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Original Article

Long non-coding RNA FOXD3-AS1 aggravates ischemia/reperfusion injury of cardiomyocytes through promoting autophagy

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Abstract: We aimed to investigate the role of long non-coding RNA (lncRNA) FOXD3 antisense RNA 1 (FOXD3-AS1) in myocardial Ischemia/reperfusion (I/R) injury. In our study, H9C2 cells were treated with oxygen-glucose deprivation and reoxygenation (OGD/R). RT-qPCR was performed to detect the expression level of lncRNA FOXD3-AS1 in OGD/R induced H9C2 cells. Then, pcDNA-lncRNA FOXD3-AS1 was transfected into H9C2 cells. The level of LC3 II was measured by immunofluorescence assay. And the expression of autophagy related genes were detected using western blot. In addition, 3-methyladenine (3 M), an autophagy inhibitor, was recruited to treat with H9C2 cells. The contents of creatine kinase (CK), CK isoenzymes (CK-MB), cardiac troponin I (cTnI), inflammation associated factors, reactive oxygen (ROS) and NO were evaluated by kits. Moreover, cell apoptosis was measured by a flow cytometry assay and the expression levels of apoptosis associated proteins were evaluated by western blot. Furthermore, the expression of NF-κB/iNOS/COX2 signaling were measured in our study. The results indicated that FOXD3-AS1 expression was increased in OGD/R-treated H9C2 cells and overexpression of FOXD3-AS1 upregulated the expression of LC3 II, Beclin1, ATG5 accompanied by a downregulated expression of p62. In addition, FOXD3-AS1 overexpression increased the levels of CK, CK-MB, cTnI, TNF-α, IL-1β, IL-6, ROS and NO, whereas the increase of above factors were reversed following treatment with 3 M. Moreover, FOXD3-AS1 overexpression enhanced the rate of apoptosis cells coupled with a decrease of Bcl-2 expression and an increase of Bax and cleaved caspase 3 expression, which were reversed by 3 M. Furthermore, FOXD3-AS1 overexpression promoted the activation of NF-κB/iNOS/COX2 signaling, which was blocked following treatment with 3 M. These findings demonstrate that overexpression of lncRNA FOXD3-AS1 aggravates myocardial I/R injury through promoting autophagy, which was regulated by activating NF-κB/iNOS/COX2 signaling.

Keywords: FOXD3-AS1, cardiomyocytes, ischemia/reperfusion, apoptosis, autophagy

Introduction

Myocardial ischemia/reperfusion (I/R) injury is still a clinical severe problem in recent years. It leads to high rates of disability and mortality following cardiac surgery, heart failure and myocardial infarction [1-3]. Myocardial I/R injury occurs when reperfusion after ischemia not only fails to restore the physiological functions of organs, but also aggravates dysfunction and causes structural damage [4]. As we all know, myocardial I/R induces cardiomyocyte apoptosis, autophagy activation and inflammation in myocardial cells [5-7]. However, there is still no effective therapy for preventing and treating myocardial I/R injury. Therefore, great efforts have been made to explore effective therapeutic targets for the treatment of myocardial I/R injury.

Abnormal regulation of autophagy is associated with a number of cardiac diseases, including heart failure, ischemic heart disease, and dilated cardiomyopathy [8-10]. Mounting evidences support that autophagy is crucially involved in cardiomyocyte, nephrocyte, and neuron I/R injury [11-13]. In addition, it has been found that autophagy could be promoted by myocardial I/R injury [14]. Long non-coding RNA (lncRNA) is an RNA molecule with a length of > 200 nucleo-

tides, which commonly do not have any protein-coding capacity [15]. However, lncRNAs have key roles in regulating inflammation, apoptosis, and autophagy [16]. It has been well documented that the expression of lncRNA FOXD3 antisense RNA 1 (FOXD3-AS1) was increased in ROS induced lung hypoxic injury, which promoted lung epithelial cell death induced by oxidative stress [17]. However, the role of lncRNA FOXD3-AS1 in the regulation of autophagy in myocardial I/R injury has not been reported yet.

In the present study, we aimed to investigate the function of lncRNA FOXD3-AS1 in myocardial ischemia/reperfusion, which may provide a novel therapeutic strategy for myocardial I/R injury.

Material and methods

Cell culture

The cardiomyocytes cell line H9C2 (Chinese Academy of Sciences, China) were cultured in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), 100 μg/ml penicillin and 100 μg/ml streptomycin (Solarbio, China) at 37°C in a humidified 5% CO_2/95% air atmosphere.

Oxygen-glucose deprivation and reperfusion (OGD/R)

When cultured cardiomyocytes reached confluency, ischemia was simulated by deprivation of oxygen and glucose for 24 h in serum/glucose-free DMEM medium at 5% CO_2, 95% N_2 in a hypoxic chamber. Subsequently, the cells were cultured in the glucose-containing DMEM with 10% FBS under normoxic conditions for 12 h to reoxygenation. Control cells were incubated under normoxic conditions.

Transfection

H9C2 cells were seeded in 6-well plates with the density of 2×10^5/well and maintained at 37°C with 5% CO_2. When cells reached confluency, overexpression vector pcDNA-lncRNA FOXD3-AS1 or its control pcDNA vector (Shanghai GenePharma Co., Ltd.; Shanghai, China) were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, MA, USA) according to the manufacturer's protocols. Subsequently, the successful transfection was measured using RT-qPCR.

