# Semaphorin-3A alleviates cardiac hypertrophy by regulating autophagy

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**Abstract.** Cardiac hypertrophy, characterized by cardiomyocyte enlargement, is an adaptive response of the heart to certain hypertrophic stimuli; however, prolonged hypertrophy results in cardiac dysfunction and can ultimately cause heart failure. The present study evaluated the role of semaphorin-3A (Sema3A), a neurochemical inhibitor, in cardiac hypertrophy, utilizing an isoproterenol (ISO) induced H9c2 cell model. Cells were stained with rhodamine-phalloidin to assess the cell surface area and reverse transcription-quantitative PCR was performed to quantify mRNA expression levels of Sema3A, brain natriuretic factor (BNF) and β-myosin heavy chain (β-MHC). The protein expression levels of the autophagy-related proteins light chain 3 (LC3), p62 and Beclin-1, and the Akt/mTOR signaling pathway associated proteins Akt, phosphorylated (p)-Akt, mTOR, p-mTOR, 4E-binding protein 1 (4EBP1) and p-4EBP1 were semi-quantified using western blotting. Rapamycin, a canonical autophagy inducer, was administered to H9c2 cells to elucidate the regulatory mechanism of Sema3A. The results indicated significantly increased cell surface area and elevated BNF and β-MHC mRNA expression levels, increased LC3II/I ratio and Beclin-1 protein expression levels and significantly decreased p62 protein expression levels after treatment of H9c2 cardiomyocytes with ISO for 24 h. Sema3A overexpression improved ISO-induced hypertrophy in H9c2 cells, indicated by decreased cell surface area and reduced BNF and β-MHC mRNA expression levels. Moreover, Sema3A overexpression inhibited ISO-induced autophagy in H9c2 cells, indicated by decreased LC3II/I ratio and Beclin-1 protein expression levels and increased p62 protein expression levels. The autophagy activator rapamycin partially inhibited the protective effect of Sema3A on ISO-induced hypertrophy. Sema3A overexpression suppressed the decrease of the protein expression levels of p-Akt, mTOR and their downstream target 4EBP1, which is induced by ISO. Collectively, these results suggested Sema3A prevented ISO-induced cardiac hypertrophy by inhibiting autophagy via the Akt/mTOR signaling pathway.

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## Introduction

Cardiac hypertrophy constitutes an adaptive response to pressure or volume overload, but prolonged cardiac hypertrophy may cause heart failure, which is implicated in a large number of cardiac disorders, including myocardial ischemia, myocardial infarction and hypertrophic cardiomyopathy (1). Therefore, studying the molecular mechanism of cardiac hypertrophy is beneficial in order for effective measures to be taken to reduce the morbidity and mortality of heart disease. There are numerous studies concerning the therapeutic strategy for cardiac hypertrophy (2,3); however, the practical therapeutic regimen remains to be explored. Cardiac hypertrophy has a complex pathophysiology, which involves numerous signaling pathways and cell factors, including inflammation, apoptosis and redox reactions (4). Autophagy, an intracellular self-degradative mechanism to maintain cellular homoeostasis by degrading and recycling cytoplasmic proteins and organelles, serves an important role in cardiac remodeling (5). However, the role of autophagy in cardiac remodeling remains controversial. Numerous reports have reported that autophagy serves a dual role, with basal autophagy being adaptive and beneficial, and excessive or insufficient autophagy being maladaptive and detrimental (6,7). Therefore, autophagy regulation in cardiac remodeling needs to be further studied and could provide novel treatment options for heart failure.

Semaphorin-3A (Sema3A), a neurochemical inhibitor, serves an important role in the distribution of sympathetic nerves, and is involved in multiple signaling pathways through interactions with neuropilin-1 (NP-1), a Semaphorin-3A receptor (8-10). Sema3A is associated with many diseases, including cancer, dermatitis, arthritis and kidney disease (11,12). Our previous study reported that Sema3A is

also expressed in cardiomyocytes and serves a potential role in the regulation of the induction of ventricular arrhythmia after infarction (13). Recently, it was reported that Sema3A reduces cardiac inflammation and improves cardiac function after myocardial infarction (14). However, the roles of Sema3A in cardiac hypertrophy have not been thoroughly studied to date.

The present study examined whether Sema3A overexpression affected cardiac hypertrophy in the context of isoproterenol (ISO) treatment. The possible mechanisms of Sema3A involvement in cardiac hypertrophy were also assessed.

## Materials and methods

*Cell culture and treatment*. The H9c2 rat embryonic ventricular myocytes cell line was purchased from the Shanghai Institute of Biochemistry and Cell Biology. Cells were cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS) (both Shanghai ExCell Biology, Inc.) at 37˚C in an incubator with 5% $CO_2$. The ISO-induced H9c2 cell model was established to study cardiomyocyte hypertrophy. Cells were pretreated with 10 $\mu$M ISO (MilliporeSigma) and 50 nM rapamycin (Cell Signaling Technology, Inc.) for 24 h before collection.

*Cell transfection*. Sema3A overexpression was performed using lentivirus transfection according to the manufacturer's instructions using a fourth-generation self-inactivating lentivirus vector (Shanghai GeneChem Co. Ltd.). H9c2 cells at 20-30% confluence (~$2x10^5$ cells per well) in a 6-well plate, were transfected with Sema3A-LV or Mock-LV (MOI, 20) containing a cytomegalovirus-driven green-fluorescent protein (GFP) reporter gene in 1 ml of a mixture of culture medium (10% FBS in DMEM) and HiTransG P (1:25). After 15 h of transfection, cardiomyocytes were cultured in 2 ml DMEM without FBS at 37˚C in an incubator with 5% $CO_2$. After treatment with ISO and rapamycin according to the aforementioned method, cells were divided into six groups including the Control (Mock lentivirus + PBS), Sema3A over (Sema3A overexpression lentivirus + PBS), Control + ISO (Mock lentivirus + ISO), Sema3A over + ISO (Sema3A overexpression lentivirus + ISO), Control + ISO + Rap (Mock lentivirus group + ISO + rapamycin) and Sema3A over + ISO + Rap (Sema3A overexpression lentivirus + ISO + rapamycin) groups. Cells treated with ISO and rapamycin were harvested after 72 h of transfection.

