

# Vaspin alleviates pathological cardiac hypertrophy by regulating autophagy-dependent myocardial senescence

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## Abstract

**Background:** Visceral adipose tissue-derived serine protease inhibitor (vaspin), a secretory adipokine, protects against insulin resistance. Recent studies have demonstrated that serum vaspin levels are decreased in patients with coronary artery disease and that vaspin protects against myocardial ischemia-reperfusion injury and atherosclerosis. However, it remains unclear whether vaspin exerts specific effects on pathological cardiac hypertrophy.

**Methods:** An in vivo study was conducted using a cardiac hypertrophy model established by subcutaneous injection of isoproterenol (ISO) in C57BL/6 and vaspin-ko mice. Rapamycin was administered intraperitoneally to mice, for further study. H9c2 cells and neonatal rat ventricular myocytes (NRVMs) were treated with ISO to induce hypertrophy. Human vaspin fusion protein, the proteasome inhibitor MG132, and chloroquine diphosphate were used for further mechanistic studies.

**Results:** Here, we provide the first evidence that vaspin knockdown results in markedly exaggerated cardiac hypertrophy, fibrosis, and cardiomyocyte senescence in mice treated with ISO. Conversely, the administration of exogenous recombinant human vaspin protected NRVMs in vitro against ISO-induced hypertrophy and senescence. Furthermore, vaspin significantly potentiated the ISO-induced decrease in autophagy. Both rapamycin and chloroquine diphosphate regulated autophagy in vivo and in vitro, respectively, and participated in vaspin-mediated cardioprotection. Moreover, the PI3K-AKT-mTOR pathway plays a critical role in vaspin-mediated autophagy in cardiac tissues and NRVMs. Our data showed that vaspin downregulated the p85 and p110 subunits of PI3K by linking p85 and p110 to NEDD4L-mediated ubiquitination degradation.

**Conclusion:** Our results show, for the first time, that vaspin functions as a critical regulator that alleviates pathological cardiac hypertrophy by regulating autophagy-dependent myocardial senescence, providing potential preventive and therapeutic targets for pathological cardiac hypertrophy.

**Keywords:** Autophagic flux, Myocardial senescence, Pathological cardiac hypertrophy, Ubiquitination, Vaspin

HR and HY contributed equally to this article.

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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## Introduction

Pathological cardiac hypertrophy, a cellular response to increased hemodynamic load or neurohumoral stimuli,<sup>[1]</sup> is a common result of the harmful development of diseases such as hypertension and myocardial infarction. Hypertrophy leads to several severe cardiac complications, including increased susceptibility to ischemia-reperfusion injury (IRI), arrhythmias, cardiomyopathy, heart failure, and sudden death.<sup>[2,3]</sup> Despite the progress in pathological cardiac hypertrophy therapy, it remains one of the primary causes of death worldwide. Hence, understanding the mechanisms underlying the development of myocardial hypertrophy is vital to explore efficient therapeutic strategies for the prevention and treatment of pathological cardiac hypertrophy.

Visceral adipose tissue-derived serine protease inhibitor (vaspin), a member of the serine protease inhibitor family, was first identified in the visceral white adipose tissues of the Otsuka Long-Evans Tokushima fatty rat<sup>[4]</sup> and later found in the visceral and subcutaneous adipose tissues of obese humans.<sup>[5]</sup> Previous reports have shown that vaspin alleviates insulin resistance, obesity, metabolic disturbances, and hepatic steatosis through autocrine and paracrine mechanisms.<sup>[6]</sup> Recently, vaspin was shown to prevent the development of atherosclerosis and reduce the risk of cardiovascular disease.<sup>[7,8]</sup> Vaspin also plays a protective role against myocardial injury caused by ischemia/reperfusion and diabetes through the regulation of apoptosis, inflammation, and autophagy.<sup>[9,10]</sup> For example, our previous study found that vaspin exerts protective effects against myocardial IRI by upregulating autophagic flux.<sup>[11]</sup> However, the effect of vaspin

on pathological cardiac hypertrophy remains unknown and requires further investigation.

