'-UCUGAUCGCACGUAUGAUC-3'. H9c2 cells (1x10<sup>5</sup> cells/well) were transfected with the recombinants (1  $\mu$ g) using Lipofectamine<sup>®</sup> 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions for 6 h at room temperature. After 48 h cells underwent H/R injury.

**Cell viability assay.** The Cell Counting Kit-8 (CCK-8) assay was utilized to detect cell viability (27). Briefly, at 48 h following transfection, H9c2 cells were seeded into 96-well plates at a density of 5x10<sup>3</sup> cells/well and treated with H/R for 8 h (4 h hypoxia followed by 4 h reperfusion) at 37°C. Subsequently, each well was supplemented with 10  $\mu$ l CCK-8 solution (Beyotime Institute of Biotechnology) and cells were incubated at 37°C for a further 2 h. The absorbance of each well was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

**Detection of lactate dehydrogenase (LDH) activity.** H9c2 cells were seeded into 96-well plates at a density of 5x10<sup>3</sup> cells/well and cultured for 4 h of hypoxia and 4 h of reperfusion at 37°C. The cell supernatants were harvested using centrifugation at 1,000 x g for 15 min at 4°C to quantify the secretory levels of LDH using the LDH Assay Kit (cat. no. A020-2-2; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol.

**ELISA.** The secretory levels of TNF- $\alpha$  and IL-6 were measured using the TNF- $\alpha$  Assay Kit (cat. no. H052-1; Nanjing Jiancheng Bioengineering Institute) and IL-6 Kit (cat. no. H007-1-1; Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocol. IL-1 $\beta$  levels were detected using the Human IL-1 $\beta$  ELISA Kit (cat. no. ab214025; Abcam) according to the manufacturer's protocol. Culture supernatants from H9c2 cells (2x10<sup>4</sup> cells/well) were cultured at 37°C under 4 h of hypoxia and 4 h of reperfusion and were collected. Subsequently, 50  $\mu$ l supernatant was added to each well. The optical density was detected at 450 nm using a microplate reader (BioTek Instruments, Inc.). A total of eight parallel wells were used for each ELISA.

**Cell apoptosis.** The TUNEL assay was performed to evaluate cell apoptosis (28). Briefly, following treatment, H9c2 cells ( $2 \times 10^6$  cells/well) were washed twice with PBS. Following fixation with 4% paraformaldehyde for 15 min at room temperature, cells were incubated with 15  $\mu\text{g/ml}$  proteinase K for 15 min at 37°C and endogenous peroxidase activity was blocked using 3%  $\text{H}_2\text{O}_2$  for 15 min at room temperature. After washing, cells were treated with TUNEL working solution for 60 min at 37°C, followed by co-labeling with DAPI (0.5  $\mu\text{g/ml}$ ) for 5 min at room temperature. The cells were mounted in Antifade Mounting Medium (Beyotime Institute of Biotechnology) and cells from five randomly selected fields of view were captured using a fluorescence microscope (Leica Microsystems GmbH). Apoptosis was quantified using ImageJ v1.8.0 software (National Institutes of Health).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from murine myocardial tissues and H9c2 cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, RNA was reverse transcribed into first-strand cDNA using the PrimeScript RT Master Mix (Takara Bio, Inc.) according to the manufacturer's instructions. qPCR was performed using the SYBR Premix ExTaq Kit (Takara Bio, Inc.) using the ABI PRISM 7900 Real-Time System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: initial denaturation at 95°C for 30 sec; 40 cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec and extension at 72°C for 60 sec. The program was terminated via final extension at 72°C for 1 min and cooling at 40°C. The primer sequences used for qPCR were as follows: CTRP12 F, 5'-CCTGTCCTGGCCGATTTA-3' and R, 5'-CAGGGACGTATGACGGTGAC-3'; KLF15 F, 5'-GGGATCGTGGAGGAGAGCCT-3' and R, 5'-CCAGCTGAGAGCTGGCTACA-3'; and GAPDH F, 5'-GTCAAGGCTGAGAACGGGAA-3' and R 5'-AAATGAGCCCGAGCCTTCTC-3'. The mRNA expression levels were quantified using the  $2^{-\Delta\Delta\text{C}_q}$  method and were normalized using the internal reference gene GAPDH (29).

**Western blotting.** Total protein was extracted from myocardial tissues and H9c2 cells using RIPA buffer (Auragene; Hunan Aijia Biotechnology Co., Ltd.). Total protein (30  $\mu\text{g}$  protein/lane) was separated via SDS-PAGE (Bio-Rad Laboratories, Inc.) on a 10% gel. Separated protein was transferred onto a PVDF membrane (MilliporeSigma). Following blocking with 5% skimmed milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies targeting CTRP12 (1:1,000; cat. no. ab177991), KLF15 (1:1,000; cat. no. ab2647), Bax (1:2,000; cat. no. ab182733), cleaved caspase-3 (1:500; cat. no. ab32042), cleaved poly (ADP-ribose) polymerase (PARP; 1:1,000; cat. no. ab32064), Bcl-2 (1:1,000; cat. no. ab194583) and GAPDH (1:3,000; cat. no. ab125247) (all purchased from Abcam). Subsequently, the membranes were washed four times with PBS and incubated with horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibodies (1:3,000; cat. no. 7074S; Cell Signaling Technology, Inc.) for 1 h at room temperature. The protein bands were visualized using an Amersham<sup>™</sup> ECL<sup>™</sup> Prime Western Blotting Detection

Reagent (Amersham; Cytiva) and analyzed using ImageJ software v1.8.0 (National Institutes of Health).

**Bioinformatic analysis.** The binding site between KLF15 and the CTRP12 promoter was analyzed using the JASPAR database 2018 (<https://jaspar.genereg.net/>). The JASPAR database is an open-access database containing manually curated, non-redundant transcription factor (TF) binding profiles for TFs across six taxonomic groups.

