Effects of Sirt3-autophagy and resveratrol activation on myocardial hypertrophy and energy metabolism

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Abstract. The aim of the present study was to examine the role of sirtuin 3 (Sirt3)-autophagy in regulating myocardial energy metabolism and inhibiting myocardial hypertrophy in angiotensin (Ang) II-induced myocardial cell hypertrophy. The primary cultured myocardial cells of neonatal Sprague Dawley rats were used to construct a myocardial hypertrophy model induced with Ang II. Following the activation of Sirt3 by resveratrol (Res), Sirt3 was silenced using small interfering (si)RNA-Sirt3, and the morphology of the myocardial cells was observed under an optical microscope. Reverse transcription-polymerase chain reaction was used to detect the mRNA expression of the following myocardial hypertrophy markers; atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), Sirt3, medium-chain acyl-CoA dehydrogenase (MCAD) and pyruvate kinase (PK). Western blot analysis was used to detect the protein expression of Sirt3, light chain 3 (LC3) and Beclin1. Ang II may inhibit the protein expression of Sirt3, LC3 and Beclin1. Res, an agonist of Sirt3, may promote the protein expression of Sirt3, LC3 and Beclin1. Res inhibited the mRNA expression of ANP and BNP, and reversed the Ang II-induced myocardial cell hypertrophy. The addition of siRNA-Sirt3 decreased the protein expression of Sirt3, LC3 and Beclin1, increased the mRNA expression of ANP and BNP, and weakened the inhibitory effect of Res on myocardial cell hypertrophy. Res promoted the mRNA expression of MCAD, inhibited the mRNA expression of PK, and reversed the influence of Ang II on myocardial energy metabolism. siRNA-Sirt3 intervention significantly decreased the effect of Res in eliminating abnormal myocardial energy metabolism. In conclusion, Sirt3 may inhibit Ang II-induced myocardial hypertrophy and reverse the Ang II-caused abnormal myocardial energy metabolism through activation of autophagy.

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Introduction

Myocardial hypertrophy is an independent risk factor of cardiovascular diseases (1). At the molecular level, myocardial hypertrophy is caused by an imbalance of protein synthesis and degradation. Persistent external stimuli lead to overexpression of the signaling pathway for myocardial protein synthesis and inhibition of the metabolic pathway (2). Other effects include increased myocardial cell volume, increased protein synthesis and muscle fibre and myocardial remodeling (3). Furthermore, abnormal energy metabolism, which turns from the oxygenolysis of fatty acids to the utilization of glucose, is additionally accompanied (4). Such a long-term alteration will lead to the reduction of energy production efficiency, the accumulation of fatty acids in myocardial cells, and the increase of the anaerobic glycolysis of glucose, which reduces the myocardial cell contraction and accelerates the occurrence and development of heart failure (5-7). The present study examined the regulation of myocardial cell energy metabolism and the autophagy signaling pathway to determine a mechanism that may inhibit myocardial hypertrophy and may be used as a novel target of treatment.

The primary regulatory factors in cell energy metabolism are AMP-activated protein kinase (AMPK) and members of the silent information regulator family (7,8). Sirtuin3 (Sirt3) belongs to the latter and is a histone deacetylase (HDAC) III (8). As a nicotinamide adenine dinucleotide (NAD)-dependent HDAC primarily existing in the mitochondria, Sirt3 not only regulates the energy metabolism of cells; however, additionally serves an important role in apoptosis, tumor growth and cardiovascular diseases (9). Previously, an increasing number of studies have reported that Sirt3 serves a key role in myocardial hypertrophy. Sundaresan *et al* (10) observed that Sirt3 may downregulate mitogen-activated protein kinases/extracellular signal-regulated kinases and the phosphoinositide 3-kinase/protein kinase B signaling pathways through inhibition of the oxygen radical-mediated renin activity. This inhibition occurs by activating forkhead box protein O3 (FoxO3), manganese superoxide dismutase and catalase, and by inhibiting myocardial hypertrophy (10). Pillai *et al* (11) determined that myocardial hypertrophy may be inhibited through activation of the Sirt3-liver

kinase B1 (LKB1)-AMPK pathway (11). It was observed that Sirt3 gene knockout mice demonstrated myocardial hypertrophy (12). All of these studies suggest that Sirt3 is involved in the occurrence and development of myocardial hypertrophy.

Autophagy is a biological phenomenon widely available in eukaryocytes. Autophagy is additionally an important channel of waste elimination, structure reconstruction, and growth and development of cells (13). Reduced autophagy has been identified in myocardial hypertrophy since the early 1980s; Dämmrich and Pfeifer (14) identified decreased autophagy in an aortic coarctation model. Nakai *et al* (15) observed significantly decreased autophagy in a myocardial hypertrophy model induced by aortic coarctation. A previous study published in 2014 (16) additionally demonstrated that the autophagy level decreased significantly in an *in vitro* myocardial hypertrophy model induced by angiotensin (Ang) II, which suggests that autophagy may inhibit the development of myocardial hypertrophy.