Premature myocardial senescence, observed in stress-induced pathological cardiac hypertrophy, has been suggested as a significant cause of cardiac dysfunction.<sup>[12,13]</sup> Myocytes undergoing senescence show distinctive functional alterations, including increased expression of cell cycle regulatory molecules, senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) activity, and a senescence-associated secretory phenotype. Cardiomyocyte senescence and hypertrophy share certain features including increased cardiomyocyte size and enhanced protein synthesis.<sup>[14]</sup> Dysfunctional cardiac autophagy, which results in the accumulation of misfolded proteins or toxic metabolites in the heart, is an important mechanism in both cardiomyocyte senescence and pathological hypertrophy.<sup>[15–18]</sup> However, the involvement of premature cardiomyocyte senescence in stress-induced pathological cardiac hypertrophy owing to abnormal autophagy should be considered.

In this study, we evaluated the role of vaspin in pathological cardiac hypertrophy and explored its underlying mechanisms. We hypothesized that vaspin would alleviate pathological cardiac hypertrophy by regulating autophagy-dependent myocardial senescence.

## Materials and methods

### Animals and experimental procedures

The target exon DNA sequence of *Tec* was cut using the CRISPR system, creating a double-strand break, resulting in a frameshift mutation via the nonhomologous end joining DNA repair mechanism, resulting in *Serpina12* knockout mice. C57BL/6 male mice (8–10 weeks old) were purchased from Huafukang Company (Beijing, China) and housed at the Experimental Animal Center of Qilu Hospital, Shandong University (Jinan, China). The mice were housed in rooms with free access to food and water at a constant temperature with 12-hour light-dark cycles. Experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Qilu Hospital of Shandong University. An *in vivo* study was conducted using a cardiac hypertrophy model established by the subcutaneous injection of isoproterenol (ISO) (5 mg/kg per day, dissolved in saline; Sigma-Aldrich, I5627, St. Louis, MO, USA) once daily for 9 days, according to previous studies.<sup>[19]</sup> Rapamycin (2 mg/kg per day, S1039; Selleck, Shaerworthy County, CA, USA) or vehicle was administered intraperitoneally to wild-type (WT) and vaspin-ko mice, as previously described.<sup>[20]</sup>

### Cell culture and treatments

The H9c2 cells were cultured in Dulbecco modified eagle medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. The neonatal rat ventricular myocytes (NRVMs) were cultured in Dulbecco modified eagle medium, less sugar supplemented with 10% newborn bovine serum, 6% horse serum, and 1% bromodeoxyuridine in a humidified incubator with 5% CO<sub>2</sub> at 37 °C. Cells were treated with ISO (10  $\mu$ mol/L, dissolved in phosphate-buffered saline; Sigma-Aldrich, I5627) for 48 hours to induce hypertrophy, as previously described.<sup>[21]</sup> Human vaspin fusion protein (100  $\mu$ g/L, Ag11621; Proteintech, Chicago, IL, USA) or chloroquine diphosphate (CQ) (20  $\mu$ M S4157, Selleck) was added 1 hour before ISO stimulation. The proteasome inhibitors MG132 (10  $\mu$ M, S2619; Selleck) and CQ were added 1 hour before stimulation with human vaspin fusion protein. Neonatal rat ventricular myocytes were treated with cycloheximide (100  $\mu$ g/mL, castanospermine [CST], 2112S) and vaspin (100  $\mu$ g/L) for the indicated time periods.

### Echocardiography

Cardiac function was measured using the Visual Sonics high-resolution Vevo 2100 system (Visual Sonics, Toronto, Ontario, Canada). After removing hair from the anterior chest, the mice were anesthetized with 1.5% isoflurane. Related parameters were obtained from the left ventricular parasternal long axis (B-mode) and short axis (M-mode). Three images from consecutive cardiac cycles were analyzed and averaged. The depth of anesthesia was set to maintain the heart rate at approximately 550 beats per minute.

