Latent-transforming growth factor β-binding protein 2 accelerates cardiac fibroblast apoptosis by regulating the expression and activity of caspase-3

CHUNLAI SHI, XUAN LI, FEI HONG, XUEYAN WANG, TINGTING JIANG, BINGBING SUN and SHUANG LI

Cardiovascular Department, Jinqiu Hospital of Liaoning Province, Shenyang, Liaoning 110016, P.R. China

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Abstract. Cardiac fibrosis is a core process in the development of heart failure. However, the underlying mechanism of cardiac fibrosis remains unclear. Recently, a study found that in an isoproterenol (ISO)-induced cardiac fibrosis animal model, there is high expression of latent-transforming growth factor β-binding protein 2 (LTBP2) in cardiac fibroblasts. Whether LTBP2 serves a role in cardiac fibrosis is currently unknown. In the present study, mouse cardiac fibroblasts (MCFs) were treated with 100 μM/l ISO for 24, 48, or 72 h, and small interfering RNAs (siRNAs) were used to knockdown LTBP2. Reverse transcription-quantitative PCR and western blotting were used to determine gene and protein expression levels, respectively. Caspase-3 serves a key role in cell apoptosis and is related to cardiac fibrosis-induced heart failure. Caspase-3 activity was therefore determined using a caspase-3 assay kit, CCK8 was used to determine the rate of cell proliferation and apoptosis rates were quantified using a cell death detection ELISA kit. The present study demonstrated that cell apoptosis and LTBP2 expression increased in MCFs treated with 100 μM/l ISO in a time-dependent manner. Expression and activity of caspase-3 also increased in MCFs treated with 100 μM/l ISO for 48 h compared with the control group. In addition, ISO stimulation-induced MCF apoptosis, along with the increased expression of caspase-3 were partly abolished when LTBP2 was knocked down. In conclusion, LTBP2 expression increased in ISO-treated MCFs and accelerated mouse cardiac fibroblast apoptosis by enhancing the expression and activity of caspase-3. LTBP2 may therefore be a potential therapeutic target for treating patients with cardiac fibrosis.

Correspondence to: Professor Chunlai Shi, Cardiovascular Department, Jinqiu Hospital of Liaoning Province, 317 Xiaonan, Shenyang, Liaoning 110016, P.R. China
E-mail: shiclsy78@aliyun.com

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Introduction

Chronic heart failure is a critical end-complication and comorbidity of various cardiovascular diseases, such as hypertension and coronary heart disease (1). Cardiac fibrosis is an inevitable intermediate pathological process during the development of heart failure (2). Cardiac fibrosis is characterized by a net accumulation of extracellular matrix proteins (including fibrillar collagen types I and III, Elastin and Fibronectin) in the cardiac interstitium and contributes to both systolic and diastolic dysfunction in myocardial remodeling and heart failure (3,4). Cardiac fibroblasts (CFbs), the most prevalent cell type in the heart, serve a key role in the adverse cardiac fibrosis that occurs with heart failure (5,6). CFbs have been recognized to have a fundamental contribution to the cardiac response of a variety of injuries (7). For example, when ischemia-reperfusion injury occurs in the myocardium, triggering an inflammatory response, the inflammasome is activated in cardiac fibroblasts but not in cardiac myocytes (8). Furthermore, the inflammatory response generated by this injury is the key pathophysiological process in myocardial remodeling and the progression of heart failure (9). In the fibrotic heart, excessive cardiac fibroblasts impair the electromechanical coupling of cardiomyocytes, hence increasing the risk of arrhythmogenesis and mortality (7). Hence, CFbs are regarded as a therapeutic target for heart failure (10).

Recently, a study demonstrated genetic variation in cardiac fibrosis by characterizing the response of CFbs from multiple inbred mouse strains to isoproterenol (ISO) treatment (8). In addition, the study identified latent-transforming growth factor β-binding protein 2 (LTBP2) as a marker for cardiac fibrosis (11). LTBP2 is a protein expressed in elastic tissues, such as heart and muscle (12), interact with a number of matrix components, including collagen and fibronectin (13) and is a member of a superfamily of proteins comprising extracellular proteins fibrillins and latent-transforming growth factor β-binding proteins (13), however whether LTBP2 serves a role in cardiac fibrosis remains unclear. Recent studies have shown that LTBP2 is associated with apoptosis (14,15); however, whether the upregulation of LTBP2 expression promotes or inhibits apoptosis remains controversial. Liang *et al* (14) demonstrated that LTBP2 knockdown alleviated apoptosis in

osteosarcoma cells *in vitro*, while Suri *et al* (15) demonstrated that LTBP2 deficiency induced apoptosis in trabecular meshwork cells *in vitro* (16). Hence, whether LTBP2 affects cells apoptosis in cardiac fibrosis and its role in CFb apoptosis are important to understand in the context of cardiac fibrosis as well as heart failure development.