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

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific, MA, USA) from H9-C2 cells. Then, cDNA was synthesized using a RevertAid First Strand cDNA Synthesis kit (K1622; Thermo Fermentas, USA) according to the manufacturer's instructions. RT-qPCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA). GAPDH served as the reference gene. The gene expression was quantified by the method of $2^{-\Delta\Delta Cq}$ [18].

Measurement of creatine kinase (CK), CK isoenzymes (CK-MB), cardiac troponin I (cTnI) and reactive oxygen (ROS)

Following the OGD/R experiment, the cell culture medium was collected at the end of the reoxygenation stage, and levels of CK, CK-MB, cTnI and ROS in the cell culture were measured by corresponding assay kits (all from Nanjing Jiancheng Bioengineering Institute, Nanjing, china) in accordance with the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA)

Culture supernatant was collected and concentrations of interleukin (IL)-6, IL-1β, TNF-α (tumor necrosis factor-alpha) and NO were measured by enzymelinked immunosorbent assay (ELISA) following the manufacturer's protocols (R&D Systems, Abingdon, UK). Above ELISA kits were all purchased from Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China).

Apoptosis assay

Cell apoptosis was detected by Annexin V-FITC/PI apoptosis double staining kit (Becton, Dickinson and Company, NJ, USA). Cells were harvested and washed twice with pre-cooled PBS, and then stained successively with propidium iodide (10 μl) and Annexin V-FITC (10 μl; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). After 15 min of incubation, a flow cytometer (FACSVantage SE; BD Biosciences, San Jose, CA, USA) was applied to sample analysis and cell number in each classification was presented.

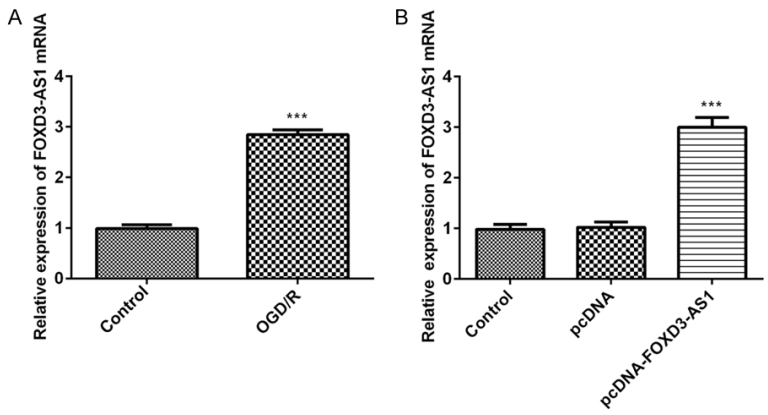

Figure 1. The expression of lncRNA FOXD3-AS1 was upregulated in OGD/R-induced H9C2 cells. A. The expression of FOXD3-AS1 was determined by RT-qPCR. ***P < 0.001 vs. Control. B. H9C2 cells were transfected with FOXD3-AS1 and its control plasmids. ***P < 0.001 vs. pcDNA. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion.

Immunofluorescence assay

H9C2 cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 20 min at room temperature. After being blocked in 5% bovine serum albumin for 1 h, cells were incubated with anti-LC3II (Cell Signaling Technology, Boston, MA, USA) overnight at 4°C. After washing with PBS, cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Boster Biological Technology). Images were acquired using a fluorescence microscope (Nikon Corporation) after staining the cell nuclei with DAPI (Boster Biological Technology). Magnification, × 400.

Western blot analysis

H9C2 cells were collected and lysed on ice in RIPA Lysis Buffer (Beyotime, Shanghai, China). The concentration of the proteins was measured using Enhanced BCA Protein Assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Protein samples of 40 μg per lane were isolated by SDS-PAGE, and subsequently electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Then, the membranes were blocked for 2 h in 5% skimmed milk at room temperature, and incubated overnight at 4°C with primary antibody. Then, the membranes were washed with TBST three times and incubated with HRP-labeled Goat Anti-Mouse IgG (H+L) antibody (A0216, Beyotime, Shanghai, China) at room temperature for 1 h. Subsequently, the blots were detected using an enhanced chemiluminescence (ECL) reagent and analyzed with ImageJ software. Anti-Beclin1 (3495T), anti-ATG5 (12994T), anti-Bcl-2 (4223T), anti-Bax (5023T), anti-cleaved caspase-3 (9579T), anti-caspase-3 (14220T), anti-NF-κB p65 (8801S), anti-p-NF-κB p65 (3033T), anti-COX2 (12282T), anti-iNOS (13120S) and anti-GAPDH (5174S) were the products of Cell Signaling Technology (Boston, MA, USA).

Statistical analysis

All experiments were performed with at least three replicates and all the data are presented as mean ± SD. Statistical analysis was performed by SPSS software 16.0. Statistical comparisons were made by on-way ANOVA followed by a post hoc Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Results

OGD/R treatment increases the expression level of FOXD3-AS1 in H9C2 cells

To investigate the potential correlation of FOXD3-AS1 in I/R injury, we first evaluate whether OGD/R treatment affected the expression level of FOXD3-AS1 in H9C2 cells. The result was presented in **Figure 1A**, the expression level of FOXD3-AS1 was markedly upregulated after OGD/R treatment. The results suggest that a potential role of FOXD3-AS1 in regulating I/R injury.