At present, the Sirt3-autophagy pathway is more frequently reported, including in hepatic diseases (17-19), the nervous system (20,21), tumors (22,23) and skeletal muscle (24). In myocardial ischemia reperfusion, Sirt3 may protect the heart by promoting autophagy (25). However, there is no study at present, to the best of the authors' knowledge, on the Sirt3-autophagy pathway in myocardial hypertrophy.

Materials and methods

Experimental animals. In total, 12 clean Sprague Dawley rats (6 male and 6 female) born within 1-2 days were provided by the Experimental Animal Centre of Shantou University Medical College (Shantou, China) from the same race and brood. The temperature was maintained at 25˚C and the humidity at was maintained at 50% with 12-h light/dark cycle. All the rats had free access food and water. Differences pertaining to a comparison among age, weight (average 5-6 g) and health state were not statistically significant (P>0.05). The present study was approved by the Medical Ethics Committee of Shantou University.

Primary reagents. Dulbecco's modified Eagle's medium (DMEM)-F12 and fetal calf serum (FCS; HyClone; GE Healthcare Life Sciences), trypsin and collagenase I (Gibco; Thermo Fisher Scientific, Inc.), BrdU (Sigma-Aldrich; Merck KGaA), Ang II (AnaSpec), antibodies against Sirt3 [Cell Signaling Technology (CST), Inc.; C73E3 Rabbit mAb; cat. no. 2627S], light chain (LC)3I/II (CST, Inc.; D11, XP Rabbit mAb; cat. no. 3868S), Beclin1 (CST, Inc.; D40C5, Rabbit mAb; cat. no. 3495S), GADPH (CST, Inc.; D16H11, XP Rabbit mAb; cat. no. 5174S) and β-actin (CST, Inc.; 13E5, Rabbit mAb; cat. no. 4970T), goat anti-rabbit immunoglobulin G-horseradish peroxidase (HRP) secondary antibody (cat. no. R11412; Bellancom), a western blotting kit (EMD Millipore), nuclease-free water, RNAase inhibitor, deoxyribonucleotide mixture, reverse transcriptase and random primers (Takara Bio, Inc.), and resveratrol (Shanghai Sangon Pharmaceutical Co., Ltd.) were used.

Separation and culture of primary myocardial cells of neonatal rats. The heart was removed by thoracotomy under aseptic conditions and submerged in D-Hank's solution. Subsequent to the bloodiness being cleaned, the connective tissue of the cardiac base and the atrial tissue were removed. The ventricle was cut off to remove the extravasated blood. The myocardial tissue was placed into a 15 ml centrifuge tube and cut into blocks of 1 mm^3. After 0.5 g/l collagenase type I was added, the tube was gently oscillated in a 37˚C thermostatic bath for 2 h to lyse the tissue block. Subsequently, 0.125 g/l pancreatin was added to digest the tissue and this was conducted 2-3 times (5 min/time). The digested cells were collected with medium containing 100 ml/l FCS, inoculated into a culture dish, and placed into a 5% CO_2 incubator for 1 h at 37˚C. Fibroblasts were eliminated by differential adhesion. Myocardial cells were inoculated into a 6-well plate at a density of $1x10^6$/ml. A total of 0.03 g/l BrdU was added to inhibit fibroblast growth. A total of 0.1 g/l penicillin and 0.1 g/l streptomycin were added to prevent bacterial contamination. Subsequently, the plate was cultured in a 50 ml/l CO_2 incubator. At 24 h after inoculation and when the cells fused and contracted synchronously, the serum-free medium was used. After 24 h, intervention of the different groups was conducted (26).

Protein expression is detected by western blot analysis. Following the completion of the aforementioned treatment, myocardial cells were lysed and the total protein was extracted using SDS buffer (Jianglai Bio, Inc.). A total of 30 μl of protein, determined using bicinchoninic acid method, was loaded in each lane of a 12% of SDS-PAGE gel for electrophoresis. Subsequent to electrophoresis, the protein was transferred to the polyvinylidene difluoride (PVDF) membranes (80 V; 120 min); after 1 h of blocking at 25˚C using 5% bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.), the protein was incubated with the following antibodies overnight at 4˚C; Sirt3 (1:1,000), LC3I/II (1:1,000), Beclin1 (1:1,000), medium-chain acyl-CoA dehydrogenase (MCAD; 1:1,000), GADPH (1:20,000) and β-actin (1:10,000). Subsequent to washing with Tris-buffered saline with 15 ml of 1X Tween-20 the following day, the PVDF membranes were incubated with HRP-labelled goat anti-rabbit (1:10,000) antibody for 1 h at room temperature. Western blot analysis was conducted using enhanced chemiluminescence substrate kit (Shanghai Yaxin Biotechnology Co., Ltd.). Quantity One image analysis software (version 4.5; Bio-Rad Laboratories, Inc.) was used to detect the grey value of the protein bands. The grey value ratio of the target band and GAPDH or β-actin was used to indicate the expression level of the target protein (27).

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The mRNA was extracted by routine TRIzol® lysis (Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA. Reverse transcription was performed using a PrimeScript RT Reagent kit (Takara Bio, Inc.). GAPDH was used as an internal control. The PCR reaction conditions were as follows: Initial denaturation at 42˚C for 60 min followed by 99˚C for 5 min and 4˚C for 5 min. Relative expression of the target gene was calculated using the $2^{-\Delta\Delta Cq}$ method (28): $\Delta\Delta Cq$ = Cq target gene - Cq reference gene (experimental group), Cq target gene - Cq reference gene (control group). Cq was the number of quantification cycles at which the fluorescence exceeded the threshold. Each experiment was performed in triplicate.

