Submitted: 05 June, 2025 Accepted: 05 August, 2025 Published: 08 October, 2025 DOI:10.22514/sv.2025.151

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ORIGINAL RESEARCH

Knockdown of *LTBP4* alleviates OGD/R-induced cardiomyocyte apoptosis and mitochondrial dysfunction, and attenuates the TGF-β signaling pathway

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Abstract

Background: Heart failure (HF) is a prevalent cardiovascular condition that significantly compromises patient health and survival. Latent transforming growth factor-beta binding protein 4 (*LTBP4*), an extracellular matrix protein abundantly expressed in the heart, has been implicated in various pathological processes. But, its specific role in HF pathogenesis is insufficiently characterized. **Methods**: We evaluated protein expression through Western blotting, assessed cell viability through the Cell Counting Kit-8 (CCK-8) assay, and quantified apoptosis by flow cytometry. Additionally, oxidative stress and mitochondrial function were examined by measuring reactive oxygen species (ROS) and adenosine triphosphate (ATP) levels using commercial kits, while mitochondrial membrane potential (MMP) was inspected via JC-1 staining. **Results**: The findings revealed a marked upregulation of *LTBP4* expression in oxygen-glucose deprivation/reperfusion (OGD/R). This elevation in *LTBP4* coincided with a significant increase in apoptosis, mitochondrial dysfunction, and activation of the transforming growth factor-β (TGF-β) signaling pathway. Notably, silencing *LTBP4* effectively mitigated these pathological changes, as evidenced by reduced apoptotic rates, decreased ROS accumulation, preserved ATP production, stabilized MMP, and suppressed TGF-β signaling activity. **Conclusions**: Taken together, these results provide evidence that *LTBP4* knockdown confers protective effects against OGD/R-induced cardiac damage by alleviating apoptosis and mitochondrial dysfunction while concurrently attenuating TGF-β pathway activation, hinting that *LTBP4* may represent a helpful therapeutic target in the amelioration of HF.

Keywords

Heart failure; *LTBP4*; OGD/R; Mitochondrial dysfunction

1. Introduction

Heart failure (HF), a chronic and progressive cardiovascular disorder, is primarily induced by persistent pressure overload [1, 2]. Its development involves a complex interplay of pathophysiological mechanisms, including hyperactivation of the sympathetic nervous system, stimulation of the renin-angiotensin-aldosterone system, generation of pro-inflammatory markers, and progressive cardiomyocyte apoptosis, all of which contribute to cardiac dysfunction and adverse remodeling [3]. Although therapeutic advances have improved symptom management, the overall prognosis for patients with HF remains poor [4], highlighting the urgent need to identify viable molecular targets capable of modulating cardiomyocyte function and thereby mitigating disease progression.

Latent transforming growth factor-beta binding protein 4 (*LTBP4*), an extracellular matrix protein abundantly expressed in the heart, lungs, and other tissues [5, 6], has been implicated in the development of several diseases. Previous studies have clarified that *LTBP4* influences mitochondrial function to ameliorate renal fibrosis [7] and regulates both angiogenesis and mitochondrial integrity in similar contexts [8]. In cardiovascular pathology, specific insertion/deletion of polymorphisms in the *LTBP4* gene can modulate the increased risk of sudden cardiac death in individuals with coronary artery disease [9]. Additionally, *LTBP4* appears to delay cellular senescence and inflammation while modulating mitochondrial dysfunction during the progression of emphysema [10]. Importantly, elevated *LTBP4* expression has been observed in myocardial tissues from HF patients [11], although the precise functional significance of *LTBP4* in the context of HF pathogenesis remains inadequately defined.

Mitochondria represent a central target in hypoxia-induced

cellular injury [12]. Mitochondrial dysfunction disrupts adenosine triphosphate (ATP) production, impairs the oxidative-antioxidative balance, and facilitates the release of pro-apoptotic factors, ultimately leading to apoptosis and further myocardial damage [7]. Despite evidence of *LTBP4* involvement in mitochondrial regulation in other disease models, its role in modulating cardiomyocyte apoptosis and mitochondrial dysfunction remains largely undefined.

In this project, we demonstrated that silencing *LTBP4* effectively mitigates cardiomyocyte apoptosis and mitochondrial dysfunction evoked by oxygen-glucose deprivation/reperfusion (OGD/R). These findings implied that *LTBP4* may become one potential target for HF treatment.

2. Materials and methods

2.1 Cell lines and cell culture

Rat H9C2 cardiomyoblast cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). OGD/R was performed by incubating H9C2 cells in glucose-free medium under hypoxic conditions (1% O_2, 5% CO_2, and 37 °C) for 2 h, and reaeration for 6 h [13–15].

