F-box and WD repeat-containing protein 7 ameliorates angiotensin II-induced myocardial hypertrophic injury via the mTOR-mediated autophagy pathway

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Abstract. Myocardial hypertrophy is a common heart disease that is closely associated with heart failure. The expression of F-box and WD repeat-containing protein 7 (FBW7) is significantly downregulated in angiotensin (Ang) II-induced cardiac fibroblasts, suggesting that it may possess an important function in cardiac development. The present study attempted to explore the role of FBW7 in Ang II-induced myocardial hypertrophic injury and its associated mechanism of action. Reverse transcription-quantitative PCR and western blotting were used to determine the expression levels of FBW7 in Ang II-induced H9C2 cells. The expression levels of autophagy-related and mTOR signaling pathway-related proteins were detected using western blotting. Cell viability was assessed using the Cell Counting Kit-8 assay. The apoptosis rate of H9C2 cells was detected using TUNEL assay and western blotting. Cellular hypertrophy and fibrosis were assessed using phalloidin staining and western blotting. Levels of inflammatory factors were examined using ELISA and western blotting, whereas levels of oxidative stress-related markers were detected by corresponding kits. The results indicated that FBW7 expression was downregulated in Ang II-induced H9C2 cells. FBW7 upregulation enhanced the expression levels of autophagy-related proteins and activated mTOR-mediated cellular autophagy. FBW7 overexpression promoted the cell viability, inhibited Ang II-induced apoptosis, cellular hypertrophy and fibrosis in H9C2 cells via the autophagic pathway, as well as inflammation and oxidative stress. Overall, the data indicated that FBW7 overexpression ameliorated Ang II-induced hypertrophic myocardial injury via the mTOR-mediated autophagic pathway.

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Introduction

Myocardial hypertrophy is an independent risk factor for cardiovascular disease and may develop into heart failure (1). Myocardial hypertrophy is marked by increased myocyte volume, ventricular wall thickness and myocardial contractility under early overload stress conditions (2). Persistent myocardial hypertrophy is often accompanied by severe myocardial interstitial fibrosis, leading to dilated cardiomyopathy and even sudden death (3). The current pharmacological treatment for cardiac hypertrophy is based on anti-hypertension, such as angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, calcium antagonists and nitric oxide donors (4). However, the toxic side effects of the drugs used inhibit efficient treatment of myocardial hypertrophy. Results of previous studies have demonstrated that myocardial hypertrophy exhibits multiple damaging effects on the heart, including apoptosis, cellular hypertrophy and fibrosis, oxidative stress, and overproduction of inflammation (5-8). Consequently, the reduction in the hypertrophic damage of the cardiomyocytes is one of the most important ways to improve the symptoms of heart disease.

F-box and WD repeat domain-containing protein 7 (FBW7), also known as FBXW7, is a member of the F-box protein family (9). Previous studies have revealed that FBW7 exerts a notable regulatory role in cardiomyocytes. For example, microRNA (miR)-25 promotes cardiomyocyte proliferation by targeting FBXW7 (10). MiR-195-5p promotes cardiomyocyte hypertrophy by targeting mitofusin 2 and FBXW7 (11). In addition, the dysregulation of FBW7 exerts a key role in the progression of hematological tumors (12-14). A previous study indicated that FBW7 expression is significantly downregulated in Ang II-induced cardiac fibroblasts and that miR-27b promotes cardiac fibrosis by targeting the FBW7/Snail pathway (15). In addition, mice lacking FBW7 have defective cardiovascular development (16). This evidence suggests that FBW7 may have a notable regulatory role in cardiac development.

By contrast, mTOR is a serine/threonine kinase, which performs a key role in the regulation of autophagy (17). It has been documented that FBW7 levels are reduced in high glucose-induced renal thylakoid cells, whereas their overexpression increases cellular autophagy by inhibiting the mTOR signaling pathway (18). Diabetic nephropathy is highly correlated with cardiac hypertrophy (19). Ang II is the most important active component of the renin-angiotensin system and can directly promote the effects of cardiomyocyte hypertrophy (20). Based on these findings, the presents study investigated the hypothesis that FBW7 may be involved in Ang II-induced myocardial hypertrophic injury through the mTOR-mediated autophagic pathway.

In the present study, the present study aimed to explore the biological role of FBW7 and mechanism involved in Ang II-induced myocardial hypertrophic injury. The expression levels of FBW7 were explored in Ang II-treated H9C2 cells and the association of this protein with mTOR was explored. Following FBW7 overexpression, the induction of apoptosis, hypertrophy, fibrosis, inflammation and oxidative stress were investigated in Ang II-induced H9C2 cells. The current study comprehensively described the roles of FBW7 in Ang II-treated H9C2 cells, which provided a novel therapeutical target in the treatment of myocardial hypertrophy.

Materials and methods

Cell culture and treatment. The rat cardiomyocyte cell line H9C2 was purchased from Procell Life Science & Technology Co., Ltd. The cells were maintained in Dulbecco's modified Eagle's Medium containing 10% fetal bovine serum and 100 U/ml penicillin with 100 mg/ml streptomycin at 37˚C in the presence of 5% CO_2 and 95% air.

The establishment of an *in vitro* model of cardiomyocyte hypertrophy was performed by culturing H9C2 cells in a 6-well plate overnight. Following starvation, the cells were incubated with serum-free medium for 4 h and Ang II (Sigma-Aldrich; Merck KGaA) was used at the concentrations of 0.5, 1, 5 and 10 μM to induce H9C2 cells for 24 h at 37˚C.

The autophagic pathway was examined by pre-treatment of H9C2 cells with 10 μM 3-methyladenine (3-MA) for 3 h at 37˚C prior to the establishment of an *in vitro* model of myocardial hypertrophy.