According to the standard curve: i) Using cDNA of the control group as the template, the cDNA was diluted to 1:10, 1:50, 1:1,00 and 1:1,000; ii) ligand system: The total volume

Table I. Primer sequences for the polymerase chain reaction assays.

Gene	Primer	Primer sequences
Sirt3	F	5'-TGCACGGTCTGTCGAAGGTC-3'
	R	5'-AGGTTTCACAACGCCAGTA-3'
ANP	F	5'-CGTATACAGTGCGGTGTCCA-3'
	R	5'-GATCTATCGGAGGGGTCCCA-3'
BNP	F	5'-TCCTTAATCTGTCGCCGCTG-3'
	R	5'-CGCCGATCCGGTCTATCTTC-3'
MCAD	F	5'-AGCCCTGGACGAAGCTACTA-3'
	R	5'-GCGAGCTGGTTGGCAATATC-3'
GAPDH	F	5'-TGCCACTCAGAAGACTGTGG-3'
	R	5'-TTCAGCTCTGGGATGACCTT-3'
β-actin	F	5'-GAACCCTAAGGCCAACCGTGAAAAGAT-3'
	R	5'-ACCGCTCGTTGCCAATAGTGATG-3'
PK	F	5'-AATCCCGGCAGATACAGACT-3'
	R	5'-GGAGTTCCACACCCTGCTAT-3'

Sirt3, sirtuin 3; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; MCAD, medium-chain acyl-CoA dehydrogenase; PK, pyruvate kinase; F, forward; R, reverse.

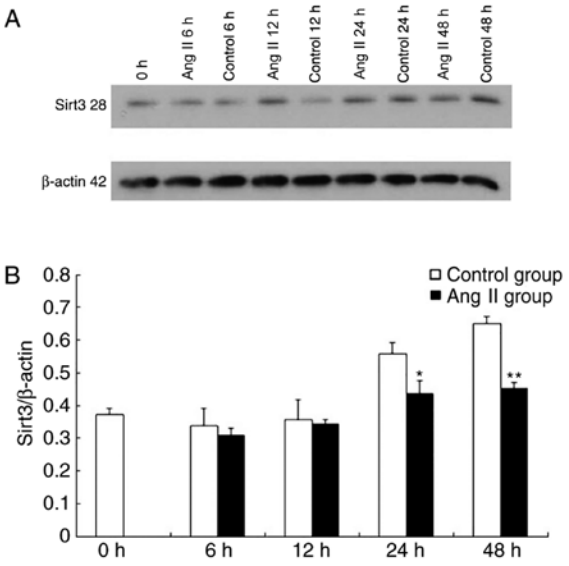

Figure 1. Influence of Ang II on Sirt3 protein expression at different time-points. (A) Shows the representative western blot of Sirt3 protein expression at 4 different time points in the control group and AngII group. (B) Sirt3/β-actin ratio figure at 4 different time points. n=4. *P<0.05, **P<0.01 vs. respective control. Ang II, angiotensin II; Sirt3, sirtuin 3.

was 10 μl and each sample had three wells (4 μl cDNA + 0.3 μl Primer F + 0.3 μl Primer R + 0.4 μl H_2O + 5 μl SYBR-Green (Takara Bio, Inc.) x number of wells; iii) centrifugation: 999 x g for 4 min at 4˚C; and iv) real-time PCR analysis.

The quantitative PCR was performed according to a previous study (28). First, the cDNA solution was diluted 50 times. Subsequently, the samples (4 μl) were loaded. The rest was the same as that above. Samples were run in triplicate. The system was put into the Roche PCR analyzer (Roche Molecular Diagnostics). The reaction conditions were pre-denaturation at 95˚C for 5 min followed by processing at 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 20 sec for a total of 45 cycles. Following completion of the reaction, DataAssist™ software (v3.01) (Thermo Fisher Scientific, Inc.) was used for analysis. The analysis was repeated three times. The data were used for statistical analysis (Table I).

Area of myocardial cells. Following treatment with Ang II (20 μM) for 48 h at 37˚C, the myocardial cells were fixed with 4% paraformaldehyde at 4˚C for 30 min and imaged using a fluorescent microscope, magnification, x400. Fifty views of the same size were taken for each group. The image analysis software, Image pro plus 6.0 (National Institutes of Health), was used to calculate the area of the cells (27).

Transfection. A transfection kit was purchased from Foregene Co., Ltd. (TransEasy™). Ad-siRNA-Sirt3 was constructed by Sangon Biotech (Shanghai) Co., Ltd. In the 6-well plate used to culture 1x10^6 myocardial cells, Ad-siRNA-Sirt3 virus (1x10^8 pfu/virus) was used to infect the cells [multiplicity of infection (MOI) =100]. The cells were collected 24 h after infection. Subsequent to lysis by the protein lysate, the protein was extracted. A total of 50 μg protein of each group was detected by western blot analysis. Whether the recombination of adenovirus Ad-siRNA-Sirt3 was successful was determined utilizing the specific Sirt3 antibody and according to the expression alteration of the Sirt3 protein in each group.