2.2 Cell transfection

Short hairpin RNAs (shRNAs) targeting *LTBP4* (shLTBP4) and a non-targeting negative control (shNC) were purchased from GenePharma (Shanghai, China). The pcDNA3.1 plasmids targeting *LTBP4* (LTBP4) and a non-targeting negative control (OE-NC) were also performed. Transfection for these constructed plasmids was made through Lipofectamine 2000 reagent (11668019, Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol.

2.3 Cell viability assay

H9C2 cells were placed in 96-well plates, and cell viability was measured using the Cell Counting Kit-8 (CCK-8, CK04, Dojindo Laboratories, Kumamoto, Japan). Briefly, 10 μL of CCK-8 solution was added to each well, and absorbance was measured using a spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, Waltham, MA, USA) to evaluate cell viability.

2.4 Reactive oxygen species (ROS) assay

Intracellular ROS levels were assessed through a commercial ROS assay kit (E004-1-1, Nanjing Jiancheng Technology Co., Ltd., Nanjing, China). Cells were treated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA), and ROS levels were subsequently quantified according to the manufacturer's instructions.

2.5 ATP examination

Cellular ATP content was determined using an ATP detection kit (A095, Jiancheng Bioengineering Institute, Nanjing, China). After treatment, H9C2 cells were harvested, centrifuged, and the supernatant was collected. The optical density at 636 nm was measured using a spectrophotometer (Thermo Fisher Scientific, MA, USA).

2.6 Mitochondrial membrane potential (MMP) analysis using JC-1 staining

The cells were treated with 0.5 mL of JC-1 dye solution (46007ES01, Yeasen, Shanghai, China). After staining and centrifugation, cells were resuspended and visualized under a fluorescence microscope (BX41, Olympus, Tokyo, Japan) to evaluate JC-1 aggregate and monomer fluorescence.

2.7 Flow cytometry

Apoptotic cell death was inspected through the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (556547, BD Biosciences, Franklin Lakes, NJ, USA). Cells were stained with 5 μL of FITC Annexin V and 10 μL of propidium iodide (PI), followed by analysis using a flow cytometer (FACSCanto II, BD Biosciences, San Jose, CA, USA).

2.8 Western blot analysis

Total protein was extracted from H9C2 cells through radio immunoprecipitation assay (RIPA) lysis buffer (89901, Thermo Fisher Scientific, Inc., Waltham, MA, USA), and protein concentrations were determined using a bicinchoninic acid assay (BCA) kit. Equal amounts of protein were separated via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Beyotime, Shanghai, China). Following blocking with 5% non-fat milk, membranes were incubated with primary antibodies overnight at 4 °C. Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; ab7090, Abcam, Shanghai, China) for 1 h at room temperature. Protein bands were visualized using a chemiluminescence detection kit (89880, Thermo Fisher Scientific, Inc., Waltham, MA, USA).

The following primary antibodies were used: LTBP4 (1:1000; ab222844, Abcam, Shanghai, China), Bax (1:1000; ab32503, Abcam, Shanghai, China), B-cell lymphoma-2 (BCL-2, 1:500; ab194583, Abcam, Shanghai, China), cleavedcaspase-3 (1:500; ab32042, Abcam, Shanghai, China), caspase-3 (1:2000; ab184787, Abcam, Shanghai, China), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:500; ab8245, Abcam, Shanghai, China) as a loading control.

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

Total RNA was extracted from H9C2 cells through TRIzol reagent (15596018, Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the PrimeScript™ RT Reagent Kit (RR037A, Takara, Dalian, China). Quantitative PCR was conducted using the SYBR Green PCR kit (QPK-201, Toyobo, Osaka, Japan), and the relative expression of *LTBP4* mRNA was calculated through the $2^{-\Delta\Delta Ct}$ method.

2.10 Statistical analysis

All data are presented as the mean ± standard deviation (SD) from at least three independent experiments. Statistical analysis was conducted using GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA). Differences between two groups were assessed by Student's *t*-test, while comparisons among multiple groups were done by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test. A *p*-value of less than 0.05 was set as statistically significant.

3. Results

3.1 LTBP4 expression is upregulated in cardiomyocytes subjected to OGD/R

As displayed in Fig. 1A, LTBP4 protein expression was increased following OGD/R treatment. Consistently, *LTBP4* mRNA levels were also markedly elevated after OGD/R stimulation (Fig. 1B). These findings indicate that OGD/R induces augmentation of *LTBP4* expression in cardiomyocytes.

3.2 LTBP4 knockdown attenuates OGD/R-evoked cardiomyocyte apoptosis

The increased protein expression of LTBP4 under OGD/R condition was effectively reduced by shRNA-mediated knockdown of *LTBP4* (Fig. 2A). OGD/R stimulation significantly decreased cell viability, whereas silencing *LTBP4* restored cell viability to near-baseline levels (Fig. 2B). Flow cytometry revealed that OGD/R markedly increased cardiomyocyte apoptosis, and this response could be alleviated by *LTBP4* knockdown (Fig. 2C). At the molecular level, OGD/R treatment led to upregulation of pro-apoptotic Bax and cleaved caspase-3, accompanied by a reduction in anti-apoptotic BCL-2, while caspase-3 expression remained unchanged; these OGD/R-induced changes were reversed upon *LTBP4* inhibition (Fig. 2D). Collectively, these results suggest that *LTBP4* knockdown mitigates OGD/R-induced apoptosis in cardiomyocytes.