**Dual-luciferase reporter assay.** The activity of the CTRP12 promoter was evaluated using a dual-luciferase reporter assay (30). Briefly, the CTRP12 promoter covering predicted binding sites was cloned into the pGL3 basic plasmid (Shanghai Genomeditech Co., Ltd.) with the luciferase/*Renilla* reporter gene. The Ov-KLF15 vector or the empty vector and luciferase/*Renilla* plasmids were co-transfected into H9c2 cells ( $1 \times 10^5$  cells/well) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at room temperature. Each well was then supplemented with 100  $\mu\text{l}$  *Renilla* Luciferase Reporter Gene Assay Cell Lysis Buffer (Beyotime Institute of Biotechnology) and the lysed cells were centrifuged at 5,000  $\times$  g for 1 min at 4°C to collect the supernatants. Subsequently, the cells were supplemented with 20  $\mu\text{l}$  lysis buffer and then with 100  $\mu\text{l}$  1X firefly and 1X *Renilla* luciferase reaction solutions (Wuhan Scithera Microbiological Technologies, Inc.) to detect firefly and *Renilla* luciferase activity, respectively. After 48 h, luciferase activity was measured using the Dual Luciferase Reporter System, purchased from Promega Corporation, according to the manufacturer's instructions. The results are presented as the ratio of luciferase to *Renilla* activity.

**Statistical analysis.** All data are presented as the mean  $\pm$  SD. All statistical analyses were carried out using GraphPad Prism 6 software (GraphPad Software, Inc.) using unpaired two-tailed Student's t-tests or a one-way ANOVA followed by a Bonferroni's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**CTRP12 is downregulated in I/R-induced myocardial tissues and H/R-induced cardiomyocytes.** To explore the role of CTRP12 in myocardial I/R injury, a myocardial I/R mouse model was established. H&E staining demonstrated that mice who underwent the sham operation exhibited normal myocardial tissue structure, whereas those in the I/R group displayed damaged myocardial fiber structure, broken vascular walls and hemocyte and inflammatory molecule infiltration in the myocardial tissue (Fig. 1A), indicating that the model was successfully established. Subsequently, the mRNA expression levels of CTRP12 were determined in myocardial tissue samples and H9c2 cardiomyocytes using RT-qPCR and western blotting. The mRNA and protein expression levels of CTRP12 were significantly decreased in both I/R-induced myocardial tissues and H/R-induced H9c2 cells compared with the corresponding control groups (Fig. 1B-E). These results suggested that the decreased expression of CTRP12 was associated with myocardial I/R injury.

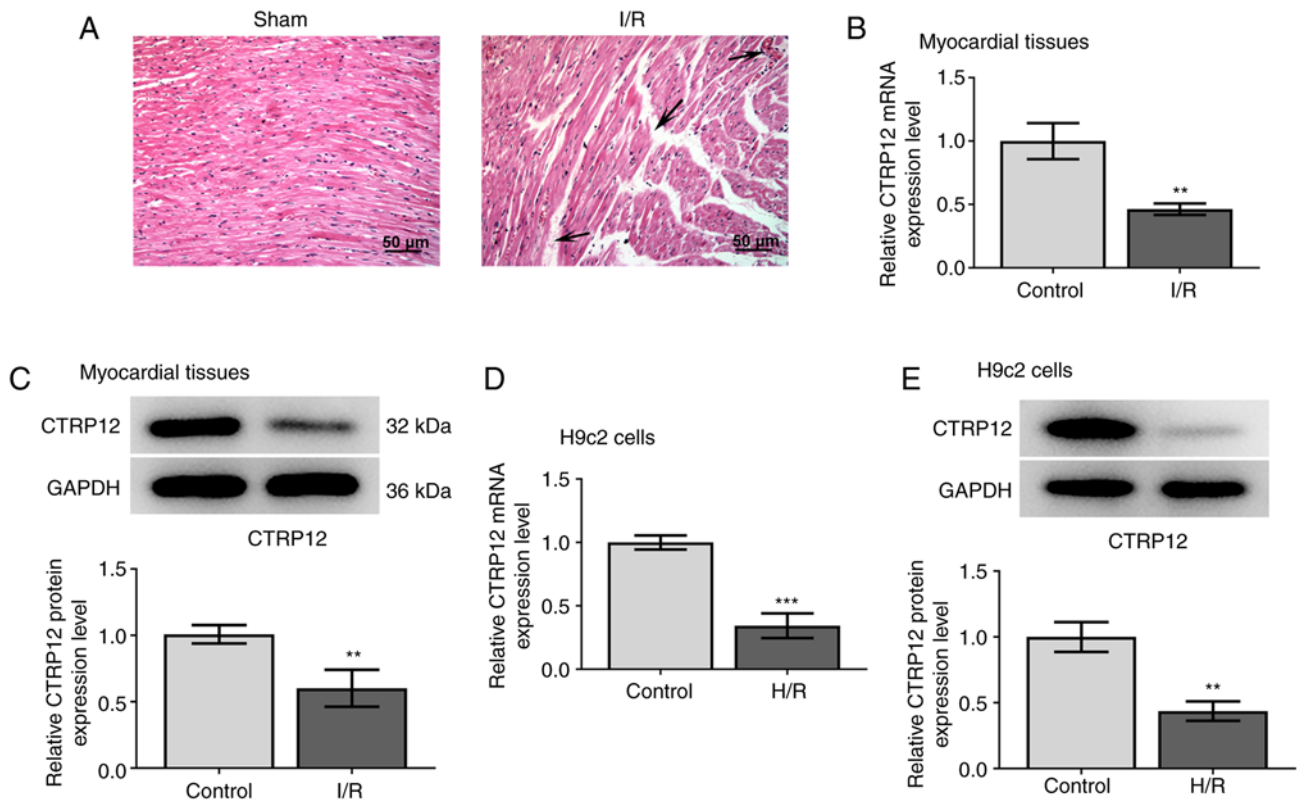


Figure 1. CTRP12 expression is downregulated in I/R-induced myocardial tissue and H/R-induced cardiomyocytes. (A) Representative images of myocardial tissue in control or I/R-induced mice. The arrows indicate lesions in the tissue. Scale bar, 50  $\mu$ m. (B) mRNA and (C) protein expression levels of CTRP12 were detected in myocardial tissue in I/R-induced mice via RT-qPCR and western blotting, respectively. (D) mRNA and (E) protein expression levels of CTRP12 were detected in H/R-induced H9c2 cells via RT-qPCR and western blotting respectively. Results are presented as the mean  $\pm$  SD analyzed using three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control. CTRP12, C1q/TNF-related protein 12; I/R, ischemic/reperfused; RT-qPCR, reverse transcription-quantitative PCR, H/R, hypoxic/reoxygenated.