More importantly, the mechanism through which LTBP2 affects cell apoptosis has not been elucidated. Mitochondria-related apoptosis is a key process in cardiac fibrosis (17,18), and caspase-3, the execution protein of mitochondrial-induced cell apoptosis serves a pivotal role in heart failure (19). A previous study illustrated that transforming growth factor β (TGF-β) induces apoptosis by targeting caspase-3 activity and LTBP2 has been identified to form latent complexes with TGFβ (20). Based on these aforementioned results, it was hypothesized that LTBP2 may promote CFb apoptosis by activating caspase-3. The current study was conducted to explore the role and mechanism of LTBP2 in ISO-induced apoptosis in CFb to provide a potential therapeutic target for heart failure.

Materials and methods

Mouse cell fibroblasts (MCFs) culture and treatment. MCFs (cat. no. CP-M074) were obtained from Procell Life Science & Technology Co. Ltd. Cells were cultured in 30 ml of DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and placed in a 37˚C, 5% CO_2 incubator for 90 min of absolute static incubation. When changing the culture medium, a cross was used to shake the dish and the principle of differential cell adhesion was used to wash and discard non-adherent cells

After repeated washing with PBS at 37˚C, 10 ml of DMEM containing 10% FBS was added to the cells. Cells were then placed in an incubator for static culturing and the medium was changed every 3 days. Cells were passaged when 90% confluency was reached. MCFs were then treated with 100 μM/l ISO (8) (Sigma-Aldrich; Merck KGaA) in a 37˚C, 5% CO_2 incubator for 0, 24, 48 or 72 h, while the control group was treated with an equivalent amount of PBS.

Small interfering (si) RNA transfection. LTBP2 knockdown by siRNA reverses myocardial oxidative stress injury, fibrosis and remodelling during dilated cardiomyopathy. Therefore, LTBP2 and scrambled NC siRNAs were obtained from Shanghai Shenggong Biology Engineering Technology Service, Ltd. MCFs were grown in 12-well plates at a density of 6-8x10^5 and transfected in a 37˚C, 5% CO_2 incubator for 12 h. Samples were then transfected with a mixture containing 4 μl of Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific Inc.) and 3 μl of siRNA in 125 μl of Opti-MEM (Invitrogen; Thermo Fisher Scientific Inc.). Cells were then incubated at 37˚C in 5% CO_2 for another 12 h, after which the medium was changed to DMEM containing 100 μM/l ISO or PBS. Knockdown efficiency was determined by western blotting. The primer sequences used were as follows: siRNA LTBP2 forward, 5'-CCGGGUUAUAAGCGGGUUAUU-3' and reverse, GAACCAAACGUCUGCGCAAUU-3'; scrambled NC siRNA forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACGUGACACGUUCGGAGAATT-3'.

Reverse transcription-quantitative (RT-q)PCR. The thermocycling conditios for OCR were as follows: Pre-denaturation 95˚C, 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 60 sec and melting curve analysis at 60-95˚C, with a 0.3˚C rise every 15 sec. the $2^{-\Delta\Delta Cq}$ method was used for quantification (21). Experimental methods and conditions were performed according to the instructions of the PrimeScript RT Master Mix kit (Takara Bio, Inc.; cat. no. RR036A). Experimental group MCFs were treated with 100 μM/l ISO at 37˚C in 5% CO_2, while control group cells were treated with an equivalent amount of PBS. After 72 h, all cells were harvested. Total RNA was isolated from MCFs using a RNAiso reagent (Takara Bio, Inc.). The PrimeScript RT reagent kit (Takara Bio Inc.) was used to transcribe isolated RNA to cDNA according to the manufacturer's protocol. qPCR was performed using a SYBR Premix Ex Taq II kit (Takara Bio Inc.). The mRNA level of β-actin was used as an internal control. The primer sequences used were: LTBP2 forward, 5'-TTACAAGCAGAGACTCAC-3'; reverse, 5'-ACAACAGAAGAGACCAGAT-3'; caspase-3 forward, 5'-ACAGCACCTGGTTACTATTC-3', reverse, 5'-CAGTTCTTTCGTGAGCAT-3'; and β-actin, forward, 5'-GCTGCGTGTGGCCCCTGAG-3', reverse, 5'-ACGCAGGATGGCATGAGGGA-3'.

