VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 83, No. 1, pp. 125–138
DOI: 10.5603/FM.a2023.0002

ISSN 0015–5659
eISSN 1644–3284
journals.viamedica.pl

FOXO6 transcription inhibition of CTRP3 promotes OGD/R-triggered cardiac microvascular endothelial barrier disruption via SIRT1/Nrf2 signalling

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[Received: 31 October 2022; Accepted: 21 December 2022; Early publication date: 17 January 2023]

Background: *C1q/TNF-related protein 3 (CTRP3) has been clarified to display its protective roles in cardiac function. The current study is concentrated on exploring the impacts of CTRP3 on myocardial ischaemia.*

Materials and methods: *Oxygen and glucose hypoxia/reoxygenation (OGD/R) model was constructed in human cardiac microvascular endothelial cells (HCMECs). Reverse transcription-quantitative polymerase chain reaction and western blot analysis of CTRP3 expression were conducted. CCK-8 assay was to estimate cell activity and lactate dehydrogenase (LDH) assay kit was to test LDH release. TUNEL assay and western blot were to judge apoptosis. Endothelial barrier function was detected by in vitro vascular permeability assay kit. Zonula occludens-1 (ZO-1) expression was evaluated by immunofluorescence assay. The interaction between CTRP3 promoter and Forkhead Box O6 (FOXO6) was predicted by JASPAR database and verified by chromatin immunoprecipitation and luciferase reporter assays. After OGD/R-induced HCMECs were co-transfected with CTRP3 overexpression and FOXO6 overexpression plasmids, the above functional experiments above were conducted again. Lastly, the expression of sirtuin 1 (SIRT1)/nuclear factor erythroid 2-related factor 2 (Nrf2) signalling-related proteins was examined by western blot.*

Results: *CTRP3 was down-regulated in OGD/R-induced HCMECs. CTRP3 enhanced the viability and barrier integrity while reduced the apoptosis and permeability of OGD/R-insulted HCMECs. This process may be regulated by FOXO6 transcription. Also, FOXO6 inhibition-mediated CTRP3 up-regulation activated the SIRT1/Nrf2 signalling.*

Conclusions: *FOXO6 transcription inhibition of CTRP3 promotes OGD/R-triggered cardiac microvascular endothelial barrier disruption via SIRT1/Nrf2 signalling.* (Folia Morphol 2024; 83, 1: 125–138)

Keywords: myocardial ischaemia, cardiac microvascular endothelial cells, barrier disruption, CTRP3, FOXO6, SIRT1/Nrf2 signalling

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INTRODUCTION

Cardiovascular disease (CVD) is recognized as a prevalent and threatening worldwide health problem accompanied with increasing morbidity and mortality [32]. Notably, myocardial ischaemia is a dominant contributor responsible for the high death rate of CVD patients and the incidence of myocardial ischaemia is increasingly elevated nowadays [25]. Myocardial ischaemia is referred to as a pathological condition of myocardial anoxia, metabolic changes, cardiac dysfunction as well as damaged myocardial structure due to reduced blood perfusion to the heart [1]. Moreover, cardiac microvascular endothelial cells (CMECs) injury represents an earlier and severer phenomenon during the process of myocardial ischaemia. Therefore, the protection for CMECs is indispensable for reducing the risk of myocardial ischaemia.

C1q/TNF-related protein (CTRP) family, which contains a group of adiponectin paralogous proteins, can regulate the proliferation, apoptosis and metabolism in malignant tumours as well as other human diseases [15]. CTRP3, a well-studied member of CTRP family, was initially identified as a secretory protein expressed in the cartilage and kidney of adult mice [23]. Notably, CTRP3 has been discovered to display abnormal expression in CVD [9]. It has reported that CTRP3 protects mesenchymal stem cells from hypoxia and serum deprivation induced apoptosis through PI3K/Akt pathway [11]. Moreover, CTRP3 alleviates ox-LDL-induced inflammatory response and endothelial dysfunction in mouse aortic endothelial cells by activating PI3K/Akt/eNOS pathway, thus improving atherosclerosis [2]. However, the study of CTRP3 on cardiac microvascular endothelial cells in myocardial ischaemia has not been reported so far.

JASPAR database predicts the binding of the transcription factor FOXO6 to the CTRP3 promoter. FOXO6 belongs to the Forkhead box O (FOXO) family of transcription factors that are implicated in multiple cellular pathways via posttranslational modifications [20, 24]. Previous study showed that FOXO6 is up-regulated in hypoxia-exposed cardiomyocytes and its silencing mitigates apoptosis and oxidative stress through elevating silent information regulator 2 homolog 6 (SIRT6) expression and activating nuclear factor E2-related factor (Nrf2) [14]. Nonetheless, whether FOXO6 exerts influence on CMECs remains obscure.

Silent information regulator 2 homolog 1 (SIRIT1), a histone deacetylase dependent on nicotinamide adenosine dinucleotide (NAD), has been supported to be related to a variety of cell metabolisms, such as inflammation, carcinogenesis, oxidative stress and so on [42]. The activation of SIRT1 signalling participates in endothelial barrier function [34]. Besides, elevation of SIRT1 reduces endothelial tight junction permeability to alleviate LPS-stimulated lung injury [7]. The regulation of SIRT1 signalling pathway can promote angiogenesis of rat brain microvascular endothelial cells after oxygen and glucose hypoxia/reoxygenation (OGD/R) injury [29].

Transcription factor Nrf2 is regarded as a decisive downstream target of SIRT1 signalling [37]. Intriguingly, it is also well documented that Nrf2 is involved in endothelial barrier function through mediating oxidative stress [19]. And activation of SIRT1/Nrf2 signalling pathway can inhibit oxidative stress and inflammatory response, and reduce OGD/R injury of human umbilical vein endothelial cells [18]. Moreover, CTRP3 can protect against doxorubicin-induced cardiac dysfunction, inflammation and cell death via activation of SIRT1 [40]. So we made a hypothesis that CTRP3 is regulated by FOXO6 transcription to affect the SIRT1/Nrf2 pathway, thus affecting the function of OGD/R-induced cardiac microvascular endothelial cells.

Herein, this paper is aimed at exploring the impacts of CTRP3 on OGD/R-treated CMECs and probing into the interaction among CTRP3, FOXO6 as well as SIRT1/Nrf2 signalling. Our study provides a theoretical basis for the pathogenesis and clinical treatment of myocardial ischaemia.