Cell transfection. The FBW7 overexpression plasmid (Oe-FBW7) and its negative control (Oe-NC) were obtained from Guangzhou RiboBio Co., Ltd. Oe-FBW7 consisted of a pcDNA3.1 vector containing the full-length FBW7 cDNA sequence, with empty pcDNA3.1 as the negative control (Oe-NC). These vectors were transfected into H9C2 cells using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at a concentration of 50 ng/ml for 48 h at 37˚C. The overexpression transfection efficiency was determined using reverse transcription-quantitative (RT-q)PCR and western blotting 48 h post-transfection.

RT-qPCR. The extraction of total RNA was conducted from H9C2 cells by employing TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The reverse transcription of cDNA was carried out using total RNA as a template by PrimeScript RT Master Mix obtained from Takara (Takara Bio, Inc.) according to the manufacturer's instructions. cDNA was amplified with qPCR using the SYBR Green I dye detection kit (Takara Bio, Inc.) according to the instructions provided by the manufacturer. The reaction was performed on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc.). The amplification conditions for this reaction were as follows: 95˚C For 30 sec, followed by 40 cycles of 60˚C for 30 sec and 72˚C for 30 sec. The primer sequences were designed by Sangon Biotech Co., Ltd. The primer sequences are listed as follows: FBW7 forward, 5'-TTG GCTTGGGACAACAGACT-3', and reverse, 5'-TGGAAG ATAACCAGCCCGTG-3'; GAPDH forward, 5'-GTCGTG GAGTCTACTGGCGTCTTCA-3, and reverse, 5-TCGTGG TTCACACCCATCACAAACA-3'. The relative expression of FBW7 was normalized to that of the expression of GAPDH by the $2^{-\Delta\Delta Cq}$ method (21).

Western blotting. Whole-cell protein lysates were extracted from H9C2 cells with an ice-cold radioimmunoprecipitation (Beyotime Institute of Biotechnology) buffer containing protease inhibitors and phenylmethylsulfonyl fluoride. Subsequently, the protein concentration levels were detected using the bicinchoninic acid protein assay kit. Each protein sample (30 μg per lane) was resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis using 15% SDS gels. The proteins were transferred to PVDF membranes, which were subsequently blocked with 5% non-fat milk and incubated at 4˚C overnight with the primary antibodies against FBW7 (1:2,000; cat. no. s-5996R; BIOSS), microtubule-associated proteins 1A/1B light chain 3B-II/I (LC3B-II/I; 1:3,000; cat. no. ab51520; Abcam), p62 (1:2,000; cat. no. FNab06086; Fine Test), Beclin-1 (1:2,000; cat. no. ab207612; Abcam), p phosphorylated (p)-mTOR (1:10,000; cat. no. ab109268; Abcam), mTOR (1:10,000; cat. no. ab134903; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam), Bax (1:10,000; cat. no. ab32503; Abcam), cleaved caspase 3 (1:500; cat. no. ab32042; Abcam), beta-myosin heavy chain (β-MHC; bs-4298R; cat. no. 1:2,000; BIOSS), brain natriuretic peptide (BNP; 1:20,000; cat. no. ab92500; Abcam), atrial natriuretic factor (ANF; 1:2,000; cat. no. ATA24633; AtaGenix), α-smooth muscle actin (α-SMA; 1:1,000; cat. no. YT680; Bjbalb), fibronectin (1:1,000; cat. no. ab268020; Abcam), vimentin (1:1,000; cat. no. ab92547; Abcam), collagen I (1:10,000; cat. no. ab34710; Abcam), collagen III (1:1,000; cat. no. ab184993; Abcam), inducible nitric oxide synthase (iNOS; 1:1,000; cat. no. ab178945; Abcam) and cyclooxygenase-2 (COX-2; 1:500; cat. no. 252153; Signalway Antibody LLC) followed by the incubation with a goat anti-rabbit IgG secondary antibody conjugated to HRP (1:2,000; cat. no. ab6721; Abcam) for an additional 1 h at room temperature. The protein bands were visualized using the enhanced chemiluminescence kit (MilliporeSigma) and the ChemiDoc imaging system (Bio-Rad Laboratories, Inc.), and were semi-quantified using ImageJ software (version 1.48v; National Institutes of Health).

Cell viability assay. The cell viability of H9C2 cells was detected using the Cell Counting Kit (CCK)-8 method. H9C2 cells were incubated in 96-well plates at a density of $5x10^3$ cells/well at 37˚C in the presence of 5% CO_2 for 24 h.

Subsequently, 10 μl CCK-8 solution (Beyotime Institute of Biotechnology) was added into the wells and incubated for 2 h according to the manufacturer's instructions. The absorbance was detected at 450 nm using a microplate reader (Molecular Devices, LLC).

Detection of apoptosis. Apoptosis was examined using the TUNEL assay. Briefly, H9C2 cells were fixed in a 1% formaldehyde solution for 15 min at room temperature. Subsequently, these cells were mixed with 0.2% Triton X-100 and analyzed with the ApopTag® Plus Fluorescein In Situ Apoptosis Detection kit (cat. no. S7111; Millipore Sigma) containing TUNEL reagent, according to the manufacturer's instructions. Subsequently, the cells were incubated with DAPI at 37˚C for 2-3 min and mounted in an anti-fade reagent (Beijing Solarbio Science & Technology Co., Ltd.). The stained cells were observed in three random fields of view with the application of a fluorescence microscope (Nikon TE200-U; Nikon Corporation).

Cytoskeletal assay. H9C2 cells were incubated on slides with medium at 37˚C, in the presence of 5% CO_2. When the cells were cultured to 80-90% confluence, they were washed twice with PBS (pH 7.4). The cells were fixed with 4% formaldehyde for 10 min at room temperature and washed twice with PBS for 10 min. Subsequently, the cells were permeabilized with 0.5% Triton X-100 solution for 5 min at room temperature. Following washing twice with PBS for 10 min, 200 μl rhodamine-labeled phalloidin solution (5 μg/ml; Sigma-Aldrich; Merck KGaA) was incubated for 30 min with the cells at room temperature in the dark. Following a second wash with PBS twice for 5 min each time, the nuclei were re-stained with 200 μl DAPI solution at 37˚C for 30 sec. Finally, the cell length was observed after 24 h using a Smartproof 5 confocal microscope (Zeiss AG).