LncRNA FOXD3-AS1 overexpression promotes autophagy in OGD/R-induced H9C2 cells

To further explore the regulatory mechanisms of FOXD3-AS1 on I/R injury in cardiomyocytes, FOXD3-AS1 was overexpressed in our study. The results showed that the expression of FOXD3-AS1 was upregulated obviously following transfection with pcDNA-FOXD3-AS1 com-

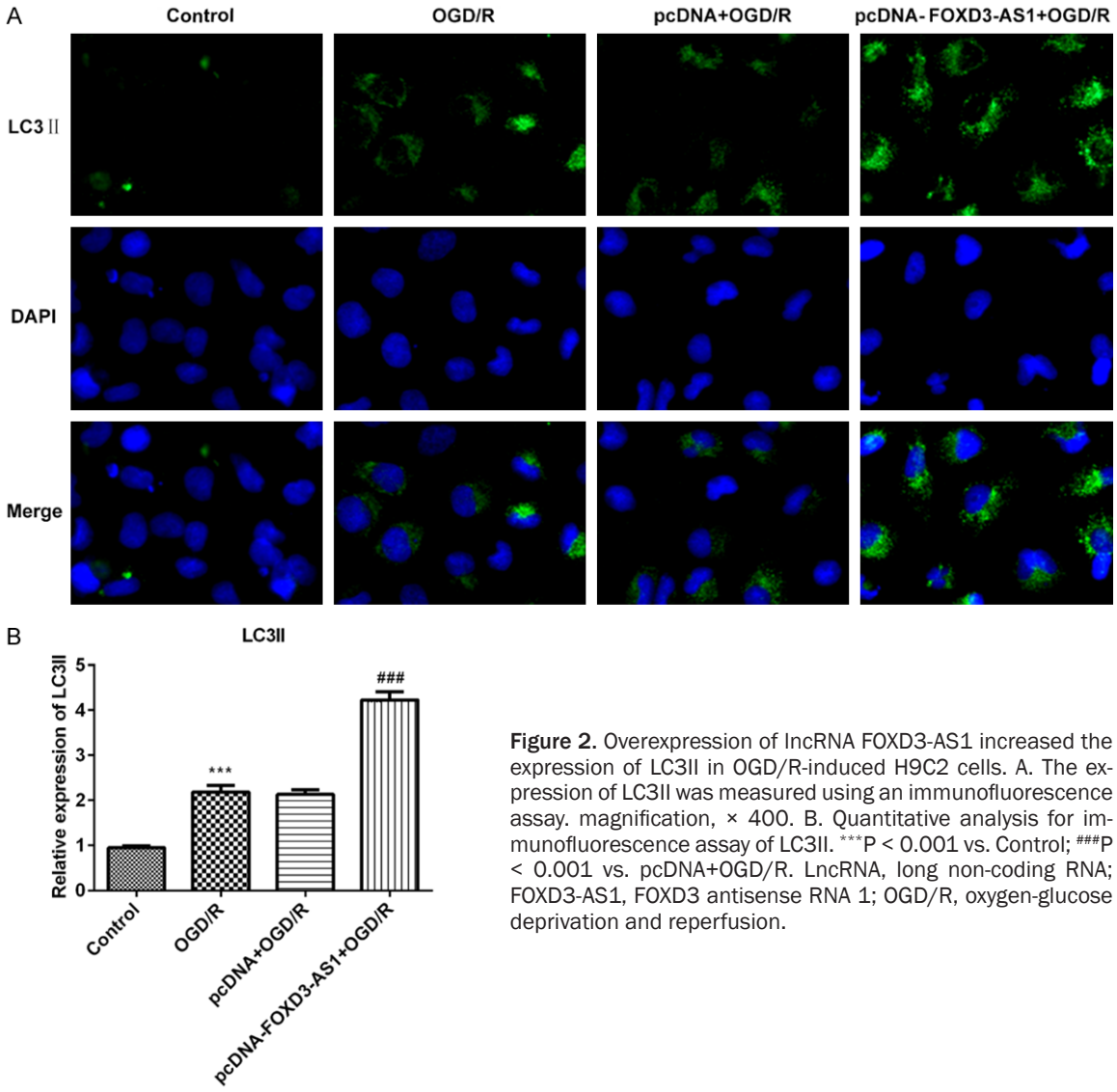

Figure 2. Overexpression of lncRNA FOXD3-AS1 increased the expression of LC3II in OGD/R-induced H9C2 cells. A. The expression of LC3II was measured using an immunofluorescence assay. magnification, × 400. B. Quantitative analysis for immunofluorescence assay of LC3II. ***P < 0.001 vs. Control; ###P < 0.001 vs. pcDNA+OGD/R. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion.

pared with transfection with empty vector, which indicated that FOXD3-AS1 overexpression was performed successfully (**Figure 1B**). Then, the levels of autophagy-related genes were measured. We found that the expression level of LC3II was significantly increased in OGD/R group compared with control group (**Figure 2**). Following transfection with pcDNA-FOXD3-AS1, the level of LC3II was enhanced markedly in comparison with empty vector. At the same time, the expression of Beclin1 and ATG5 were notably upregulated accompanied by a downregulation of p62 expression compared with empty vector, which were all autophagy-associated genes (**Figure 3**). These observations reveal that lncRNA FOXD3-AS1 overexpression promoted autophagy in OGD/R-induced H9C2 cells.