*CTRP12 overexpression alleviates H/R-induced cell injury in H9c2 cells.* To uncover the biological role of CTRP12 in hypoxia-induced cardiomyocytes, H9c2 cells were transfected with Ov-CTRP12 to enhance CTRP12 expression. The transfection efficiency of Ov-CTRP12 was evaluated using RT-qPCR and it was demonstrated that the mRNA expression levels of CTRP12 were significantly elevated compared with the Ov-NC group (Fig. 2A). The results demonstrated that CTRP12 mRNA and protein expression levels were significantly downregulated under H/R conditions compared with the control group, which was significantly reversed following transfection with Ov-CTRP12 in H/R cells compared with the H/R only group (Fig. 2B and C). The CCK-8 assay was performed to measure the effects of CTRP12 overexpression on cell viability and the results demonstrated that H/R injury significantly reduced cell viability compared with the control. However, CTRP12 overexpression significantly restored the H/R-mediated reduced cell viability compared with the H/R group (Fig. 2D). Furthermore, the levels of LDH were measured to determine the severity of cardiomyocyte injury. The results demonstrated that LDH activity was significantly enhanced in hypoxic cardiomyocytes compared with the control, whereas CTRP12 overexpression significantly reversed this effect compared with the H/R group (Fig. 2E). The aforementioned findings indicated that CTRP12 overexpression exerted a protective role against H/R-induced myocardial cell injury.

*CTRP12 overexpression relieves the secretion of inflammatory factors and apoptosis in H/R-induced cells.* Subsequently, the present study aimed to investigate whether CTRP12 overexpression could potentially protect H9c2 cells from H/R-induced inflammation and apoptosis. The secretory levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly elevated by H/R injury compared with the control, whereas their levels were significantly reduced following CTRP12 overexpression compared with the H/R group (Fig. 3A-C). Furthermore, the TUNEL assay results demonstrated that H/R treatment significantly promoted cardiomyocyte apoptosis compared with the control, whereas CTRP12 overexpression significantly prevented the effects of H/R induction on cardiomyocyte apoptosis compared with the H/R group (Fig. 3D and E). Moreover, western blotting demonstrated that compared to the control the protein expression levels of Bax, cleaved caspase-3 and cleaved PARP were significantly increased, whereas those of Bcl-2 were significantly decreased in H/R-treated cells. However, CTRP12 overexpression significantly reversed the effects of H/R exposure on the expression profile of the aforementioned proteins, compared with the H/R group (Fig. 3F). These results suggested that CTRP12 overexpression may ameliorate hypoxia-induced H9c2 cell injury via attenuating inflammation and apoptosis.

*KLF15 enhances the activity of the CTRP12 promoter and regulates CTRP12 expression levels.* To elucidate whether KLF15 was involved in the regulation of CTRP12 expression