Enzyme-linked immunosorbent assay (ELISA). H9C2 cells were treated with Ang II (0.5, 1, 5 and 10 μM) at 37˚C for 24 h and centrifuged at 2,000 x g for 5 min at 4˚C. The supernatants were collected for the determination of the expression levels of the inflammatory cytokines TNF-α, IL-6 and IL-1β. TNF-α (cat. no. ab236712), IL-6 (cat. no. ab234570) and IL-1β (cat. no. ab255730) ELISA kits (all from Abcam) were used and the protocol was performed as determined by the manufacturer's instructions.

Measurement of superoxide dismutase (SOD) activity and malondialdehyde (MDA) levels. The activity of SOD (cat. no. BC0170) and the MDA (cat. no. BC0025) levels (markers of oxidative stress) were assessed using the corresponding commercial kits (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The absorbance values were recorded at 560 and 532 nm using a microplate reader for the determination of the SOD activity and the MDA levels, respectively.

Statistical analysis. All data are presented as mean ± standard deviation from at least three independent experiments. Statistical analysis was conducted using SPSS 19.0 (SPSS, Inc.). Significant differences were noted between multiple groups and the data were analyzed using one-way analysis of variance followed by a Bonferroni post hoc comparisons test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FBW7 expression is downregulated in Ang II-induced H9C2 cells and its upregulation activates mTOR-mediated autophagy. The protein and mRNA expression levels of FBW7 were significantly reduced in H9C2 cells treated by Ang II at the concentrations of 0.5, 1, 5 and 10 μM compared with those in the control group (Fig. 1A and B). FBW7 expression was the lowest when the concentration of Ang II was 10 μM. Therefore, 10 μM Ang II was selected as the concentration required for the induction experiments. By transfecting Oe-FBW7 into H9C2 cells, the expression levels of FBW7 were successfully significantly elevated compared with those of the negative control (Fig. 1C and D). The expression levels of the autophagy-related proteins LC3B-II and Beclin-1 were significantly reduced in the Ang II group (vs. control), whereas they were significantly increased in the Ang II+Oe-FBW7 group (vs. Oe-NC). The expression levels of LC3B-I and p62 indicated the opposite trends to the aforementioned two proteins in each group (Fig. 1E). In addition, western blotting was used to detect a significantly increased expression of p-mTOR in the Ang II group compared with that of the control group. A significantly decreased expression of p-mTOR was noted in the Ang II+Oe-FBW7 group in comparison with the Oe-NC group (Fig. 1F). The expression levels of mTOR remained the same in each group. Therefore, FBW7 expression was downregulated in Ang II-induced H9C2 cells and the upregulation of FBW7 expression activated mTOR-mediated cellular autophagy.

FBW7 inhibits Ang II-induced apoptosis in H9C2 cells via the activation of autophagy. To determine whether FBW7 affects Ang II-induced H9C2 cell viability via induction of autophagy, the autophagy inhibitor 3-MA was added to the cells. The viability of H9C2 cells was decreased by ~45% in the Ang II group compared with that of the control group. It was also increased by ~30% in the Ang II+Oe-FBW7 group compared with that of the negative control group. The addition of 3-MA significantly decreased the cell viability of H9C2 cells compared with the Ang II+Oe-FBW7 group (Fig. 2A). This result indicated that FBW7 overexpression could largely prevent Ang II-induced loss of cell viability, whereas 3-MA reversed partly the effect of FBW7 on H9C2 cells. The apoptotic rate in the Ang II group was estimated to be ~25%, which was markedly higher than that in the control group (Fig. 2B and C). FBW7 overexpression reduced this percentage to ~10% (vs. Oe-NC), whereas 3-MA increased it further to ~20% compared with the Oe-FBW7 group. In addition, the expression level of the anti-apoptotic protein Bcl-2 in H9C2 cells was significantly decreased, whereas the levels of Bax and cleaved caspase 3 were increased in the Ang II group compared with those in the control group (Fig. 2D). The expression level of Bcl-2 was significantly elevated, and the levels of Bax and cleaved caspase 3 were decreased in Ang II-treated H9C2 cells transfected with Oe-FBW7 compared with those in the negative control group, whereas the Bcl-2 level was reduced and the levels of Bax and cleaved