### Immunostaining

After fixation with 4% paraformaldehyde at room temperature for 30 minutes, NRVMs were washed 3 times with ice-cold phosphate-buffered saline, permeabilized with 0.1% Triton X-100 for 30 minutes, blocked with 5% bovine serum albumin for 30 minutes, and incubated with primary antibodies in blocking medium overnight at 4 °C. The samples were then incubated with a secondary antibody at room temperature for 2 hours. DAPI (4',6-diamidino-2-phenylindole dihydrochloride) was added for nuclear counterstaining. The levels of P16 (anti-P16, ab51243; Abcam, 1:100, Boston, MA, USA), LC3B (anti-LC3B, ab48394; Abcam, 1:1000), and  $\alpha$ -actinin (anti- $\alpha$ -actinin, A7811; Sigma, 1:1000, St. Louis, MO, USA) in cells were evaluated in images obtained using confocal laser scanning fluorescence microscopy (LSM780; Carl Zeiss AG, Oberkochen, Germany).

### Histological analysis

For histological analysis, hearts were fixed in 4% paraformaldehyde at room temperature for more than 24 hours. Fixed hearts were embedded in paraffin and cut transversely into 5- $\mu$ m sections. The heart sections were immunohistochemically stained using standard protocols. Serial heart sections were stained with wheat germ agglutinin to measure the myocyte cross-sectional areas. The degree of collagen deposition was detected using a picrosirius red staining kit and Masson staining kit.

### Electron microscopy

Samples (H9c2 cells and heart tissue) were fixed immediately in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at 4 °C. The samples were immersed in 1% osmium tetroxide in cacodylate buffer (0.1 mmol/L) for 1 hour after rinsing in cacodylate buffer (0.1 mmol/L with 1% tannic acid). After rinsing, the samples were dehydrated with alcohol and embedded in the Epon 812 medium. The samples were examined using a transmission electron microscope.

### SA- $\beta$ -gal staining

An SA- $\beta$ -gal staining kit (CST, #9860) was used *in vitro* according to the manufacturer's instructions.

### Enzyme-linked immunosorbent assay

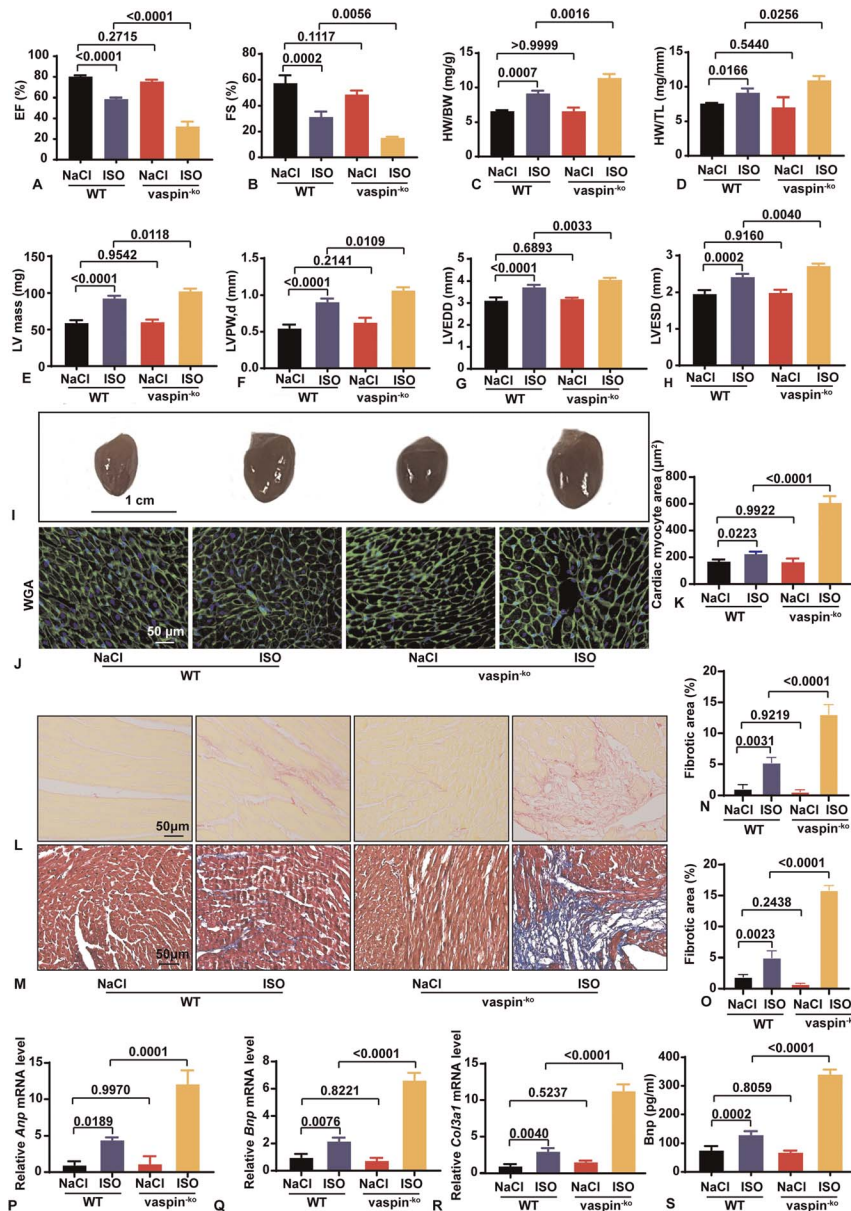
Brain natriuretic peptide (SEA541Mu; Cloud-Clone Corp, Houston, TX, USA) concentrations in the samples (serum) were measured using enzyme-linked immunosorbent assay kits.