MATERIALS AND METHODS

Cell culture

The culture medium for human cardiac microvascular endothelial cells (HCMECs) purchased from ScienCell was endothelial cell medium (ECM; ScienCell). The medium was supplemented 10% fetal bovine serum (FBS; Atlanta Biologicals; Bio-Techne Corporation) and placed in a humid atmosphere at 37°C with 5% CO_2. To mimic cardiac ischaemia/reperfusion (I/R) injury *in vitro*, HCMECs were grown in serum/glucose-free ECM under the condition of 95% N_2 and 5% CO_2 at 37°C for 4 h. After that, the cells were subjected to re-oxygenation in the normoxic incubator (5% CO_2 at 37°C) for 24 h. Untreated HCMECs that cultured under normoxic conditions for 4 days were referred to the control group.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

With the aid of Omega Bio-tek E.Z.N.A. Total RNA kit (Doraville, GA, USA), total RNA was prepared from HCMECs and then subjected to reverse transcription using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Shanghai, China). SYBR® Green PCR master mix (Bio-Rad Laboratories, Inc.) was adopted to perform PCR reactions on the MX3000p PCR system (Agilent, Santa Clara, CA). The calculation of relative gene expression was achieved by $2^{-\Delta\Delta Ct}$ method [30]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was adopted for normalization. Primer sequences were as follows: CTRP3 forward: 5'-ATGCTTTGGAGGCAGCTCAT-3', reverse: 5'-TCACCTTTGTCGCCCTTCTC-3'; FOXO6 forward: 5'-TCTACGACTGGATGGTCCGT-3', reverse: 5'-GGGTCTTCCCTGTCTTTCCG-3'; GAPDH forward: 5'-AATGGGCAGCCGTTAGGAAA-3', reverse: 5'-GCGCCCAATACGACCAAATC-3'.

Western blot

Total proteins that extracted from HCMECs utilizing RIPA buffer (JRDUN Biotechnology) were ascertained with the application of BCA protein assay kit (CoWin, Beijing, China). Following, PVDF membranes were to shift protein samples that resolved by 10% SDS-PAGE. Non-specific interaction was ensured after the membranes were incubated with 5% non-fat milk. Afterwards, primary antibodies as well as goat anti-rabbit HRP antibody (cat. no. ab205718; 1/2000; Abcam) were respectively probed with the membranes overnight at 4°C and for 1 h. The blots were visualized by the ECL western blotting system (Amersham; Cytiva) and analysed by Image Quant LAS 500 (GE Healthcare) [36]. CTRP3 (cat. no. GTX85505; 1:1000; GeneTex), B cell lymphoma-2 (Bcl-2; cat. no. ab32124; 1/1000; Abcam), BCL-2-associated X (Bax; cat. no. ab32503; 1/1000; Abcam), cleaved caspase 3 (cat. no. ab2302; 1/1000; Abcam), cytochrome c (cyto-C; cat. no. ab133504; 1/5000; Abcam), zonula occludens-1 (ZO-1; cat. no. ab216880; 1/5000; Abcam), Occludin (cat. no. ab216327; 1/1000; Abcam), VE-cadherin (cat. no. ab33168; 1/1000; Abcam), Claudin-5 (cat. no. ab131259; 1/1000; Abcam), FOXO6 (cat. no. 19122-1-AP; 1/500; Proteintech), SIRT1 (cat. no. ab189494; 1/1000; Abcam), Nrf2 (cat. no. ab62352; 1/1000; Abcam), GAPDH (cat. no. ab9485; 1/2500; Abcam) antibodies were utilized here.

Plasmid transfection

PcDNA3.1 expression vector containing full-length human CTRP3 (pcDNA3.1-CTRP3) as well as corresponding negative control (pcDNA3.1-NC), pcDNA3.1(+) FOXO6 overexpression vector (Ov-FOXO6) and Ov-NC were all provided by GenePharma (Shanghai, China). OGD/R-induced cells overexpressing CTRP3 were divided into control, OGD/R, OGD/R+pcDNA3.1 and OGD/R+pcDNA3.1-CTRP3 groups. After both CTRP3 and FOXO6 were overexpressed, the cells were grouped into control, OGD/R, OGD/R+pcDNA3.1, OGD/R+pcDNA3.1-CTRP3, OGD/R+pcDNA3.1-CTRP3+OV-NC and OGD/R+pcDNA3.1-CTRP3+OV-FOXO6 groups. Above plasmids were transduced into cells employing Lipofectamine™ 3000 (Takara, Kusatsu, Japan). Cells were obtained for ensue assays 48 h later.

CCK-8 assay

Human cardiac microvascular endothelial cells were inoculated into 96-well plates at a density of 3,000 cells/well and then cultivated overnight at 37°C. A total of 10 μL cell counting Kit-8 (CCK-8) solution (Beijing TransGen Biotech Co., Ltd.) was added into each well and the cells were cultivated at 37°C for another 2 h. The measurement of the absorbance at 450 nm was implemented with a microplate reader (Beckman Coulter, Inc.) [33].

Detection of LDH release

Briefly, lactate dehydrogenase (LDH) concentration in HCMECs was ascertained by means of LDH assay kit (cat. no. BC0685; Solarbio) in the light of the manufacturer's guidance. Before incubating with supernatant (60 μL) and LDH substrate solution (30 μL) for another 30 min, cell supernatant was obtained after centrifugation at 300 × g for 10 min. With the application of a microplate reader (Beckman Coulter, Inc.), at a wavelength of 440 nm, LDH activity was calculated [38].

TUNEL

Cell apoptosis was appraised applying a terminal-deoxynucleoitidyl transferase mediated nick end labelling (TUNEL) Apoptosis kit (cat. no. BA27A; Nanjing Biobox Biotech Co., Ltd.) in the light of the manufacturer's guidance. In short, 4% paraformaldehyde was added to HCMECs for immobilization, following which was the permeabilization with 0.1% Triton X-100. Subsequently, the cells were cultivated with TUNEL reaction reagent for 1 h and the nuclei were

labelled with 10 mg/mL DAPI for 10 min. Finally, the images were acquired under a fluorescence microscope (UltraVIEW VoX; PerkinElmer, Inc.) [12].

Detection of HCMECs permeability

The permeability of HCMECs was detected using a commercial *in vitro* permeability assay kit (Millipore, Billerica, MA, USA). In brief, a tight monolayer was formed after 1×10^3 HCMECs that plated onto collagen-coated inserts were incubated for 3 days. After indicated treatment, each receiver plate well was supplemented with 500 μL glucose-free ECM. To permeate the monolayers, 2.5% FITC (fluorescein isothiocyanate)-dextran (40 kDa) solution was added for 20 min. The medium in the receiver wells was then thoroughly mixed. The intensity of FITC fluorescence was captured under a fluorescence spectrometer (MV06744, MoleCular Devices, Shanghai, China). The excitation wavelength was 482 nm and the detection wavelength was 525 nm [16].

Immunofluorescence staining

Following OGD/R treatment, HCMECs were subjected to immobilization and then probed with 0.2% Triton X-100 for 20 min. 1% BSA was used for blocking after cells were rinsed in PBS. Then ZO-1 (Abcam, 1:100, cat. no. ab221547) antibody was supplemented overnight at 4°C, after which was the cultivation with goat anti-rabbit IgG/Alexa Fluor 555 (Beijing Biosynthesis Biotechnology Co., Ltd., 1:100, cat. no. bs-0295G-A555) was used. Nuclear staining with DAPI (OriGene Technologies, Inc.) was performed. A fluorescence microscope (UltraVIEW VoX; PerkinElmer, Inc.) was to monitor the images [3].