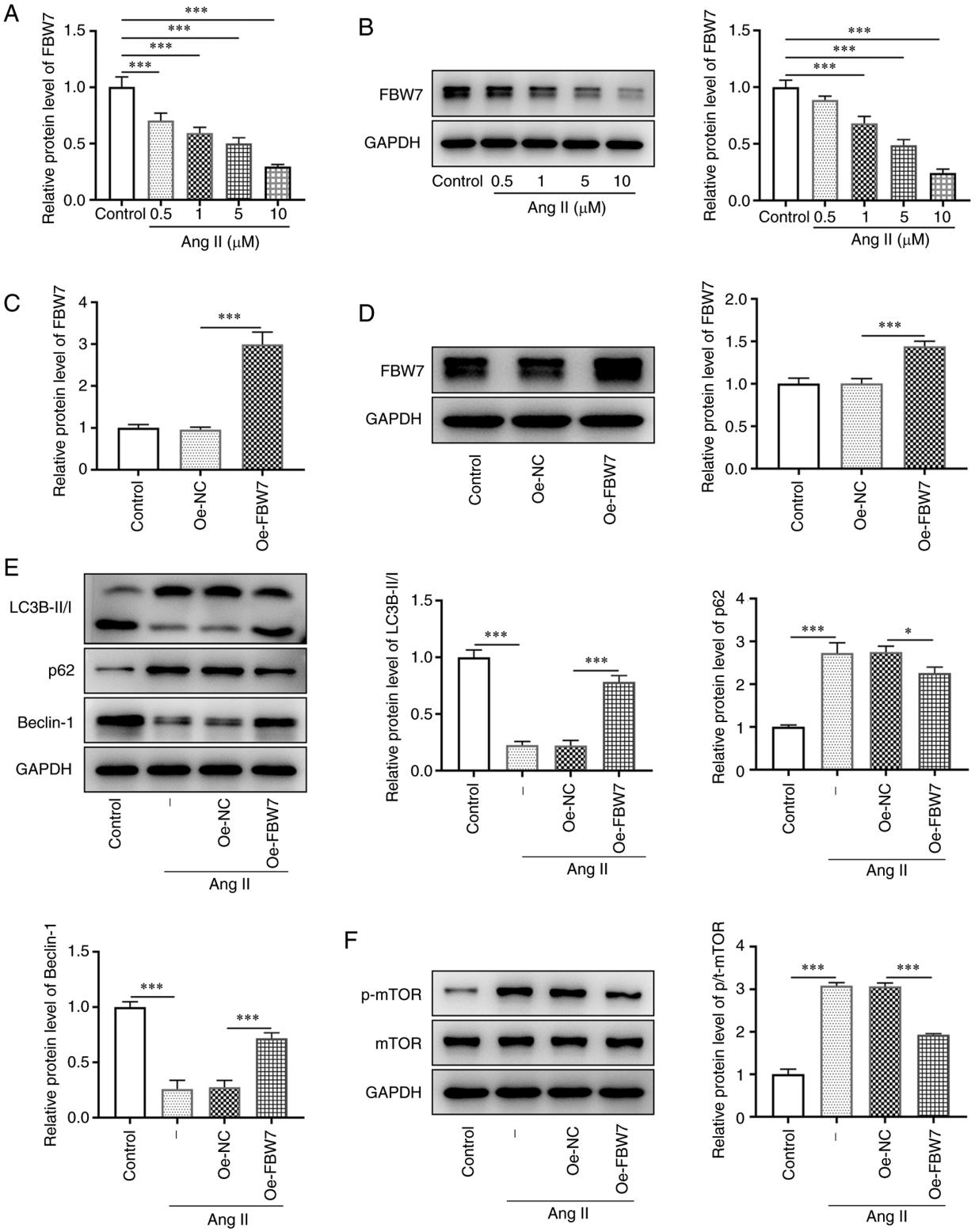

Figure 1. FBW7 expression is downregulated in Ang II-induced H9C2 cells and its upregulation activates mTOR-mediated cell autophagy. (A) mRNA expression of FBW7 in the control and Ang II (0.5, 1, 5 and 10 μM) groups. (B) Protein expression level of FBW7 in the control and Ang II (0.5, 1, 5 and 10 μM) groups. Overexpression efficiency of FBW7 in the control, Oe-NC and Oe-FBW7 groups was measured using (C) RT-qPCR and (D) western blotting. (E) Protein expression levels of LC3B-II/I, P62 and Beclin-1 related to autophagy in the control, Ang II, Ang II+Oe-NC and Ang II+Oe-FBW7 groups. (F) Protein levels of p-mTOR and mTOR related to mTOR signaling pathway in the control, Ang II, Ang II+Oe-NC and Ang II+Oe-FBW7 groups. *P<0.05, ***P<0.001. FBW7, F-box and WD repeat-containing protein 7; Ang, angiotensin; Oe, overexpression; NC, negative control; LC3B, microtubule-associated proteins 1A/1B light chain 3B; p-, phosphorylated; -, Ang II treatment without transfection.

caspase 3 were elevated in the Ang II+Oe-FBW7+3-MA group compared with in the Ang II+Oe-FBW7 group (Fig. 2D). This evidence suggested that FBW7 could inhibit Ang II-induced apoptosis in H9C2 cells via the autophagic pathway.

