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TGF-β1 inhibits apoptosis of cardiomyocytes H9c2 by regulating autophagy and ERK pathway

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Abstract. This study aimed to explore the expression and mechanism of transforming growth factor β1 (TGF-β1) in oxygen glucose deprivation reperfusion (OGD/R)-induced ischemia/reperfusion (I/R) injury. An OGD/R model was established in cardiomyocytes H9c2, resulting in upregulation of Beclin-1 and LC3II/LC3I expression. Upon overexpression of TGF-β1, the viability of OGD/R-induced H9c2 cells was enhanced, while apoptosis was suppressed by downregulating Bax and upregulating Bcl-2. Additionally, TGF-β1 overexpression promoted autophagy in OGD/R-induced H9c2 cells by further upregulating the levels of Beclin-1 and LC3II/LC3I. Importantly, treatments with 3-methyladenine (3-MA), an autophagy inhibitor, and U0126, an extracellular signal-related kinases 1 and 2 (ERK1/2) inhibitor, significantly inhibited cell viability, increased intracellular reactive oxygen species levels, promoted cell apoptosis (by upregulating Bax and downregulating Bcl-2), and inhibited cell autophagy (by downregulating Beclin-1 and LC3II/LC3I) in OGD/R-induced H9c2 cells with TGF-β1 overexpression. Additionally, OGD/R induction significantly increased the levels of p-ERK, p-P38, and p-JNK, which were further enhanced by TGF-β1 overexpression. U0126 treatments significantly downregulated the p-ERK compared to OGD/R-induced H9c2 cells with TGF-β1 overexpression. Our study suggests that TGF-β1 could inhibit the growth of cardiomyocytes H9c2 by regulating autophagy and ERK pathways, providing a new theoretical basis for the treatment and prevention of OGD/R in clinical practice.

Key words: TGF-β1 — OGD/R — Autophagy — Apoptosis — ERK pathway

Introduction

Cardiovascular disease (CVD) is the global leading cause of morbidity and mortality, with 17.9 million deaths annually, constituting 31% of all registered deaths worldwide, according to the latest World Health Organization data from 2017 (Rodrigo et al. 2022). Ischemia/reperfusion (I/R) injury emerges as a significant factor leading to heart failure, CVD, and sudden death, particularly prevalent in the Western world. The process of reperfusion, while essential, contributes to increased infarct size, triggering oxidative stress and damage. Therefore, there is an urgent need for advancements in effective cardiac treatment and novel strategies to address this critical health challenge.

Currently, there is a growing body of research focused on autophagy and apoptosis in I/R injury. Autophagy regulation has been identified to play a key role in cardiovascular function; however, excessive autophagy has been implicated in the pathogenesis of disease (Ren and Zhang 2018; Wu

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et al. 2019). Notably, instances of autophagy and apoptosis coexisting within the same cell have been observed (Fan et al. 2020). Previous studies have indicated that autophagy can suppress apoptosis by maintaining cellular homeostasis (Zheng et al. 2022), with autophagy acting in an anti-apoptotic manner and preceding apoptosis. Conversely, apoptosis can hinder autophagy through caspases (Pfister 2019). Impaired autophagy has been associated with the overaccumulation of reactive oxygen species (ROS) in the mitochondria, leading to tissue damage (Ruart et al. 2019). The autophagy program maintains normal cardiovascular function by degrading damaged organelles and molecules, thereby eliciting apoptotic signals (Cai et al. 2022). Moreover, the precise role of autophagy in CVD warrants further investigation in future studies.

Previous studies have indicated that transforming growth factor β1 (TGF-β1) is associated in various diseases, including tissue fibrosis, cancer progression, and CVD (Zhu et al. 2017). The active signaling of TGF-β1 is transmitted through specific receptors, subsequently activating downstream signaling pathways by recruiting and phosphorylating receptor-regulated Smad proteins (Sha et al. 2018). Elevated concentrations of TGF-β1 in mouse plasma have been associated with severe renal fibrosis, highlighting its significant role in disease pathogenesis (Zhu et al. 2017; Németh et al. 2019). Moreover, previous studies have associated TGF-β1 to gene transcription, skin recovery, fibrosis in various cancers, tissue fibrosis, and CVD (Yao et al. 2020; Ma et al. 2021; Weng et al. 2023). Studies have presented conflicting findings regarding the role of TGF-β1-mediated autophagy, with some suggesting a protective effect against cell apoptosis, while others indicating a potential for cell injury (Shrestha et al. 2016; Gifford et al. 2021). The precise impact of autophagy on cardiomyocytes and its underlying mechanisms warranted further investigation. In this study, we aimed to explore the relationship between TGF-β1 and autophagy in an I/R injury cell model induced by oxygen glucose deprivation reperfusion (OGD/R).

Materials and Methods

Reagents and antibodies

Cardiomyocyte H9c2 cells were procured from the Cell Bank of Shanghai Academy of Chinese Sciences (Shanghai, China). Fetal bovine serum (FBS) was sourced from Gibco (Grand Island, NY, USA), while Dulbecco's modified eagle medium (DMEM) culture medium was obtained from Hyclone (Waltham, MA, USA). Real-time quantitative polymerase chain reaction (qRT-PCR) kits and cDNA synthesis kits were acquired from Takara (Takara tech., Japan), and RIPA lysate was purchased from Invitrogen (California, USA). The Cell Counting Kit-8 (CCK-8) was obtained from Beyotime (Shanghai, China), along with the following antibodies from Abcam (Cambridge, UK): anti-ERK antibody (cat no. ab184699), anti-p-ERK antibody (cat no. ab214036), anti-p-p38 antibody (cat no. ab178867), anti-p38 antibody (cat no. ab170099), anti-JNK antibody (cat no. ab288739), anti-p-JNK antibody (cat no. 307802), anti-Bcl-2 antibody (cat no. ab182858), anti-Bax antibody (cat no. ab32503), anti-Beclin-1 antibody (cat no. ab207612), and anti-LC3 antibody (cat no. ab63817). Additionally, the corresponding horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG secondary antibody (cat no. SA00001-2) and goat anti-mouse IgG secondary antibody (cat no. SA00001-1) were procured from Proteintech (Wuhan, China).