*Cell surface area measurement using rhodamine-phalloidin staining*. H9c2 cells grown on coverslips were fixed with 4% paraformaldehyde in phosphate buffer solution (PBS) for 15 min at room temperature. After a PBS wash, cells were further incubated with 0.1% Triton X-100 (Sangon Biotech Co., Ltd.) for 10 min. Cells were then washed with PBS and incubated with 100 nM rhodamine-phalloidin for 30 min (Sigma-Aldrich; Merck KGaA) at room temperature, shielded from light. After three PBS washes, cells were further stained with 100 nM DAPI (Sigma Aldrich; Merck KGaA) at 4˚C. An Olympus FV3000 laser scanning confocal microscope (Olympus Corporation) was used for imaging. Cell surface areas in randomly selected fields (50 for each group) were assessed using ImageJ (version 180; National Institutes of Health).

*Reverse transcription-quantitative PCR (RT-qPCR)*. Sema3A, brain natriuretic factor (BNF) and β-myosin heavy chain (β-MHC) mRNA expression levels were quantified using RT-qPCR. Total RNA from H9c2 cells was extracted using SevenFast Total RNA Extraction Kit for Cells [Seven Innovation (Beijing) Biotechnology Co., Ltd.] according to the manufacturer's protocol. cDNA was synthesized in a 20 $\mu$l reaction using the PrimeScript™ RT reagent Kit containing gDNA Eraser (Perfect Real Time) (Takara Bio, Inc.). Amplification with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara Bio, Inc.) was performed on an Applied Biosystems Prism 7500 Fast Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with thermocycling at 95˚C for 30 sec followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. The primers used were as follows: Sema3A forward (F), 5'-AGACGACAGGATATA AGGAATGG-3' and reverse (R), 5'-GCAGACTACGAAGCA GGAG-3'; BNF forward, 5'-GTGCTGCCCCAG ATGATT CT-3' and R, 5'-GCAGCTTCTGCATCGTGGAT-3'; β-MHC F, 5'-TGCTCTACAATCTCAAGGAGAGGT-3' and R, 5'-TGT TGACGGTCTTACCAGCTC-3'; and GAPDH F, 5'-GTCGGT GTGAACGGATTTGG-3' and R, 5'-GCTCCTGGAAGA TGGTGATGG-3'. All primers were synthesized by Shanghai GenePharma Co., Ltd. Triplicate reactions were analyzed using the $2^{-\Delta\Delta Cq}$ method (15), with GAPDH used for normalization.

*Western blotting*. The protein expression levels of the autophagy-related proteins light-chain 3 (LC3), p62 and Beclin-1, and the Akt/mTOR signaling associated proteins Akt, phosphorylated (p)-Akt, mTOR, p-mTOR, 4E-binding protein 1 (4EBP1) and p-4EBP1 were assessed using western blotting. H9c2 cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) and the protein concentration was assessed using a BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). Equal amounts of total protein (30 $\mu$g per lane) were separated by SDS-PAGE (5% stacking gel, 10% separating gel) and transferred onto PVDF membranes. After blocking with 5% nonfat milk at 37˚C for 2 h, the membranes were incubated with primary antibodies against LC3 (1:2,000; cat. no. 12741S; Cell Signaling Technology, Inc.), p62 (1:2,000; cat. no. 39749T; Cell Signaling Technology, Inc.), Beclin-1 (1:2,000; cat. no. 3495S; Cell Signaling Technology, Inc.), Akt (1:2,000; cat. no. 4691T; Cell Signaling Technology, Inc.), p-Akt (1:2,000; cat. no. 9271S; Cell Signaling Technology, Inc.), mTOR (1:2,000; cat. no. 2983T; Cell Signaling Technology, Inc.), p-mTOR (1:2,000; cat. no. 5536S; Cell Signaling Technology, Inc.), 4EBP1 (1:2,000; cat. no. 9644T; Cell Signaling Technology, Inc.), p-4EBP1 (1:2,000; cat. no. 2855S; Cell Signaling Technology, Inc.) and GAPDH (1:2,500; cat. no. 2118S; Cell Signaling Technology, Inc.) in 5% BSA at 4˚C overnight. Membranes were then washed three times with TBST (0.1% Tween 20) and incubated with HRP-Conjugated AffiniPure Goat Anti-rabbit secondary antibodies (1:2,000; cat. no. BA1054; Wuhan Boster Biological Technology Co., Ltd.) in 5% non-fat milk at 37˚C for 1 h. After washing, proteins were visualized using an ECL luminescence kit (cat. no. AR1173; Wuhan Boster Biological Technology Co.,