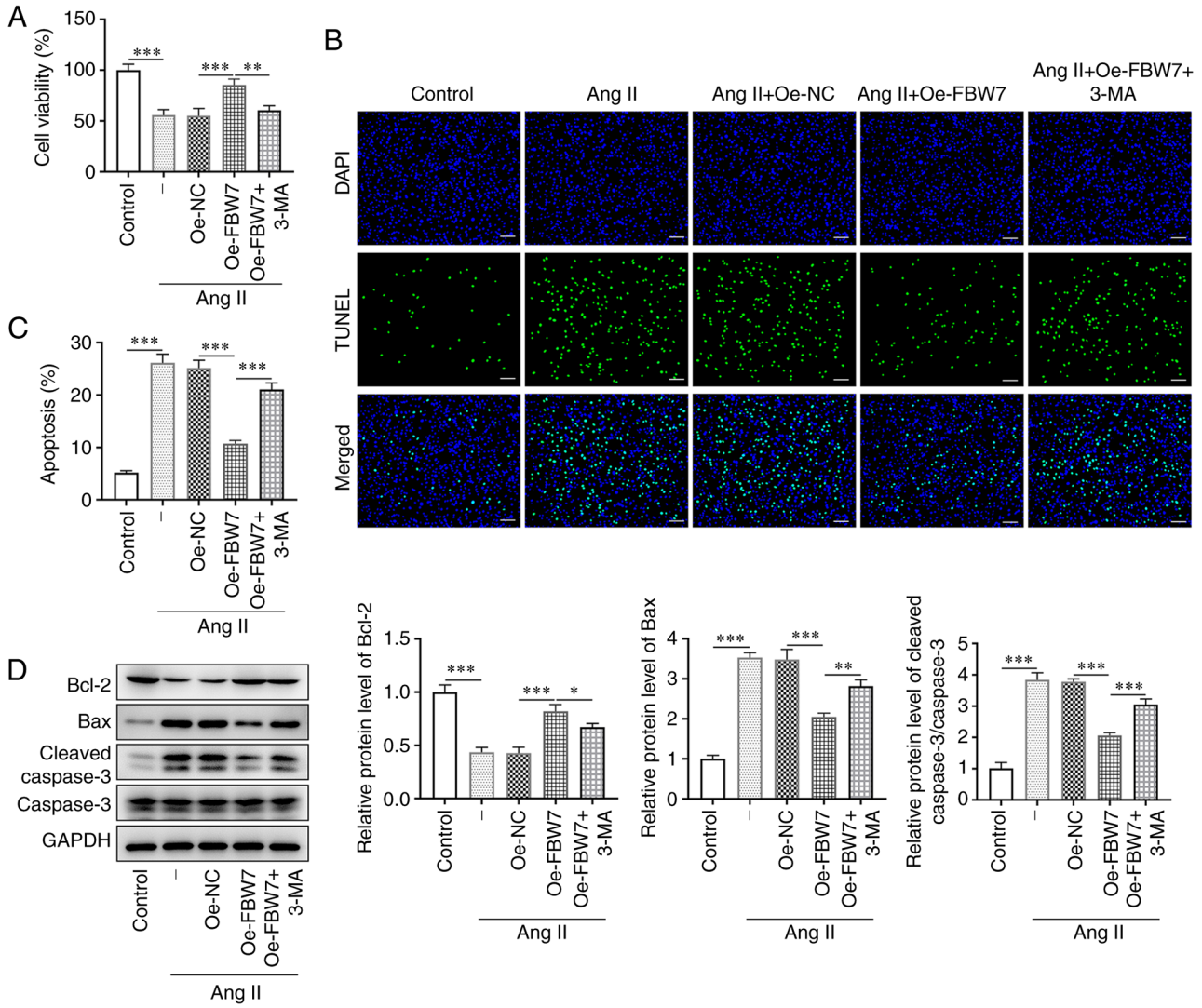

Figure 2. FBW7 inhibits Ang II-induced apoptosis in H9C2 cells via autophagy. (A) Cell viability of H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. (B and C) Apoptosis of H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. Scale bar, 50 μm. (D) Protein levels of Bcl-2, Bax and cleaved caspase 3 associated with apoptosis in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. *P<0.05, **P<0.01, ***P<0.001. FBW7, F-box and WD repeat-containing protein 7; Ang, angiotensin; Oe, overexpression; NC, negative control; 3-MA, 3-methyladenine; -, Ang II treatment without transfection.

FBW7 inhibits Ang II-induced hypertrophy and fibrosis in H9C2 cells via induction of autophagy. The size of the cytoskeleton was observed using confocal microscopy to assess whether the Ang II-induced cardiomyocyte hypertrophy model was successfully established. Ang II effectively induced H9C2 cytoskeletal expansion as determined by an increase in cell length, whereas FBW7 overexpression successfully prevented the Ang II-stimulated irease in the cytoskeletal size (Fig. 3A). The length of Ang II-induced H9C2 cells transfected with Oe-FBW7 was increased again following treatment with 3-MA. In addition, the protein levels of the hypertrophic markers β-MHC, BNP and ANF were also significantly increased in response to Ang II treatment compared with the control group, whereas they were significantly decreased following FBW7 oncverexpression compared with the Ang II+Oe-NC group. 3-MA caused a significant increase in the levels of these hypertrophic markers compared with the Ang II+Oe-FBW7 group (Fig. 3B). Concomitantly, the cells underwent fibrotic changes. The expression levels of the fibrosis-related proteins α-SMA, fibronectin, vimentin, collagen I and III were significantly increased in Ang II-induced H9C2 cells compared with the control group, whereas they were significantly decreased following FBW7 overexpression compared with the Ang II+Oe-NC group (Fig. 3C). However, the expression levels of these proteins were markedly increased again following 3-MA treatment. Collectively, these results demonstrated that FBW7 overexpression could inhibit Ang II-induced hypertrophy and fibrosis in H9C2 cells via the autophagic pathway.

FBW7 suppresses Ang II-induced inflammation and oxidative stress in H9C2 cells via induction of autophagy. In order to determine whether FBW7 affects inflammation and oxidative stress in Ang II-induced H9C2 cells, the expression levels of the inflammatory factors and the levels of oxidative stress were assessed. The levels of the pro-inflammatory cytokines TNF-α and IL-1β were significantly increased in the Ang II group compared with the control, whereas they significantly