Cell culture and transfection

The H9c2 cells were cultured in DMEM containing 10% FBS and 100 U/ml penicillin and streptomycin, and maintained in an incubator with 5% CO_2 at 37°C.

For cell transfection, H9c2 cells were transfected with pcDNA3.1-NC (empty vector, 4 μg) or pcDNA3.1-TGF-β1 (overexpression vector, 4 μg) using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) based on the manufacturer's instructions, as previously reported (Kim et al. 2020). Briefly, H9c2 cells were seeded into a 96-well plate at a density of 1×10^4 cells/well, and cultured overnight. On the next day, the cell medium was changed to serum-free medium, and then were transfected with 4 μg pcDNA3.1-TGF-β1 or 4 μg pcDNA3.1-NC using Lipofectamine 2000. After cultured for 6 hours, the medium was replaced with complete medium. After cultured for another 48 hours, total RNA was isolated from the cells with different treatment, and the expression of TGF-β1 were determined using quantification real-time pCR (qRT-PCR) to assess the transfection efficiency.

To investigate the effects of TGF-β1 on an I/R injury cell model induced by OGD/R, rat cardiomyocytes H9c2 were divided into three groups: control, OGD/R+pcDNA3.1, and OGD/R+pcDNA3.1-TGF-β1 groups. For cells in the OGD/R+pcDNA3.1 and OGD/R+pcDNA3.1-TGF-β1 groups, cardiomyocytes H9c2 were firstly transfected with pcDNA3.1 or pcDNA3.1-TGF-β1 for 6 hours, and then were transferred into sugar-free, serum-free, and hypoxic DMEM medium (nitrogen pre-anoxia for 10 min), as well as cultured in an incubator filled with 95% nitrogen and 5% CO_2 at 37°C for 3 hours. Following this, the medium was replaced with normal medium, and the cells were reoxygenated for 3 hours in a 37°C incubator with 95% air + 5% CO_2.

To investigate the roles of ERK pathways in TGF-β1 regulation of OGD/R-induced I/R injury cells in H9c2 cells, the cells were divided into three groups: OGD/R+pcDNA3.1-TGF-β1, OGD/R+pcDNA3.1-TGF-β1+3-MA (an autophagy

inhibitor), and OGD/R+pcDNA3.1-TGF-β1+U0126 (an ERK1/2 inhibitor, 10 μm) groups.

Flow cytometry analysis

The cell apoptosis detection kit (for flow cytometry) was used to measure the ratio of apoptotic cells, following the instructions provided with the kit. Briefly, PI/FITC was diluted using a phosphate-buffered solution (PBS). Upon collection, cell pellets were treated with propidium iodide (PI) solution. The cell samples were filtered through a 200-mesh filter and analyzed using a flow cytometer (BD Accuri C6 plus).

Cell viability assay

Cell Counting Kit-8 (CCK-8) was employed for cell proliferation detection following the manufacturer's protocol. The cells were seeded into 6-well plates and treated with the respective treatments. Subsequently, they were incubated with CCK-8 solution. The cell viability was assessed on a microplate reader at 450 nm.

Electron microscopic observation

Samples were fixed with 4% paraformaldehyde overnight and rinsed with PBS three times. Subsequently, the cells underwent osmication, dehydration, and embedding in pale gold thin sections. Images were captured using the AMT XR611 camera attached to the microscope.

Western blot analysis

After the cells were stimulated with OGD/R, the proteins were extracted and quantified using the bicinchoninic acid assay (BCA) method. The extracted proteins underwent sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were subsequently transferred to a polyvinylidene difluoride (PVDF) membrane. After washing, the membranes were incubated with the corresponding primary antibodies at 4°C overnight. Following three washes, the HRP-secondary antibody was diluted at 1:5000 and incubated for 1 hour at room temperature. In this experiment, β-actin was used as an internal reference, and ImageJ software was employed for image analysis.

qRT-PCR

The total RNAs of the cultured cells were extracted according to the manufacturer's instructions using Trizol reagent. The primeScript 1st Strand cDNA Synthesis Kit (Takara tech., Japan) was used for first-strand cDNA synthesis, facilitated by the proFlex^{TM} 3×32-well PCR System (Applied Biosystems). Subsequently, the StepOne Real-Time PCR System (Applied Biosystems) and SYBR Green PCR kit were utilized to measure the expression levels of the target genes. The forward and reverse primers of β-actin were 5'-CGTTGACATCCGTAAAGACCTC-3' and 5'-TAGGAGCCAGGGCAGTAATCT-3', respectively. The forward and reverse primers of TGF-β1 were 5'-GTGGCTGAACCAAGGAGACG-3' and 5'-AGGTGTTGAGCCCTTTCCAG-3', respectively.