Western blotting. 5x10^6 cells MCFs were collected and homogenized in lysis buffer (Beyotime Institute of Biotechnology) containing protease and phosphatase inhibitors (Roche Diagnostics) and incubated on ice for 30 min. Whole cell lysates were centrifuged at 12,000 x g for 15 min at 25˚C and protein concentrations were determined using the bicinchoninic acid (BCA) assay (Beyotime Institute of Biotechnology). Proteins (20 μg) were then separated by 8% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). Membranes were blocked with 5% skimmed milk in Tris-buffered saline solution with 0.1% Tween-20 (TBS/T) at room temperature for 2 h and subsequently incubated with specific primary antibodies (all Abcam) against LTBP2 (1:1,000; cat. no. ab121193), caspase-3 (1:1,000; cat. no. ab184787), β-actin (1:2,000; cat. no. ab8226) at 4˚C overnight. The membranes were washed with TBS/T 3 times and then incubated with an HRP-conjugated secondary antibody (goat anti-mouse IgG; 1:2,000, Beyotime Institute of Biotechnology; cat. no. A0216) at 37˚C for 2 h. Protein bands were visualized by chemiluminescence detection (ChemiDoc MP; Bio-Rad Laboratories, Inc.) using Image Lab software for densitometry (version 5.2; Bio-Rad Laboratories, Inc.).

Caspase-3 activity. Caspase-3 activity was determined using a colorimetric Caspase-3 assay kit (Abcam). 5x10^6 MCFs were collected and resuspended in 50 μl of lysis buffer and incubated on ice for 15 min, subsequently 50 μl of the 2X reaction buffer was added to each sample. DEVD-pNA substrate (1 mM) was added, and samples were incubated for 2 h at 37˚C. Caspase-3 activity was measured at an optical density (OD) of 400 nm. Protein concentration was assayed by BCA assay (Beyotime Institute of Biotechnology), BCA assay and protein concentration were performed as aforementioned. Caspase-3 activity was normalized to the protein concentration.