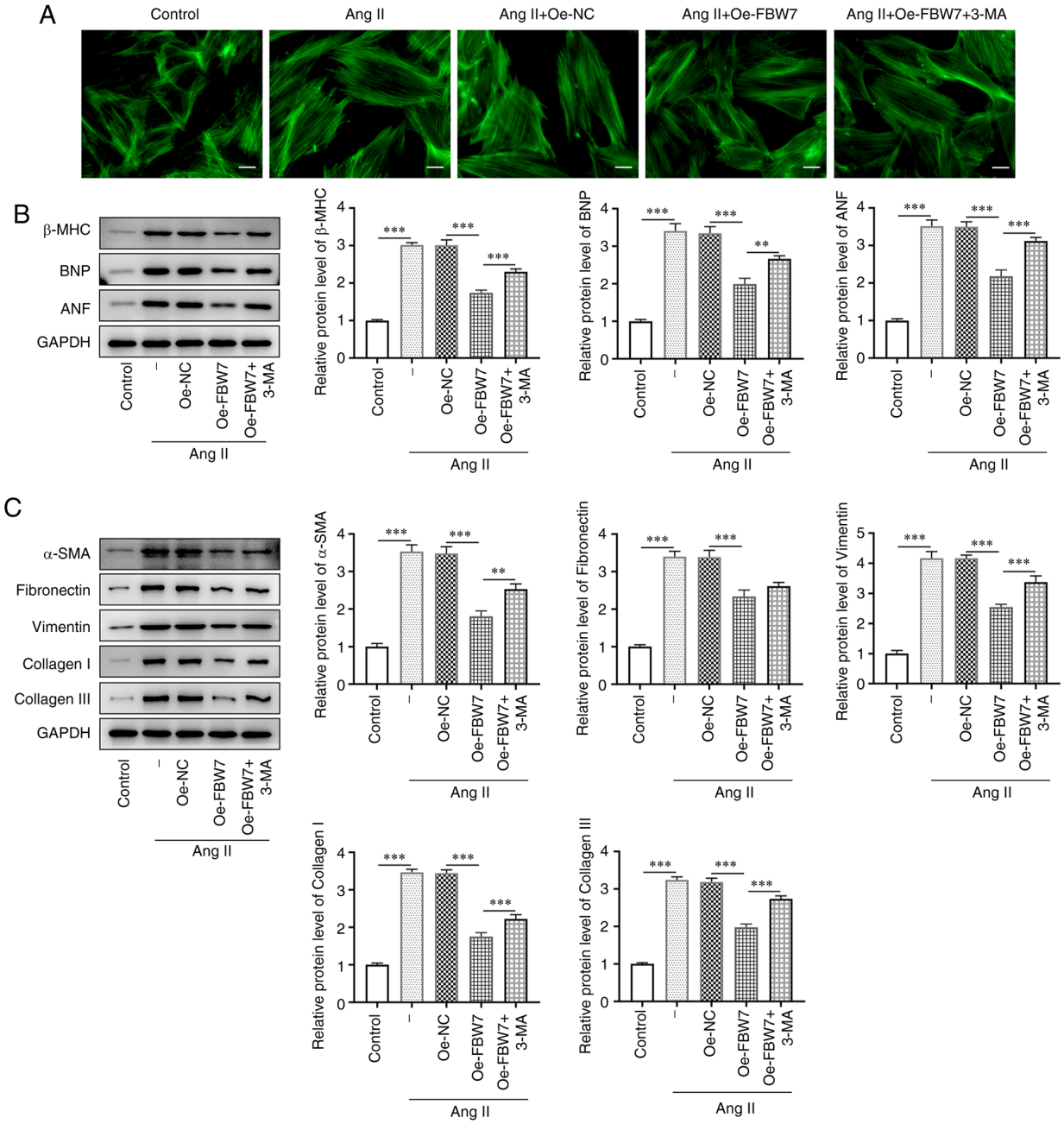

Figure 3. FBW7 inhibits Ang II-induced hypertrophy and fibrosis in H9C2 cells through autophagy. (A) Cell length of H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. Scale bar, 25 μm. (B) Protein levels of hypertrophic markers β-MHC, BNP and ANF in H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. (C) Protein levels of α-SMA, Fibronectin, Vimentin Collagen I and Collagen III associated fibrosis in H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. **P<0.01, ***P<0.001. FBW7, F-box and WD repeat-containing protein 7; Ang, angiotensin; Oe, overexpression; NC, negative control; 3-MA, 3-methyladenine; β-MHC, β-myosin heavy chain; BNP, brain natriuretic peptide; ANF, atrial natriuretic factor; atrial natriuretic factor; SMA, smooth muscle actin; -, Ang II treatment without transfection.

declined in the Ang II+Oe-FBW7 group compared with Oe-NC (Fig. 4A and B). By contrast, the expression levels of TNF-α and IL-1β were increased in the Ang II+Oe-FBW7+3-MA group compared with the Ang II+Oe-FBW7 group (Fig. 4A and B). Moreover, Ang II induced a significant increase in the protein levels of iNOS and COX-2 in H9C2 cells compared with the control group. This increase was significantly reduced by FBW7 overexpression. 3-MA partially abrogated the inhibitory effect of FBW7 on the protein levels of iNOS and COX-2 compared with the Ang II+Oe-FBW7 group (Fig. 4C). Furthermore, the activity of SOD was significantly reduced in the Ang II group compared with that of the control group, whereas it was increased in the Ang II+Oe-FBW7 group compared with that of the Oe-NC group. The activity of SOD declined in the Ang II+Oe-FBW7+3-MA group compared with the Ang II+Oe-FBW7 group, while the levels of MDA exhibited the opposite trend to that of MDA (Fig. 4D and E). Overall, these results illustrated that FBW7 could suppress Ang II-induced inflammation and oxidative stress in H9C2 cells via the autophagic pathway.