Statistical analysis

The experimental data were summarized as mean ± standard deviation (SD) of three replicates. Statistical analysis was conducted using GraphPad prism 8 software (GraphPad Software, MA, USA). For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by the Bonferroni method was performed. For comparisons between two groups, the unpaired Student's *t* test was used. A p-value < 0.05 was considered statistically significant.

Results

TGF-β1 overexpression increased the viability and inhibited the apoptosis of cardiomyocytes H9c2 induced by OGD/R.

TGF-β1 plays a key role in intestinal cell and immune regulation, and has been shown to improve mucosal IgA dysfunction and ecological dysbiosis following intestinal I/R injury in mice. However, its roles in myocardial I/R injury remain unclear. To investigate the effects of TGF-β1 on the growth and autophagy of H9c2 cells induced by OGD/R, H9c2 cells with TGF-β1 overexpression were firstly constructed with pcDNA3.1-TGF-β1 using Lipofectamine 2000 through the cell transfection method, and then treated with OGD/R. It was observed that there was no significant difference in TGF-β1 expression between the control and pcDNA3.1 groups (p > 0.05). However, upon transfection with pcDNA3.1-TGF-β1, TGF-β1 expression was significantly increased compared to the control cells (p < 0.05, Fig. 1A). These findings indicate that the H9c2 cells with TGF-β1 overexpression were successfully constructed and can be used for further experiments.

Subsequently, the viability and apoptosis of H9c2 cells induced by OGD/R were assessed by CCK-8 and PI staining followed by flow cytometry, respectively. The results revealed that OGD/R treatment significantly decreased cell viability and induced apoptosis compared to control H9c2 cells (p < 0.05). Conversely, compared to H9c2 cells treated with OGD/R alone, TGF-β1 overexpression evidently increased cell viability and inhibited apoptosis of H9c2 cells induced by OGD/R (p < 0.05, Fig. 1B,C). Additionally, Bax is a pro-apoptotic protein, while Bcl-2 is an anti-apoptotic protein. Western blot analysis demonstrated that OGD/R treatment markedly upregulated Bax expression and downregulated Bcl-2 expression compared to control H9c2 cells (p < 0.05). However, TGF-β1 overexpres-