The primary cultured myocardial cells of rats were used. The recombinant adenovirus vector Ad-siRNA-Sirt3 was used to infect the myocardial cells, and Ad-GFP and Ad-siRNA served as the control. The virus solution diluted into suitable titres was added to achieve the corresponding MOI (optimal MOI was 50 μM, according to the preliminary experiment; data not shown). After 6 h of culture at 37˚C, the virus solution was left, and DMEM culture medium containing a small amount of FCS was added for an additional 12 h of culture. After 48 h of treatment with 20 μM Ang II, the mRNA expression of myocardial hypertrophy markers, atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), MCAD (a key enzyme of energy metabolism) and pyruvate kinase (PK), and the protein expression of Sirt3, LC3 and Beclin1, were detected.

Statistical analysis. The SPSS 15.0 (SPSS, Inc.) statistical package was used for the statistical analysis. The data of each group are presented as the mean ± standard deviation. The differences between the groups and the differences between the time-points were analyzed by one-way analysis of variance, with the least significant difference method as the post hoc test. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated three times.

Results

Sirt3 expression is decreased in Ang II-induced cardiomyocyte hypertrophy. The alterations in the expression of Sirt3 were observed in Ang II-induced cardiomyocyte hypertrophy to assess the role of Sirt3. The cardiomyocytes were treated with Ang II *in vitro*, and the expressions of Sirt3 were detected at 0, 6, 12, 24 and 48 h. The results demonstrated that compared with

the control group, the Sirt3/β-actin ratio following treatment with Ang II decreased at different time-points (Fig. 1A and B). This suggested that Sirt3 is downregulated in Ang II-induced cardiomyocyte hypertrophy. The expression of LC3, which is a homologue of the autophagy-associated gene (GABA type A receptor associated protein like 2), in Ang II-induced cardiomyocyte hypertrophy decreased (data not shown). These results suggest that there is a potential association between the two (Fig. 1).

Res inhibits cardiomyocyte hypertrophy. The Sirt3 agonist Res was used to treat cardiomyocytes in Ang II-induced cardiomyocyte hypertrophy. It was observed that Res significantly inhibited cardiomyocyte hypertrophy in terms of cell surface area and the expression of hypertrophy-associated genes compared with the Ang II group (Fig. 2; $P<0.05$).

Res upregulates the expression of Sirt3, LC3 and Beclin1, and increases the energy metabolism of cardiomyocytes. The effects of Res on Sirt3 and autophagy were observed in Ang II-induced cardiomyocyte hypertrophy and the potential association between the two was assessed. The results demonstrated that in Ang II-induced cardiomyocyte hypertrophy, Res upregulated the expressions of Sirt3, autophagy-associated protein LC3 and Beclin1, which counteracted the effect of Ang II to a certain extent (Fig. 3A-C). With regard to the energy metabolism, Res increased the mRNA expression of MCAD while inhibiting the mRNA expression of PK (Fig. 3D and E).

Silencing Sirt3 reverses the effects of Res. As shown in Fig. 4, the effect of siRNA-mediated silencing of Sirt3 on cardiomyocytes and autophagy in Ang II-induced cardiomyocyte hypertrophy was observed. Following silencing, the expressions of Sirt3, LC3 and Beclin1 decreased; whereas, the mRNA expression of ANP and BNP increased. This effect counteracted the Res-induced inhibition on cardiomyocyte hypertrophy, increased the mRNA expression of PK and decreased the mRNA expression of MCAD. Consequently, the effect of Res on the energy metabolism in cardiomyocytes was reversed. These results demonstrated that the siRNA-mediated silencing of Sirt3 counteracted the promoting effect of Res on autophagy and its inhibitory effect on cardiomyocyte hypertrophy. From another perspective, it was confirmed that the Sirt3-autophagy signaling pathway served a role in cardiomyocyte hypertrophy.

Discussion

Hypertension is an important global health problem, causing myocardial cell hypertrophy (29). At present, inhibiting myocardial cell hypertrophy may block the signaling pathway of protein synthesis or promote the signal pathway of proteolysis (30). The present study examined the association between Sirt3 and autophagy in Ang II-induced myocardial cell hypertrophy, and assessed whether Sirt3 affected myocardial cell hypertrophy and energy metabolism through autophagy.