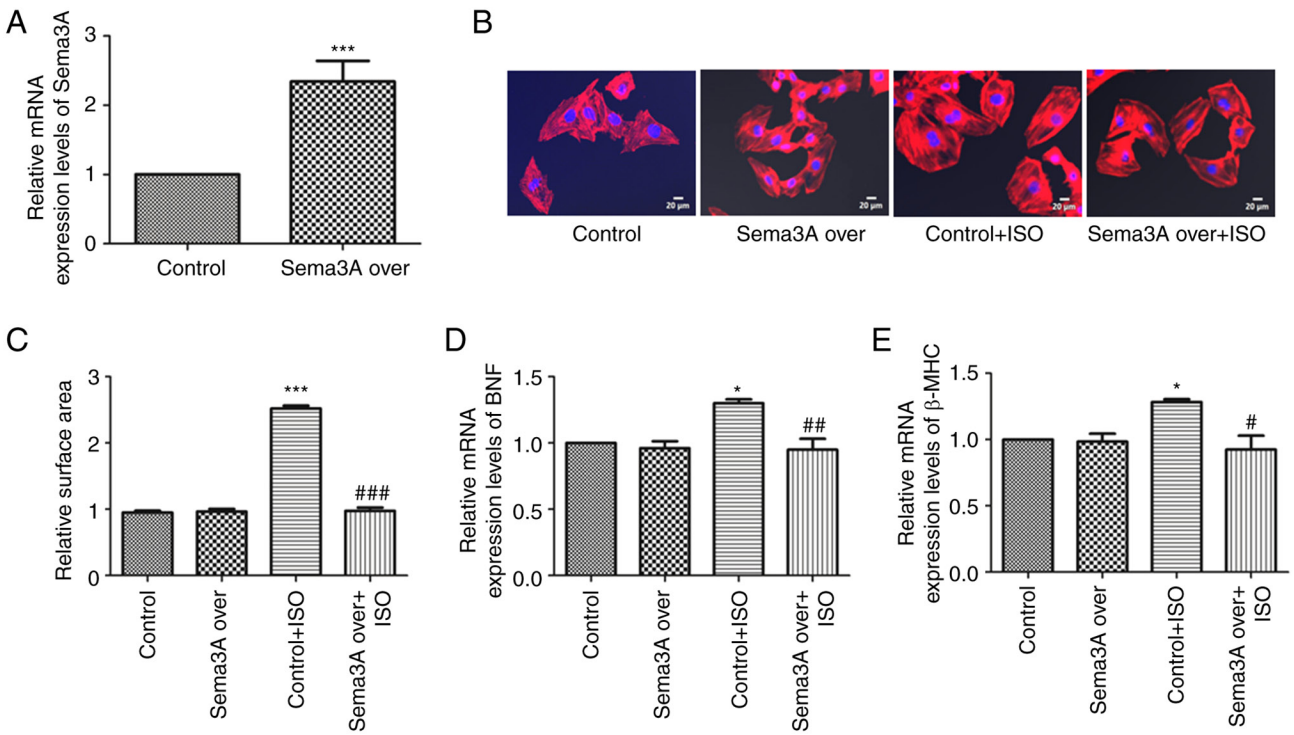


Figure 1. Sema3A protects H9c2 cells from ISO-induced hypertrophy. (A) Expression of Sema3A in H9C2 cells transfected with Sema3A overexpression lentivirus was assessed using RT-qPCR. H9c2 cells were transfected with Sema3A overexpression lentivirus followed by incubation with ISO (10 $\mu$M for 24 h). The hypertrophic responses of cardiomyocytes were detected by assessing the (B and C) cell surface area and mRNA expression levels of (D) BNF and (E) β-MHC in H9c2 cells using RT-qPCR. Data are presented as mean ± standard error of the mean compared with the control, which was set at 1. $^{*}$P<0.05 and $^{***}$P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control + ISO. Sema3A over, Semaphorin-3A overexpression lentivirus; ISO, isoproterenol; RT-qPCR, reverse transcription- quantitative PCR.

Ltd.). The ChemiDoc XRS System (Bio-Rad Laboratories, Inc.) was utilized for imaging, and images were analyzed with Image Lab™ Software 6.1 (Bio-Rad Laboratories, Inc.). The protein expression levels of LC3, P62, Beclin-1, Akt, p-Akt, mTOR, p-mTOR, 4EBP1 and p-4EBP1 were normalized to GAPDH expression.

*Statistical analysis*. Data are presented as mean ± standard error of the mean. GraphPad Prism 5.0 (Dotmatics) was used for data analysis. Two groups and multiple groups were compared using unpaired Student's t-test and one-way ANOVA followed by Tukey's post hoc test, respectively. P<0.05 was considered to indicate a statistically significant difference. Assays were performed in triplicate.

## Results

*Sema3A alleviates ISO-induced cardiomyocyte hypertrophy*. After transfection with Sema3A overexpression lentivirus for 72 h, Sema3A mRNA expression levels were assessed using RT-qPCR. Compared with the control group, Sema3A mRNA expression levels were significantly increased in the Sema3A over group (Fig. 1A), which indicated that H9c2 cells transfected with the Sema3A lentivirus significantly overexpressed Sema3A.

H9c2 cells were incubated with 10 $\mu$M ISO for 24 h to induce cardiomyocyte hypertrophy. Then, BNF and β-MHC mRNA expression levels were assessed using RT-qPCR, and cardiomyocyte surface area was assessed using rhodamine-phalloidin staining, to validate the cardiomyocyte hypertrophic model. ISO significantly increased the surface area of H9c2 cells, with cell surface areas in the Control + ISO group being 2.65-fold greater than those of the Control group. Sema3A overexpression significantly ameliorated the change induced by ISO on the surface area of myocardial cells, with cell surface area significantly decreased in the Sema3A over + ISO group compared with the Control + ISO group (Fig. 1B and C). Similarly, BNF and β-MHC mRNA expression levels were also significantly increased in cells treated with ISO compared with the Control group; Sema3A counteracted this effect, with the Sema3A over + ISO group showing decreased BNF and β-MHC mRNA expression levels compared with the Control + ISO group (Fig. 1D and E). However, Sema3A overexpression demonstrated no effect on BNF and β-MHC mRNA expression levels, or cardiomyocyte surface area in H9c2 cells without ISO treatment.

*Sema3A attenuates ISO-induced autophagy in cardiomyocytes*. To assess the effect of Sema3A on ISO-induced autophagy, the protein expression levels of the autophagy-related proteins LC3, p62 and Beclin-1 were assessed using western blotting. ISO-induced a significant increase in the LC3-II/I ratio and Beclin-1 protein expression level and a significant decreased in the p62 protein expression level in the Control + ISO group compared with the Control, these effects were all significantly attended by Sema3A (Fig. 2). In agreement with the aforementioned results of the present study, the Sema3A group and non-ISO H9c2 cells had similar autophagy levels.