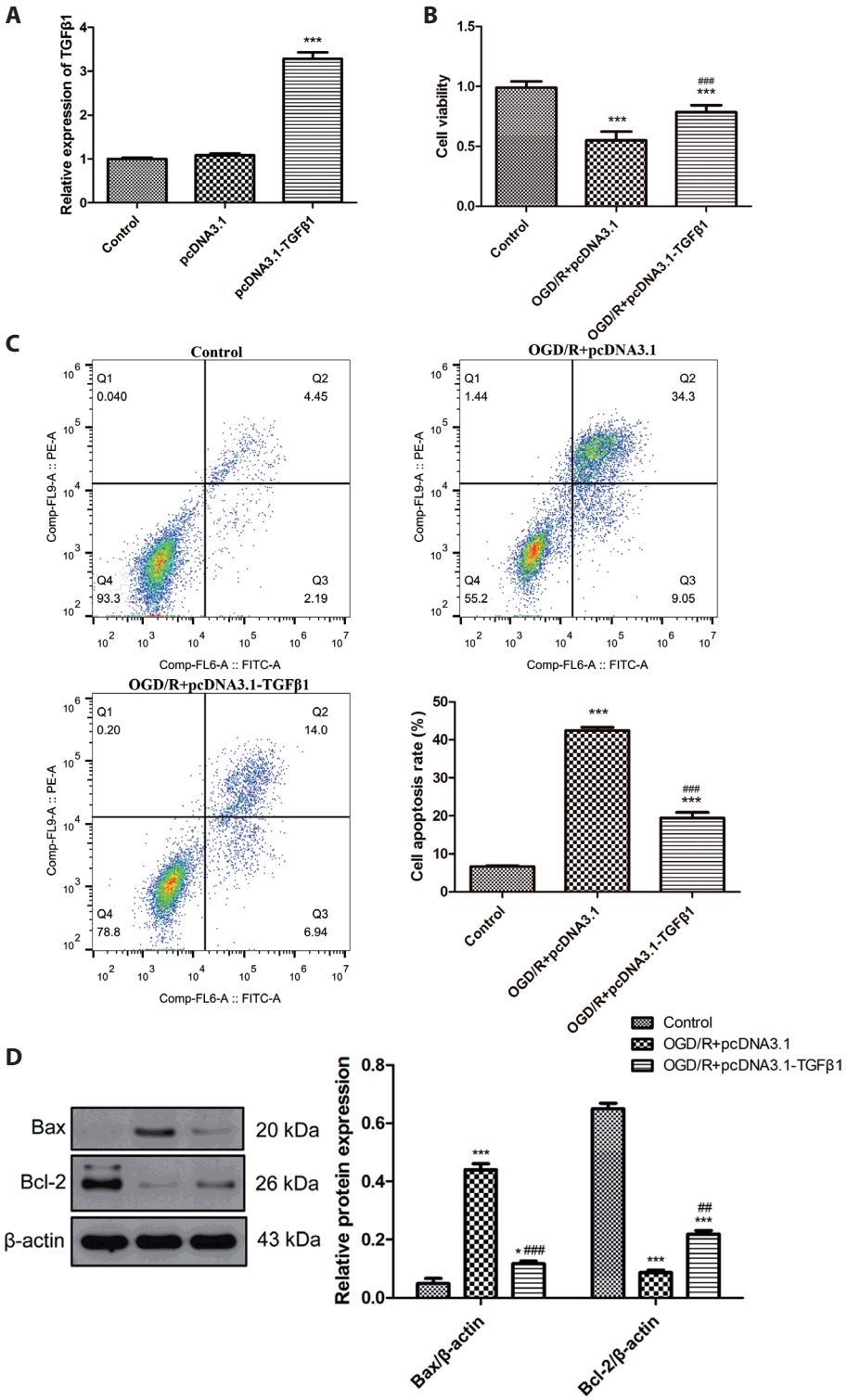

Figure 1. TGF-β1 increased the viability while inhibiting the apoptosis of cardiomyocytes H9c2 induced by oxygen glucose deprivation reperfusion (OGD/R). H9c2 cells transfected with pcDNA3.1-TGF-β1, and then treated with OGD/R. **A.** The mRNA levels of TGF-β1 in the control, pcDNA3.1, and pcDNA3.1-TGF-β1 groups (n = 3). Cell counting kit-8 (CCK-8) assay detecting the cell viability (**B**), flow cytometry detecting the apoptosis rate (**C**) and Western blot analysis detecting the protein expression levels of Bax and Bcl-2 (**D**) in the control, OGD/R+pcDNA3.1, and OGD/R+pcDNA3.1-TGF-β1 groups (n = 3). * $p < 0.05$, *** $p < 0.001$ *vs.* control group; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ *vs.*OGD/R+pcDNA3.1 group. β-actin was used as an invariant internal control for calculating protein-fold changes.

sion significantly reversed the effects of OGD/R treatment on Bax and Bcl-2 expression ($p < 0.05$, Fig. 1D).

TGF-β1 overexpression promoted autophagy of OGD/R-induced cardiomyocytes H9c2

Autophagy regulation has been demonstrated to play a key role in CVD function and can inhibit apoptosis by maintaining cell homeostasis. The autophagy of cells subjected to different treatments was observed under transmission electron microscopy. It was observed that there was no significant difference in the number of autophagosomes between the control and OGD/R+pcDNA3.1 groups ($p > 0.05$). However, the number of autophagosomes in the TGF-β1 overexpression group was significantly higher than that in the control group ($p < 0.05$, Fig. 2A). Beclin-1 and LC3I/II are proteins related to autophagy. Western blot analysis revealed that OGD/R treatment significantly increased the protein expression levels of Beclin-1 and LC3II/LC3I compared to the control group ($p < 0.05$). Furthermore, TGF-β1 overexpression further significantly enhanced their protein expression levels induced by OGD/R ($p < 0.05$, Fig. 2B).

TGF-β1 overexpression regulate the growth of OGD/R-induced H9c2 cells via ERK pathway

Mitogen-activated protein kinase (MAPK), which includes ERK, p38, and JNK, plays a crucial role in regulating various cellular activities such as growth, differentiation, and cell death. To further explore the mechanisms of TGF-β1 on the growth of OGD/R-induced H9c2 cells, the protein expression of p-ERK, p-P38, and p-JNK was detected by Western blot. As depicted in Figure 3A, we observed that compared to control H9c2 cells, the protein expression levels of p-ERK, p-P38, and p-JNK were significantly increased by OGD/R treatment ($p < 0.05$). Furthermore, these levels were further enhanced after TGF-β1 overexpression in OGD/R-induced H9c2 cells ($p < 0.05$, Fig. 3A).

Effects of autophagy and ERK pathway on TGF-β1 overexpression regulating the growth of H9c2 cells induced by OGD/R

To further elucidate the mechanisms of autophagy and the ERK pathway in regulating the growth of H9c2 cells induced by OGD/R with TGF-β1 overexpression, we treated the OGD/R-induced H9c2 cells with TGF-β1 overexpression (transfected with pcDNA3.1-TGF-β1) using 3-MA (an autophagy inhibitor) or U0126 (an ERK1/2 inhibitor). There was no significant difference observed in p-ERK expression between the OGD/R+pcDNA3.1-TGF-β1 and OGD/R+pcDNA3.1-TGF-β1+3-MA groups ($p > 0.05$). However, administration of U0126 significantly downregulated p-ERK expression compared to the OGD/R+pcDNA3.1-TGF-β1 group ($p < 0.05$, Fig. 3B).

Subsequently, the viability, intracellular ROS levels, apoptosis, and autophagy of OGD/R-induced H9c2 cells