LncRNA FOXD3-AS1 overexpression aggravates myocardial injury by promoting autophagy in OGD/R-induced H9C2 cells

To demonstrate the effect of FOXD3-AS1 on OGD/R-induced myocardial injury in H9C2 cells, the levels of CK, CK-MB and cTnI were measured in our experiment. As presented in **Figure 4A-C**, the contents of CK, CK-MB and cTnI were increased in OGD/R group compared with control normoxia group. Following overexpression of FOXD3-AS1, the levels of above factors were remarkably augmented, which were reduced after treatment with 3 M. In addition, the changes of inflammatory cytokines TNF-α, IL-1β and IL-6 levels were in accordance with the results of CK, CK-MB and cTnI (**Figure 4D-F**). Moreover, the contents of ROS and NO were said the

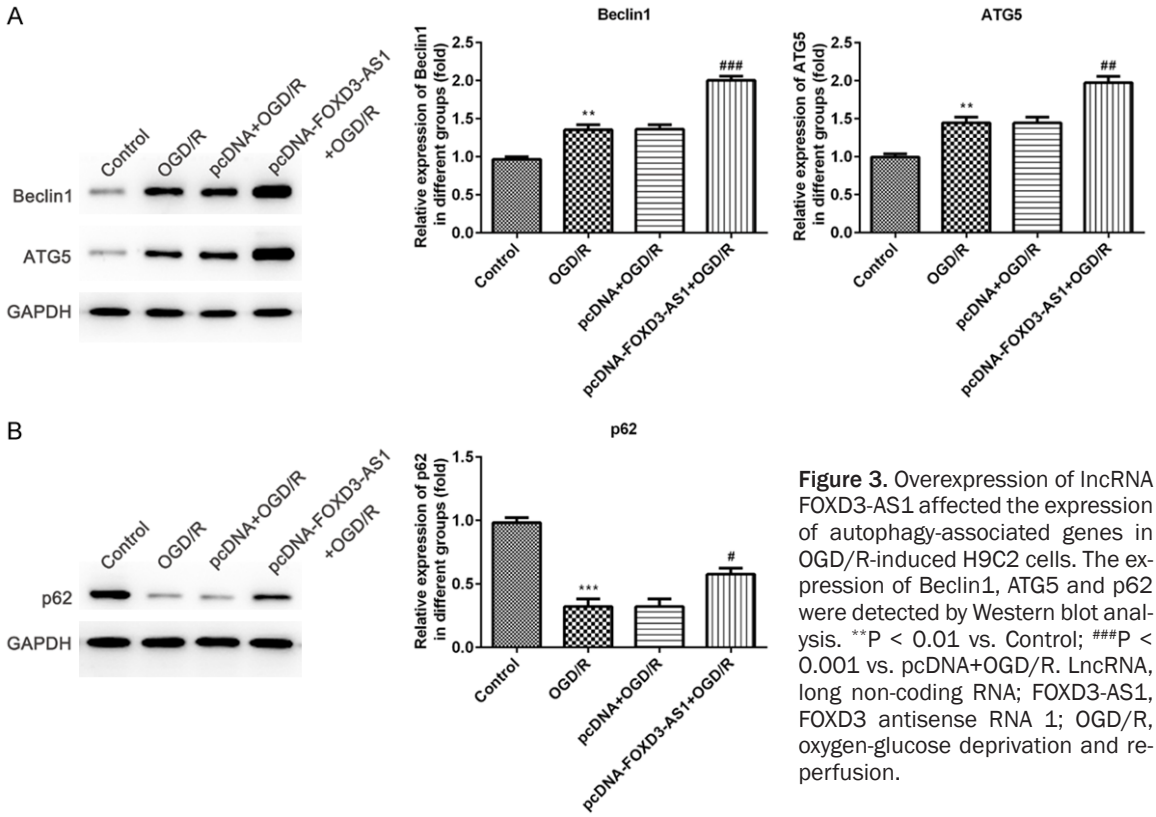

Figure 3. Overexpression of lncRNA FOXD3-AS1 affected the expression of autophagy-associated genes in OGD/R-induced H9C2 cells. The expression of Beclin1, ATG5 and p62 were detected by Western blot analysis. $^{**}P < 0.01$ vs. Control; $^{###}P < 0.001$ vs. pcDNA+OGD/R. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion.

same story with inflammatory cytokines (**Figure 4G** and **4H**). These date suggest that lncRNA FOXD3-AS1 overexpression aggravates myocardial injury by promoting autophagy in OGD/R-induced H9C2 cells.

LncRNA FOXD3-AS1 overexpression increases apoptosis of H9C2 cells by promoting autophagy in OGD/R-induced H9C2 cells

We measured the effect of FOXD3-AS1 on apoptosis of H9C2 cells treated with OGD/R. As exhibited in **Figure 5A** and **5B**, cell apoptosis rate in OGD/R group was significantly higher than control group. FOXD3-AS1 overexpression increased the number of apoptosis cell compared with OGD/R, and which was decreased when cell was treated with 3 M. Subsequently, the expression of apoptosis-associated proteins were detected by western blot analysis. We found that the expression of Bax, cleaved caspase-3 and cleaved caspase-9 were upregulated accompanied by a downregulated expression of Bcl-2 after transfection with pcDNA-FOXD3-AS1, which were reversed following intervene with 3 M (**Figure 6**). Overall, these results indicate that lncRNA FOXD3-AS1 overexpression accelerates apoptosis of H9C2 cells by promoting autophagy in OGD/R-induced H9C2 cells.