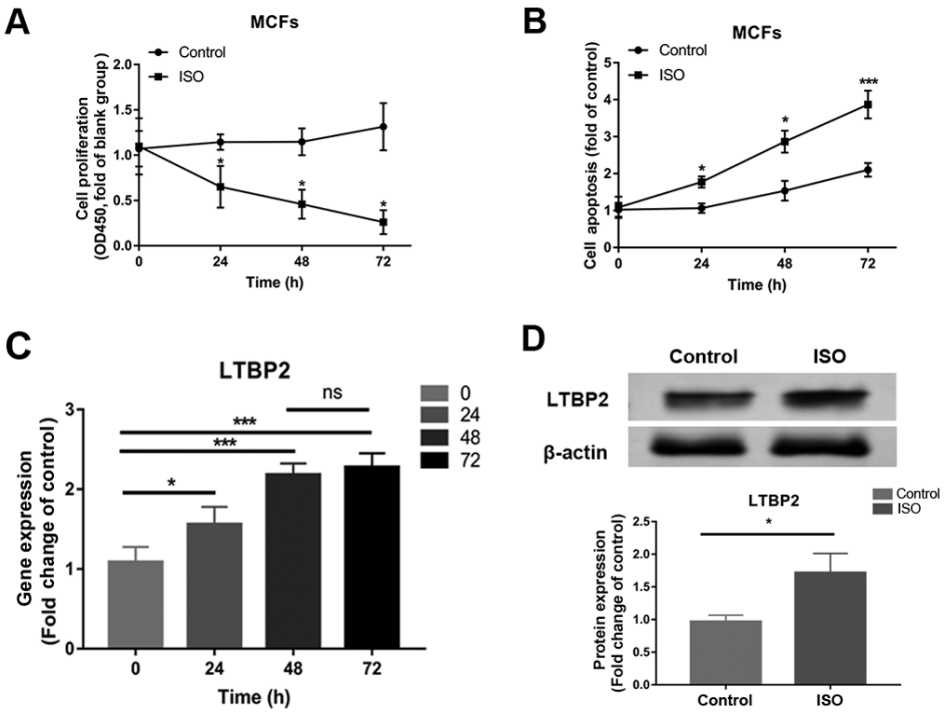

Figure 1. Cell apoptosis and LTBP2 expression increased in MCFs in response to ISO. (A) Proliferation of cells treated with ISO for varying times compared with PBS-treated cells. (B) Apoptosis of cells. (C) LTBP2 gene expression. (D) LTBP2 protein expression. (A and B) *P<0.05 and ***P<0.01 vs. control at the same time points; (C and D) *P<0.05 and ***P<0.01 vs. control; n=3 for each test, mean ± SEM. LTBP2, latent-transforming growth factor β-binding protein 2; RT-q, reverse transcription-quantitative; ISO, isoproterenol; MCFs, mouse cell fibroblasts; OD, optical density.

Cell proliferation. CCK-8 (Beyotime Insitute of Biotechnology) assay was used to determine cell proliferation. MCFs ($2x10^4$ cells/ml) were seeded onto 96-well plates at 100 μl per well (total of 56 wells) and further supplemented with 100 μl of DMEM containing 10% FBS at 37˚C. Experimental group cells were treated with 100 μM/l ISO, while controls were treated with PBS. After 24, 48, and 72 h of incubation, 100 μl of culture medium was aspirated and 10 μl of CCK-8 working solution (Beyotime Institute of Biotechnology) was added for 1 h at room temperature. The absorbance (A) values at 450 nm were measured by a microplate reader and 0 was set using blank control wells. After continuous detection for 7 days, by measuring absorbance at 450 nm, the proliferation rate of MCFs was calculated.

Cell apoptosis. Cell Death Detection ELISA kit (Roche Diagnostics GmBH) was used to evaluate the apoptotic rates according to the manufacturer's instructions. Cells ($2x10^4$ cells/ml) were treated with ISO or PBS and centrifuged at 11,000 x g at 25˚C for 10 min. After the supernatant was removed, cell pellets were incubated with 200 μl lysis buffer for 30 min at room temperature. Cytoplasmic lysates were transferred to a streptavidin-coated plate, then a mixture of anti-DNA-POD and anti-histone-biotin was added at 25˚C for 2 h. Absorbance at 405 nm was detected with a reference wavelength at 490 nm (Synergy Mx; BioTek Instruments, Inc.). Apoptotic rate was determined by measuring the absorbance at 405 nm.

Statistical analysis. SPSS 20.0 software (IBM Corp.) was used for statistical analysis. Results are expressed as mean ± standard error of mean (SEM). All experiments were repeated at least 3 times. An unpaired t-test was performed for comparison between 2 groups, multiple comparisons were compared using one-way analysis of variance (ANOVA) followed by the post hoc Tukey's test. P<0.05 was considered to indicate a statistically significant difference, while P<0.01 was considered to indicate a highly significant difference.

Results

Cell apoptosis and LTBP2 expression increased in a time-dependent manner in MCFs treated with ISO. Compared with control, MCFs treated with ISO at 100 μM/l for 24 h had decreased proliferation at 0, 24, 48 and 72 h (Fig. 1A), while cell apoptosis was enhanced in a time-dependent manner in ISO-treated cells at 24, 48 and 72 h (Fig. 1B). Similar to the changes observed in cell apoptosis, LTBP2 mRNA expression significantly increased in cells treated with ISO and demonstrated a time-dependent increase (Fig. 1C). Since there are no significant changes in LTBP2 mRNA expression at 72 h compared with 48 h, the latter timepoint was chosen for protein detection, which demonstrated increased LTBP2 protein expression following ISO treatment compared with the control (Fig. 1D). The results revealed that cell apoptosis and LTBP2 expression increased in a time-dependent manner in MCFs treated with ISO.