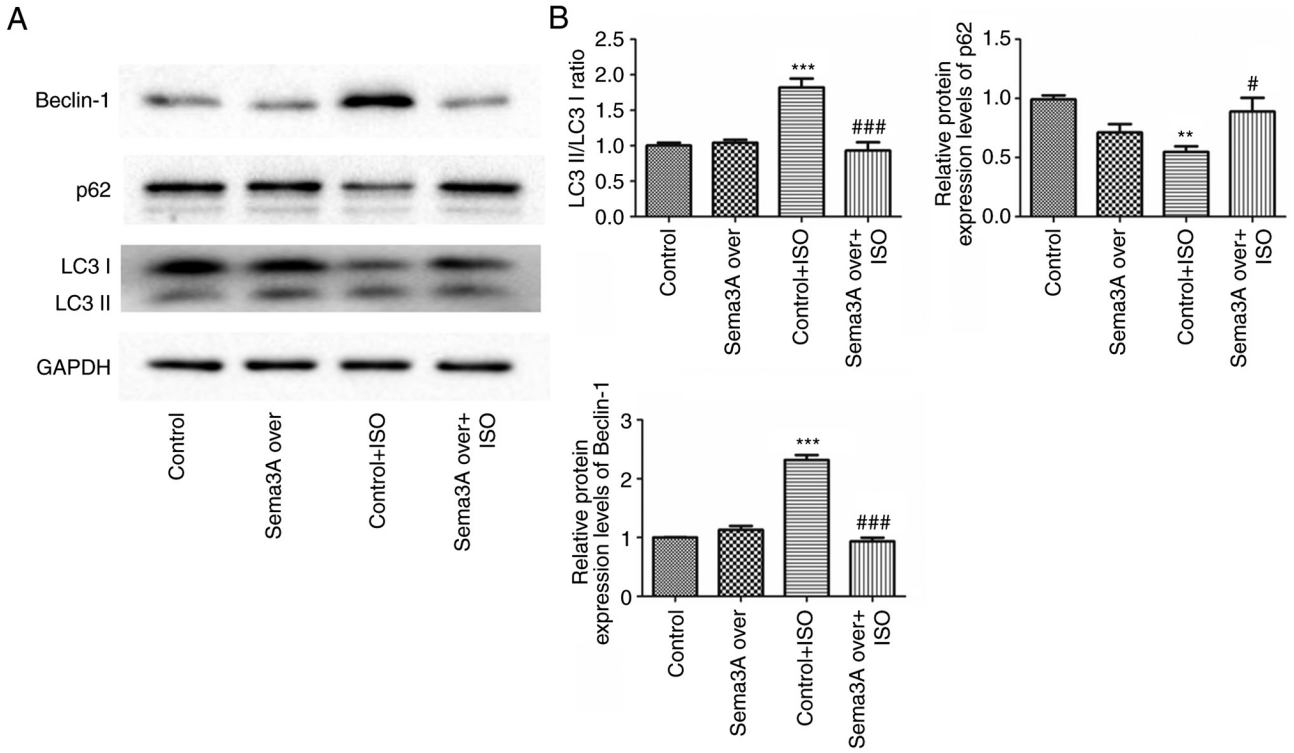


Figure 2. Sema3A attenuates ISO-induced excessive autophagy in cardiomyocytes. H9c2 cells were transfected with Sema3A or empty vector and further incubated with ISO (10 $\mu$M) for 24 h. (A) Western blotting was performed to semi-quantify the protein expression levels of exogenous LC3, p62 and Beclin-1. (B) Semi-quantification of LC3, p62 and Beclin-1. Data are presented as mean ± standard error of the mean. $^{**}$P<0.01 and $^{***}$P<0.001 vs. control; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. control + ISO. Sema3A over, Semaphorin-3A overexpression lentivirus; ISO, isoproterenol; LC3, light chain 3.

These results suggested Sema3A alleviated excessive cardiac autophagy caused by ISO.

*Sema3A ameliorates cardiomyocyte hypertrophy via suppression of autophagy*. To further evaluate whether autophagy affected by Sema3A was involved in cardiac hypertrophy, rapamycin, a potent autophagy agonist, was used to induce cardiac autophagy. In previous experiments, treatment of cells with ISO was demonstrated to exacerbate autophagy in the process of cardiomyocyte hypertrophy, which was repressed by Sema3A upregulation. Increases in cell surface area, LC3-II/I ratio, and protein expression levels of Beclin-1, BNF and β-MHC, as well as reduction in p62 protein expression levels, were all significantly greater in the Control + ISO + Rap group compared with the Control + ISO group. These data suggested rapamycin aggravated autophagy induction to worsen ISO-induced cardiac hypertrophy. Meanwhile, significant reductions of cell surface area, LC3-II/I ratio and protein expression levels of Beclin-1, BNF and β-MHC were demonstrated in the Sema3A over + ISO + Rap group compared with the Control + ISO + Rap group. Concomitantly, p62 expression was significantly upregulated in the Sema3A over + ISO + Rap group compared with the Control + ISO + Rap group. These findings indicated Sema3A upregulation still inhibited autophagy in myocardial cells and improved cardiomyocyte hypertrophy under treatment with ISO and rapamycin. Significant increases were demonstrated for cell surface area and in the LC3-II/I ratio and Beclin-1, BNF and β-MHC protein expression levels, as well as a significant decrease in p62 protein expression levels, in the Sema3A over + ISO + Rap group compared with the Sema3A over + ISO group (Fig. 3). Collectively, these results indicated rapamycin partly abolished the anti-hypertrophy effects of Sema3a by regulating autophagy, and further demonstrated that Sema3a improved ISO-induced cardiomyocyte hypertrophy by inhibiting maladaptive cardiomyocyte autophagy.

*Sema3A suppresses autophagy by activating the Akt/mTOR signaling pathway*. To evaluate the molecular mechanisms involved in the Sema3A-induced autophagic responses, protein expression levels of Akt, p-Akt, mTOR, p-mTOR, 4EBP1 and p-4EBP1 were assessed using western blotting. Compared with the control group, ISO significantly decreased p-Akt, p-mTOR and p-4EBP1 levels in H9c2 cells. Sema3A overexpression resulted in a significant increase in the p-Akt, p-mTOR and p-4EBP1 levels in ISO-treated H9c2 cells (Fig. 4A). To further assess whether Sema3A inhibited ISO-induced excessive autophagy via activation of the Akt/mTOR pathway, the mTOR activator rapamycin was used. In the present study, the levels of p-Akt, p-mTOR and p-4EBP1 were significantly decreased in the Control + ISO + Rap group compared with the Control + ISO group (Fig. 4B). Moreover, p-Akt, p-mTOR and p-4EBP1 levels were significantly increased in the Sema3A over + ISO + Rap group compared with the Control + ISO + Rap group, which indicated that Sema3A overexpression activated the Akt/mTOR signaling pathway in H9c2 cells after treatment with rapamycin and ISO. Furthermore, the Sema3A over + ISO + Rap group had significantly lower levels of p-Akt, p-mTOR and p-4EBP1 compared with the Sema3A over + ISO group, which suggested that rapamycin