Belonging to the NAD-dependent HDAC family, Sirt3 is primarily distributed in the mitochondria (31). A previous study identified that Sirt3 additionally exists in the cytoplasm and nuclei (32). The expression of Sirt3 is increased in organs with high metabolic activity, including brain tissue, the heart, liver and kidneys (33). Exercise, hunger, cold and oxidative stress may activate the expression of Sirt3 (34). Sirt3 expression was significantly decreased in people with a high fat diet and those taking metformin, as well as in tumor cells (35-37). Sirt3 is involved in the regulation of approximately all cell metabolism-associated signaling pathways, including reactive oxygen species scavenging (38), tricarboxylic acid cycle (39-43), fatty acid oxidation (34,44,45), ketogenesis (46,47), protein synthesis (48,49), cell growth and apoptosis (50-54). Sirt3 maintains the normal vital activities of the human body through multisystem and multi-link regulation, energy metabolism balance, anti-oxidative stress and the cell cycle (38). A number of previous studies demonstrated that Sirt3 may resist myocardial hypertrophy and heart failure through regulation of energy metabolism and reduction of oxidative stress. Sundaresan *et al* (10) observed that Sirt3 knockout mice demonstrated alterations of myocardial hypertrophy and myocardial fibrosis. Pillai *et al* (11) identified that myocardial hypertrophy may be inhibited by activating the Sirt3-LKB1-AMPK pathway. Chen *et al* (55) observed that Sirt3 may reduce myocardial fibrosis and improve the myocardial contraction through the transforming growth factor (TGF)-β/mothers against decapentaplegic homolog 3 pathway. Furthermore, another previous study demonstrated that Sirt3 gene knockout aggravates the lipid deposition of the heart and suggests that abnormal energy metabolism may promote myocardial hypertrophy (56).

With respect to myocardial hypertrophy, autophagy may regulate the scavenging of cells, and maintain the mechanical function of the myocardium and the quality of the ventricle (55). Nakai *et al* (15) observed that autophagy related 5 (Atg5) gene knockout rats are more liable to ventricular hypertrophy, ventricular dilatation and abnormal contraction. Ucar *et al* (57) identified that miRNA-212/132 knockout mice have upregulated autophagy and significantly decreased myocardial hypertrophy. Another previous study suggested that the heart volume caused by myocardial hypertrophy is reduced by FoxO3 through upregulation of autophagy (58). Laurent *et al* (59) observed that exchanger 1 may activate the autophagy through calcium/calmodulin-dependent protein kinase kinase β/AMPK; however, the downregulation of 3-methlyadenine and Atg5 (blockers of autophagy) may accelerate myocardial hypertrophy. It was suggested that the increase of autophagy is a reaction that balances cell hypertrophy to protect the myocardial cells.

Accumulating evidence suggests that excessive autophagy may accelerate myocardial cell death and aggravate heart failure. Zhu *et al* (60) observed that Beclin1 heterozygote ($Beclin1^{+/-}$) mice with downregulated autophagy demonstrated significantly improved ventricular remodeling and heart failure. Kostin *et al* (61) identified in heart failure subjects that moribund myocardial cells demonstrated significantly increased autophagy. Rawat *et al* (62) identified that increased active oxygen and abnormal energy metabolism may upregulate autophagy and accelerate the development of myocardial hypertrophy and diastolic heart failure. Therefore, a number of researchers suggested that cell death caused by autophagy may be the immediate cause of heart failure. Therefore, in the process of myocardial hypertrophy, moderate autophagy may