LTBP2 is involved in ISO-induced MCF apoptosis. siRNA transfection was used to knockdown the expression of LTBP2 and the role of LTBP2 in ISO-induced MCFs apoptosis was investigated. RT-qPCR and western blotting demonstrated

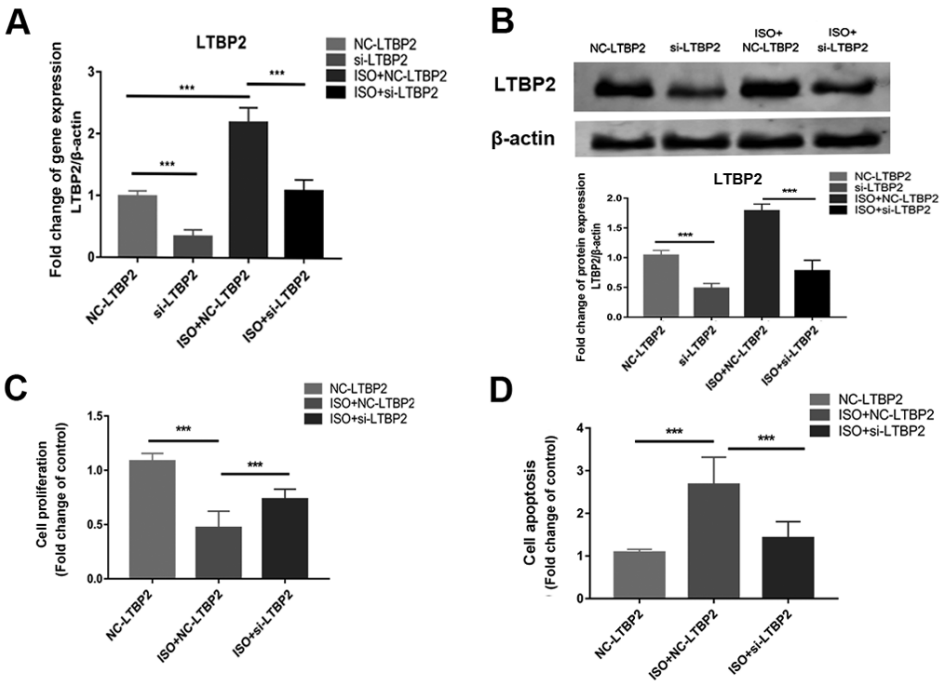

Figure 2. ISO-induced apoptosis is reduced when LTBP2 is knocked down. (A) LTBP2 gene expression. (B) LTBP2 protein expression. (C) Proliferation of cells treated with ISO for varying times compared with NC-LTBP2 group. (D) Cell apoptosis. ***P<0.01 vs. control; n=3 for each test, mean ± SEM. LTBP2, latent-transforming growth factor β-binding protein 2; RT-q, reverse transcription-quantitative; ISO, isoproterenol; si, small interfering; NC, negative control.

successful LTBP2 knockdown (Fig. 2A and B), and results of the CCK-8 assay demonstrated that cell proliferation was inhibited by ISO (Fig. 2C). Knocking down LTBP2 partially reversed the inhibition induced by ISO compared with scrambled siRNA (Fig. 2C), as well as abated the enhanced apoptosis caused by ISO treatment compared with scrambled siRNA (Fig. 2D). The results demonstrated the involvement of LTBP2 in the ISO-induced apoptosis of MCF.

Caspase-3 expression and activity increases in MCFs treated with ISO. Gene and protein expression of caspase-3 measured by RT-qPCR and western blotting respectively, increased in cells treated with ISO at 100 μM/l for 72 h compared with that in control cells (Fig. 3A and B). In addition, enhanced caspase-3 activity was demonstrated following ISO treatment compared with the control group (Fig. 3C). The results demonstrated that Caspase-3 was involved in ISO-induced MCF apoptosis.

LTBP2 increases MCF apoptosis by regulating caspase-3 expression and activity. To examine whether the effect of LTBP2 on MCF apoptosis is associated with caspase-3, LTBP2 was knocked down and the gene and protein expressions of caspase-3 was quantified in cells treated with ISO. The RT-qPCR and western blotting results demonstrated that LTBP2 deficiency partially reversed the increase in caspase-3 expression induced by ISO treatment (Fig. 4A and B). In addition, the results indicated that knocking down LTBP2 inhibited the increase in caspase-3 activity caused by ISO compared with the control group (Fig. 4C). The results revealed that LTBP2 affects MCF apoptosis induced by ISO through caspase-3.