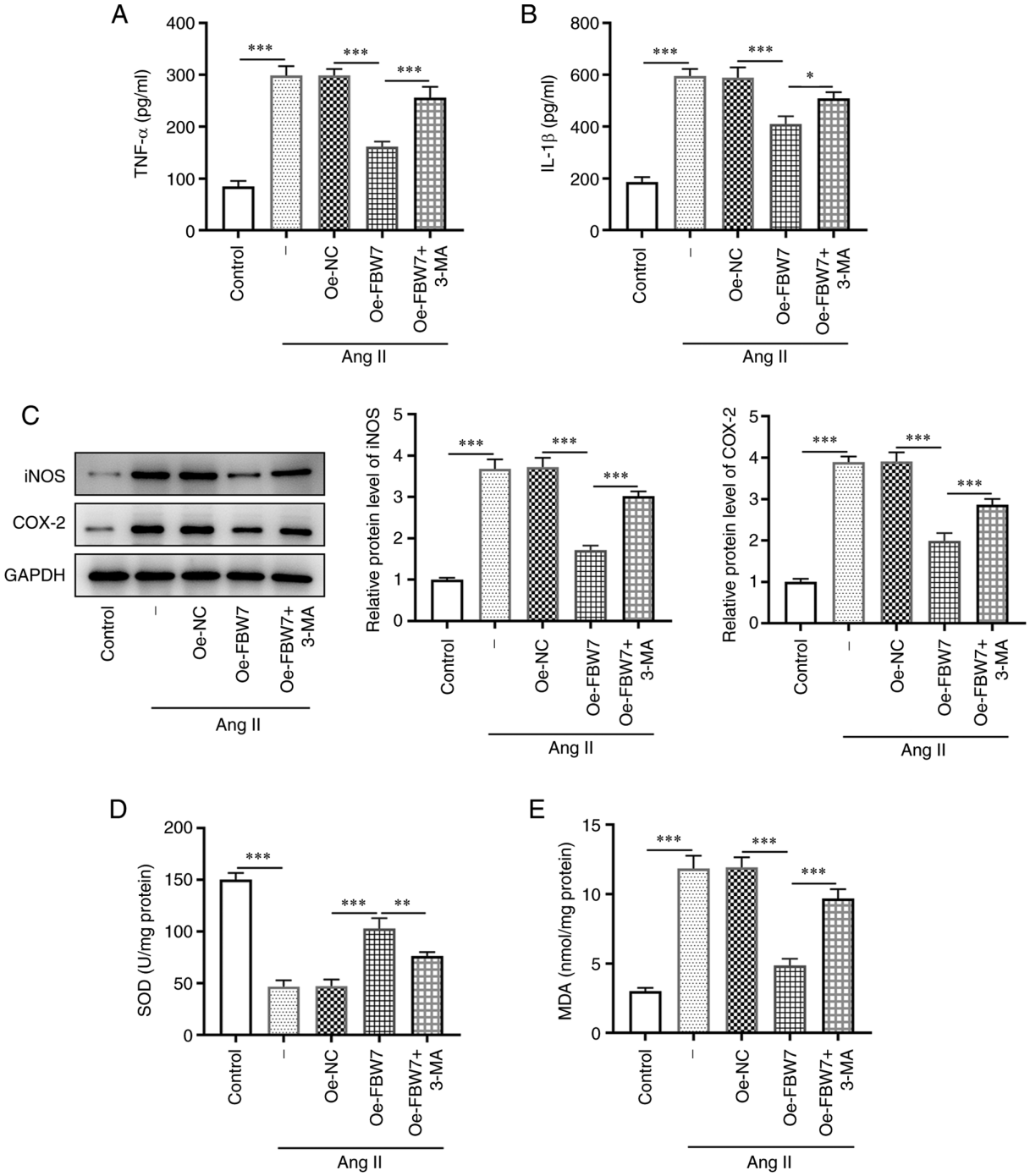

Figure 4. FBW7 suppresses Ang II-induced inflammation and oxidative stress in H9C2 cells via autophagy. Levels of inflammatory cytokines (A) TNF-α and (B) IL-1β in H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. (C) Protein levels of iNOS and COX-2 associated with inflammation in H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. Levels of (D) SOD and (E) MDA associated with oxidative stress in H9C2 cells in the control, Ang II, Ang II+Oe-NC, Ang II+Oe-FBW7 and Ang II+Oe-FBW7+3-MA groups. *P<0.05, **P<0.01, ***P<0.001. FBW7, F-box and WD repeat-containing protein 7; IL, interleukin; Ang, angiotensin; Oe, overexpression; NC, negative control; 3-MA, 3-methyladenine; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; SOD, superoxide dismutase; MDA, malondialdehyde; -, Ang II treatment without transfection.

Discussion

Myocardial hypertrophy is an adaptive response to hemodynamic stress and serves as compensation to improve cardiac performance and reduce ventricular wall tension and oxygen consumption (22). However, pathological myocardial hypertrophy is more likely to lead to a variety of cardiovascular diseases, such as myocardial infarction and sudden death (23). As aforementioned, FBW7 has been reported to exert an important regulatory role in cardiac development (10,11). Therefore, to explore the regulatory role of FBW7 in cardiac hypertrophy, the present study established a myocardial hypertrophy model using Ang II-treated cells and determined the protective role of FBW7 in Ang II-induced H9C2 cell injury.

The F-box protein FBW7, also termed FBXW7, is a component of the SCF-type E3 ubiquitin ligase (24). As

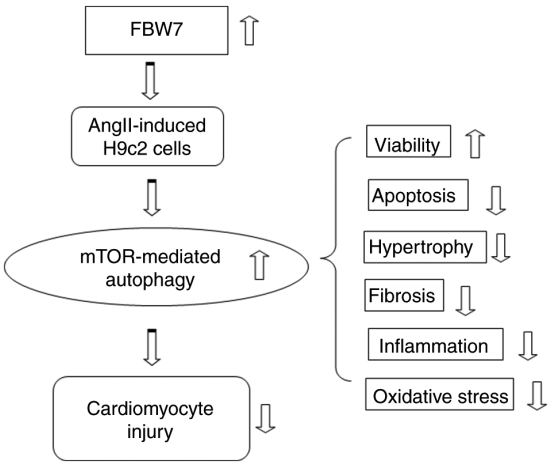

Figure 5. Schematic diagram presenting the hypothesis of the present study. FBW7, F-box and WD repeat-containing protein 7; Ang, angiotensin.

previously described, FBW7 plays a key role in cardiomyocyte development. Its dysregulated expression is considered to be a pivotal step in malignant transformation (16). The present experiments indicated that FBW7 expression was significantly downregulated in Ang II-induced H9C2 cells in a concentration-dependent manner. In addition, the induction of autophagy is associated with clearance of pathogens and antigen expression and has been indicated to be inhibited by the mTOR complex 1 (mTORC1) (25). It has been demonstrated that the induction of autophagy in cardiomyocytes plays a protective role during hemodynamic stress (26). Moreover, the FBXW7-SHOC2-RPTOR axis can regulate mTORC1-mediated autophagy (27). The present experiments revealed a significant increase in the autophagy-related proteins LC3B-II and Beclin-1, and a decrease in the levels of the proteins LC3B-I and p62 in Ang II-induced H9C2 cells following overexpression of FBW7 in comparison with the Oe-NC group. Furthermore, a drop in the protein levels of p-mTOR was noted in Ang II-induced H9C2 cells transfected with Oe-FBW7 compared with that the levels in Ang II-induced H9C2 cells transfected with Oe-NC, indicating that upregulation of FBW7 could activate mTOR-mediated autophagy in Ang II-treated H9C2 cells.