3.3 LTBP4 inhibition alleviates mitochondrial dysfunction induced by OGD/R

In Fig. 3A, intracellular ROS levels were aggrandized in cardiomyocytes following OGD/R exposure, while *LTBP4* knockdown effectively suppressed ROS accumulation. Correspondingly, ATP production was reduced under OGD/R conditions, and this decrease was weakened upon *LTBP4* suppression (Fig. 3B). Moreover, JC-1 staining revealed that OGD/R resulted into a reduction in MMP, a hallmark of mitochondrial dysfunction, which was restored by *LTBP4* knockdown (Fig. 3C). These results collectively indicate that silencing *LTBP4* improves mitochondrial function in cardiomyocytes subjected to OGD/R stress.

3.4 Downregulation of LTBP4 suppresses activation of the TGF-β signaling pathway

OGD/R treatment generated a significant increase in transforming growth factor-β (TGF-β) expression as well as elevated phosphorylation levels of mothers against decapentaplegic homolog 2 (Smad2) and Smad3, as reflected by increased p-Smad2/Smad2 and p-Smad3/Smad3 ratios (Fig. 4). These effects were significantly attenuated following *LTBP4* knockdown, indicating that downregulation of *LTBP4* inhibits activation of the TGF-β signaling pathway under OGD/R conditions.

3.5 LTBP4 affects OGD/R-induced cell apoptosis and mitochondrial dysfunction

The increased LTBP4 protein expression mediated by OGD/R was attenuated after *LTBP4* suppression, but this effect was offset after *LTBP4* overexpression (Fig. 5A). The reduced cell viability mediated by OGD/R was reversed after *LTBP4* inhibition, but this change was further counteracted after *LTBP4* amplification (Fig. 5B). In addition, the elevated cell apoptosis mediated by OGD/R was relieved after *LTBP4* knockdown, but this influence was offset after *LTBP4* upregulation (Fig. 5C). The enhanced ROS level mediated by OGD/R was offset after *LTBP4* silencing, but this impact was rescued after *LTBP4* overexpression (Fig. 5D). The decreased ATP level mediated by OGD/R was reversed after *LTBP4* silencing, but this impact was neutralized after *LTBP4* amplification (Fig. 5E). Moreover, the elevated TGF-β, p-Smad2/Smad2 and p-Smad3/Smad3 protein expression was counteracted after *LTBP4* inhibition, but this effect was further offset after *LTBP4* overexpression (Fig. 5F). In a word, *LTBP4* affects OGD/R-induced cell apoptosis and mitochondrial dysfunction.

4. Discussion

LTBP4 has been clarified in multiple diseases through its regulatory roles in extracellular matrix remodeling and mitochondrial function [7–11]. Importantly, it has been reported that elevated expression of *LTBP4* existed in HF tissue samples [11]. Consistent with these findings, this project uncovered that *LTBP4* expression was significantly upregulated in cardiomyocytes subjected to OGD/R.

Cardiomyocyte apoptosis is one pivotal contributor to the loss of functional myocardial cells, thereby exacerbating HF progression [16, 17]. Inhibiting apoptosis has been shown to reduce adverse ventricular remodeling, improve cardiac function, and enhance the overall prognosis of HF patients [18]. In this study, OGD/R treatment significantly increased cardiomyocyte apoptosis, and these effects could be substantially mitigated by knockdown of *LTBP4*, suggesting a pro-apoptotic role of *LTBP4* under ischemic conditions.

Mitochondrial dysfunction is another hallmark of HF pathophysiology, characterized by impaired bioenergetics [19]. Mitochondrial impairment leads to elevated oxidative stress and promotes cardiomyocyte injury, thereby contributing to disease progression [20]. Considerable attention has been directed toward targeting mitochondrial dysfunction as a therapeutic strategy in the progression of HF. For instance, in-