### Western blot analysis

The concentration of total protein extracted from frozen hearts or collected cells was measured using a bicinchoninic acid protein assay. Protein samples (20  $\mu$ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to poly

(vinylidene fluoride) membranes (Millipore, Boston, MA, USA). After blocking with 5% milk for 1 hour at room temperature and then incubation overnight at 4 °C with the following primary antibodies: GAPDH (CST, 97166), β-actin (Proteintech, 66009-1-Ig), LC3B (Abcam, ab48394), P62 (CST, 5114), P16 (Abcam, ab51243), P53 (Proteintech, 10442-1-AP), P21 (Abcam, ab109199), PI3K (p110, CST, 4249; p85, Proteintech, 60225-1-Ig), P-PI3K (Tyr458,

affinity, AF3242), mTOR (CST, 2983), P-mTOR (Ser2448, CST, 5536), AKT (CST, 4685), P-AKT (Ser473, CST, 4060), ubiquitin (CST, 3936), NEDD4L (Proteintech, 13690-1-AP), NEDD4 (Proteintech, 21698-1-AP), CBL (ABclonal, A7881), CBLB (Proteintech, 12781-1-AP), UBE3A (Proteintech, 10344-1-AP), FBXW7 (ABclonal, A5872), and SMURF1 (affinity, AF4623). The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary



**Figure 1.** Vaspin-ko aggravated ISO-induced pathological cardiac hypertrophy in vivo. (A) and (B) Cardiac function of wild-type (WT) and vaspin-ko mice injected with NaCl or isoproterenol (ISO) as shown by ejection fraction (EF) and fractional shortening (FS) obtained from echocardiography. (C) and (D) Heart weight-to-body weight ratio (HW/BW) and heart weight-to-tibia length (HW/TL) of WT and vaspin-ko mice. (E–H) Left ventricular mass (LV mass), left ventricular end-diastolic posterior wall thickness (LVPW,d), left ventricular end-diastolic diameter (LVEDD), and left ventricular end-systolic diameter (LVESD) of WT and vaspin-ko mice obtained from echocardiography. (I–K) Gross hearts and wheat germ agglutinin staining (scale bar = 50 μm) were performed to determine the hypertrophic growth of the hearts in WT and vaspin-ko mice injected with NaCl or ISO. (L–O) Picrosirius red staining (scale bar = 50 μm) and Masson staining (scale bar = 50 μm) were performed to determine fibrosis of the hearts in WT and vaspin-ko mice injected with NaCl or ISO. (P–R) The mRNA expression levels of markers of cardiac hypertrophy and fibrosis in WT and vaspin-ko mice injected with NaCl or ISO were assessed by real-time PCR. (S) The change of brain natriuretic peptide in serum between vaspin-ko and WT mice was assessed by enzyme-linked immunosorbent assay (ELISA). Means ± SDs are shown, with n = 6. *P* < 0.05 was considered significant. PCR, polymerase chain reaction.