Chromatin immunoprecipitation

With the adoption of the Imprint chromatin immunoprecipitation (ChIP) kit (cat. no. CHP1; Sigma-Aldrich; Merck KGaA), ChIP assay was executed in with the light of the manufacturer's guidelines. For the purpose of crosslinking the protein and DNA, HCMECs were firstly treated by 1% formaldehyde. The chromatin fragments that acquired after sonicating of cell lysates were precipitated with FOXO6 antibody (Proteintech, cat. no. 19122-1-AP) or IgG antibody (Abcam, ab6715) overnight. The purified DNA fragments were subjected to PCR analysis [5].

Luciferase reporter assay

PGL3 vectors (Huada Genomics, Shenzheng, China) containing wild type (WT) binding sequences between FOXO6 and CTRP3 promoter and the corresponding mutant type named as CTRP3-MUT were co-transfected with Ov-FOXO6 and Ov-NC into cells employing Lipofectamine™ 3000 (Takara, Kusatsu, Japan). After 48 h, with the aid of the Dual-Glo® Luciferase Reagent (Promega Corporation), the luciferase activity was evaluated.

Statistical analyses

Statistical analyses were executed employing SPSS 22.0 (IBM, Armonk, NY, USA). All data that collected 3 parallel repeat experiments were denoted as the mean ± standard deviation. Statistical significances were measured using Student's t-test or one-way ANOVA along with Tukey's post hoc test. The significance level was $p < 0.05$.

Bioinformatics tools

The potential binding sites between FOXO6 and CTRP3 promoter were predicted by JASPAR database (https://jaspar.genereg.net/) [6].

RESULTS

Elevation of CTRP3 potentiates the viability of OGD/R-insulted HCMECs

To determine the role of CTRP3 in OGD/R-treated HCMECs, CTRP3 expression was examined. It was noted from RT-qPCR and western blot that CTRP3 expression was declined in HCMECs following OGD/R treatment (Fig. 1A, B). Before assessing the impacts of CTRP3 on the behaviours of OGD/R-treated HCMECs, the overexpression efficiency of CTRP3 was tested. As Figures 1C and D illuminated, CTRP3 expression was remarkably increased after the transfection with pcDNA3.1-CTRP3. Through CCK-8 assay, it was observed that the viability of HCMECs was prominently reduced under OGD/R conditions. When CTRP3 was up-regulated, the viability of OGD/R-insulted HCMECs was obviously enhanced relative to the OGD/R+pcDNA3.1 group (Fig. 1E). Similarly, LDH production was monitored to evaluate cell cytotoxicity. LDH release was found to be stimulated in OGD/R-treated HCMECs, which was then suppressed after CTRP3 was overexpressed (Fig. 1F).

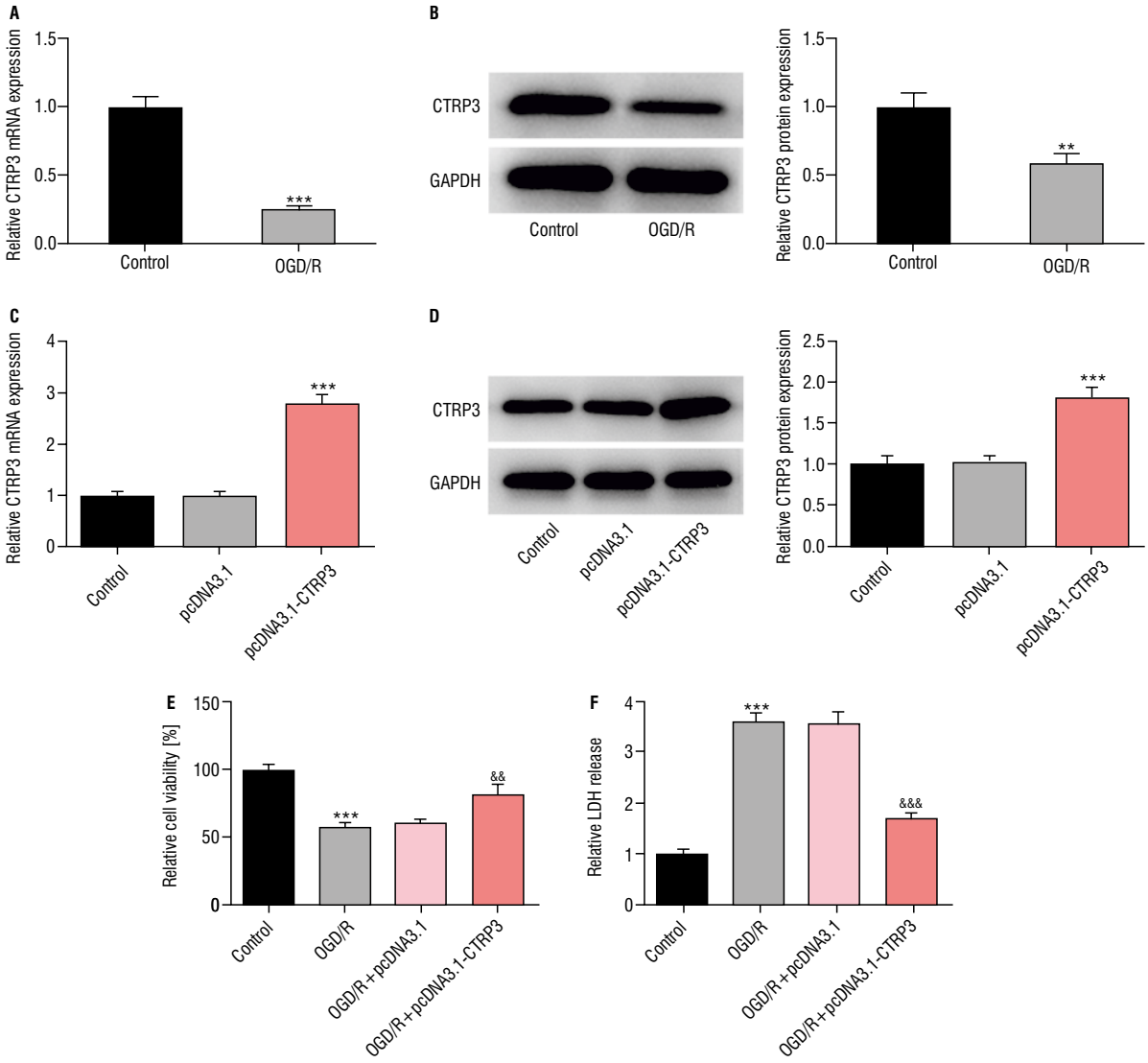

Figure 1. Elevation of C1q/TNF-related protein 3 (CTRP3) potentiates the viability of oxygen and glucose hypoxia/reoxygenation (OGD/R)-insulted human cardiac microvascular endothelial cells (HCMECs). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (**A**) and western blot (**B**) ascertained CTRP3 expression in HCMECs with the absence or presence of OGD/R treatment; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; RT-qPCR (**C**) and western blot (**D**) analysis of the overexpression efficacy of pcDNA3.1-CTRP3 plasmid; $^{***}p < 0.001$ vs. pcDNA3.1. OGD/R-exposed HCMECs activity was judged via CCK-8 assay (**E**). Lactate dehydrogenase (LDH) production was examined with LDH assay kit (**F**); $^{***}p < 0.001$ vs. control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. OGD/R+pc DNA3.1.