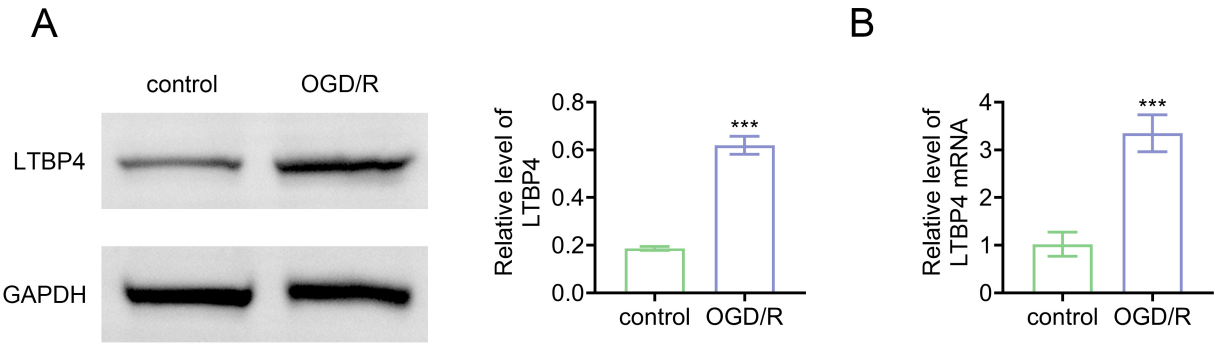

FIGURE 1. ***LTBP4*** **expression is elevated in cardiomyocytes subjected to OGD/R.** (A) Western blot analysis of LTBP4 protein expression in control and OGD/R-treated groups. (B) Quantification of *LTBP4* mRNA expression by RT-qPCR in control and OGD/R-treated groups. ***$p < 0.001$. LTBP4: Latent transforming growth factor-beta binding protein 4; OGD/R: oxygen-glucose deprivation/reperfusion; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