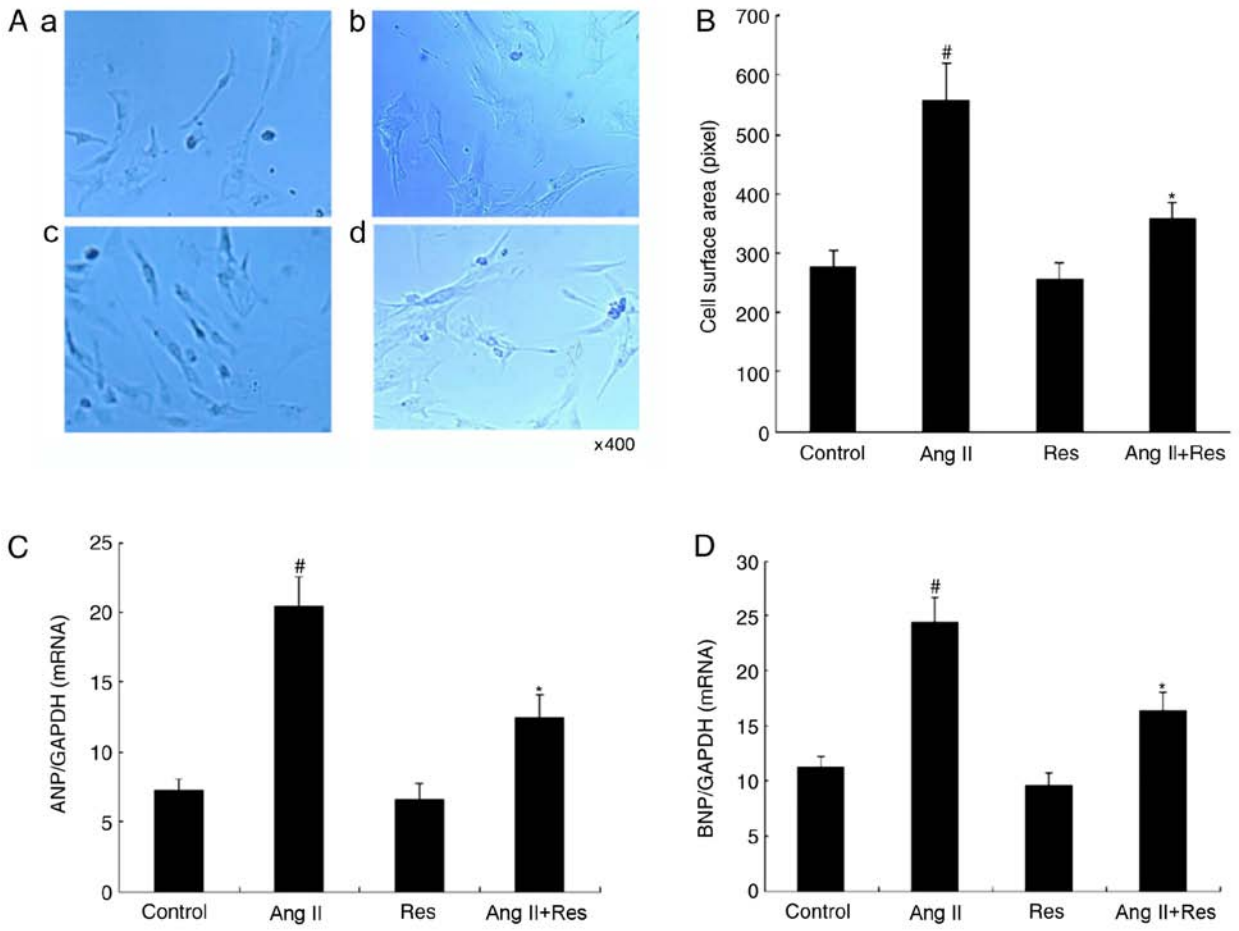

Figure 2. Influence of Res on protein expression in different treatment groups. (A) Influence of Res on the morphology of Ang II-induced hypertrophic myocardial cells. (Aa) Control group; (Ab) Ang II (20 μM); (Ac) Res (50 μM); and (Ad) initially treated with Res (50 μM) and subsequently with Ang II (20 μM) 30 min later. (B) Influence of Res on the area of Ang II-induced hypertrophic myocardial cells. (C) Influence of Res on the ANP mRNA in the Ang II-induced hypertrophic myocardial cells. (D) Influence of Res on the BNP mRNA in the Ang II-induced hypertrophic myocardial cells. n=4. $^{\#}$P<0.01 vs. respective control; *P<0.05 vs. respective Ang II. Res, resveratrol; Ang II, angiotensin II; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide.

protect the myocardial cells; whereas, excessive autophagy will lead to the death of myocardial cells and the progression of heart failure (63). Autophagy exhibits different effects under the stimulation of different conditions and factors.

The present study examined the role of Sirt3-autophagy in myocardial hypertrophy. In a previous study, it was observed that in the process of Ang II-induced myocardial cell hypertrophy, the activity of autophagy decreased and the expression of the LC3 protein decreased significantly (16). It was additionally observed that a similar trend of downregulation of the Sirt3 protein was present in the identical cell model, which may be influenced by the NAD/reduced NAD ratio (16). To clarify the association between Sirt3 and autophagy, the present study used Res, which is an agonist of Sirt3, to intervene. It was identified that Res may increase the expression of Sirt3 and autophagy proteins, and inhibit the expression of ANP and BNP (myocardial cell hypertrophy factors), which suggested that Res may resist myocardial hypertrophy through activation of Sirt3 to induce autophagy. To further verify the observations, the present study combined adenovirus transfected siRNA-Sirt3 with Res intervention. It was observed that the expression of autophagy proteins was significantly decreased and the expression of ANP and BNP (myocardial cell hypertrophy factors) was significantly increased, which suggests that Sirt3 inhibited retinal neovascularization by regulating the migration-, neovascularization- and autophagy-associated factors expression (64). Sirt3 exerts protective effects in response to various damage factors involved in endothelial dysfunction, including Ang II, TGF-β and high glucose (65). The reduced microvascular formation and VEGF expression in cardiac tissue was accompanied by a loss of mitochondrial Sirt3 during Ang II-induced cardiac remodeling (65).

In the present study, the expression of MCAD and PK was detected, and it was identified that the expression of MCAD was significantly decreased; however, the expression of PK was significantly increased in hypertrophic myocardial cells, which is consistent with a previous study (2). These results suggest that the energy utilization method of myocardial cells is altered and the efficiency of energy utilization decreases. Res intervention may reverse the alteration of MCAD and PK. However, the effect of Res decreases significantly following the combination of siRNA-Sirt3 and Res intervention. This result suggests that Sirt3 may promote fatty acid oxidation in myocardial cells, reduce the anaerobic glycolysis of glucose, lower the toxic effect of the intermediate product of glycolysis on myocardial cells and protect the heart accordingly (56).