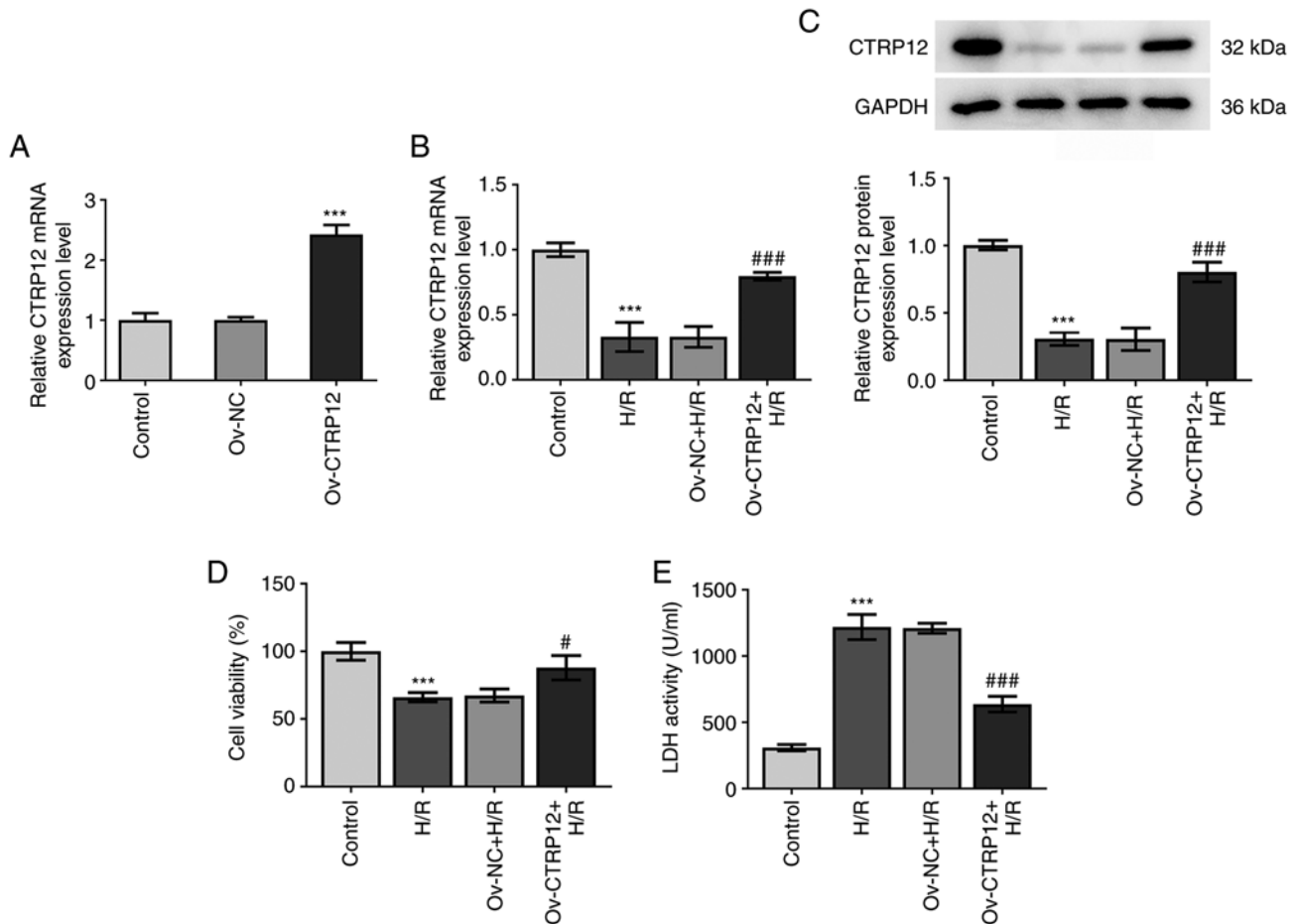


Figure 2. CTRP12 overexpression protects against H/R-induced cell injury in H9c2 cells. (A) Transfection efficiency of Ov-CTR12 was assessed via reverse transcription-quantitative PCR. (B) mRNA and (C) protein expression levels of CTRP12 in untreated or H/R-induced H9c2 cells transfected with Ov-CTR12 were detected. (D) Cell viability was assessed using the Cell Counting Kit-8 assay. (E) LDH activity levels were evaluated. Results are presented as the mean  $\pm$  SD analyzed using three independent experiments. \*\*\* $P < 0.001$  vs. control; and # $P < 0.05$ , ### $P < 0.001$  vs. H/R group. CTRP12, C1q/TNF-related protein 12; H/R, hypoxic/reoxygenated; Ov, overexpressed; LDH, lactate dehydrogenase; NC, negative control.

in myocardial I/R injury, the expression levels of KLF15 were determined in I/R-induced myocardial tissues and H/R-induced cardiomyocytes. The results demonstrated that the mRNA and protein expression levels of KLF15 were significantly reduced in both myocardial tissues and H9c2 cells compared with those in the untreated control groups (Fig. 4A-D). The transfection efficiency of Ov-KLF15 was assessed using RT-qPCR and the results demonstrated that the KLF15 mRNA expression levels were significantly elevated in the Ov-KLF15 group (Fig. 4E). Subsequently, H/R-treated H9c2 cells were transfected with Ov-KLF15 to overexpress KLF15 and the results demonstrated that KLF15 mRNA expression in the Ov-KLF15 + H/R group was significantly increased compared with the Ov-NC + H/R group (Fig. 4F and G). The mRNA and protein expression levels of CTRP12 were significantly increased in KLF15-overexpressing H9c2 cells compared with the H/R group (Fig. 4H and I). The binding site of the KLF15 and CTRP12 promoter was predicted using the JASPAR database (Fig. 4J). The results of the dual-luciferase reporter assay demonstrated that the luciferase activity was significantly enhanced in cells transfected with Ov-KLF15 after exposure to H/R compared with the H/R group (Fig. 4K). These results

therefore suggested that KLF15 overexpression may increase the promoter activity of CTRP12.

*KLF15 overexpression attenuates H/R-induced H9c2 cell injury via regulation of CTRP12.* Based on the aforementioned findings, the present study further investigated the effect of KLF15 on regulating CTRP12 expression levels in cardiomyocytes. The transfection efficiency of siRNA-CTR12 was assessed using RT-qPCR and the results demonstrated that CTRP12 mRNA expression were significantly reduced in the siRNA-CTR12 group compared with the siRNA-NC (Fig. 5A). Compared with the H/R group, KLF15 overexpression significantly rescued H/R-mediated reduced cell viability in H/R-treated H9c2 cells (Fig. 5B). Furthermore, CTRP12 silencing significantly reduced the effects of H/R on cell viability compared with the Ov-KLF15 + siRNA-NC + H/R group. LDH activity was also assessed to determine the degree of H9c2 cell injury and the results demonstrated that CTRP12 silencing significantly elevated LDH activity in H/R-treated H9c2 cells transfected with Ov-KLF15 compared with the Ov-KLF15 + siRNA-NC + H/R group (Fig. 5C). These results therefore supported the involvement of KLF15 in CTRP12-mediated H/R-induced cardiomyocyte injury.