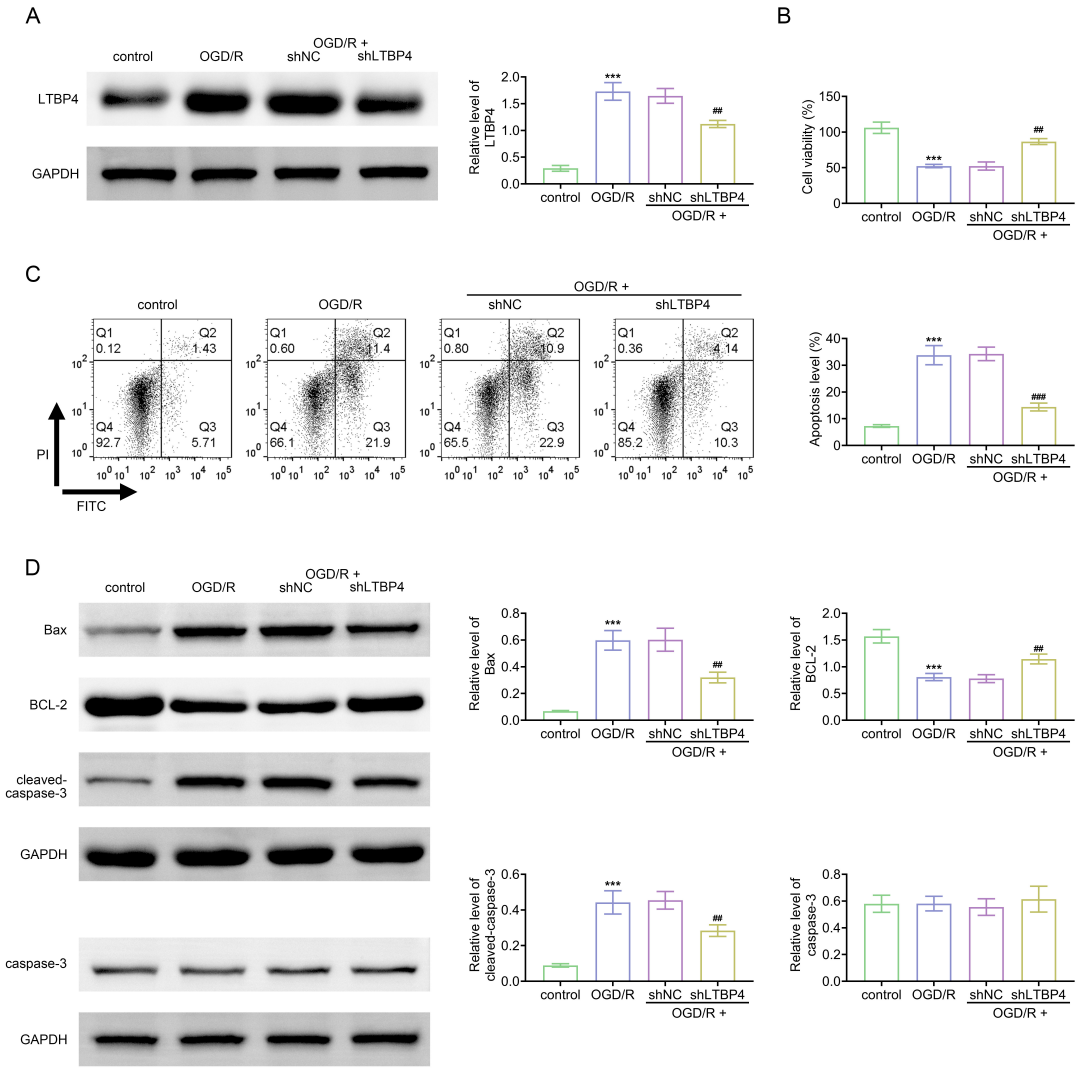

FIGURE 2. ***LTBP4*** **knockdown attenuates OGD/R-induced apoptosis in cardiomyocytes.** H9C2 cells were divided into four groups: control, OGD/R, OGD/R + shNC, and OGD/R + shLTBP4. (A) Western blot analysis of LTBP4 protein expression. (B) Cell viability assessed by CCK-8 assay. (C) Apoptosis evaluated by flow cytometry. (D) Western blot analysis of apoptosis-related proteins, including Bax, BCL-2, cleaved caspase-3, and caspase-3. ***$p < 0.001$ *vs.* control; $^{###}p < 0.001$, $^{##}p < 0.01$ *vs.* OGD/R + shNC. LTBP4: Latent transforming growth factor-beta binding protein 4; OGD/R: oxygen-glucose deprivation/reperfusion; PI: propidium iodide; shNC: Short hairpin negative control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; FITC: Fluorescein isothiocyanate; BCL: B-cell lymphoma.