Discussion

LTBP2 is a protein that associates with the extracellular matrix and binds to it in a fibrillin-dependent manner (22,23). In humans, it has been confirmed that LTBP2 serves a pivotal role in primary congenital glaucoma with defects in the trabecular network (16,24), and loss of LTBP2 also results in an autosomal recessive ocular syndrome (25). In addition, a series of clinical studies demonstrated that LTBP2 is related to coronary artery disease and heart failure (26,27), and a recent study revealed increased expression and localization of LTBP2 in the fibrotic regions of the myocardium after injury in mice and in patients with heart failure (11). Together, the findings of the aforementioned studies suggested that LTBP2 may play a role in the development of heart failure. However, its function as well as the underlying mechanism by which LTBP2 may be involved in heart failure remains unknown. Cardiac fibroblasts play a key role in heart failure (5,6); hence, there is great value in investigating the function of LTBP2 in this process.

The findings of the present study demonstrated that LTBP2 expression increased in a time-dependent manner in ISO-treated MCFs, which is consistent with a previous study (11) and implied that LTBP2 is involved in the development of cardiac fibrosis. In addition, the present study demonstrated that compared with control, ISO inhibited the proliferation of MCFs, which was alleviated by knocking down LTBP2 expression. This implies a role for LTBP2 in ISO-induced MCF apoptosis. LTBP2 has been reportedly involved in other types of cell apoptosis in recent studies, but its function in these processes varies. Liang *et al* (14) demonstrated that depletion of LTBP2 partly abolished the apoptosis of osteosarcoma cells induced by a microRNA-421 inhibitor.