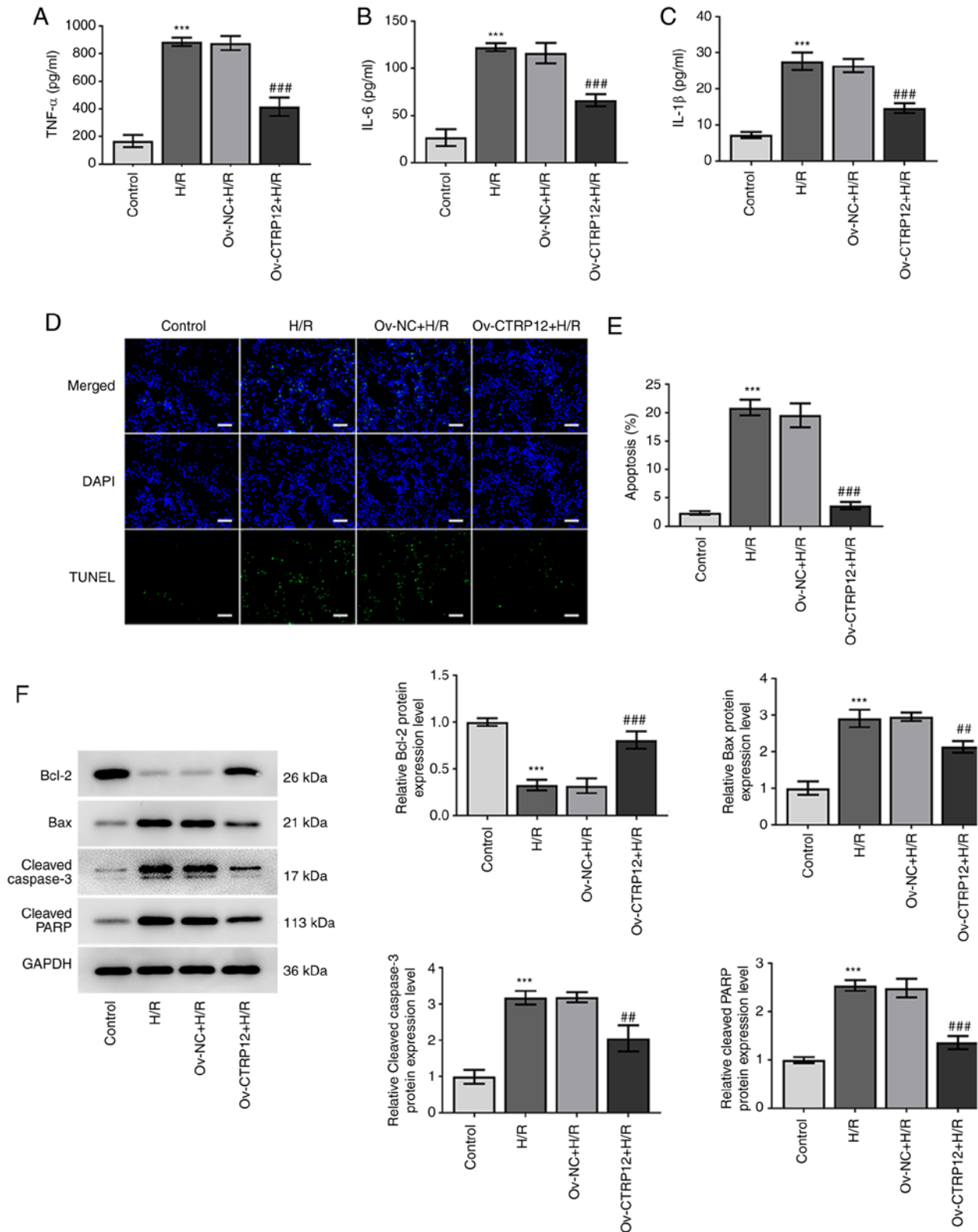


Figure 3. Overexpression of CTRP12 inhibits the release of inflammatory factors and apoptosis in H/R-induced H9c2 cells. (A) TNF- $\alpha$ , (B) IL-1 $\beta$  and (C) IL-6 levels were quantified using ELISA. (D and E) Cell apoptosis was detected using the TUNEL assay. Scale bar, 100  $\mu$ m. (F) Western blotting was performed to assess protein expression levels of Bcl-2, Bax, cleaved caspase-3 and cleaved PARP. Results are presented as the mean  $\pm$  SD analyzed using three independent experiments. <sup>\*\*\*</sup>P<0.001 vs. control; and <sup>##</sup>P<0.01, <sup>###</sup>P<0.001 vs. H/R group. CTRP12, C1q/TNF-related protein 12; H/R, hypoxic/reoxygenated; PARP, poly(ADP-ribose) polymerase; Ov, overexpressed; NC, negative control.

*KLF15* overexpression inhibits inflammation and apoptosis in H/R-induced H9c2 cells via regulation of CTRP12. The effects of KLF15 overexpression on H/R-induced inflammation and

apoptosis were evaluated in CTRP12-silenced H9c2 cells. The secretory levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly reduced by KLF15 overexpression compared with the H/R