LncRNA FOXD3-AS1 overexpression promotes autophagy via activating NF-κB/COX2/iNOS signaling pathway in OGD/R-induced H9C2 cells

To study the underlying regulatory mechanisms of FOXD3-AS1 in promoting autophagy, the expression of NF-κB/COX2/iNOS signaling pathway was evaluated by western bot analysis in our study. The results were shown in **Figure 7**. We found that the expression of p-NF-κB p65, COX-2 and INOS were upregulated remarkably following FOXD3-AS1 overexpression in comparison with empty vector, whereas the expression of above genes were downregulated after treatment with 3 M. These findings demonstrate that FOXD3-AS1 overexpression accelerates autophagy via motivating NF-κB/COX2/iNOS signaling pathway in OGD/R-induced H9C2 cells.

Discussion

Mounting evidences support that lncRNAs are implicated in regulating many human diseases

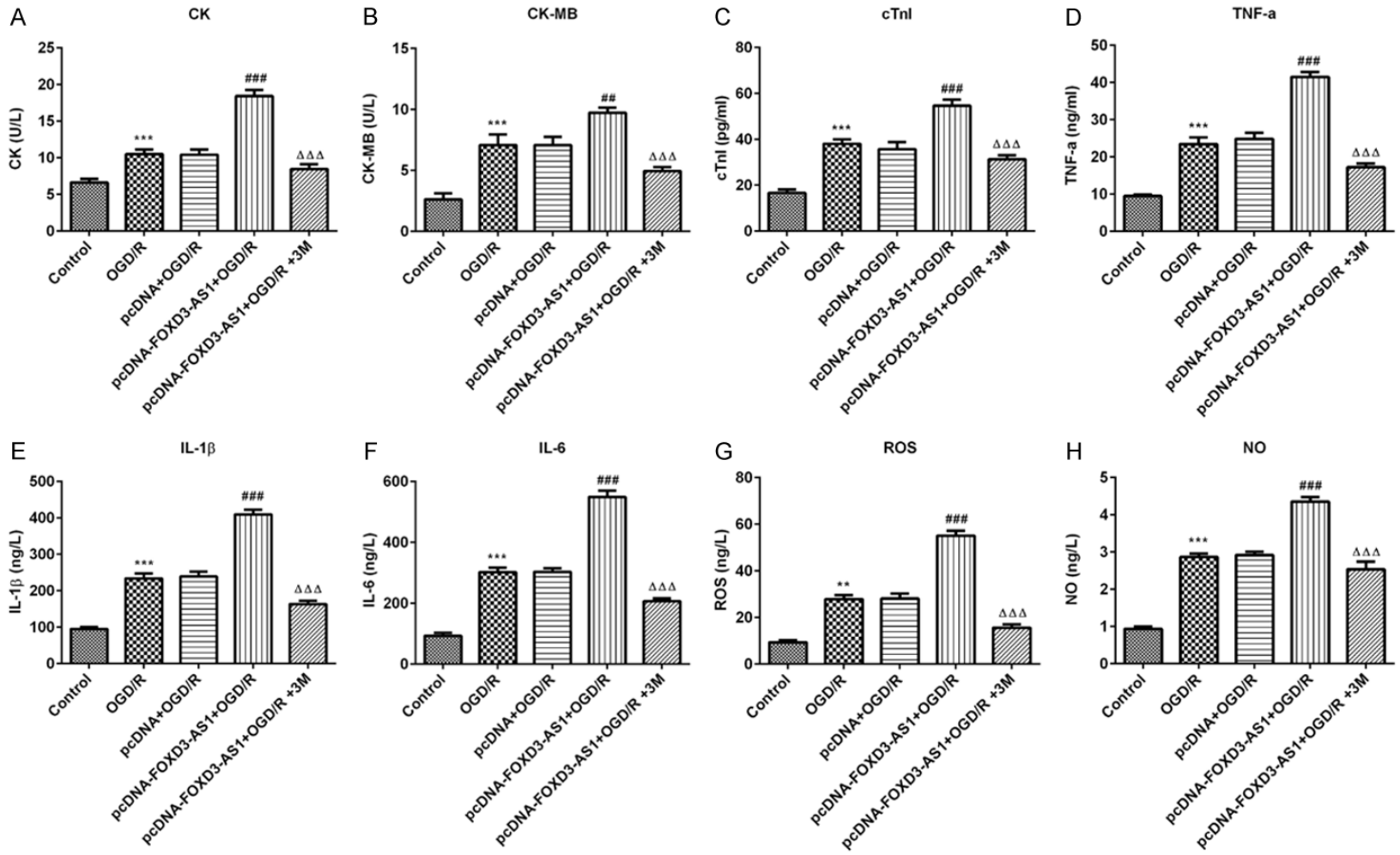

Figure 4. LncRNA FOXD3-AS1 overexpression aggravates myocardial injury by promoting autophagy in OGD/R-induced H9C2 cells. The levels of (A) CK, (B) CK-MB, (C) cTnI, (D) TNF-α, (E) IL-1β, (F) IL-6, (G) ROS and (H) NO were evaluated by kits. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. pcDNA+OGD/R; $^{\Delta\Delta\Delta}P < 0.001$ vs. pcDNA-FOXD3-AS1+OGD/R. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion; CK, creatine kinase; CK-MB, CK isoenzymes; cTnI, cardiac troponin I; IL, interleukin; TNF-α, tumor necrosis factor-alpha; ROS, reactive oxygen.