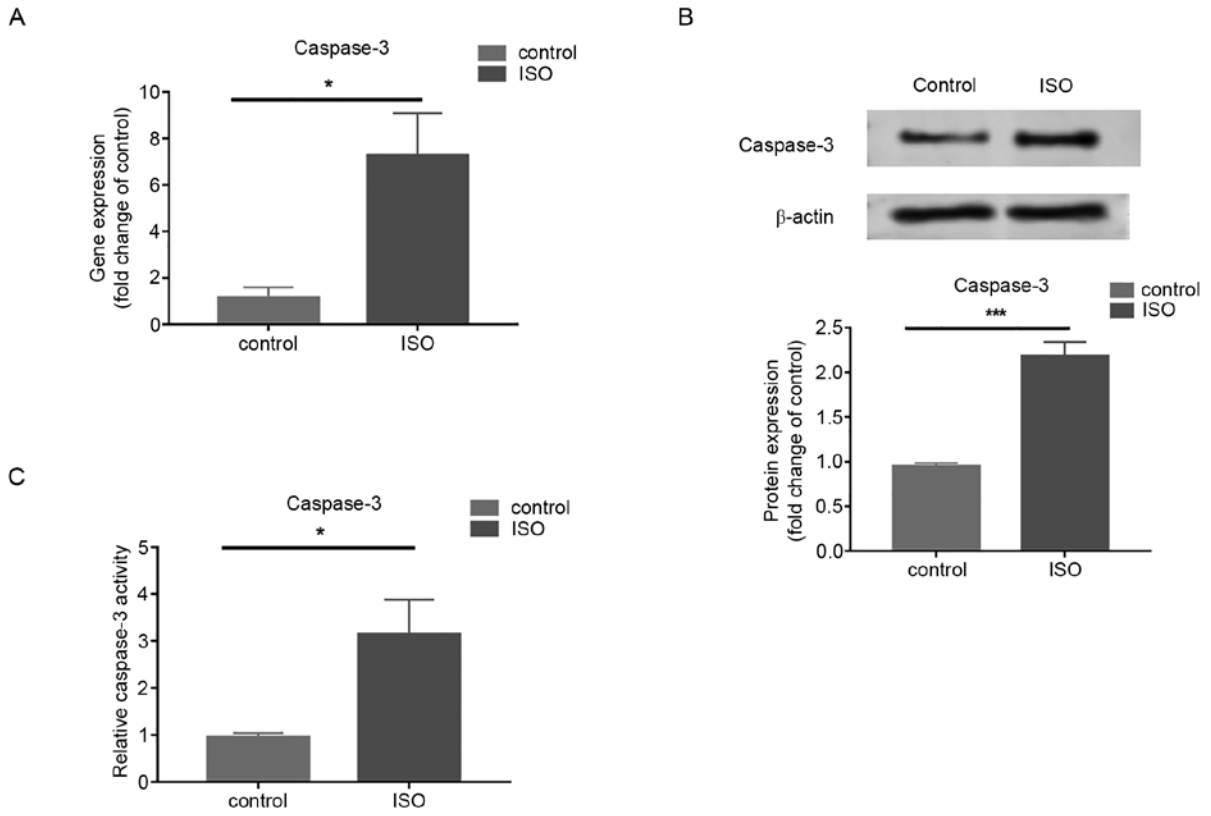

Figure 3. Caspase-3 expression and activity increased in MCFs in response to ISO. (A) caspase-3 gene expression. (B) Caspase-3 protein expression. (C) Activity of caspase-3. *P<0.05 and ***P<0.01 vs. control; n=3 for each test, mean ± SEM. LTBP2, latent-transforming growth factor β-binding protein 2; RT-q, reverse transcription-quantitative; ISO, isoproterenol; MCFs, mouse cell fibroblasts.