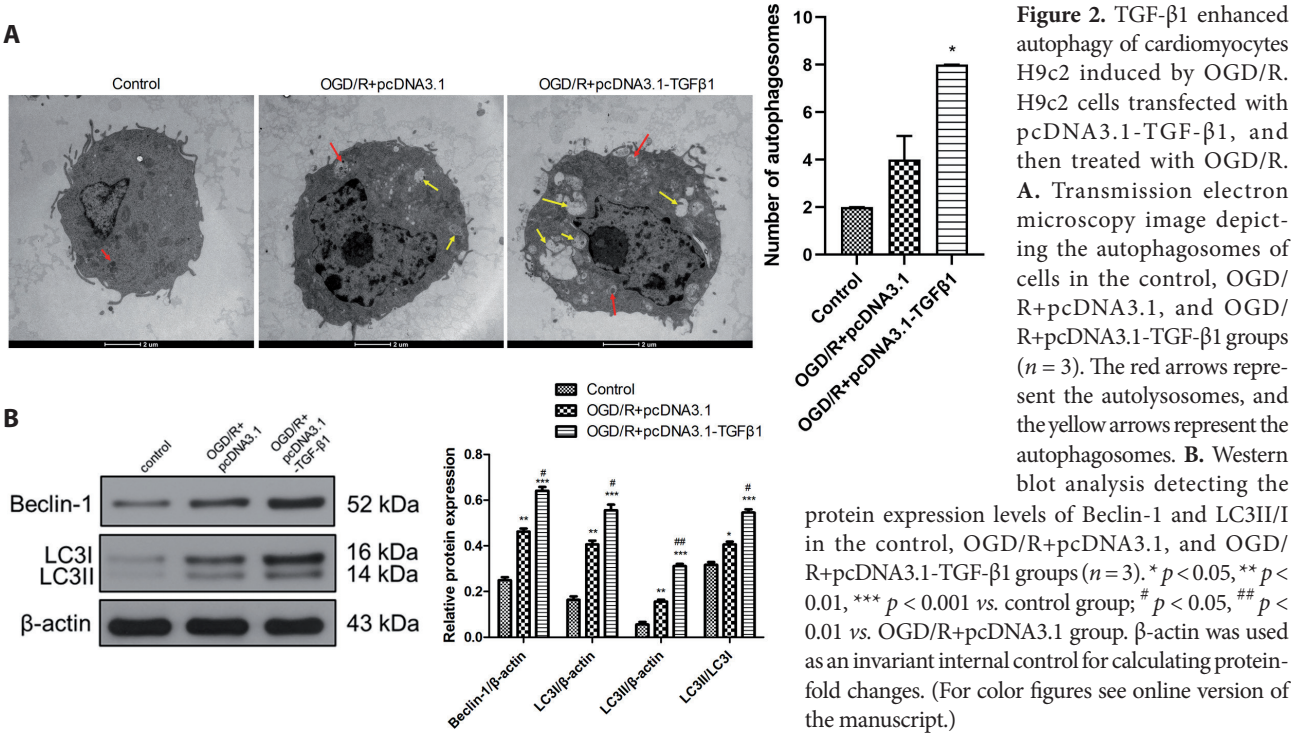

Figure 2. TGF-β1 enhanced autophagy of cardiomyocytes H9c2 induced by OGD/R. H9c2 cells transfected with pcDNA3.1-TGF-β1, and then treated with OGD/R. **A.** Transmission electron microscopy image depicting the autophagosomes of cells in the control, OGD/R+pcDNA3.1, and OGD/R+pcDNA3.1-TGF-β1 groups ($n = 3$). The red arrows represent the autolysosomes, and the yellow arrows represent the autophagosomes. **B.** Western blot analysis detecting the protein expression levels of Beclin-1 and LC3II/I in the control, OGD/R+pcDNA3.1, and OGD/R+pcDNA3.1-TGF-β1 groups ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* control group; # $p < 0.05$, ## $p < 0.01$ *vs.* OGD/R+pcDNA3.1 group. β-actin was used as an invariant internal control for calculating protein-fold changes. (For color figures see online version of the manuscript.)