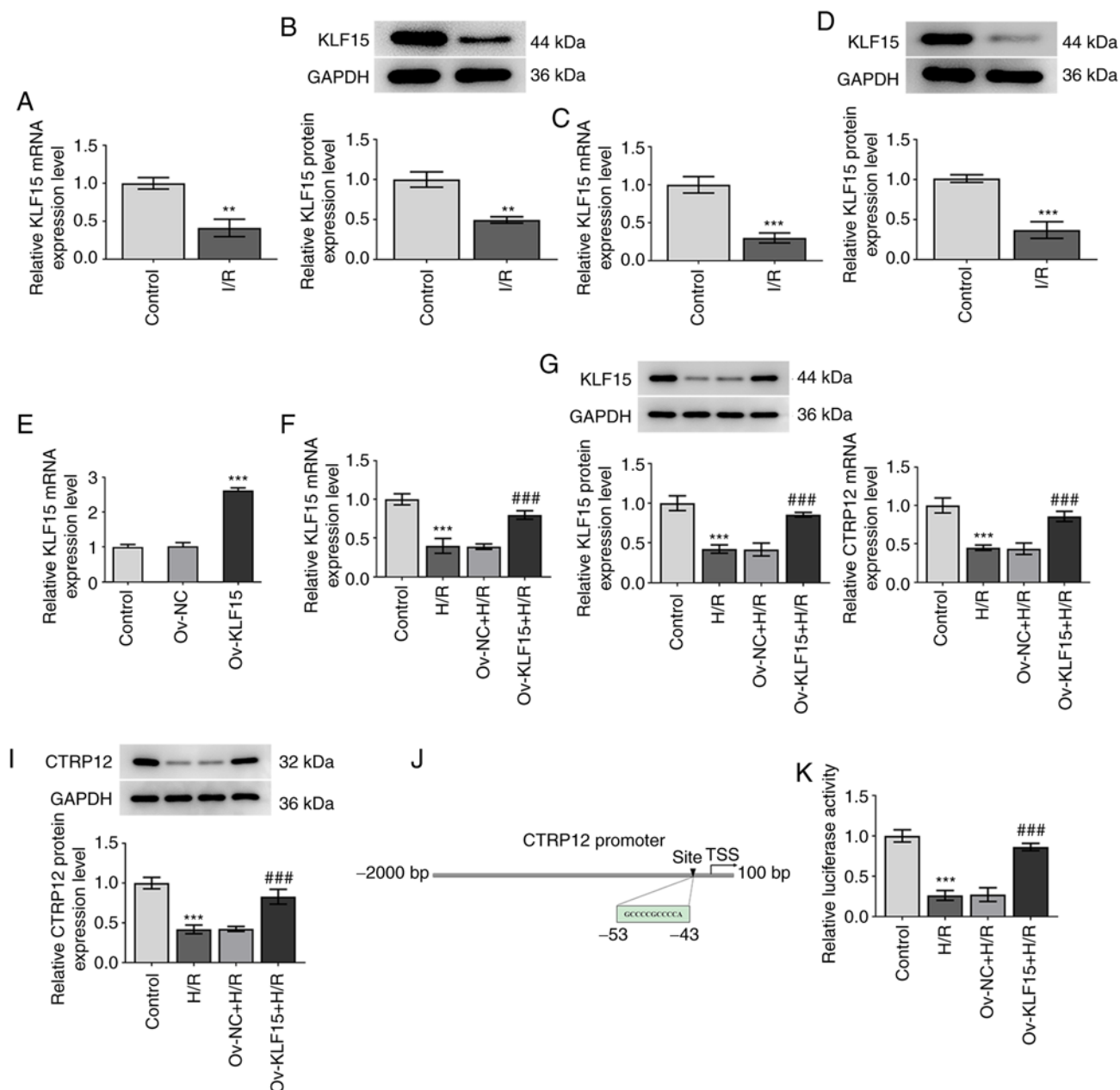


Figure 4. KLF15 regulates CTRP12 expression levels in H9c2 cells. (A) mRNA and (B) protein expression levels of KLF15 were determined via RT-qPCR and western blotting, respectively, in myocardial tissue in I/R-induced mice. (C) mRNA and (D) protein expression levels of KLF15 were determined using RT-qPCR and western blotting, respectively, in H/R-induced H9c2 cells following exposure to H/R compared with the control. (E) Transfection efficiency of Ov-KLF15 was assessed using reverse transcription-quantitative PCR. (F) mRNA and (G) protein expression levels of KLF15 in H/R cells were detected following transfection with Ov-KLF15. (H) mRNA and (I) protein expression levels of CTRP12 were assessed following transfection with Ov-KLF15. (J) Binding site of KLF15 and CTRP12 promoter was predicted using the JASPAR database. (K) CTRP12 promoter activity was determined using the dual-luciferase reporter assay. Results are presented as the mean  $\pm$  SD analyzed using three independent experiments. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. control; and ### $P < 0.001$  vs. H/R group. KLF15, Krueppel-like factor 15; CTRP12, C1q/TNF-related protein 12; I/R, ischemic/reperfused; H/R, hypoxic/reoxygenated; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpressed; NC, negative control; TSS, transcriptional start site.

group, whereas this effect was significantly reversed following CTRP12 knockdown compared with the Ov-KLF15 + siRNA-NC + H/R group (Fig. 6A-C). Furthermore, the apoptotic rate of H/R-induced cells was significantly decreased following KLF15 overexpression compared with the H/R group, whereas CTRP12 silencing resulted in a significant increase in the apoptotic rate in H/R-treated H9c2 cells transfected with Ov-KLF15 compared with the Ov-KLF15 + siRNA-NC + H/R group (Fig. 6D and E). Furthermore, western blotting demonstrated that the protein expression levels of

Bax, cleaved caspase-3 and cleaved PARP were significantly decreased, whereas Bcl-2 protein expression levels were significantly increased in the Ov-KLF15 + H/R group compared with the H/R group. However, transfection of H9c2 cells with si-CTR12 significantly upregulated Bax, cleaved caspase-3 and cleaved PARP, and significantly downregulated Bcl-2 compared with the Ov-KLF15 + siRNA-NC + H/R group (Fig. 6F). These data suggested that KLF15 overexpression may attenuate H/R-induced inflammation and apoptosis in H9c2 cells via the regulation of CTRP12 expression.

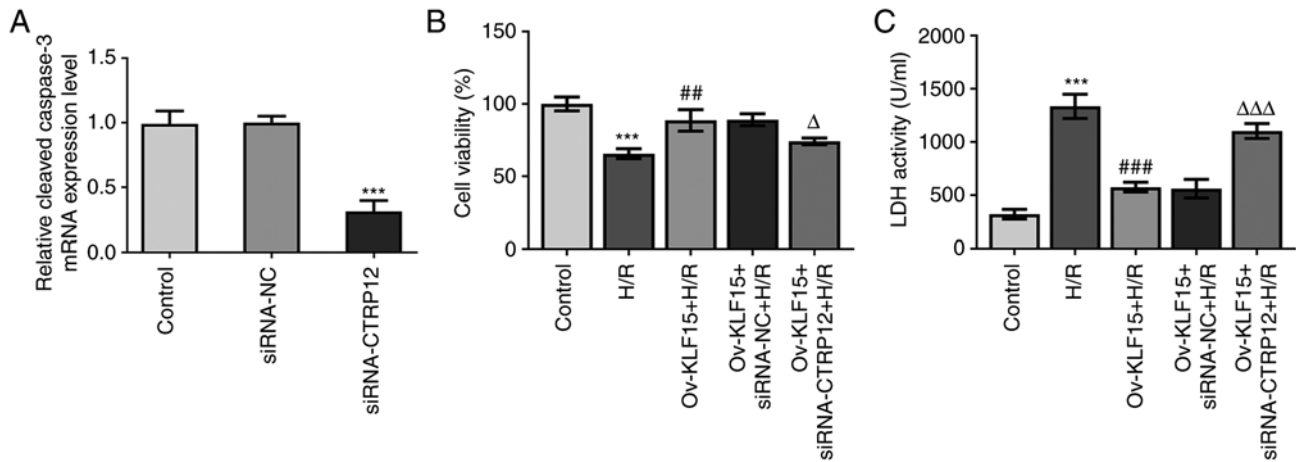


Figure 5. Overexpression of KLF15 protects H/R induced H9c2 cell injury via regulation of CTRP12. (A) Transfection efficiency of Ov-CTR12 was assessed using reverse transcription-quantitative PCR. (B) Cell viability was assessed using the Cell Counting Kit-8 assay. (C) LDH activity levels were quantified following co-transfection with siRNA-CTR12 and Ov-KLF15. Results are presented as the mean  $\pm$  SD analyzed using three independent experiments. \*\*\* $P$ <0.001 vs. control; \*\* $P$ <0.01, ### $P$ <0.001 vs. H/R group; and  $\Delta$  $P$ <0.05,  $\Delta\Delta$  $P$ <0.001 vs. Ov-KLF15 + siRNA-NC + H/R. KLF15, Krueppel-like factor= 15; CTRP12, C1q/TNF-related protein 12; H/R, hypoxic/reoxygenated; LDH, lactate dehydrogenase; siRNA, small interfering RNA; Ov, overexpressed; NC, negative control.

## Discussion

The present study demonstrated that the expression levels of CTRP12 could be modulated by cardiac I/R, while its overexpression exerted a protective effect against I/R injury via alleviating reperfusion-induced inflammation and apoptosis. Furthermore, mechanistic investigations demonstrated that CTRP12 exerted its therapeutic benefits via its KLF15-regulated enhanced promoter activity.