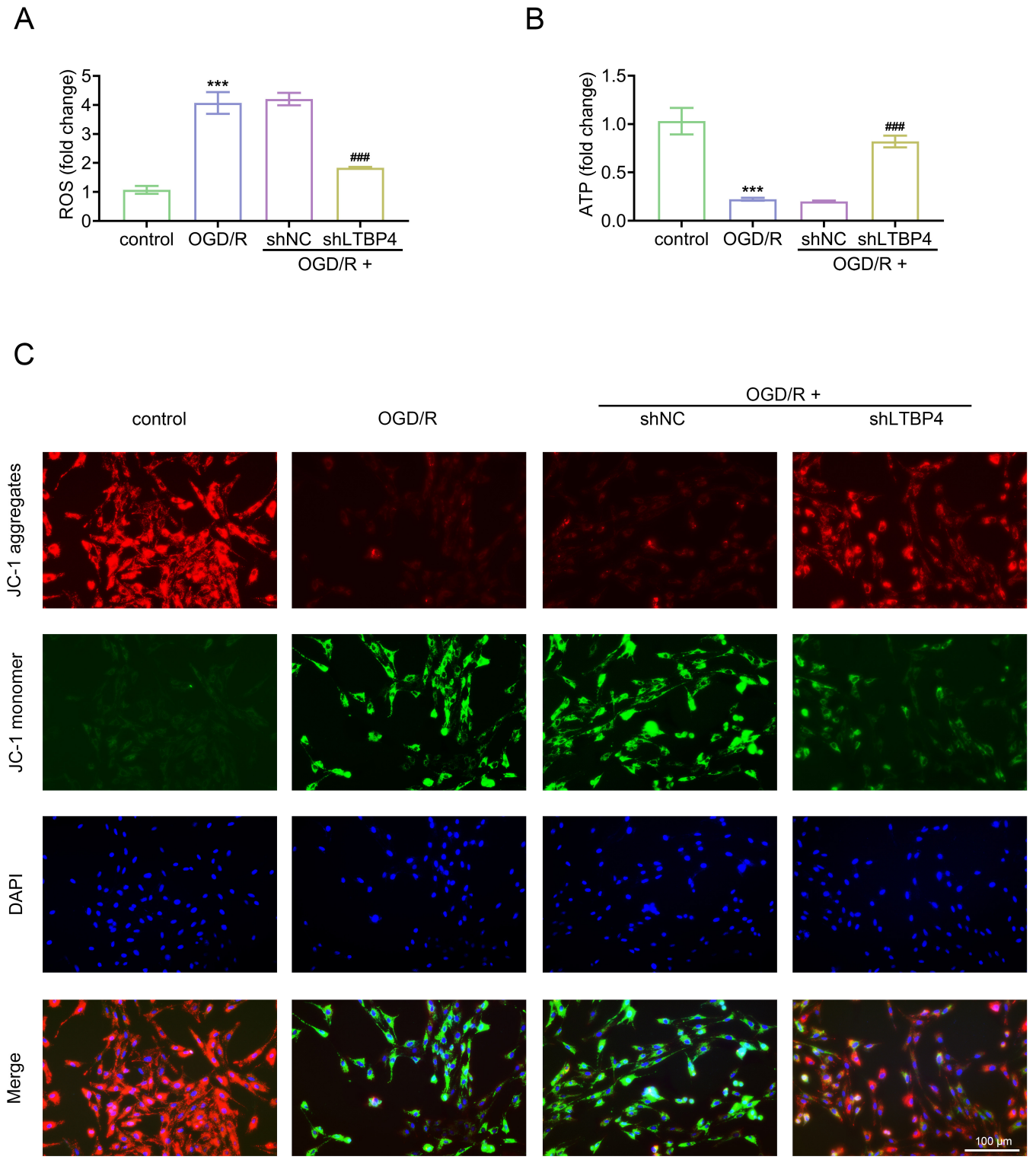

FIGURE 3. ***LTBP4*** **suppression alleviates OGD/R-induced mitochondrial dysfunction in cardiomyocytes.** The cells were divided into control, OGD/R, OGD/R + shNC, and OGD/R + shLTBP4 groups. (A) Intracellular ROS levels detected using a ROS assay kit. (B) ATP levels measured using an ATP detection kit. (C) Mitochondrial membrane potential (MMP) assessed by JC-1 staining. ***$p < 0.001$ *vs.* control; $^{###}p < 0.001$ *vs.* OGD/R + shNC. OGD/R: oxygen-glucose deprivation/reperfusion; shNC: Short hairpin negative control; LTBP4: Latent transforming growth factor-beta binding protein 4; ROS: reactive oxygen species; ATP: adenosine triphosphate; DAPI: 4′,6-diamidino-2-phenylindole.

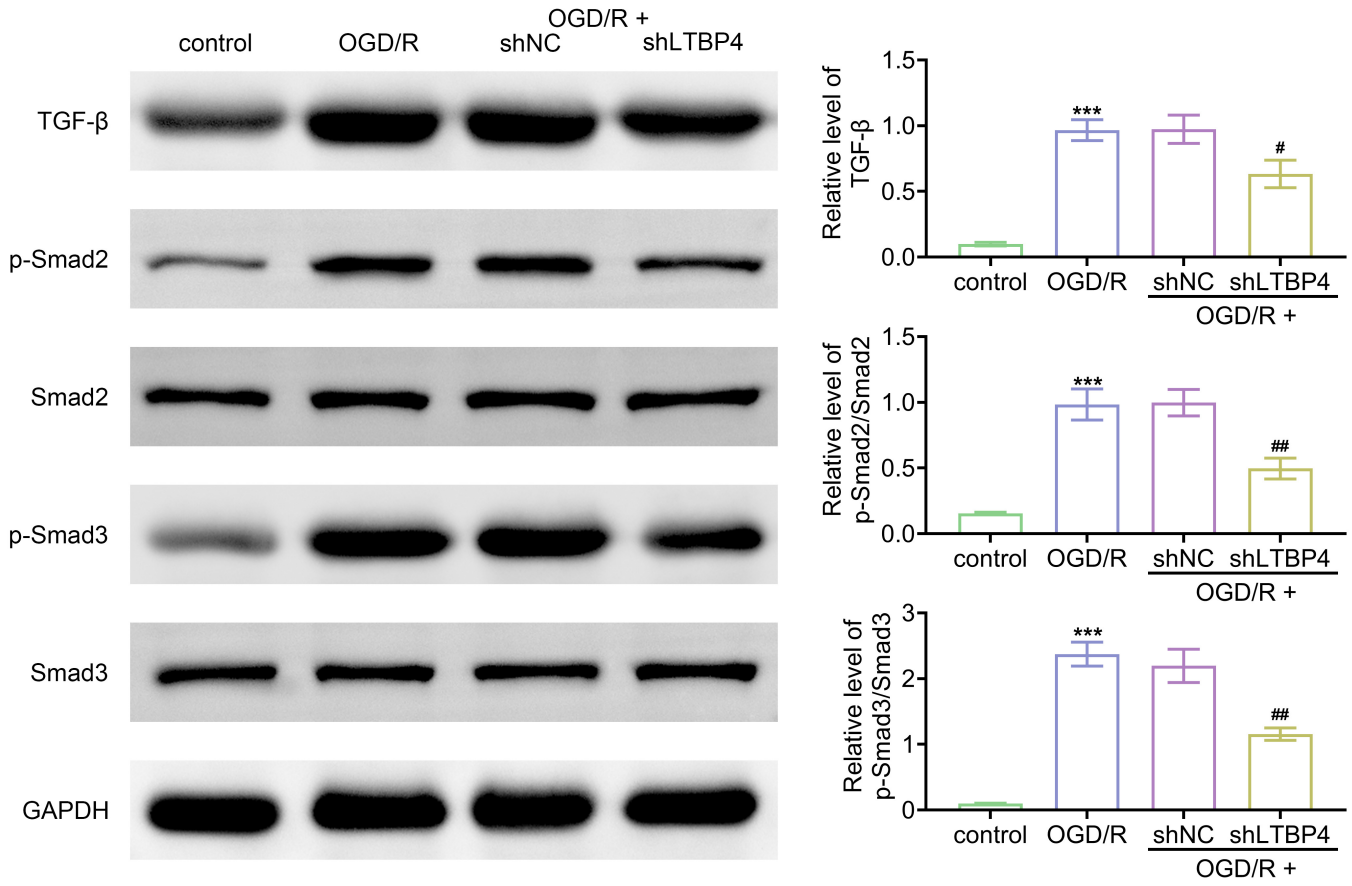

FIGURE 4. Downregulation of *LTBP4* inhibits activation of the TGF-β signaling pathway in cardiomyocytes. The cells were divided into control, OGD/R, OGD/R + shNC, and OGD/R + shLTBP4 groups. Protein expression of TGF-β, p-Smad2, Smad2, p-Smad3, and Smad3 was assessed by Western blot. ***$p < 0.001$ *vs.* control; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ *vs.* OGD/R + shNC. OGD/R: oxygen-glucose deprivation/reperfusion; shNC: Short hairpin negative control; LTBP4: Latent transforming growth factor-beta binding protein 4; TGF-β: Transforming growth factor-β; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Smad2: mothers against decapentaplegic homolog 2.