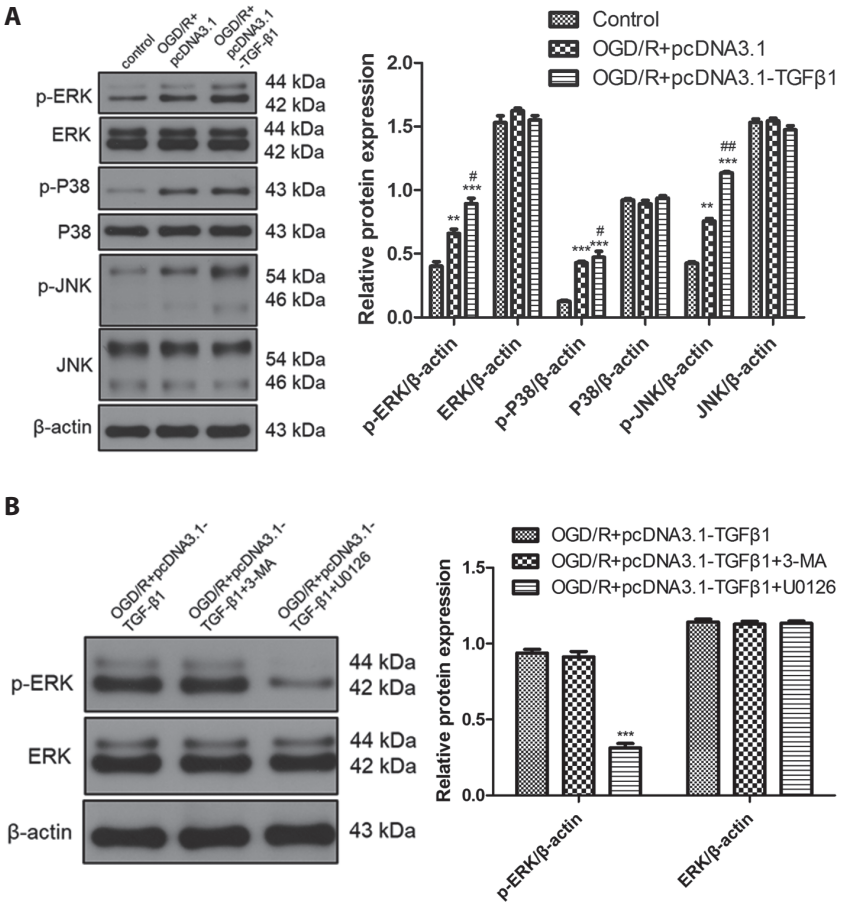

Figure 3. TGF-β1 promoted the growth of cardiomyocytes H9c2 induced by OGD/R through regulating the ERK pathway. H9c2 cells transfected with pcDNA3.1-TGF-β1, and then treated with OGD/R. **A.** Western blot analysis detecting the protein expression levels of p-ERK, p-P38, p-JNK in the control, OGD/R+pcDNA3.1, and OGD/R+pcDNA3.1-TGF-β1 groups ($n = 3$). ** $p <$ 0.01, *** $p < 0.001$ *vs.* control group; # $p <$ 0.05, ## $p < 0.01$ *vs.* OGD/R+pcDNA3.1 group. H9c2 cells transfected with pcDNA3.1-TGF-β1, and treated with OGD/R, followed by 3-MA (an autophagy inhibitor) or U0126 (an ERK1/2 inhibitor). **B.** Western blot analysis detecting the protein expression levels of p-ERK in the OGD/R+pcDNA3.1-TGF-β1, OGD/R+pcDNA3.1-TGF-β1+3-MA, and OGD/R+pcDNA3.1-TGF-β1+ U0126 groups ($n = 3$). *** $p < 0.001$ *vs.* OGD/R+pcDNA3.1-TGF-β1 group. β-actin was used as an invariant internal control for calculating protein-fold changes.

were determined. It was evident that compared with the OGD/R+pcDNA3.1-TGF-β1 group, the cell viability after 3-MA or U0126 treatments was significantly decreased ($p < 0.05$, Fig. 4A), while the intracellular ROS levels were notably increased after treated with 3-MA or U0126 ($p < 0.05$, Fig. 4B). Additionally, both 3-MA and U0126 upregulated the expression of Bax and downregulated the expression of Bcl-2 compared to OGD/R-induced cells with TGF-β1 overexpression ($p < 0.05$, Fig. 4C). Moreover, transmission electron microscopy revealed that 3-MA or U0126 treatment reduced the number of autophagosomes in the OGD/R-induced cells with TGF-β1 overexpression (Fig. 4D). Furthermore, both 3-MA and U0126 significantly downregulated the expression of Beclin-1 and LC3II/LC3I compared to the OGD/R-induced cells with TGF-β1 overexpression ($p < 0.05$, Fig. 4E).

Discussion

Myocardial tissue I/R injury in patients is associated with poor clinical outcomes (Du et al. 2020). OGD/R in cardiomyocytes served as a model to stimulate I/R injury *in vitro*. In our study, we constructed an I/R injury model using OGD/R and observed that OGD/R decreased the expression of TGF-β1, inhibited the viability of H9c2 cells, and induced their apoptosis and autophagy. However, overexpression of TGF-β1 reversed the effects of OGD/R on cell viability and apoptosis, while further promoting autophagy. Furthermore, treatment with 3-MA or U0126 upregulated the expression of p-ERK, p-P38, and p-JNK, inhibited cell viability, increased intracellular ROS levels, and suppressed autophagy in the OGD/R-induced H9c2 cells with TGF-β1 overexpression.

Macro-autophagy is responsible for the degradation of cytoplasmic proteins and intracellular organelles, while micro-autophagy is considered a protective mechanism during I/R injury (Fleming et al. 2022). LC3II/LC3I and Beclin-1 are well-known autophagy-related markers, and an increased LC3-II/LC3-I ratio and Beclin-1 expression are considered hallmarks of autophagy (Tanida et al. 2008; Wang 2015). Following OGD/R injury, mitochondrial autophagy may be enhanced (Zhang et al. 2019; Zhang and Hao 2020). Our research found that OGD/R injury induced autophagy, as evidenced by increased expression of Beclin-1 and LC3, and this trend was further enhanced by TGF-β1 overexpression. Autophagy and apoptosis often occur in