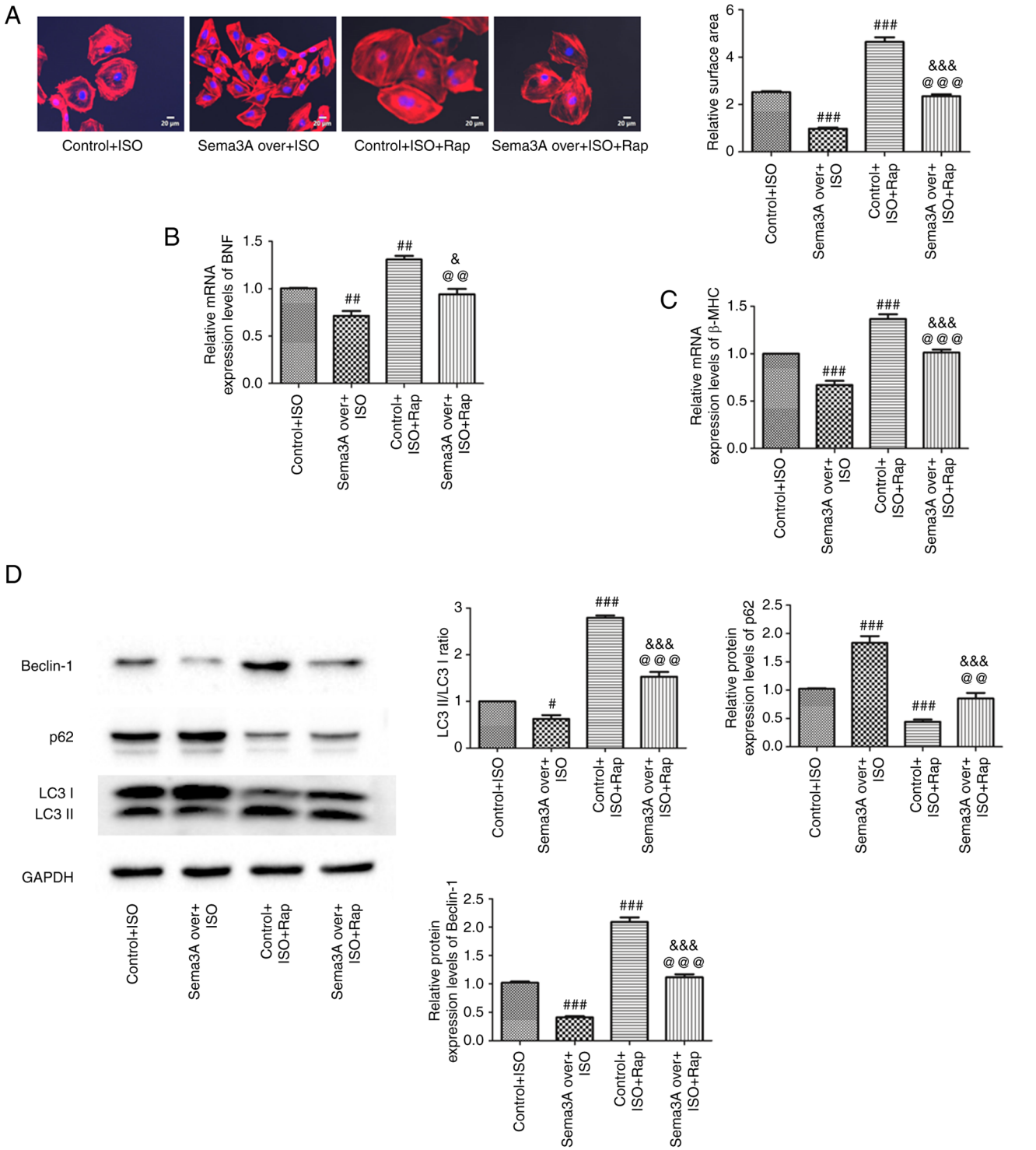


Figure 3. Overactivation of autophagy reduces the protective effect of Sema3A in ISO-induced cardiomyocyte hypertrophy. H9c2 cells were transfected with Sema3A overexpression lentivirus and further incubated with 10 $\mu$M ISO for 24 h in the presence of rapamycin (50 nM). (A) Cell surface area was assessed using rhodamine-phalloidin staining and the mRNA levels of the hypertrophy biomarkers (B) BNF and (C) β-MHC were assessed using revers transcription-quantitative PCR. (D) Western blotting was performed to assess LC3, p62 and Beclin-1 protein expression levels. Data are presented as mean ± standard error of the mean. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control + ISO; $^{\&}$P<0.05 and $^{\&\&\&}$P<0.001 vs. Sema3A over + ISO; $^{@@}$P<0.01 and $^{@@@}$P<0.001 vs. Control + ISO + Rap. Sema3A over, Semaphorin-3A overexpression lentivirus; ISO, isoproterenol; Rap, rapamycin; BNF, brain natriuretic factor; β-MHC, β-myosin heavy chain; LC3, light chain 3.

partially abolished the autophagic level change induced by Sem3A overexpression via inhibition of Akt/mTOR signaling. These results further demonstrated that Sema3A suppressed ISO□induced autophagy through regulation of the Akt/mTOR signaling pathway.

## Discussion

Cardiac hypertrophy, a major pathological feature of certain cardiovascular disorders, including myocardial ischemia, diabetic cardiomyopathy and hypertrophic cardiomyopathy,