CTRP3 elevation attenuates OGD/R-elicited HCMECs apoptosis

On the contrary, the experimental results of TUNEL assay uncovered that OGD/R treatment-stimulated apoptosis of HCMECs was significantly suppressed by up-regulation of CTRP3 (Fig. 2A). In addition, western blot was to analyse the expression of apoptosis-associated factors. As expected, OGD/R treatment resulted in decreased Bcl-2 expression and increased Bax, cleaved caspase 3 and cyto-C expression, whereas these effects were offset by enhancive CTRP3 (Fig. 2B).

Up-regulation of CTRP3 reduces permeability and stabilizes tight junction in HCMECs following OGD/R treatment

Further, cell permeability was detected by *in vitro* permeability assay kit and the results indicated that the raised permeability of OGD/R-induced HCMECs was greatly decreased after the transfection with pcDNA3.1-CTRP3 (Fig. 3A). Besides, as analysed by immunofluorescence assay, the down-regulated expression of ZO-1 in OGD/R-insulted HCMECs was elevated again after CTRP3 was overexpressed (Fig.

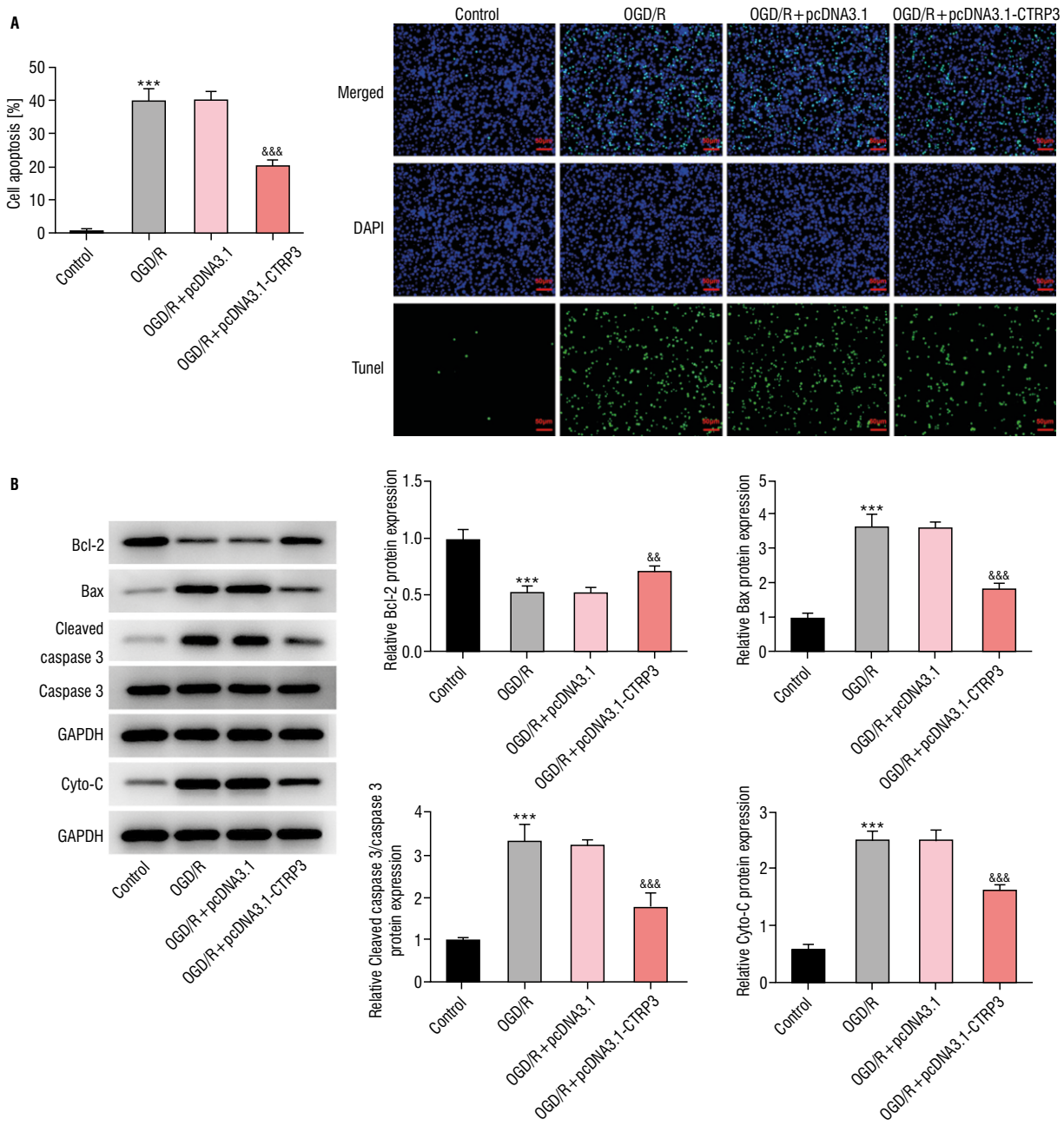

Figure 2. C1q/TNF-related protein 3 (CTRP3) elevation attenuates oxygen and glucose hypoxia/reoxygenation (OGD/R)-triggered human cardiac microvascular endothelial cells (HCMECs) apoptosis. The apoptotic ability of OGD/R-insulted HCMECs was evaluated by TUNEL assay (**A**); Western blot analysed the expression of apoptosis-associated factors (**B**); ***$p < 0.001$ vs. control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. OGD/R+pc DNA3.1; Bcl-2 — B-cell lymphoma 2; Bax — BCL-2 associated X; Cyto-c — cytochrome c.

3B). Western blot also analysed that the expressions of tight junctions including ZO-1, Occludin, VE-cadherin, Claudin-5 were cut down by OGD/R treatment in HCMECs, which were then increased by CTRP3 up-regulation (Fig. 3C).

FOXO6 is a transcription inactivator of CTRP3

Interestingly, JASPAR database predicted that CTRP3 promoter had a potential binding with FOXO6 (Fig. 4A). Moreover, FOXO6 was discovered to display high expression in OGD/R-exposed HCMECs (Fig. 4B, C). After FOXO6 was overexpressed by transfection with Ov-FOXO6 (Fig. 4D, E), the experimental results from luciferase reporter assay elaborated that elevation of FOXO6 distinctly lessened the luciferase activity of CTRP3-WT instead of that of CTRP3-MUT (Fig. 4F). Further, ChIP assay testified the high enrichment of CTRP3 promoter in FOXO6 antibody (Fig. 4G). Also,