Myocardial I/R injury is involved in complicated pathophysiological mechanisms and is considered to be a significant cause of heart diseases, which makes it a severe global burden (31). Myocardial ischemia may lead to hypoxia, thus resulting in cell injury and increased cytotoxicity, which in turn can promote cell apoptosis and oxidative stress (32,33). In the present study, a myocardial I/R mouse model was established after LAD ligation and reperfusion (34). H&E staining demonstrated typical myocardial injury, characterized by damaged myocardial fiber structure, broken vascular walls and hemocyte infiltration. Furthermore, H9c2 cells were treated with H/R to construct an *in vitro* myocardial I/R model. Subsequently, the mRNA and protein expression levels of CTRP12 in I/R-exposed myocardial tissues and H/R-induced cardiomyocytes was determined. The results demonstrated that CTRP12 was significantly downregulated both *in vivo* and *in vitro* models. CCK-8 assays and the determination of LDH activity are sensitive tools for evaluating myocardial cell viability and injury, respectively (35). In the present study, following CTRP12 overexpression, cell viability was significantly improved and LDH activity was significantly reduced, supporting the hypothesis that CTRP12 has a protective effect against I/R-induced cardiomyocyte injury, which is consistent with previous studies (17,36).

It has also been reported that CTRP12 regulates inflammation, glucose metabolism, vascular remodeling and cardiac fibrosis (15,37,38). Zhou *et al* (17) demonstrated that CTRP12 ameliorated LPS-induced inflammatory responses and cell

apoptosis in cardiomyocytes in a nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent manner. Nrf2, a critical antioxidant gene in scavenging reactive oxygen species (ROS) and maintaining redox homeostasis, could mediate redox balance (39-41). Moreover, Fadaei *et al* (16) reported that the serum levels of CTRP12 were decreased and negatively associated with TNF- $\alpha$  and IL-6 in patients with coronary artery disease. Consistent with previously published findings (36), the results of the present study demonstrated that the secretory levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were significantly increased in cell culture supernatants from H/R-treated cells, whereas CTRP12 overexpression significantly abrogated this effect. These findings indicated that CTRP12 overexpression may protect cardiomyocytes against H/R-induced inflammation.

Myocardial cell apoptosis contributes to cardiomyocyte loss in ischemic heart diseases (42). A previous study suggested that cell apoptosis is a crucial 'blasting fuse' for hypoxia-induced cardiac ischemic injury (43). Therefore, in the present study a TUNEL staining assay and western blotting were carried out to assess the effects of CTRP12 overexpression on H/R-induced cardiomyocyte apoptosis. The results demonstrated that CTRP12 significantly attenuated cardiomyocyte apoptosis induced by H/R, accompanied by the significant upregulation of the anti-apoptotic protein Bcl-2 and significantly decreased expression of the pro-apoptotic proteins, Bax, cleaved caspase-3 and cleaved PARP. The aforementioned findings suggested that CTRP12 exhibited an inhibitory effect on the apoptosis of cardiomyocytes with H/R injury. However, it was also demonstrated that cell apoptosis is not completely reversed by CTRP12 silencing. These results therefore suggested that CTRP12 silencing may reverse cell apoptosis in part and the degree of reversal is associated with numerous factors such as concentration of transfection reagent, transfection efficiency, cell type and culture time.

It has previously been reported that the zinc finger transcription factor KLF15 serves a significant role in heart diseases, including pathological forms of left ventricular hypertrophy