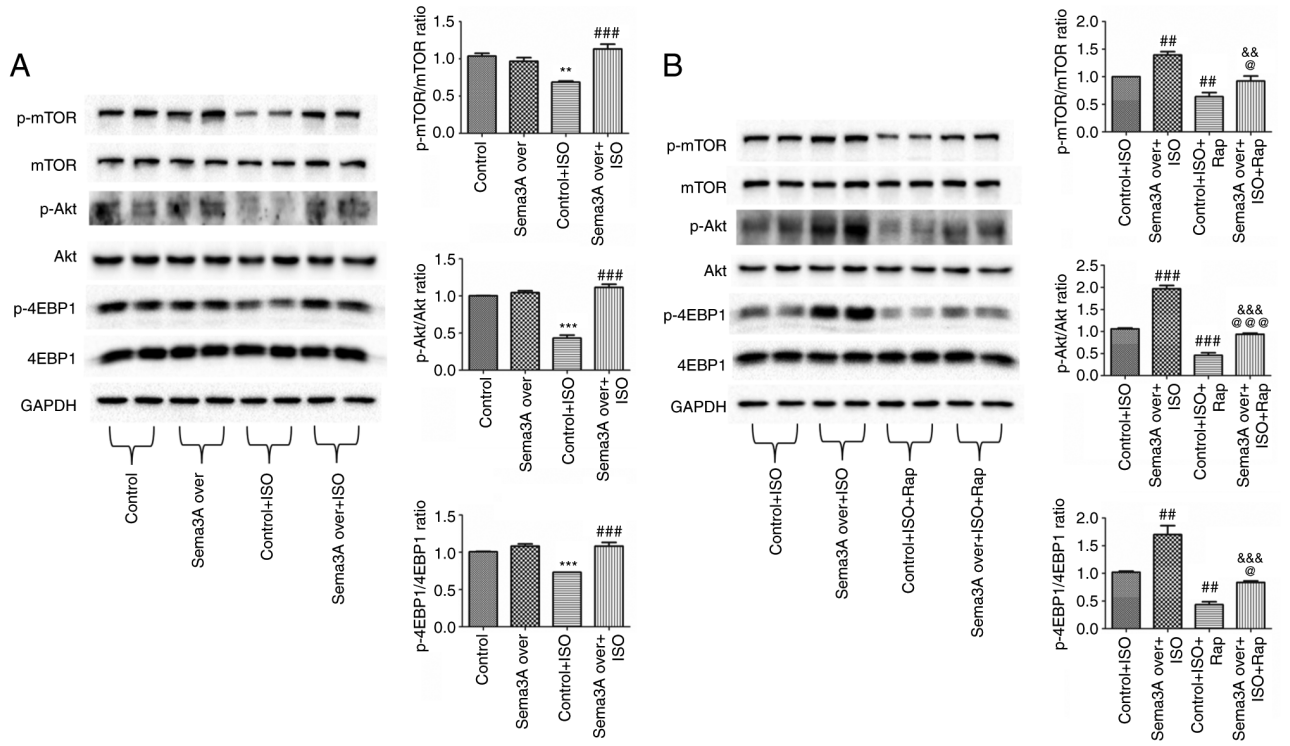


Figure 4. Sema3A inhibits autophagy by activating the Akt/mTOR signaling pathway. H9c2 cells transfected with Sema3A overexpression lentivirus were incubated with 10 $\mu$M ISO for 24 h in the absence of (A) rapamycin or (B) after pretreatment with 50 nM rapamycin. Protein expression levels of p-mTOR, mTOR, p-Akt, Akt, p-4EBP1 and 4EBP1 were semi-quantified using western blotting. Protein bands were semi-quantified by densitometry, with GAPDH used for normalization. Data are presented as mean ± standard error of the mean. $^{**}$P<0.01 and $^{***}$P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. control + ISO; $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. Sema3A over + ISO; $^{@}$P<0.05 and $^{@@@}$P<0.001 vs. Control + ISO + Rap. Sema3A over, Semaphorin-3A overexpression lentivirus; ISO, isoproterenol; Rap, rapamycin; p, phosphorylated; 4EBP1, eukaryotic translation initiation factor 4E-binding protein 1.

is closely related to heart failure (16,17). The mechanisms of cardiac hypertrophy are complex and could be regulated by numerous factors, including inflammation, apoptosis and redox reactions (4). Sema3A, a secreted axon guidance molecule, serves as a chemorepellent during axonal guidance (11,12). Previous studies have reported that Sema3A was associated with myocardial injury and malignant arrhythmia after myocardial injury (18-20). However, the role of Sema3A in cardiac hypertrophy remains unknown. In the present study, the regulatory function of Sema3A in ISO-induced cardiac hypertrophy in cardiomyocytes was investigated.

The results demonstrated that ISO induced cardiomyocyte hypertrophy, with markedly increased cardiomyocyte area. Meanwhile, the protein expression levels of BNF and β-MHC were significantly higher compared with controls. These data indicated that Sema3A overexpression significantly decreased cardiomyocyte size, as well as BNF and β-MHC protein expression levels in cardiomyocytes, which suggested that Sema3A could protect cardiomyocytes from ISO-induced hypertrophy. Multiple studies have assessed Sema3A in cardiovascular disease (13,14,19,21), but the role of Sema3A remains unclear. Our previous study reported that Sema3A overexpression after myocardial infarction reduced the inducibility of ventricular arrhythmia as a result of attenuated sympathetic reinnervation (13). Similarly, Wen *et al* (21) reported that Sema3A ameliorated electrical remodeling at infarct border zones after myocardial infarction. Rienks *et al* (14) reported that Sema3A promoted the resolution of cardiac inflammation after myocardial infarction, to maintain cardiac function. All the previous studies noted above indicated a protective role for Sema3A in cardiovascular disease. Another study reported that Sema3A deficiency improved hypoxia-induced myocardial injury by alleviating inflammation and cardiomyocytes apoptosis (19). However, little is known about the function of Sema3A in cardiac hypertrophy. In the present study, Sema3A inhibited ISO-induced myocardial hypertrophic remodeling. Cardiomyocyte area and, BNF and β-MHC protein expression levels demonstrated no significant changes in the Sema3A over group without ISO treatment, which indicated Sem3A overexpression had no effect on cardiac function under normal conditions.

Autophagy, a conserved homeostatic process for recycling cellular waste and biologically active monomers, serves an important role in pathophysiological changes in numerous diseases, including cancer, neurodegeneration, aging and heart disease (22-24). However, the roles of autophagy in cardiac remodeling and heart failure remain controversial. Studies assessing the role of autophagy in ISO-induced cardiac hypertrophy have reported different outcomes, and whether autophagy activation or inhibition is protective may depend on the model, stimuli, signaling pathway, and/or disease stage and severity (25-27). A recent study suggested that excessive or uncontrolled autophagy induced by pressure stress can be detrimental (28). In the present study, the autophagic markers LC3-II/I ratio, Beclin-1 and p62 were assessed to evaluate autophagic

level. It is known that transformation from LC3-I to LC3-II is important in autophagosome formation and autophagy activation (29). Beclin-1, also named Atg6, can positively regulate autophagy by combining with PI3KCIII and other positive or negative co-factors, which are required for the initiation of the formation of the autophagosome in autophagy (30). p62, a linker between LC3 and ubiquitinated substrates, may be degraded during autophagy (31). These data demonstrated that autophagy was significantly enhanced in the ISO group, indicated by significantly increased LC3II/I ratio and Beclin-1 protein expression levels and significantly decreased p62 protein expression levels, while Sem3A overexpression significantly reduced the LC3II/I ratio and Beclin-1 protein expression levels and significantly increased p62 protein expression levels. As shown previously, ISO-induced cardiomyocyte hypertrophy and excessive cardiomyocyte autophagy are greatly attenuated by Sema3A overexpression. These results indicated a potential protective role for Sema3A in the development of cardiomyocyte hypertrophy by normalizing the autophagic process. However, autophagy in the Sema3A over group without ISO treatment remained unchanged compared with the control group, which suggested that Sema3A overexpression did not regulate autophagy under non-diseased conditions. It was considered that Sema3A may serve as a signaling molecule, which may not be initiated or activated in the normal myocardial cell environment, which indicated that Sema3A could affect autophagy and myocardial hypertrophy in H9c2 cells under pathological conditions, such as after induction by ISO. Therefore, it could be hypothesized that Sema3A could exert similar effects in myocardial hypertrophy induced by stress load in animal models. The role of Sema3A in cardiac hypertrophy required further, *in vivo*, study.