ducible nitric oxide synthase (iNOS) has been shown to impair mitochondrial function, thereby exacerbating HF pathology [10]. Conversely, Jin-Xin-Kang, a traditional Chinese medicinal formulation, has been reported to alleviate mitochondrial dysfunction and improve cardiac outcomes in HF models [21]. In addition, DEAD-box helicase 17 (DDX17) is connected with maintaining mitochondrial homeostasis and enhancing cardiac function in HF [22], while empagliflozin, a sodium-glucose co-transporter 2 inhibitor, modulates mitochondrial dynamics and has demonstrated cardioprotective effects in murine models of HF [23]. Consistent with these prior findings, this work manifested that knockdown of *LTBP4* mitigated OGD/R-induced mitochondrial dysfunction in cardiomyocytes, further supporting the potential of targeting mitochondrial pathways in managing HF.

The TGF-β signaling pathway has been shown to be involved in the development of HF. Several therapeutic agents have shown efficacy in modulating this pathway to mitigate cardiac dysfunction. For example, Shexiang Tongxin Dropping Pill has been reported to suppress TGF-β signaling and improve chronic HF in murine models [24], while HBI-8000 alleviates HF progression by regulating the TGF-β1/mitogen-activated protein kinase (MAPK) axis [25]. Additionally, runt-related transcription factor 1 (RUNX1) has been shown to modulate TGF-β-mediated cardiac remodeling, thereby accelerating HF development [26], and Shenfu injection exerts protective effects against HF by influencing the TGF-β/Smad pathway [27]. There is a complex interconnection between mitochondrial dysfunction and TGF-β signaling, with both influencing each other [28]. On one hand, TGF-β can regulate energy metabolism by controlling mitochondrial metabolism and can also induce cardiomyocyte apoptosis, with its mechanism closely related to mitochondrial dysfunction [29, 30]. On the other hand, mitochondrial dysfunction can activate the TGF-β signaling pathway [31]. Notably, TGF-β is also known to regulate mitochondrial biogenesis, which further underscores its relevance to cardiac pathophysiology [32]. Importantly, *LTBP4* has been shown to enhance TGF-β/Smad signaling in the context of scleroderma [33], suggesting its potential upstream role in modulating this pathway. However, the regulatory impacts of *LTBP4* in modulating the TGF-β signaling pathway have not been clearly defined. In this study, we demonstrated that downregulation of *LTBP4* attenuated the evoking of the TGF-β/Smad pathway, suggesting that *LTBP4* positively regulates this signaling cascade in cardiomyocytes under ischemic stress.