The previous studies and the present results suggest that Sirt3 activation may improve autophagy and inhibit myocardial

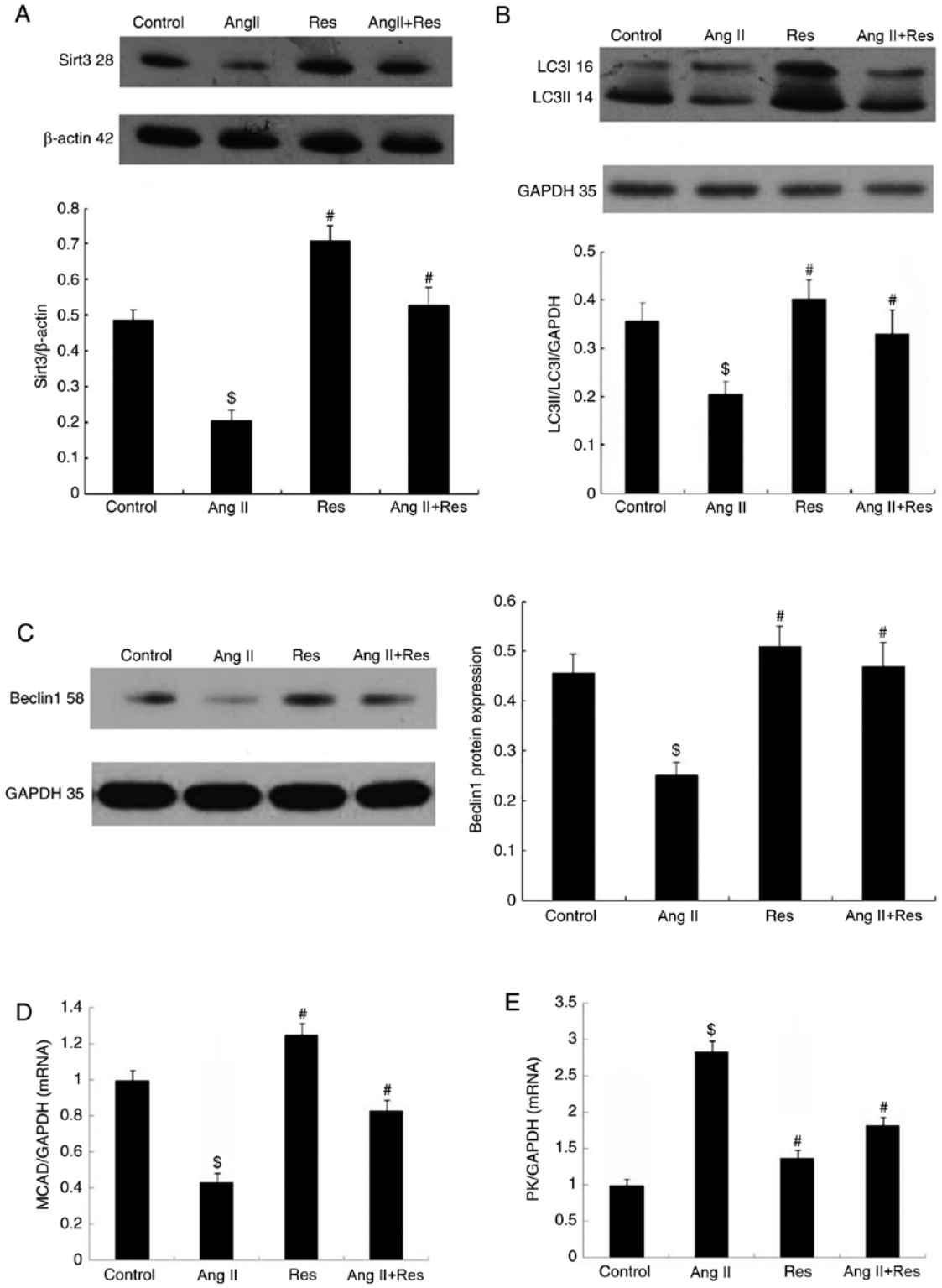

Figure 3. Influence of Res on protein expression in hypertrophic myocardial cells. (A) Influence of Res on the Sirt3 protein expression in the hypertrophic myocardial cells. (B) Influence of Res on the LC3 protein expression in the hypertrophic myocardial cells. (C) Influence of Res on the Beclin1 protein expression in the hypertrophic myocardial cells. (D) Influence of Res on the MCAD mRNA expression in the hypertrophic myocardial cells. (E) Influence of Res on PK mRNA in the hypertrophic myocardial cells. n=4. $^{\$}$P<0.01 vs. the control group; $^{\#}$P<0.01 vs. the Ang II group. Res, resveratrol; Sirt3, sirtuin 3; LC3, light chain 3; MCAD, medium-chain acyl-CoA dehydrogenase; PK, pyruvate kinase.

hypertrophy. In addition, Sirt3 protects the cell by reversing the abnormal energy metabolism caused by Ang II. As Sirt3 is vital in regulating cellular energy metabolism, cell growth and apoptosis, an increasing number of studies have identified that Sirt3 is closely associated with cardiovascular diseases, and Sirt3 may become a novel target for the treatment of cardiovascular diseases. Sirt3 is highly valuable for scientific research with strong potential for clinical application. The present study demonstrated that Sirt3 may inhibit myocardial cell hypertrophy by regulating autophagy; however, further