Apoptosis is a unique form of cell death that can cause excessive cardiomyocyte death, leading to cardiac dysfunction. Apoptosis is also involved in the transition from adaptive cardiac hypertrophy to pathological cardiac hypertrophy (28). The process of apoptotic dysregulation is associated with a decrease in the expression levels of the anti-apoptotic protein Bcl-2 and an increase in the expression levels of the pro-apoptotic protein Bax as well as in the levels of cleaved caspase 3 (29). A previous study demonstrated that FBW7 can regulate apoptosis by targeting induced myeloid leukemia cell differentiation protein MCL1 for ubiquitylation and destruction (30). The present experiments demonstrated that Ang II induced a significantly lower cell viability, reduced the rate of apoptosis and the expression levels of the anti-apoptotic protein Bcl-2 in H9C2 cells. These effects were inhibited by overexpression of FBW7. Subsequently, the autophagy inhibitor 3-MA was used to pretreat H9C2 cells for 3 h. 3-MA significantly reversed the inhibitory effects of FBW7 on Ang II-induced apoptosis in H9C2 cells. These results suggested that the inhibitory effects of FBW7 on the Ang II-induced apoptosis in H9C2 cells were achieved via the autophagic pathway.

Pathological cardiac hypertrophy is often accompanied by myocardial dysfunction and fibrosis (31). β-MHC, BNP and ANF are markers of cardiomyocyte hypertrophy. Previous studies have indicated that FBXW7 negatively regulates physiological cardiac hypertrophy by inhibiting the pro-hypertrophic hypoxia-inducible factor-1α-post signaling pathway and promoting the inactivation of Akt (32). In the present study, cell length and the levels of the hypertrophic marker proteins β-MHC, BNP and ANF were increased by Ang II in H9C2 cells. The expression levels of these markers were markedly decreased following FBW7 overexpression, whereas following the addition of 3-MA they were elevated.

Furthermore, circular RNA circFBXW4 inhibits hepatic fibrosis by targeting the miR-18b-3p/FBXW7 axis (33). Ang II induced increased expression levels of the fibrosis-associated proteins α-SMA, fibronectin, vimentin, collagen I and III in H9C2 cells, whereas these effects were decreased by FBW7 overexpression and the latter effect was partially reversed by the addition of 3-MA to the cells. This evidence suggested that FBW7 could inhibit Ang II-induced hypertrophy and fibrosis in H9C2 cells via the autophagic pathway.

According to previous studies, a reduction in oxidative stress levels is beneficial in mitigating the development of cell hypertrophy (34,35). In addition, inflammation also plays an important role in the pathogenesis of hypertrophy (36). This is due to the ability of the inflammatory cytokine TNF-α to suppress cardiac contractility and to provoke myocardial hypertrophy, cardiac fibrosis and cardiomyocyte apoptosis (37-39). Moreover, IL-1β, which is a downstream factor of caspase-1 plays an important role in cardiac hypertrophy (40). A previous study demonstrated that FBW7β contributes to the protection of cells from oxidative stress (41). FBW7 overexpression can increase autophagy by inhibiting mTOR signaling and ameliorating inflammation (18). In the present study, the expression levels of the inflammatory cytokines TNF-α and IL-1β and of the inflammation-associated proteins iNOS and COX-2 were significantly increased by Ang II in H9C2 cells, whereas they were decreased following overexpression of FBW7 and partially reversed by application of 3-MA.

Furthermore, the level of the antioxidant enzyme SOD was decreased in Ang II-treated H9C2 cells, whereas it was decreased following FBW7 overexpression. However, the level of SOD was decreased again following the addition of 3-MA. These results revealed that FBW7 overexpression could inhibit Ang II-induced inflammation and oxidative stress in H9C2 cells via the autophagic pathway.

Notably, Gao *et al* (42) revealed that FBXW7 promotes pathological cardiac hypertrophy by targeting EZH2-SIX1 signaling, which shows discrepant results with the present results. However, another study used the same cell line as that in the current study and demonstrated results that are consistent with the present paper (11). In addition, several studies have demonstrated that FBW7 is beneficial for cardiovascular physiological activity and metabolism (16,32,43). Thus, the roles of FBW7 in cardiac hypertrophy still need to be explored. Furthermore, Ang II concentration used in the present *in vitro* study was different compared with that of plasma level of

human, thus the result of the present study may not be suitable for the phenomenon of *in vivo*. Future *in vivo* experiments will be performed to confirm the role of FBW7 and the involvement of mTOR-mediated autophagic pathway in myocardial hypertrophy in further study.

The studies presented so far provide evidence that FBW7 inhibits Ang II-induced H9C2 apoptosis, hypertrophy and fibrosis, inflammation and oxidative stress via the mTOR-mediated autophagic pathway (Fig. 5). The findings may provide a novel fundamental insight into how FBW7 ameliorates myocardial hypertrophy and enhance the potential applicability of FBW7 in clinical practice.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QL and WZ designed the study, drafted and revised the manuscript. CH, XW and JZ analyzed the data and searched the literature. QL, CH and XW performed the experiments. All authors read and approved the final manuscript. QL and CH 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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