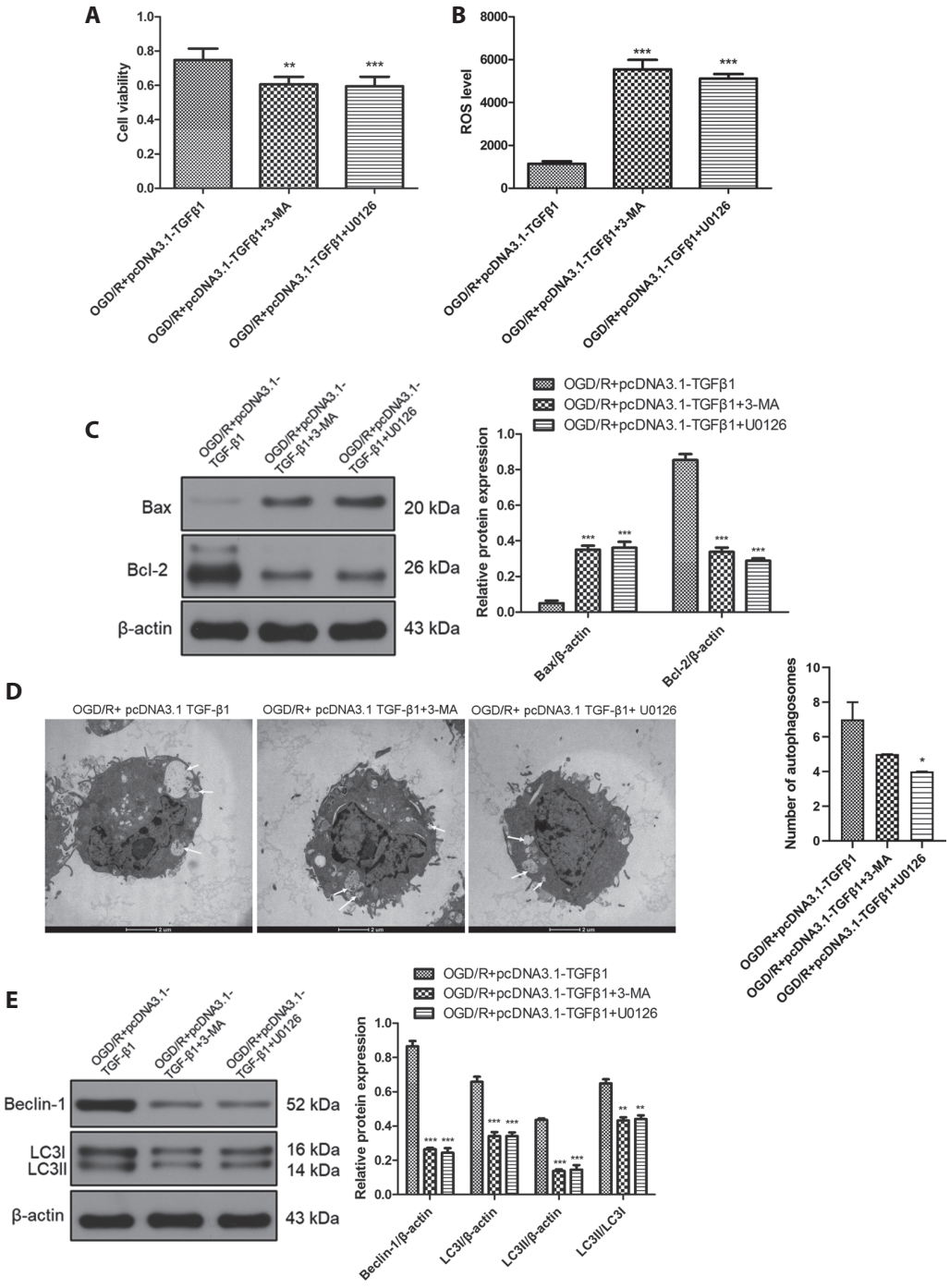

Figure 4. TGF-β1 promoted the growth of cardiomyocytes H9c2 induced by OGD/R by regulating autophagy. H9c2 cells transfected with pcDNA3.1-TGF-β1, and then treated with OGD/R, followed by 3-MA (an autophagy inhibitor) or U0126 (an ERK1/2 inhibitor). CCK-8 assay detecting the cell viability (**A**), the reactive oxygen species (ROS) levels (**B**) and Western blot analysis detecting the protein expression levels of Bax and Bcl-2 (**C**) in the OGD/R+pcDNA3.1-TGF-β1, OGD/R+pcDNA3.1-TGF-β1+3-MA, and OGD/R+pcDNA3.1-TGF-β1+ U0126 groups ($n = 3$). **D.** Transmission electron microscopy depicting the autophagosomes of cells in the OGD/R+pcDNA3.1-TGF-β1, OGD/R+pcDNA3.1-TGF-β1+3-MA, and OGD/R+pcDNA3.1-TGF-β1+ U0126 groups ($n = 3$). The red arrows represent the autolysosomes, and the yellow arrows represent the autophagosomes. **E.** Western blot analysis detecting the protein expression levels of Beclin-1 and LC3II/I in the OGD/R+pcDNA3.1-TGF-β1, OGD/R+pcDNA3.1-TGF-β1+3-MA, and OGD/R+pcDNA3.1-TGF-β1+ U0126 groups ($n = 3$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* OGD/R+pcDNA3.1-TGF-β1 group. β-actin was used as an invariant internal control for calculating protein-fold changes. (For color figures see online version of the manuscript.)

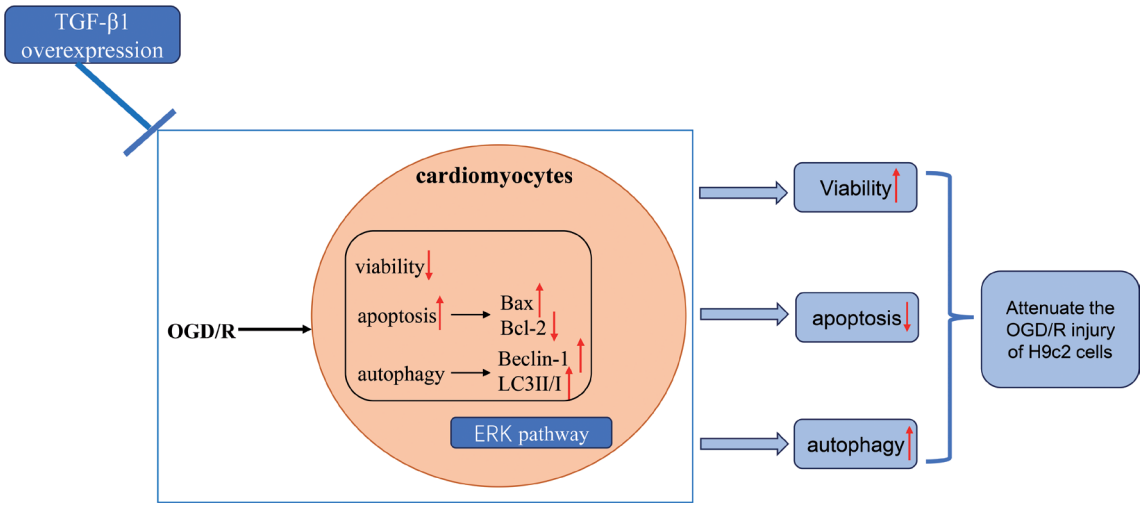

Figure 5. Diagram illustrating the hypothesized mechanism of this research. OGD/R inhibited the viability of H9c2 cells and induced apoptosis through upregulating Bax and downregulating Bcl-2. However, TGF-β1 overexpression could enhance the viability and inhibit the apoptosis (downregulating Bax and upregulating Bcl-2) of OGD/R-induced H9c2 cells, and promote their autophagy by further upregulated the levels of Beclin-1, and LC3II/LC3I, thereby alleviating the OGD/R-induced injury through the ERK pathway.