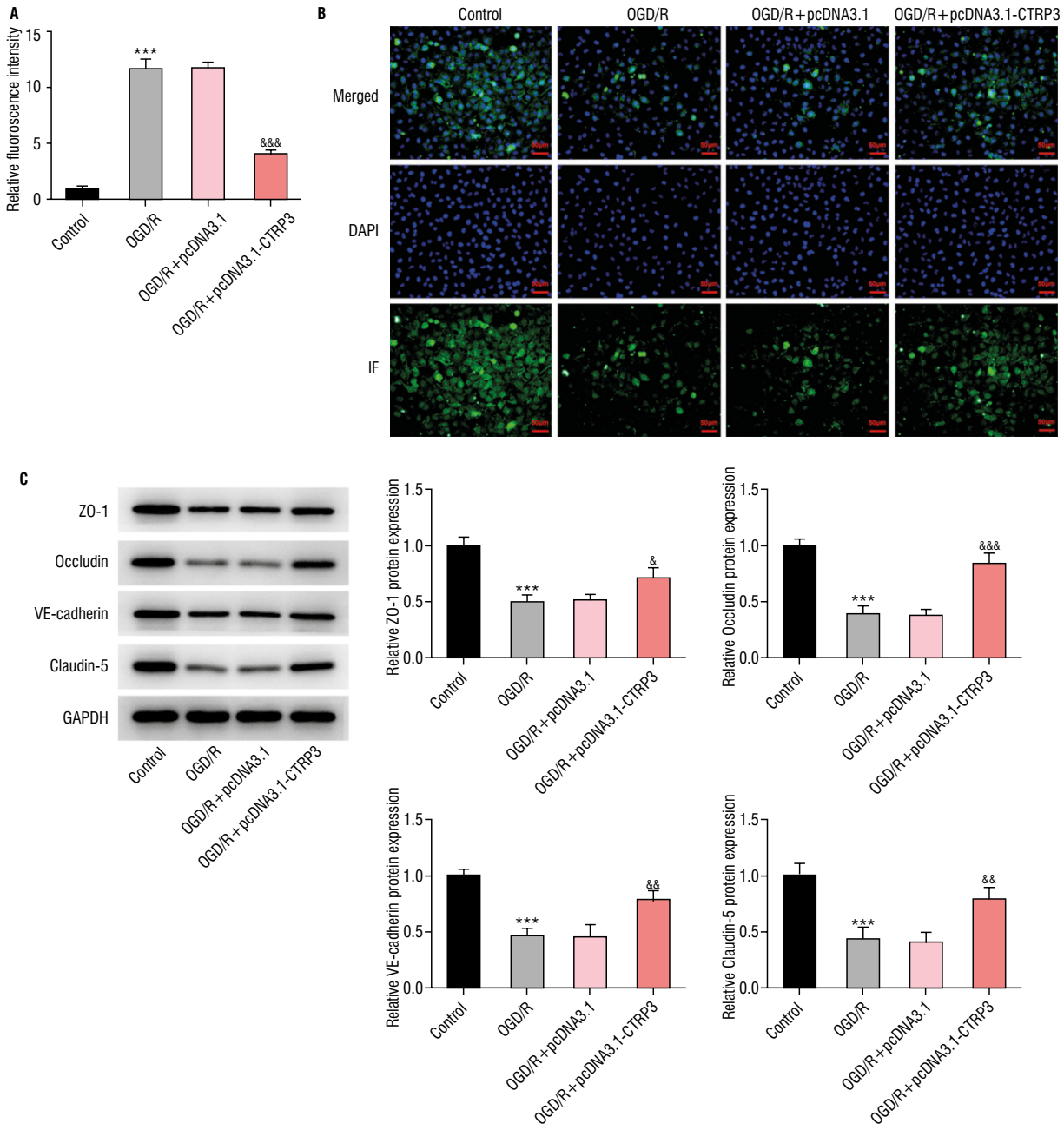

Figure 3. Up-regulation of C1q/TNF-related protein 3 (CTRP3) reduces permeability and stabilizes tight junction in human cardiac microvascular endothelial cells (HCMECs) following oxygen and glucose hypoxia/reoxygenation (OGD/R) treatment; **A.** *In vitro* permeability assay kit was to estimate the permeability of OGD/R-insulted HCMECs; **B.** Immunofluorescence (IF) assay was to examine zonula occludens-1 (ZO-1) expression; **C.** Western blot tested ZO-1, Occludin, VE-cadherin and Claudin-5 expression; ***$p < 0.001$ vs. control; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. OGD/R+pc DNA3.1.

RT-qPCR and western blot analysis indicated that the up-regulated CTRP3 expression in OGD/R-insulted HCMECs transfected with pcDNA3.1-CTRP3 was declined again when FOXO6 was up-regulated (Fig. 4H, I). In all, CTRP3 was transcriptionally suppressed by FOXO6.

FOXO6 overexpression reverses the protective role of CTRP3 in OGD/R-evoked HCMECs injury

To validate the mechanism mediated by FOXO6 and CTRP3 in OGD/R-induced cardiac microvascular endothelial barrier disruption, Ov-FOXO6 plasmid was