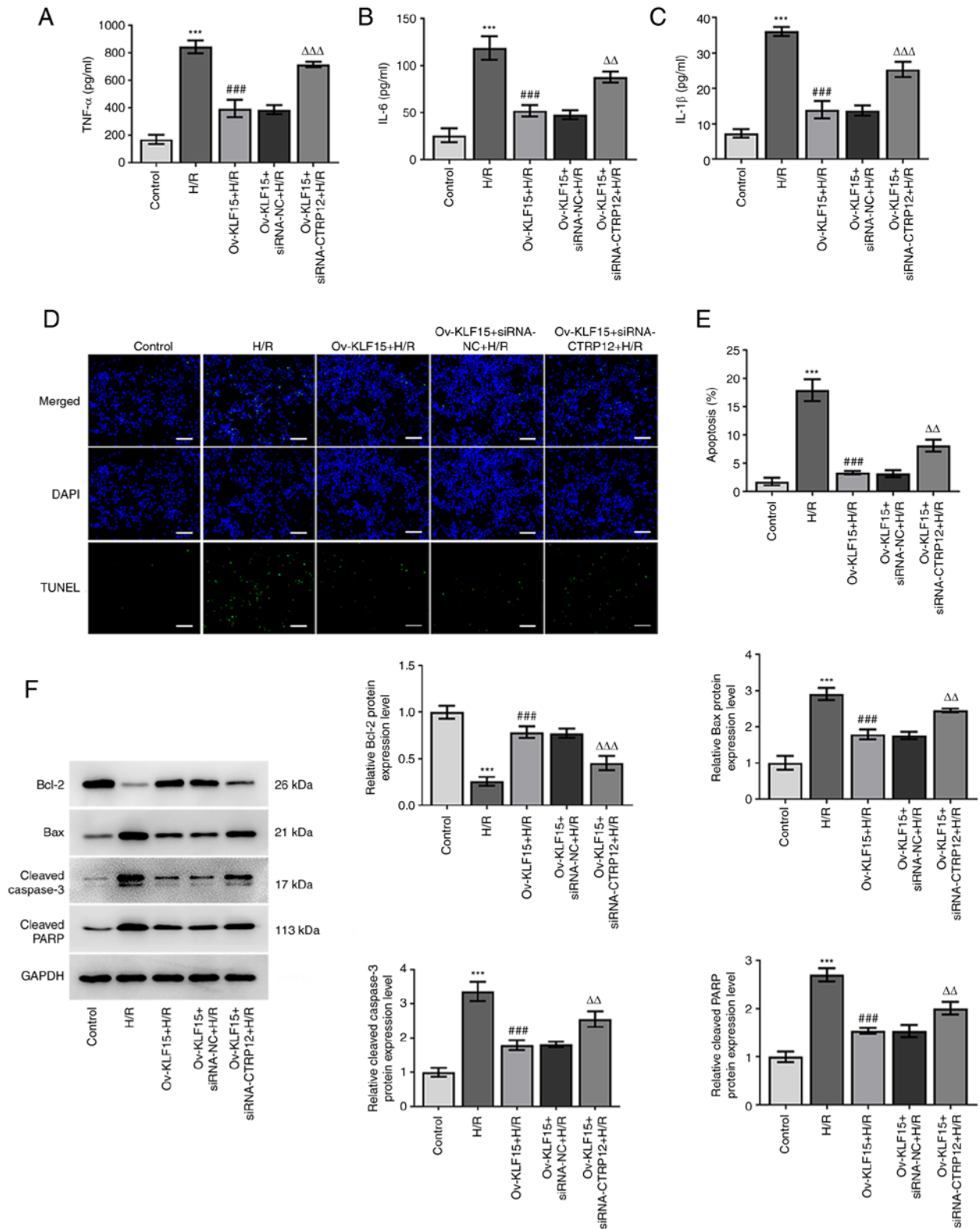


Figure 6. KLF15 overexpression suppresses H/R induced inflammation and apoptosis in H9c2 cells by regulating CTRP12. (A) TNF-α, (B) IL-1β and (C) IL-6 levels were quantified using ELISA. (D and E) Cell apoptosis was detected using the TUNEL assay. Scale bar, 100 μm. (F) Western blotting was performed to assess protein expression levels of Bcl-2, Bax, cleaved caspase-3 and cleaved PARP. Results are presented as the mean ± SD analyzed using three independent experiments. \*\*\*P<0.001 vs. control; ###P<0.001 vs. H/R group; and ΔΔP<0.01, ΔΔΔP<0.001 vs. Ov-KLF15 + siRNA-NC + H/R. KLF15, Krueppel-like factor 15; CTRP12, C1q/TNF-related protein 12; H/R, hypoxic/reoxygenated; PARP, poly (ADP-ribose) polymerase; Ov, overexpressed; siRNA, small interfering RNA; NC, negative control.

and heart failure (44,45). Furthermore, it has been proposed that KLF15 protects cardiomyocytes via attenuating hypertrophic remodeling, regulating cardiomyocyte gene expression

and lipid oxidation and inhibiting cardiomyocyte apoptosis and oxidative stress (20,45,46). In the present study the mRNA and protein expression levels of KLF15 were significantly

downregulated in both I/R-exposed myocardial tissues and H/R-induced cardiomyocytes. Moreover, the results determined that KLF15 overexpression significantly promoted the mRNA and protein expression of CTRP12, which therefore indicated that KLF15 may have exerted a regulatory role on CTRP12 expression. Furthermore, the dual-luciferase reporter assay was performed and demonstrated KLF15 overexpression increased CTRP12 promoter activity. It was therefore hypothesized that KLF15 regulated CTRP12 expression at the transcriptional level by transcriptionally activating the CTRP12 promoter. Rescue experiments demonstrated that KLF15 overexpression significantly relieved cardiomyocyte cell viability and ameliorated H/R-induced cardiomyocyte inflammation and apoptosis. However, CTRP12 silencing significantly reversed the effects of KLF15 overexpression on H/R-mediated cardiomyocyte injury. These data provided direct evidence to suggest that KLF15 may be involved in the CTRP12-mediated protection of cardiomyocytes against H/R injury.

There are several limitations of the present study. First, due to the aim of the present study investigating the biological role of CTRP12 in myocardial I/R injury, the expression levels of other CTRPs were not explored in the *in vivo* and *in vitro* models. Furthermore, although the relationship between CTRP12 and KLF15 was verified in the present study, whether other factors are related to the pathological mechanism in myocardial I/R injury is unclear. Moreover, whether the KLF15/CTRP12 signaling pathway affects other apoptotic proteins by endoplasmic reticulum stress-related or mitochondria-related apoptotic signaling is unknown. To the best of our knowledge, there are no studies that have reported the association between other CTRPs and KLFs, especially in myocardial I/R injury. Therefore, it is necessary to further study the associations of other CTRPs and KLFs in myocardial I/R injury and identify the downstream genes of the KLF15/CTRP12 signaling pathway in myocardial I/R injury. Second, even though the expression changes of CTRP12 and KLF15 in I/R tissues were explored, localization studies of CTRP and KLF15 proteins were not performed and the expression level of KLF15 in CTRP12-overexpressed H/R cells was not investigated. In the present study KLF15 overexpression and its effects were investigated; however, the role of downregulated KLF15 was not and therefore the expression of the KLF15 inhibitor microRNA-223-3p will be investigated to assess the effects of KLF15 silencing both *in vivo* and *in vitro*. Future work will also explore more signaling pathways that are potentially controlled by KLF15 in myocardial I/R injury. Third, in the present study, it was demonstrated that KLF15 and KLF15-mediated CTRP12 significantly regulated the production of inflammatory cytokines, but the mechanism which decreases inflammatory cytokine levels by CTRP12 was not explored. Fourth the interaction between the CTRP12 promoter and KLF15 was preliminarily verified using the dual-luciferase reporter assay but needs to be further confirmed. Finally, both KLF15 and CTRP12 expression should be considered following hypoxia or reperfusion alone, in order to evaluate the effect of reperfusion-induced ROS. Furthermore, the effects of CTRP12 and KLF15 on different parts of the heart, such as the infarct, the border zone and on the volume of infarcted heart, were not explored, as well as the effects of the time and degree of I/R and H/R treatment on the

expression and role of CTRP12 and KLF15. These issues are important and will be investigated in future studies.

In conclusion, the present study demonstrated that CTRP12 may potentially exert a protective effect against H/R-induced H9c2 cell injury via attenuating inflammation and apoptosis. These effects were regulated by KLF15. Overall, the aforementioned findings suggested that CTRP12 may serve as a novel target for treating ischemic heart diseases.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

BL and XT designed the study, performed the experiments, wrote and revised the manuscript. BL analyzed the datasets. XT searched the literature. Both authors read and approved the final manuscript. BL and XT confirm the authenticity of all the raw data.

### Ethics approval and consent to participate

This study was approved by the Animal Care and Use Committee of Shenzhen Peking University, The Hong Kong University of Science and Technology Medical Center (Shenzhen, China; approval no. 2020-630).

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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