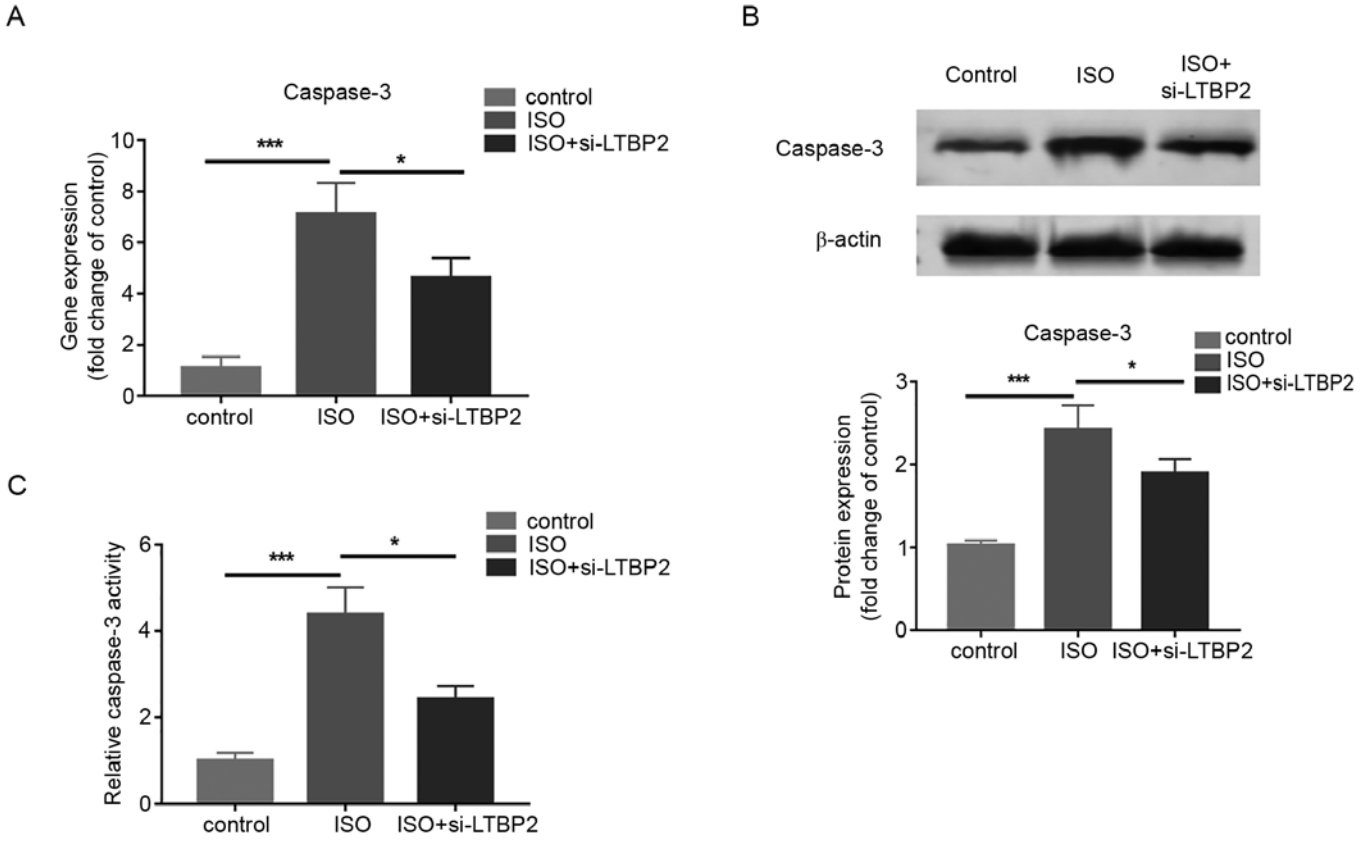

Figure 4. LTBP2 regulates caspase-3 expression and activity. (A) Caspase-3 gene expression. (B) Caspase-3 protein expression. (C) Caspase-3 activity. *P<0.05 and ***P<0.01 vs. control; n=3 for each test, mean ± SEM. LTBP2, latent-transforming growth factor β-binding protein 2; RT-q, reverse transcription-quantitative; ISO, isoproterenol; si, small interfering; NC, negative control.

Suri *et al* (15) demonstrated that knockdown of LTBP2 induced apoptosis in trabecular meshwork cells compared with the control group. The present study demonstrated that LTBP2 deficiency partially reversed ISO-induced apoptosis, which is consistent with the results of Liang *et al* (14), but is contrary to that of Suri *et al* (15). The reason behind these contradictory results remains unknown; a possible explanation for this may be that LTBP2 is regulated by different pathways in different cells or when treated with different factors. Further studies are required to confirm this.

In addition, the findings of the present study revealed that LTBP2 enhances MCF apoptosis by regulating caspase-3 expression and activity which has not been previously reported. Caspase-3 plays a key role in cell apoptosis (28) and activation of caspase-3 is related to heart failure caused by cardiac fibrosis (19,29). The results of the present study demonstrated that knocking down LTBP2 led to a decrease in caspase-3 expression and inhibition of caspase-3 activity in MCFs treated with ISO. The mechanism through which LTBP2 regulates caspase-3 expression and activity is unclear. As mentioned previously, TGF-β may serve as a mediator between LTBP2 and caspase-3 and is a potential link that needs further studies to be substantiated.

Although studies have shown that serum levels of LTBP2 are increased in patients with heart failure, the effects of LTBP2 on heart failure are currently not being investigated (27). Appropriate proliferation of cardiac fibroblasts is a means through which the heart repairs itself in response to damage and restores function (7). However, excessive fibroblast proliferation can also trigger cardiac fibrosis and lead to the progression of heart failure (7). Whether the apoptosis of cardiomyocytes triggered by upregulation of LTBP2 promotes cardiac fibrosis and the progression of heart failure or aids in its recovery needs to be further verified in animal studies. In addition, since the TGFβ pathway is closely associated with cardiac fibrosis (30), the effect of LTBP2 on cardiac fibrosis may also be related to changes in the expression of TGFβ.

There are several limitations of the present study. Firstly, apoptosis was detected using the ELISA instead of flow cytometry. CCK-8 assay can be used to determine cell viability, proliferation and cytotoxicity, but is not a good measure of apoptosis (31). Secondly, the *in vitro* results of the present study were not verified in animal models. Further research should be undertaken to investigate whether LTBP2 deficiency in animal models can lead to cardiac fibrosis as well as heart failure, and these results may provide new targets for treatment and prevention of heart failure.

In conclusion, the results revealed that LTBP2 and caspase-3 expression was increased in cells during ISO-induced MCF apoptosis. Inhibition of LTBP2 expression alleviated ISO-induced MCF apoptosis by suppressing caspase-3 expression. The results also indicated that LTBP2 may influence the development of cardiac fibrosis by regulating caspase-3-induced cardiac fibroblast apoptosis. The results of the current study may provide novel ideas for mitigating the progression of heart failure.

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Availability of data and materials

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

Authors' contributions

CS designed the study, conducted most of the experiments and drafted the manuscript. XL, FH, XW and TJ conducted experiments. BS and SL collected and analyzed the data. All authors read and approved the final manuscript. All authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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