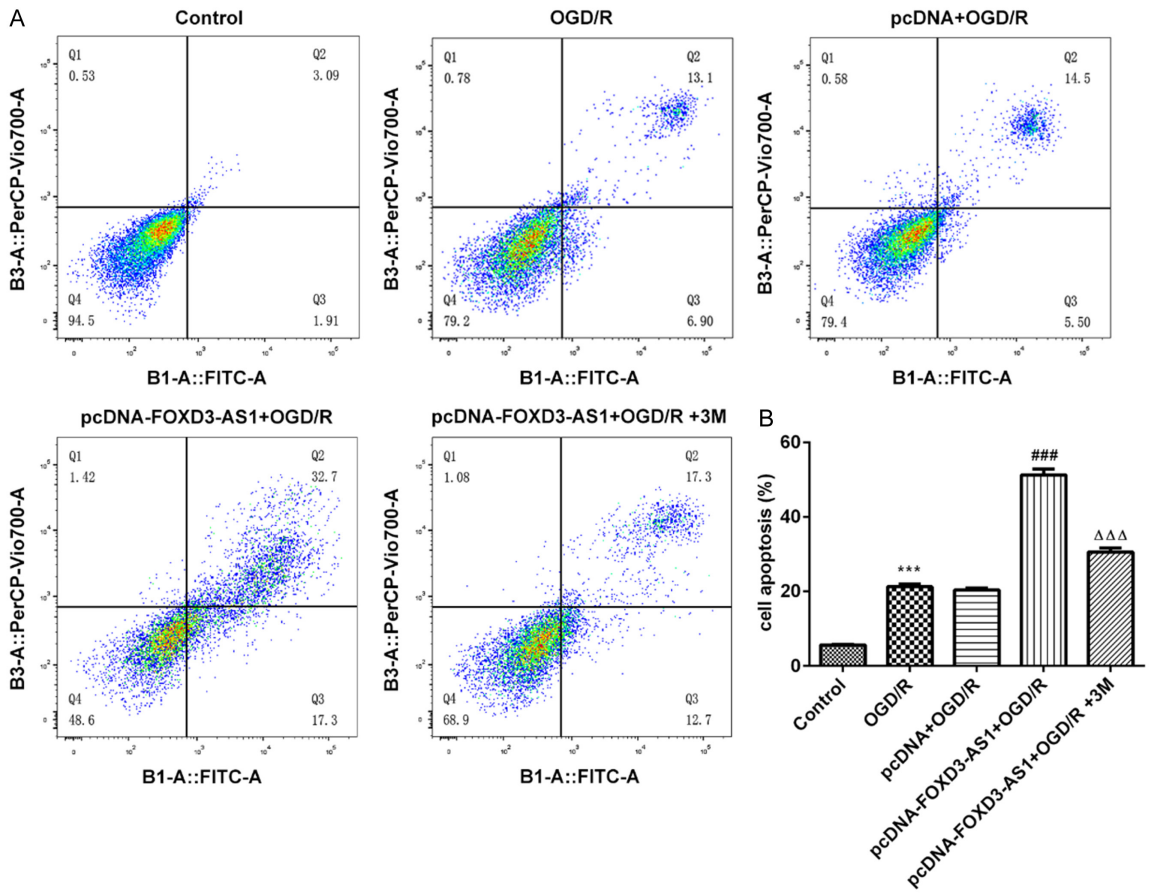

Figure 5. LncRNA FOXD3-AS1 overexpression increases cell apoptosis by promoting autophagy in OGD/R-induced H9C2 cells. A. Cell apoptosis was assessed by flow cytometry analysis. B. Cell apoptosis was quantified. $^{***}P < 0.001$ vs. Control; $^{###}P < 0.001$ vs. pcDNA+OGD/R; $^{ΔΔΔ}P < 0.001$ vs. pcDNA-FOXD3-AS1+OGD/R. LncRNA, long non-coding RNA; TUG1, taurine up-regulated gene 1; OGD/R, oxygen-glucose deprivation and reperfusion. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion.

including myocardial disease, and myocardial I/R injury is famous as a major cause of death around the world [19]. It is still the focus of intense research to enough understand the mechanisms of myocardial I/R injury and seek novel therapeutic strategies. In view of this, we performed a study to explore the regulatory effect of lncRNA FOXD3-AS1 in myocardial I/R injury, and our study ascertain that lncRNA FOXD3-AS1 is able to aggravate I/R injury of cardiomyocytes through promoting autophagy.

Autophagy is a conserved catabolism process, which has been reported plays a crucial role in the process of I/R injury [20]. Report has demonstrated previously that lncRNA AK088388 could regulate autophagy by competitively bind to miR-30a and eventually led to cell damage in I/R-induced myocardial injury [21]. In addition, a previous study unveiled that suppression of lncRNA-HRIM could enhance cell viability by decreasing autophagy during I/R [22]. Importantly, it has been well reported that the expression of lncRNA FOXD3-AS1 was increased in lung hypoxic injury, which promoted lung epithelial cell death [17]. Based on the above studied, whether lncRNA FOXD3-AS1 can regulate myocardial I/R injury by affecting autophagy draw our research interests. In our study, we found that FOXD3-AS1 overexpression augmented the levels of autophagy-associated genes LC3II, Beclin1 and ATG5 accompanied by a reduction of p62 expression, which were in accordance with previous studies [23, 24]. The finding confirmed that FOXD3-AS1 promoted autophagy in OGD/R-induced H9C2 cells.