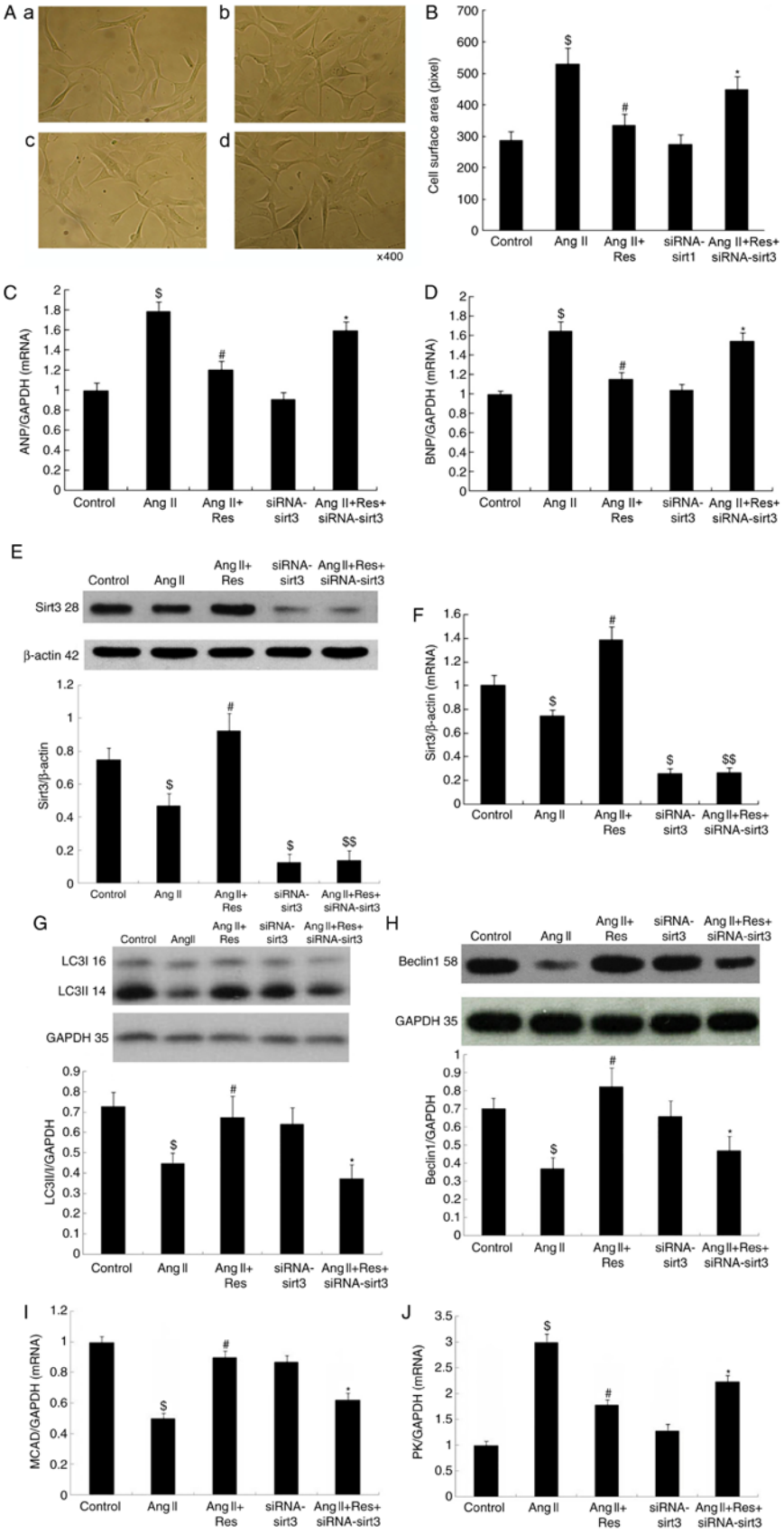

Figure 4. Influence of siRNA-Sirt3 in different treatment group. (A) Influence of siRNA-Sirt3 on the morphology of Ang II-induced hypertrophic myocardial cells. (Aa) Control group; (Ab) Ang II (20 μM); (Ac) Res (50 μM); and (Ad) initially treated firstly with Res (50 μM) and subsequently with Ang II (20 μM) 30 min later. (B) Influence of siRNA-Sirt3 on the area of Ang II-induced hypertrophic myocardial cells. (C) Influence of siRNA-Sirt3 on (C) ANP and (D) BNP mRNA in the Ang II-induced hypertrophic myocardial cells. Influence of siRNA-Sirt3 on Sirt3 (E) protein and (F) mRNA in the Ang II-induced hypertrophic myocardial cells. Influence of siRNA-Sirt3 on (G) LC3 and (H) Beclin1 protein expression in the Ang II-induced hypertrophic myocardial cells. Influence of siRNA-Sirt3 on (I) MCAD and (J) PK mRNA expression in the Ang II-induced hypertrophic myocardial cells. n=4. $^{\$}$P<0.05, $^{\$\$}$P<0.01 vs. the control group; $^{\#}$P<0.01 vs. the Ang II group; *P<0.05 vs. the Ang II+Res group. siRNA, small interfering RNA; Sirt3, sirtuin 3; Ang II, angiotensin II; Res, resveratrol; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; MCAD, medium-chain acyl-CoA dehydrogenase; PK, pyruvate kinase.

investigations are required to determine the specific molecular mechanism of Sirt3-mediated regulation of autophagy.

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Availability of data and materials

The data generated in the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HNW and JLL designed the study and performed the experiments. TX and HQY performed the statistical analysis, GHC and JH were involved in designing the study, drafting the manuscript and conducted important revisions to the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Shantou University (Shantou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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