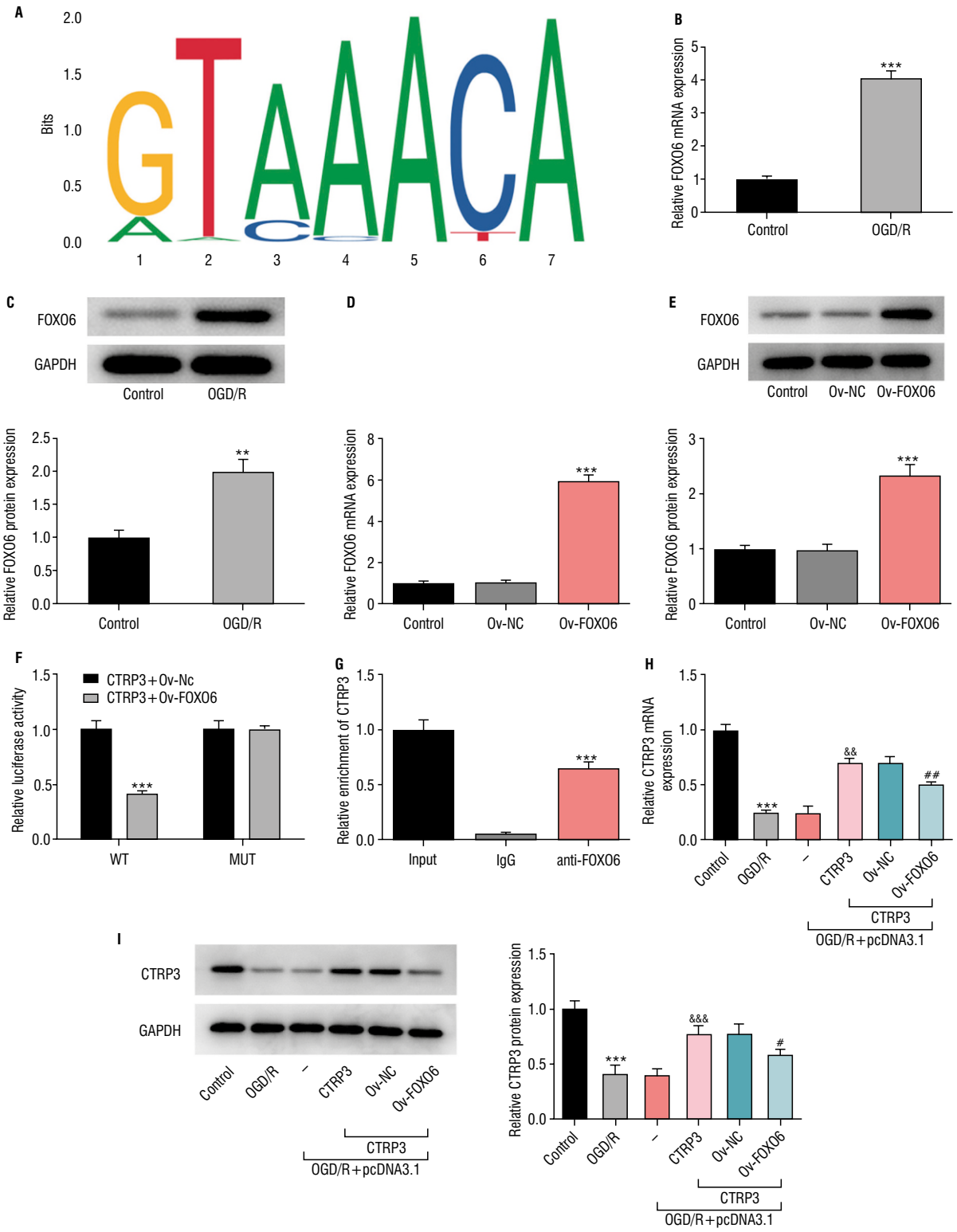

Figure 4. Forkhead Box O6 (FOXO6) is a transcription inactivator of C1q/TNF-related protein 3 (CTRP3); **A.** The potential binding sites between CTRP3 promoter and FOXO6 were predicted by JASPAR database; Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (**B**) and western blot (**C**) ascertained FOXO6 expression in human cardiac microvascular endothelial cells (HCMECs) with the absence or presence of oxygen and glucose hypoxia/reoxygenation (OGD/R) treatment; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. RT-qPCR (**D**) and western blot (**E**) analysis of the overexpression efficacy of Ov-FOXO6 plasmid; $^{***}p < 0.001$ vs. Ov-NC. Luciferase reporter assay verified the luciferase activity of CTRP3-WT and CTRP3-MUT (**F**); $^{***}p < 0.001$ vs. CTRP3+Ov-NC; Chromatin immunoprecipitation (ChIP) assay identified the abundance of CTRP3 promoter in FOXO6 antibody (**G**); $^{***}p < 0.001$ vs. IgG. RT-qPCR (**H**) and western blot (**I**) ascertained CTRP3 expression in OGD/R-induced HCMECs co-transfected with pcDNA3.1-CTRP3 and Ov-FOXO6 plasmids; $^{***}p < 0.001$ vs. control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. OGD/R + pcDNA3.1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs OGD/R + pcDNA3.1-CTRP3 + Ov-NC.

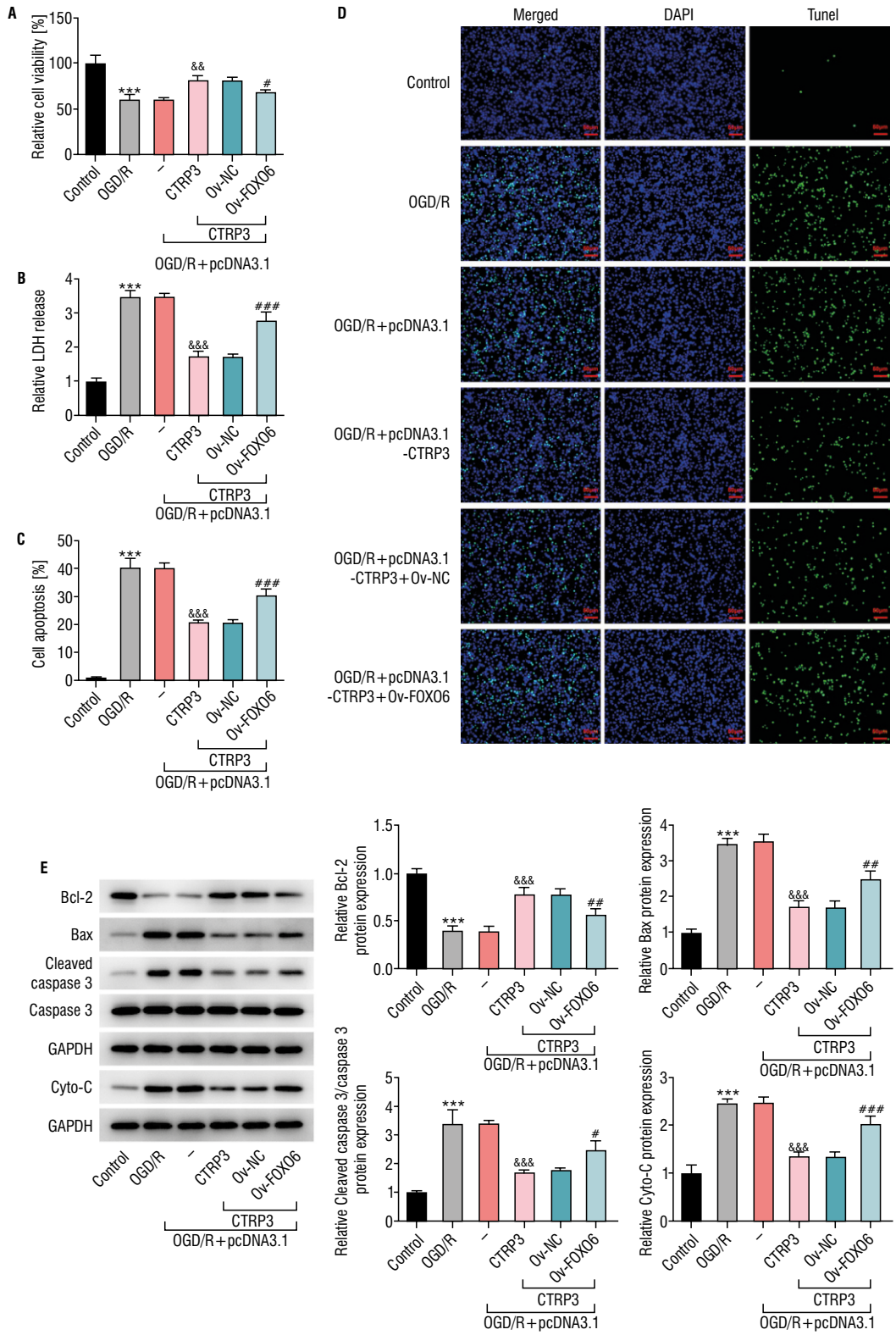

Figure 5. Forkhead Box O6 (FOXO6) overexpression reverses the impacts of C1q/TNF-related protein 3 (CTRP3) on oxygen and glucose hypoxia/reoxygenation (OGD/R)-evoked human cardiac microvascular endothelial cells (HCMECs) proliferation and apoptosis. OGD/R-exposed HCMECs activity was judged via CCK-8 assay (**A**). Lactate dehydrogenase (LDH) production was examined with LDH assay kit (**B**). The apoptotic ability of OGD/R-insulted HCMECs was evaluated by TUNEL assay (**C**, **D**). Western blot analysed the expression of apoptosis-associated factors (**E**); ***$p < 0.001$ vs. control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. OGD/R+pcDNA3.1; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. OGD/R+pcDNA3.1-CTRP3+Ov-NC; Bcl-2 — B-cell lymphoma 2; Bax — BCL-2 associated X; Cyto-c — cytochrome c.