the same cells, with autophagy frequently inhibiting apoptosis (Sorice 2022). In our study, the ratio of Bax/Bcl-2 was upregulated by OGD/R injury but partly rescued by TGF-β1 overexpression. This study ultimately concluded that upregulation of TGF-F1 could inhibit apoptosis and promote the autophagy of H9c2 cells. Additionally, treatment with 3-MA suppressed autophagy-related gene expression, the number of autophagosomes, and autolysosomes, while U0126 exhibited a similar effect on these changes. The content of LC3II/I protein is positively correlated with the number of autophagosomes, reflecting the extent of autophagy to some degree (Tanida et al. 2008). Binding of Bcl-2 to Beclin-1 inhibits Beclin-1-mediated autophagy by sequestering Beclin-1 away from class III pI3K (Xu and Qin 2019). Moreover, Beclin-1 is necessary for the formation of autophagosomes and induction of autophagy flux (Xu et al. 2017). In summary, OGD/R could increase H9c2 cell injury, while TGF-β1 overexpression could attenuate the growth of OGD-R-induced cell injury and promote autophagy by regulating the expression of apoptosis-related proteins (Bax, and Bcl-2) and autophagy-related markers (Beclin-1, and LC3II/I).

ERK1/2 is a serine/threonine kinase with a variety of intracellular targets that directly mediate stress and growth responses (Mutlak and Kehat 2015). Up to 30% of ERK1/2 proteins in cells are attached to components of the cytoskeleton, and localized to focal adhesion complexes (Yao and Seger 2009). ERK1/2 signaling is the basis of cardiomyocyte growth dynamics, which may be related to changes in cytoskeletal proteins (Le Dour et al. 2022). A previous study has shown that ERK1/2 signaling could affect the cardiomyocyte non-sarcomeric actin cytoskeletal network, thereby regulating the ability of cardiomyocytes to grow in length and/or width (Grimes et al. 2024). Another study demonstrated that crosstalk between ERK1/2 and MAPK signaling pathways is involved in cell migration, differentiation, apoptosis, proliferation, motility, and neuronal development, thereby regulating various physiological functions, such as inflammation, cardiovascular dysfunction, cancer, and cardiac fibrosis (Koga et al. 2019). Therefore, ERK1/2 MAPK signaling is a widely recognized as a modulator in the cardiovascular system (Koga et al. 2019), suggesting its potential as a therapeutic target for cardiovascular diseases. Our findings confirmed this notion, as TGF-β1 overexpression was shown to promote cell viability and reduce ROS levels. Conversely, inhibition of ERK1/2 by U0126 significantly impaired cell viability and increased intracellular ROS levels. Additionally, U0126 treatment enhanced the upregulation of Bax expression and the downregulation of Bcl-2 expression induced by TGF-β1 overexpression, indicating that ERK1/2 may act upstream of Bax and Bcl-2. These results suggest that TGF-β1 inhibits apoptosis of cardiomyocytes H9c2 by regulating ERK pathways, which was consistent with the findings of previous studies (Chong et al. 2018; Zhang et al. 2022). ROS bursts in mitochondrial complex I are considered a major contributor to I/R injury, with a previous study demonstrating that various factors, such as lipopolysaccharide, can increase the sensitivity of H9c2 cells to glucose and hypoxia/reoxygenation, promoting cell damage through increased ROS production and inducing NLRP3 inflammasome-mediated pyroptosis

(Qiu et al. 2019). In a study by Liu et al. demonstrating the roles of DUSP8 in controlling basal and acute stress (including aging and pathological stress stimulation, oxidative stress, and neurohormonal activation)-induced ERK1/2 signaling in adult cardiomyocytes, influencing cardiomyocyte growth dynamics, contractility, ventricular remodeling, and disease susceptibility (Liu et al. 2016). Another research reported that calpain activation could mediate microgravity-induced myocardial abnormalities in mice through the p38 and ERK1/2 MAPK pathways (Liang et al. 2020). In light of our results, it can be inferred that TGF-β1 overexpression may promote the growth of H9c2 cells induced by OGD/R through the regulation of autophagy and ERK pathways.

However, a few limitations need to be acknowledged, despite the study's promising findings on the protective benefits of TGF-β1 overexpression in OGD/R-induced damage. First, our results may not be as applicable to primary cardiomyocytes or human cell lines due to the dependence on H9c2 cells as the major model system, as well as need to be validate in primary cardiomyocytes and other human cardiomyocyte cell lines, such as AC16 cells. Additionally, to validate the functions and underlying mechanisms of TGF-β1 in I/R injury, more validation using *in vitro* and *in vivo* models is required.

In conclusion, in the I/R injury model induced by OGD/R, TGF-β1 overexpression demonstrated a protective effect against OGD/R-induced injury. Furthermore, TGF-β1 may induce autophagy to promote the apoptosis of H9c2 cells via ERK pathways (Fig. 5). This study provides a theoretical basis for the development of individualized treatment strategies for patients with I/R injury.

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Conflict of interest. The authors declare that they have no conflict of interest.

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