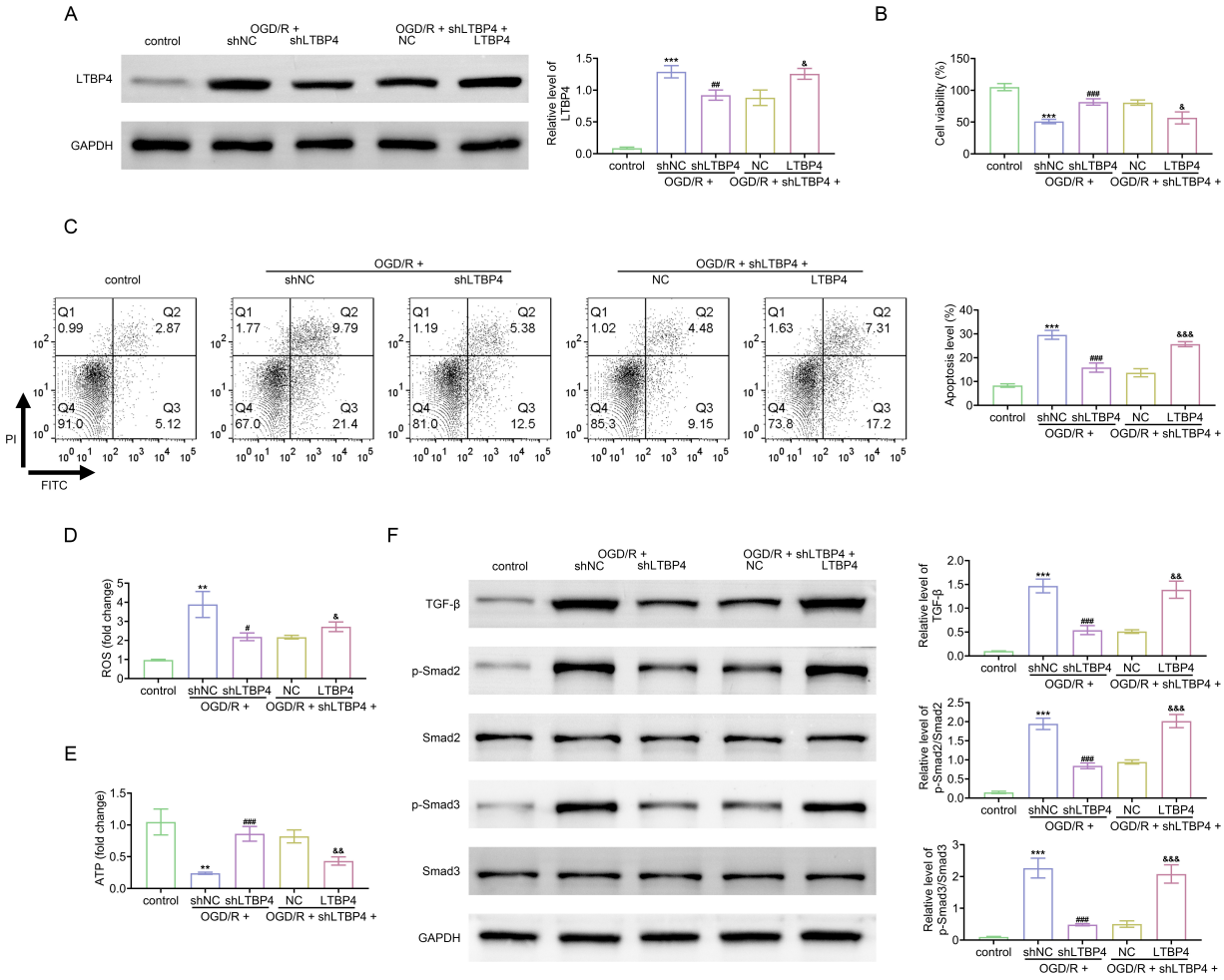

FIGURE 5. ***LTBP4* affects OGD/R-induced cell apoptosis and mitochondrial dysfunction.** The cells were divided into control, OGD/R + shNC, OGD/R + shLTBP4, OGD/R + shLTBP4 + NC and OGD/R + shLTBP4 + LTBP4 groups. (A) The protein expression of LTBP4 was examined through Western blot. (B) The cell viability was tested through CCK-8 assay. (C) The cell apoptosis was measured through flow cytometry. (D) The ROS level was detected through the ROS assay kit. (E) The ATP level was inspected through the ATP kit. (F) The protein expressions of TGF-β, p-Smad2, Smad2, p-Smad3, and Smad3 were detected through Western blot. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* the OGD/R + shNC group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ *vs.* the OGD/R + shLTBP4 + NC group. LTBP4: Latent transforming growth factor-beta binding protein 4; OGD/R: oxygen-glucose deprivation/reperfusion; PI: propidium iodide; shNC: Short hairpin negative control; ROS: reactive oxygen species; ATP: adenosine triphosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; FITC: Fluorescein isothiocyanate; TGF-β: Transforming growth factor-β; Smad2: mothers against decapentaplegic homolog 2.

5. Conclusions

This study demonstrated that the knockdown of *LTBP4* alleviates OGD/R-induced cardiomyocyte apoptosis and mitochondrial dysfunction, while also suppressing the TGF-β signaling pathway. However, several limitations should be acknowledged, including the absence of *in vivo* validation, evaluation of additional cellular phenotypes, clinical correlation, and the lack of *LTBP4* overexpression experiments. Importantly, the absence of standalone *LTBP4* overexpression experiments limits the direct evidence of *LTBP4*'s pro-pathological role in OGD/R-induced damage. Due to the resource constraints in this study, future studies incorporating animal models, clinical samples, and gain-of-function approaches are warranted to further elucidate the role of *LTBP4* in HF and to validate its translational potential.

AVAILABILITY OF DATA AND MATERIALS

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

AUTHOR CONTRIBUTIONS

XQW and LY—designed the study and carried them out, supervised the data collection, analyzed the data, interpreted the data, prepare the manuscript for publication and reviewed the draft of the manuscript. Both authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This article does not contain any studies with human participants or animals performed by any of the authors.

ACKNOWLEDGMENT

Not applicable.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Xiaoqing Wu, Li Yuan. Knockdown of *LTBP4* alleviates OGD/R-induced cardiomyocyte apoptosis and mitochondrial dysfunction, and attenuates the TGF-β signaling pathway. Signa Vitae. 2025; 21(10): 139-146. doi: 10.22514/sv.2025.151.