antibodies for 1 hour at room temperature. The membranes were detected using an Amersham Imager 600 and analyzed using ImageJ (National Institutes of Health, Bethesda, MD).

**RNA extraction and real-time polymerase chain reaction**

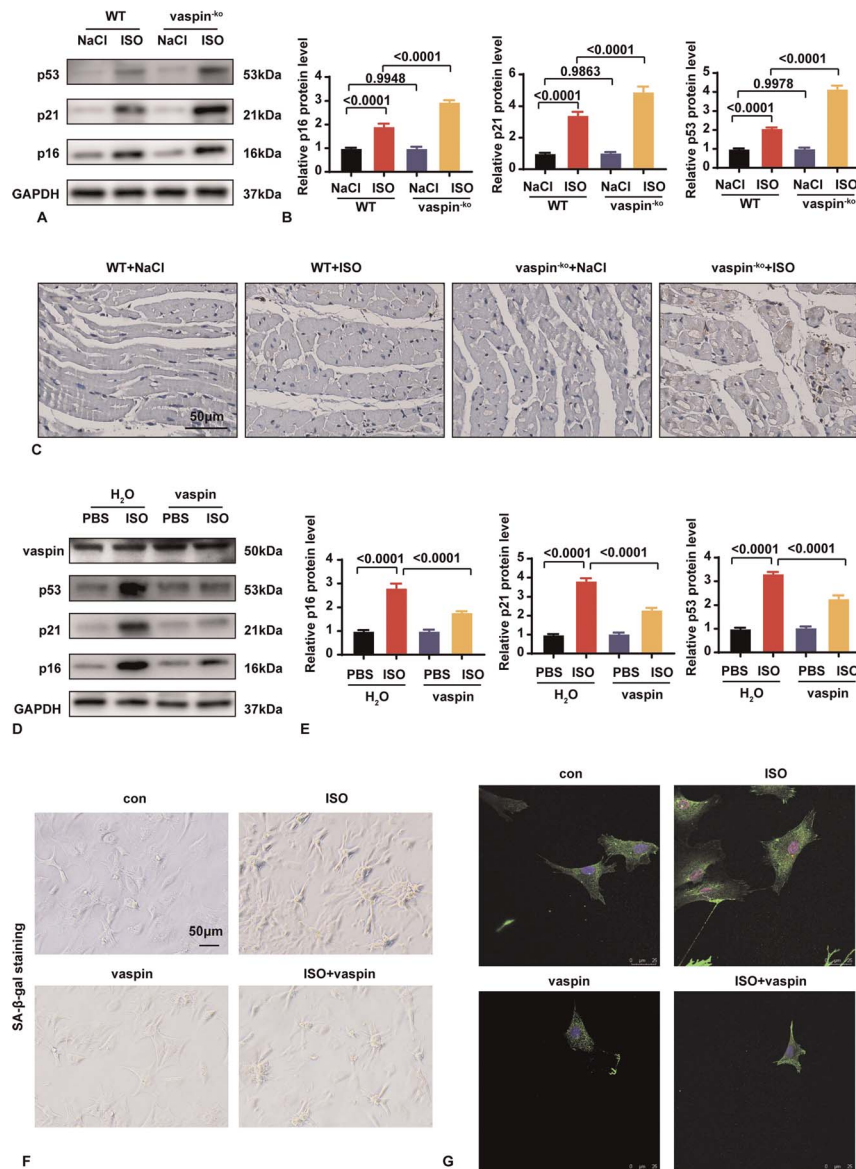
Total RNA was extracted using TRIzol (Life Technologies, 15596018) and reverse-transcribed into cDNA using the Prime Script RT reagent kit (Takara, R047A, Otsu City, Shiga Prefecture, Japan). reverse transcriptase–quantitative polymerase chain reaction (PCR) was performed using a Takara kit (RR420A) on an ABI7500 Real-time PCR System. Relative mRNA levels were calculated using the comparative C<sub>T</sub> method, with 18S mRNA as the invariant control.