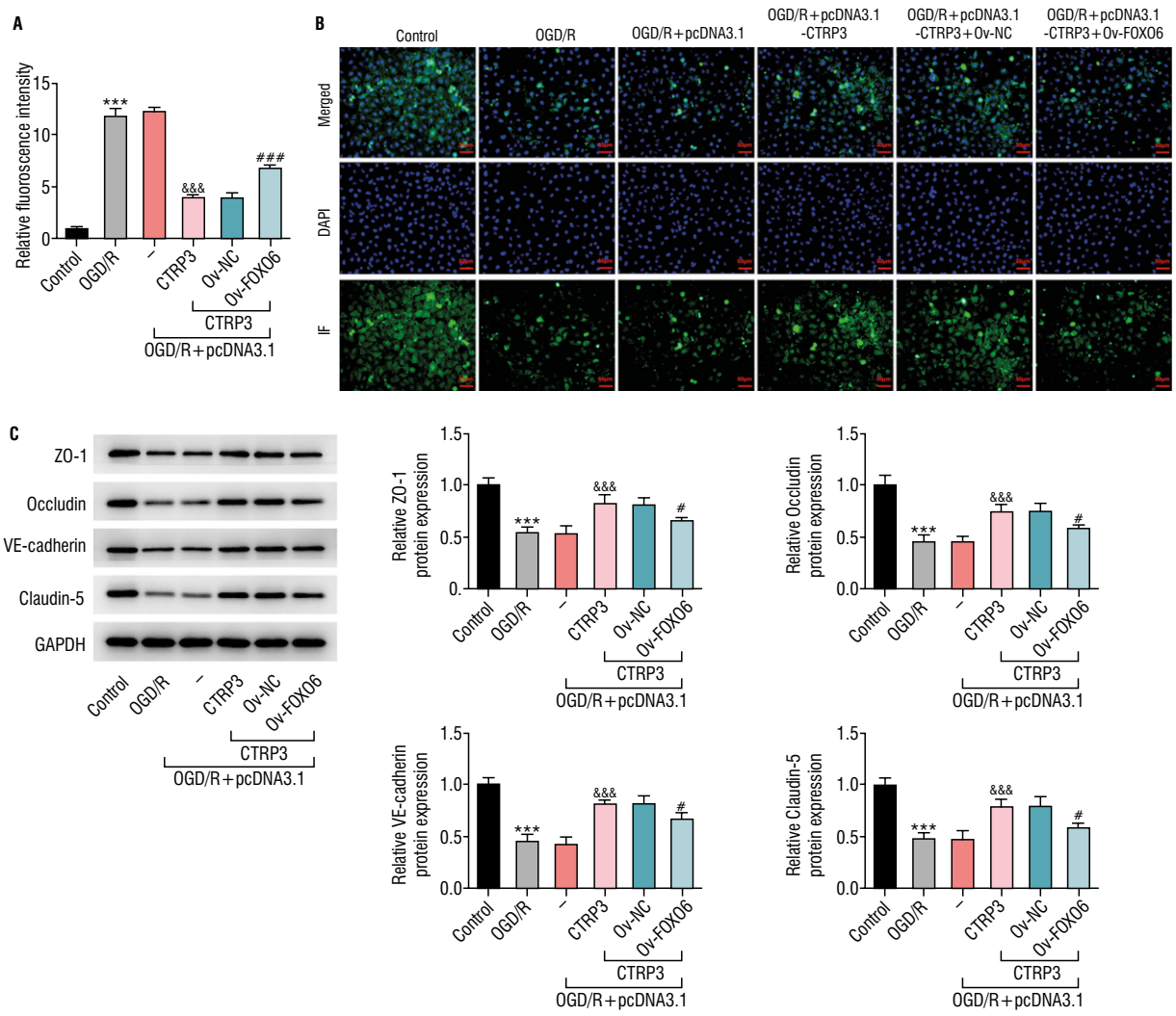

Figure 6. Forkhead Box O6 (FOXO6) overexpression reverses the protective role of C1q/TNF-related protein 3 (CTRP3) in glucose hypoxia/reoxygenation (OGD/R)-evoked cardiac microvascular endothelial barrier dysfunction; **A.** *In vitro* permeability assay kit was to estimate the permeability of oxygen and OGD/R-insulted human cardiac microvascular endothelial cells (HCMECs); **B.** Immunofluorescence (IF) assay was to examine zonula occludens-1 (ZO-1) expression; **C.** Western blot tested ZO-1, Occludin, VE-cadherin and Claudin-5 expression; ***$p < 0.001$ vs. control; &&&$p < 0.001$ vs. OGD/R+pcDNA3.1; #$p < 0.05$, ###$p < 0.001$ vs. OGD/R+pcDNA3.1-CTRP3+Ov-NC.

transfected into CTRP3-overexpressed HCMECs with OGD/R induction and then functional experiments were conducted again. The results of CCK-8 assay revealed that the stimulated viability of OGD/R-insulted HCMECs caused by CTRP3 was abrogated after FOXO6 was overexpressed (Fig. 5A). Also, the impeded LDH release caused by CTRP3 elevation was abated again when FOXO6 was up-regulated (Fig. 5B). Conversely, as Figures 5C and D depicted, FOXO6 strengthened the weakened apoptotic capacity of OGD/R-exposed HCMECs caused by CTRP3. Besides, the enhanced Bcl-2 protein level as well as declined Bax, cleaved caspase 3 and cyto-C protein levels caused by CTRP3 were also restored after upregulating FOXO6 (Fig. 5E). Additionally, CTRP3 led to decreased permeability of OGD/R-treated HCMECs, whereas this effect was reversed by Ov-FOXO6 (Fig. 6A). Compared with OGD/R+pcDNA3.1 group, the higher expression of ZO-1 in OGD/R-exposed HCMECs transfected with pcDNA3.1-CTRP3 was diminished again after FOXO6 was overexpressed (Fig. 6B). In the same way, FOXO6 up-regulation cut down CTRP3-stimulated protein levels of ZO-1, Occludin, VE-cadherin and Claudin-5 (Fig. 6C). Taken together, the suppressive role of CTRP3 in OGD/R-induced injury in HCMECs was counteracted by FOXO6.