Mounting evidence supported that CK, CK-MB and cTnI were markers reflecting myocardial injury and were widely used to evaluate the

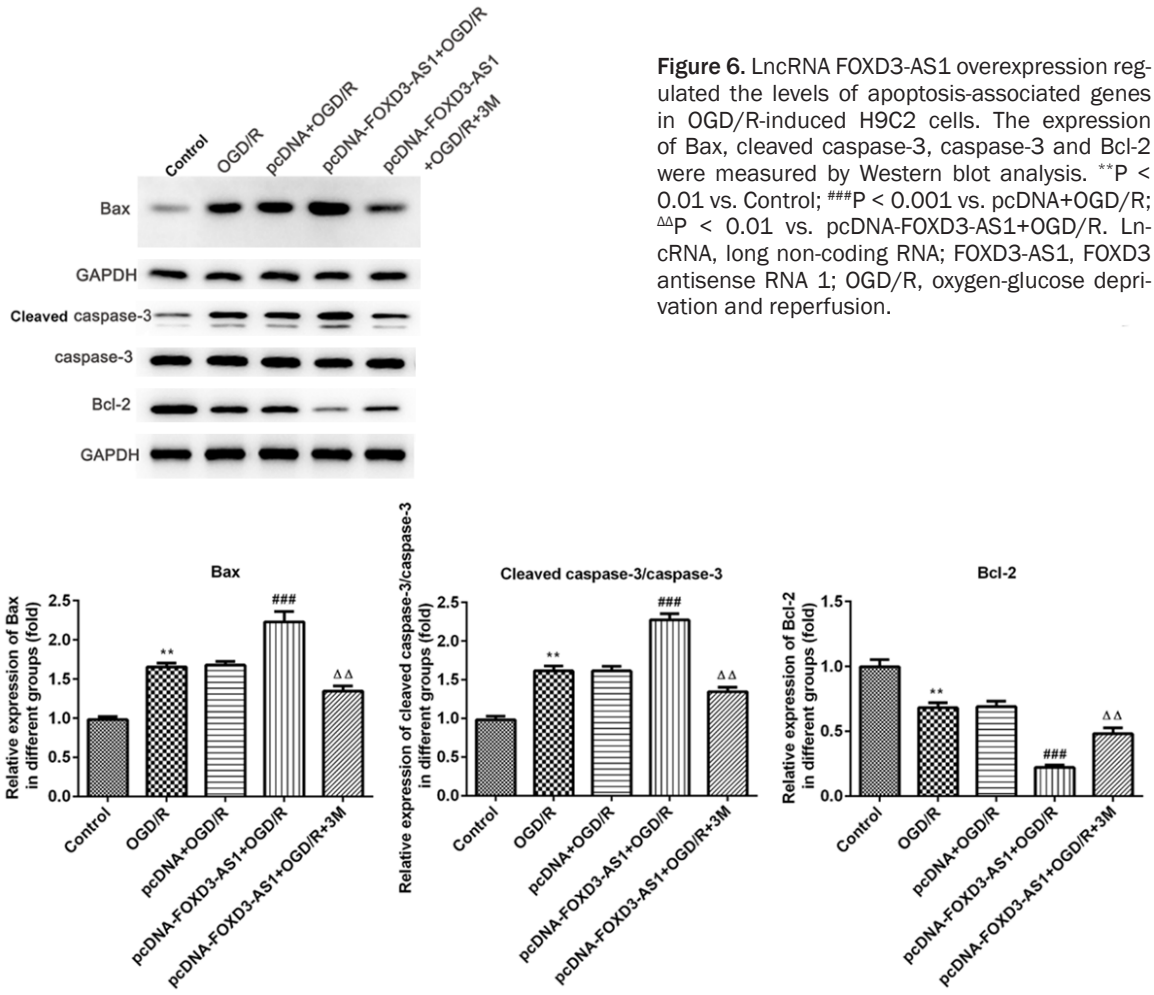

Figure 6. LncRNA FOXD3-AS1 overexpression regulated the levels of apoptosis-associated genes in OGD/R-induced H9C2 cells. The expression of Bax, cleaved caspase-3, caspase-3 and Bcl-2 were measured by Western blot analysis. $^{**}P < 0.01$ vs. Control; $^{###}P < 0.001$ vs. pcDNA+OGD/R; $^{ΔΔ}P < 0.01$ vs. pcDNA-FOXD3-AS1+OGD/R. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion.

extent of myocardial injury in many studies [25, 26]. A significantly increasing trend of CK, CK-MB and cTnI were found in the culture medium of OGD/R-induced H9C2 cells compared with normoxia group, which was in accordance with the previous study [25]. Following overexpression of FOXD3-AS1, the levels of above factors were remarkably augmented, which were reduced after treatment with autophagy inhibitor 3 M. During myocardial ischemia-reperfusion, a large amount of pro-inflammatory cytokines were released and plenty of ROS and NO were produced, which play a crucial role in the etiopathogenesis of myocardial IR injury and lead to severe cardiac damage [27-29]. We found that intervene with 3 M reversed the increase of TNF-α, IL-1β, IL-6, ROS and NO levels induced by FOXD3-AS1 overexpression in OGD/R-induced H9C2 cells, which were in line with the results of CK, CK-MB and cTnI. These results ascertain that FOXD3-AS1 aggravates myocardial injury by promoting autophagy in OGD/R-induced H9C2 cells.