The roles of autophagy in cardiac remodeling and heart failure remain controversial, and autophagy seems to have a dual effect in cardiac hypertrophy (6), which requires further research. It has been suggested autophagy is an adaptive response to myocardial hypertrophy (32,33), and that excessive autophagy exacerbates stress-induced myocardial hypertrophy (34). A previous study reported that excessive or uncontrolled autophagy induced by ISO may be harmful. In cardiac hypertrophy induced by transverse aortic constriction and angiotensin II (Ang II), Beclin-1 and LC3II/I ratio increased significantly, and p62 protein levels significantly decreased compared with the control group. The significant increase in autophagy suggested that excessive autophagy exacerbated myocardial hypertrophy (28). Qi *et al* (35) reported that the use of abdominal aortic coarctation (AAC) and Ang II also induced myocardial hypertrophy and excessive autophagy, and that inhibition of excessive autophagy could significantly alleviate heart failure and myocardial hypertrophy. Another study reported that the LC3II/I ratio increased over time within a 48 h period, in cardiac hypertrophy induced by ISO (36). The results of the present study indicated that Sema3A inhibited excessive autophagy induced by ISO to ameliorate cardiac hypertrophy. Rapamycin, an autophagy inducer, was used to assess whether autophagy participated in cardiac hypertrophy alleviated by Sema3A. In the present study, rapamycin treatment markedly enhanced autophagy, with rapamycin treatment significantly increasing the LC3II/I ratio and Beclin-1 protein expression levels, and significantly downregulating the protein expression levels of p62. The results showed that Sema3A overexpression reduced cardiomyocyte area, BNF, β-MHC and Beclin-1 protein expression levels and the LC3II/I ratio and significantly increased p62 protein expression levels in cardiomyocytes after ISO treatment. Similar to previous reports, in the present study Sema3A overexpression exhibited the same protective effects in cardiomyocytes treated with both ISO and rapamycin, including the prevention of cardiomyocyte hypertrophy progression and the inhibition of excessive cardiomyocyte autophagy. The present study also demonstrated that rapamycin significantly attenuated p62 upregulation and impeded decreases in cardiomyocyte area, LC3II/I ratio and Beclin-1, as well as BNF and β-MHC downregulation induced by Sema3A. In other words, the effects of Sema3A on cardiomyocyte hypertrophy were partly abolished by rapamycin via autophagy regulation. These results indicated that Sema3A ameliorated cardiomyocyte hypertrophy by inhibiting autophagy.

Autophagy in cardiomyocytes is a complex process involving numerous signaling pathways. To elucidate the mechanism by which Sema3A suppressed autophagy, the present study evaluated the Akt/mTOR signaling pathway causing autophagy inhibition (37). Activation of Akt/mTOR signaling, demonstrated by enhanced phosphorylation of Akt, mTOR and its downstream effector 4EBP1, downregulates autophagy. The Akt/mTOR signaling pathway serves a vital role in most cellular processes, while recent studies have reported Akt/mTOR signaling pathway suppression contributes to autophagy and apoptotic cell death (38,39). Wang *et al* (40) also reported that ISO downregulated p-mTOR, which led to excessive autophagy. Fan *et al* (36) reported that ISO could inhibit activation of the Akt/mTOR signaling pathway, and that the Traditional Chinese Medicine Qili Qiangxin significantly increased the levels of p-Akt and p-mTOR to inhibit excessive autophagy and alleviate ISO induced myocardial hypertrophy. In the present study, decreased phosphorylation of Akt, mTOR and 4EBP1 was demonstrated in the Control + ISO group, whereas Sema3A overexpression increased levels of p-Akt, p-mTOR and p-4EBP1. Rapamycin, an mTOR specific inhibitor, blunted the effects of Sema3A on the Akt/mTOR signaling pathway. These results indicated that Sema3A inhibited excessive autophagy, which may have been via activation of the Akt/mTOR signaling pathway. In the present study, the effect of Sema3A on myocardial hypertrophy was only demonstrated *in vitro* and further work is required to demonstrate the role of Sema3A in cardiac hypertrophy in animals. Moreover, numerous signaling pathways are known to regulate autophagy, so further study of the molecular mechanisms related to autophagy is also required.

In conclusion, Sema3A alleviated ISO-induced cardiac hypertrophy, at least in part, by inhibition of excessive cardiac autophagy via the Akt/mTOR signaling pathway. These findings suggested Sema3A may be a potential target for the prevention and treatment of cardiac hypertrophy and 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**

YS and JY designed the study. YS, XC and JY performed the experiments and analyzed the data. YS drafted the manuscript and interpreted the data. JY and JD revised the manuscript for important intellectual content. JD, JW and BL supervised the project and contributed to conception and design. JD and JY confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