CTRP3 negatively regulated by FOXO6 transcription factor activates SIRT1/Nrf2 signalling

Notably, western blot analysed that overexpression of CTRP3 increased the protein levels of SIRT1

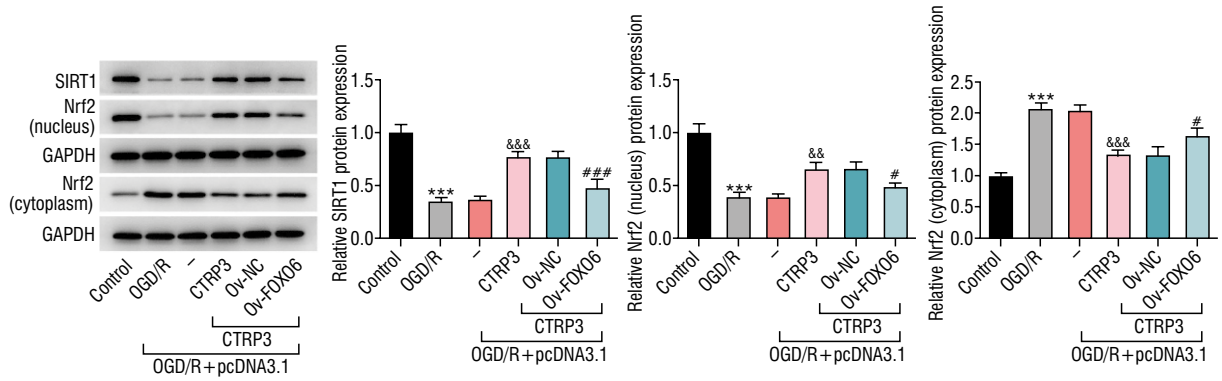

Figure 7. C1q/TNF-related protein 3 (CTRP3) negatively regulated by Forkhead Box O6 (FOXO6) transcription factor activates silent information regulator 2 homolog 1/nuclear factor E2-related factor (SIRT1/Nrf2) signalling. Western blot tested cytoplasmic Nrf2, nuclear Nrf2 and SIRT1 expression; $^{***}p < 0.001$ vs. control; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. OGD/R+pcDNA3.1; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. OGD/R+pcDNA3.1-CTRP3+Ov-NC.

and nuclear Nrf2 but decreased the protein level of cytoplasmic Nrf2. However, the consequence was reversed when FOXO6 was up-regulated. Collectively, CTRP3 that mediated by FOXO6 silencing served as a transcription activator of SIRT1/Nrf2 signalling.

DISCUSSION

Cardiac microvascular endothelial cells, an element of coronary circulation microvessels, are some of the predominant cells prone to immediately suffer damage after myocardial ischaemia injury [28]. Disruption of epithelial barriers that mediated by CMECs injury is considered as an initiating factor of CVD [17], even heart failure [4]. During the process of myocardial ischaemia injury, endothelial barrier injury is commonly characterized by autoregulatory dysfunction, eventually bringing about increased endothelial permeability[27]. Hence, OGD/R was utilized in the present study to induce endothelial barrier breakdown model, aiming to explore the proliferation, apoptosis and permeability of HCMECs.

In recent years, the impact of CTRP3 on cardiac events has attracted much attention. CTRP3 alleviates pressure overload induced cardiac hypertrophy by inhibiting the P38/CREB pathway and endoplasmic reticulum stress induced by P38 [41]. CTRP3 also relieves inflammation and endothelial dysfunction in atherosclerosis [2]. Melatonin alleviates obesity-induced myocardial oxidative stress and apoptosis by promoting the secretion of CTRP3 in adipose tissue, while the loss of CTRP3 largely eliminates melatonin-mediated cardiac protection [22]. However, the effect of CTRP3 on HCMECs in myocardial ischaemia has not been reported so far. In our experiment, it was found that the expression of CTRP3 in OGD/R-induced HCMECs decreased significantly, and the overexpression of CTRP3 could significantly improve the activity of OGD/R-induced HCMECs and inhibit cell apoptosis. The knockdown of endothelial barrier can be attributed to the altered cell junction protein levels; meanwhile, the unique property of tight junction in HCMECs is of great significance in controlling permeability [10]. As important mediators of endothelial adherence junction, the expressions of ZO-1, Occludin, VE-cadherin and Claudin-5 were also examined here. It was observed that OGD/R exposure led to decreased ZO-1, Occludin, VE-cadherin and Claudin-5 expressions, which were subsequently raised by overexpression of CTRP3.

JASPAR database and mechanism assays in this study predicted and verified the binding of FOXO6 to CTRP3 promoter. FOXO family transcription factors can affect microvascular endothelial cells, induce oxidative stress response pathways, and thus participate in cell injury and apoptosis [26]. FOXO6 is a member of the FOXO family. Previous study showed that FOXO6 contributes to hypoxia-exposed cardiomyocyte apoptosis and oxidative stress [14]. Therefore, we have reason to guess that FOXO6 can transcriptionally regulate CTRP3 and thus regulate the function of HCMECs. Our data suggested that FOXO6 was up-regulated in OGD/R-treated HCMECs. Moreover, the elevation of CTRP3 in CTRP3-overexpressed HCMECs following OGD/R injury was cut down after overexpressing FOXO6, implying that CTRP3 was transcriptionally inhibited by FOXO6. Further, functional experiments corroborated that the impacts of CTRP3

on the viability, apoptosis, permeability and tight junction of HCMECs upon exposure to OGD/R treatment were all reversed by overexpression of FOXO6.

Increasing previous studies have supported that the activation of SIRT1/Nrf2 signalling protects against myocardial ischaemia injury mainly through the regulation of apoptosis, inflammation, oxidative stress and endoplasmic reticulum stress [21, 35]. SIRT1/Nrf2 signalling mitigates intestinal barrier injury [39]. And targeting SIRT1 can inhibit apoptosis of rat retinal vascular endothelial cells and improve blood retinal vascular barrier permeability [13]. Moreover, FOXO6 contributes to hypoxia-exposed cardiomyocyte apoptosis and oxidative stress via inactivating Nrf2 [14]. CTRP3 inhibits OGD/R-induced hippocampal neuron injury through SIRT1 signalling pathway [8]. CTRP3 can protect against doxorubicin-induced cardiac dysfunction, inflammation and cell death via activation of SIRT1 [40]. However, in HCMECs of myocardial ischaemia, the regulation of FOXO6 transcriptional regulation of CTRP3 on SIRT1/Nrf2 has not been reported. Our study proved that OGD/R exposure down-regulated SIRT1 and nuclear Nrf2 protein levels but up-regulated cytoplasmic Nrf2 protein level. Meanwhile, in OGD/R-treated HCMECs, the stimulated SIRT1 expression and Nrf2 nuclear translocation imposed by CTRP3 were restored by FOXO6.

Limitations of the study

This article also has some limitations. The response of HCMECs to inflammatory response has not been covered and *in vivo* models of myocardial ischaemia induced by coronary ligation in mice also require further study. The research on the role of SIRT1/Nrf2 pathway in downstream pathway of CTRP3 is not in-depth enough. In future experiments, we will further explore the mechanism by adding SIRT1/Nrf2 pathway inhibitors or pathway activators.

CONCLUSIONS

In conclusion, CTRP3 was inhibited by FOXO6 transcription factor at posttranscriptional level. CTRP3 relieved OGD/R-evoked cardiac microvascular endothelial barrier disruption through activating SIRT1/Nrf2 signalling. This finding might provide a theoretical basis for the pathogenesis of myocardial ischaemia and provide substantial evidence for the efficacy of novel targeted therapy based on CTRP3-mediated molecular mechanism. At the same time, accumulating evidence has elucidated that endothelial barrier dysfunction is associated with inflammatory response and clinical outcomes [31].

Conflict of interest: None declared

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