Numerous studies unveiled that autophagy and apoptosis are associated with myocardial I/R injury [30, 31]. A previous study reported that sevoflurane can protect against cardiomyocyte apoptosis induced by hypoxia/reoxygenation via suppressing autophagy [32]. Compound K is able to defense I/R injury via suppressing autophagy-mediated apoptosis [33]. Importantly, emerging evidence supports the notion that lncRNA FOXD3-AS1 regulates apoptosis and promotes cell death in lung hypoxic injury [17]. In the current study, the protein levels of Bax, cleaved caspase-3 and Bcl-2 were significantly altered after treatment with 3 M compared with transfection with pcDNA-FOXD3-AS1 alone, which indicated that FOXD3-AS1 increases apoptosis of H9C2 cells by promoting autophagy. In addition, reports have demonstrated previously that NF-κB/iNOS/COX-2 signaling suppression could alleviate autophagy and apoptosis of Hippocampus [34]. In our study, we found that overexpression of FOXD3-AS1 increased the expression of p-NF-κB p65,

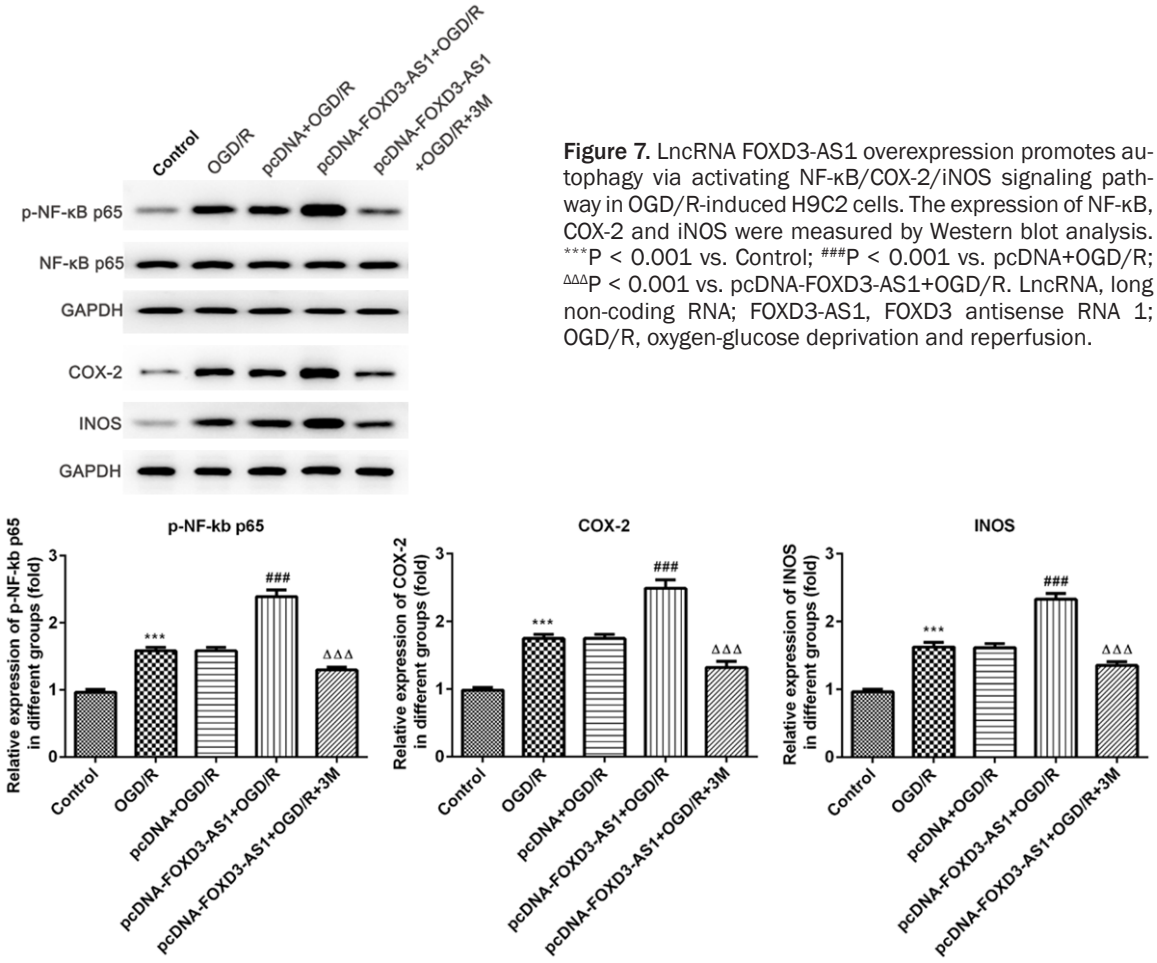

Figure 7. LncRNA FOXD3-AS1 overexpression promotes autophagy via activating NF-κB/COX-2/iNOS signaling pathway in OGD/R-induced H9C2 cells. The expression of NF-κB, COX-2 and iNOS were measured by Western blot analysis. $^{***}P < 0.001$ vs. Control; $^{###}P < 0.001$ vs. pcDNA+OGD/R; $^{ΔΔΔ}P < 0.001$ vs. pcDNA-FOXD3-AS1+OGD/R. LncRNA, long non-coding RNA; FOXD3-AS1, FOXD3 antisense RNA 1; OGD/R, oxygen-glucose deprivation and reperfusion.

iNOS and COX-2, and intervene with 3 M reversed this increase. Overall, we confirm that FOXD3-AS1 promotes autophagy via activating NF-κB/COX2/iNOS signaling pathway in OGD/R-induced H9C2 cells.

Conclusion

In summary, the present study demonstrated that FOXD3-AS1 aggravates myocardial I/R injury of cardiomyocytes through promoting autophagy, which is regulated by activating NF-κB/iNOS/COX2 signaling pathway. This study provides insights into the pathogenesis of myocardial I/R injury involving lncRNAs, which potentially serve as novel targets for the development of diagnostic and therapeutic agents.

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Disclosure of conflict of interest

None.

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