**siRNA and transfection**

Rat siNC and siNEDD4L were purchased from BioSune (Shanghai, China) and transduced into NRVMs using Lipofectamine RNAiMAX, according to the manufacturer’s recommendations. The NEDD4L-siRNA sequence was 5’-CCCUGAUUCUCAGAGUAAATT-3’. After 48-hour siRNA treatment, the vehicle or vaspin was added for 12 hours. At the end of the experiments, the cells were collected, and the level of protein expression was evaluated by Western blotting.

**Coimmunoprecipitation**

The target proteins of the NRVMs were precipitated using the respective antibodies and protein A/G beads (Merck, LSKMAGAG10,



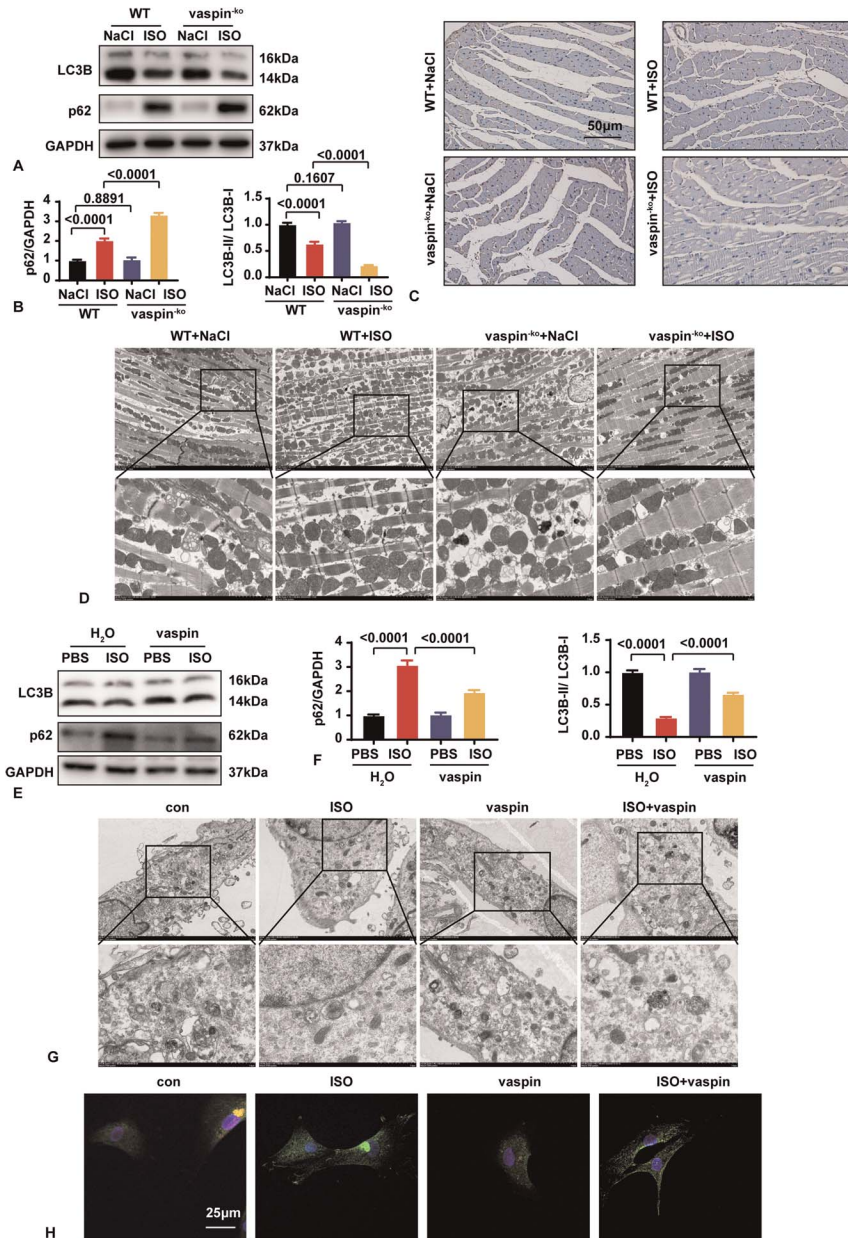
**Figure 2.** Vaspin-ko exacerbated ISO-induced premature myocardial senescence in vivo; vaspin overexpression attenuated premature senescence and hypertrophy remodeling in NRVMs. (A) and (B) Representative Western blot bands and quantification of P16, P21, and P53 protein expression in wild-type (WT) and vaspin-ko mice injected with NaCl or isoproterenol (ISO). (C) P16 immunohistochemistry staining in the heart sections of WT and vaspin-ko mice injected with NaCl or ISO. (D) and (E) Representative Western blot bands and quantification of P16, P21, and P53 protein expression in neonatal rat ventricular myocytes (NRVMs). (F) and (G) SA-β-gal staining pictures (scale bar = 50 μm) and representative immunofluorescence staining images for DAPI (blue), P16 (red), and α-Actinin (green) in NRVMs (scale bar = 25 μm). Means ± SDs are shown, with n = 6. P < 0.05 was considered significant. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; PBS, phosphate-buffered saline.