**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.

**References**

1. Haque Z and Wang D: How cardiomyocytes sense pathophysiological stresses for cardiac remodeling. Cell Mol Life Sci 4: 983-1000, 2017.
2. Qin W, Du N, Zhang L, Wu X, Hu Y, Li X, Shen N, Li Y, Yang B, Xu C, *et al*: Genistein alleviates pressure overload-induced cardiac dysfunction and interstitial fibrosis in mice. Br J Pharmacol 172: 5559-5572, 2015.
3. Ma ZG, Dai J, Zhang WB, Yuan Y, Liao HH, Zhang N, Bian ZY and Tang QZ: Protection against cardiac hypertrophy by geniposide involves the GLP-1 receptor/AMPKα signalling pathway. Br J Pharmacol 173: 1502-1516, 2016.
4. Fan W, Zhang B, Wu C, Wu H, Wu J, Wu S, Zhang J, Yang X, Yang L, Hu Z and Wu X: Plantago asiatica L. seeds extract protects against cardiomyocyte injury in isoproterenol-induced cardiac hypertrophy by inhibiting excessive autophagy and apoptosis in mice. Phytomedicine 91: 153681, 2021.
5. Wu QQ, Xiao Y, Yuan Y, Ma ZG, Liao HH, Liu C, Zhu JX, Yang Z, Deng W and Tang QZ: Mechanisms contributing to cardiac remodelling. Clin Sci (Lond) 131: 2319-2345, 2017.
6. Wang EY, Biala AK, Gordon JW and Kirshenbaum LA: Autophagy in the heart: Too much of a good thing? J Cardiovasc Pharmacol 60: 110-117, 2012.
7. Rifki OF and Hill JA: Cardiac autophagy: Good with the bad. J Cardiovasc Pharmacol 60: 248-252, 2012.
8. Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, *et al*: Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell 99: 71-80, 1999.
9. Soker S, Miao HQ, Nomi M, Takashima S and Klagsbrun M: VEGF165 mediates formation of complexes containing VEGFR-2 and neuropilin-1 that enhance VEGF165-receptor binding. J Cell Biochem 85: 357-368, 2002.
10. Bagnard D, Vaillant C, Khuth ST, Dufay N, Lohrum M, Puschel AW, Belin MF, Bolz J and Thomasset N: Semaphorin 3A-vascular endothelial growth factor165 balance mediates migration and apoptosis of neural progenitor cells by the recruitment of shared receptor. Neurosci 21: 3332-3341, 2001.
11. Neufeld G, Cohen T, Shraga N, Lange T, Kessler O and Herzog Y: The neuropilins: Multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med 12: 13-79, 2002.
12. He Z and Tessier-Lavigne M: Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell 90: 739-751, 1997.
13. Chen RH, Li YG, Jiao KL, Zhang PP, Sun Y, Zhang LP, Fong XF, Li W and Yu Y: Overexpression of Sema3a in myocardial infarction border zone decreases vulnerability of ventricular tachycardia post-myocardial infarction in rats. J Cell Mol Med 17: 608-616, 2013.
14. Rienks M, Carai P, Bitsch N, Schellings M, Vanhaverbeke M, Verjans J, Cuijpers I, Heymans S and Papageorgiou A: Sema3A promotes the resolution of cardiac inflammation after myocardial infarction. Basic Res Cardiol 112: 42, 2017.
15. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
16. Balakumar P and Jagadeesh G: Multifarious molecular signaling cascades of cardiac hypertrophy: Can the muddy waters be cleared? Pharmacol Res 62: 365-383, 2010.
17. McKinsey TA and Kass DA: Small-molecule therapies for cardiac hypertrophy: Moving beneath the cell surface. Nat Rev Drug Discov 6: 617-635, 2007.
18. Liu X, Zhou N, Sui X, Pei Y, Liang Z and Hao S: Hrd1 induces cardiomyocyte apoptosis via regulating the degradation of IGF-1R by sema3a. Biochim Biophys Acta Mol Basis Dis 1864: 3615-3622, 2018.
19. Zhao C, Liu J, Zhang M and Wu Y: Semaphorin 3A deficiency improves hypoxia-induced myocardial injury via resisting inflammation and cardiomyocytes apoptosis. Cell Mol Biol (Noisy-le-grand) 62: 8-14, 2016.
20. Ieda M, Kanazawa H, Kimura K, Hattori F, Ieda Y, Taniguchi M, Lee JK, Matsumura K, Tomita Y, Miyoshi S, *et al*: Sema3a maintains normal heart rhythm through sympathetic innervation patterning. Nat Med 13: 604-612, 2007.
21. Wen HZ, Jiang H, Li L, Xie P, Li JY, Lu ZB and He B: Semaphorin 3A attenuates electrical remodeling at infarct border zones in rats after myocardial infarction. Tohoku J Exp Med 225: 51-57, 2011.
22. Levine B and Kroemer G: Autophagy in the pathogenesis of disease. Cell 132: 27-42, 2008.
23. Sagrillo-Fagundes L, Bienvenue-Pariseault J and Vaillancourt C: Melatonin: The smart molecule that differentially modulates autophagy in tumor and normal placental cells. PLoS One 14: e0202458, 2019.
24. Esteves AR, Palma AM, Gomes R, Santos D, Silva DF and Cardoso SM: Acetylation as a major determinant to microtubule-dependent autophagy: Relevance to Alzheimer's and Parkinson disease pathology. Biochim Biophys Acta Mol Basis Dis 1865: 2008-2023, 2019.
25. Nemchenko A, Chiong M, Turer A, Lavandero S and Hill JA: Autophagy as a therapeutic target in cardiovascular disease. J Mol Cell Cardiol 51: 584-593, 2011.
26. Nakai A, Yamaguchi O, Takeda T, Higuchi Y, Hikoso S, Taniike M, Omiya S, Mizote I, Matsumura Y, Asahi M, *et al*: The role of autophagy in cardiomyocytes in the basal state and in response to hemodynamic stress. Nat Med 13: 619-624, 2007.
27. Xu X and Ren J: Unmasking the janus faces of autophagy in obesity-associated insulin resistance and cardiac dysfunction. Clin Exp Pharmacol Physiol 39: 200-208, 2012.
28. Liu L, Wang C, Sun D, Jiang S, Li H, Zhang W, Zhao Y, Xi Y, Shi S, Lu F, *et al*: $Calhex_{231}$ ameliorates cardiac hypertrophy by inhibiting cellular autophagy in vivo and in vitro. Cell Physiol Biochem 36: 1597-1612, 2015.
29. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y and Yoshimori T: LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19: 5720-5728, 2000.
30. Tukaj C: The significance of macroautophagy in health and disease. Folia Morphol (Warsz) 72: 87-93, 2013.

31. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Øvervatn A, Bjørkøy G and Johansen T: p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131-24145, 2007.
32. Xu X, Hua Y, Nair S, Bucala R and Ren J: Macrophage migration inhibitory factor deletion exacerbates pressure overload-induced cardiac hypertrophy through mitigating autophagy. Hypertension 63: 490-499, 2014.
33. McMullen JR, Sherwood MC, Tarnavski O, Zhang L, Dorfman AL, Shioi T and Izumo S: Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. Circulation 109: 3050-3055, 2004.
34. Zhu H, Tannous P, Johnstone JL, Kong Y, Shelton JM, Richardson JA, Le V, Levine B, Rothermel BA and Hill JA: Cardiac autophagy is a maladaptive response to hemodynamic stress. J Clin Invest 117: 1782-1793, 2007.
35. Qi H, Ren J, Ba L, Song C, Zhang Q, Cao Y, Shi P, Fu B, Liu Y and Sun H: MSTN attenuates cardiac hypertrophy through inhibition of excessive cardiac autophagy by blocking AMPK /mTOR and miR-128/PPARγ/NF-κB. Mol Ther Nucleic Acids 19: 507-522, 2020.
36. Fan C, Tang X, Ye M, Zhu G, Dai Y, Yao Z and Yao X: Qi-Li-Qiang-Xin alleviates isoproterenol-induced myocardial injury by inhibiting excessive autophagy *via* activating AKT/mTOR pathway. Front Pharmacol 10: 1329, 2019.
37. Li L, Xu J, He L, Peng L, Zhong Q, Chen L and Jiang Z: The role of autophagy in cardiac hypertrophy. Acta Biochim Biophys Sin (Shanghai) 48: 491-500, 2016.
38. Chen L, Liu P, Feng X and Ma C: Salidroside suppressing LPS induced myocardial injury by inhibiting ROS-mediated PI3K/Akt/mTOR pathway in vitro and in vivo. J Cell Mol Med 21: 3178-3189, 2017.
39. Tang ZB, Lei Y and Zhang XS: Vitexin mitigates myocardial ischemia reperfusion-induced damage by inhibiting excessive autophagy to suppress apoptosis via the PI3K/Akt/mTOR signaling cascade. RSC Adv 7: 56406- 56416, 2017.
40. Wang H, Wang H, Liang EY, Zhou LX, Dong ZL, Liang P, Weng QF and Yang M: Thrombopoietin protects H9C2 cells from excessive autophagy and apoptosis in doxorubicin-induced cardiotoxicity. Oncol Lett 15: 839-848, 2017.