Kenilworth, NJ, USA). The immunoprecipitates were washed, resuspended in 1× sample buffer, boiled for 10 minutes, and analyzed by Western blotting.

**Statistical analysis**

All statistical analyses and graphs were generated using the GraphPad Prism 8 software (GraphPad, La Jolla, CA, USA). All data are expressed as mean ± SD. All experiments were replicated at least 3

times. The normality of the distribution of continuous variables was confirmed using the Shapiro-Wilk normality test and visualized using a Q-Q plot. Homoscedasticity was confirmed using the *F* test. For continuous variables, based on the normal distribution and similar variances, unpaired 2-tailed Student *t* test (2 groups) and 1-way analysis of variance followed by the Bonferroni post hoc test (≥3 groups) were used; if variances differed between the groups, Welch correction was used; if data showed a significantly abnormal distribution, the Mann-Whitney *U* test (2 groups) and Kruskal-Wallis test followed



**Figure 3.** Vaspin mediated autophagy activation in pathological cardiac hypertrophy. (A) and (B) Representative Western blot bands and quantification of P62 and LC3B protein expression in wild-type (WT) and vaspin-ko mice injected with NaCl or isoproterenol (ISO). (C) LC3B immunohistochemistry staining in the heart sections of WT and vaspin-ko mice injected with NaCl or ISO. (D) Electron microscopy visualized autophagosomes and autolysosomes in WT and vaspin-ko mice injected with NaCl or ISO. (E) and (F) Representative Western blot bands and quantification of P62 and LC3B protein expression in neonatal rat ventricular myocytes (NRVMs). (G) Electron microscopy visualized autophagosomes and autolysosomes in H9c2 cells. (H) Representative immunofluorescence staining images for DAPI (blue), LC3B (red), and α-actinin (green) in NRVMs (scale bar = 25 μm). Means ± SDs are shown, with n = 6. *P* < 0.05 was considered significant. DAPI, 4',6-diamidino-2-phenylindole dihydrochloride.

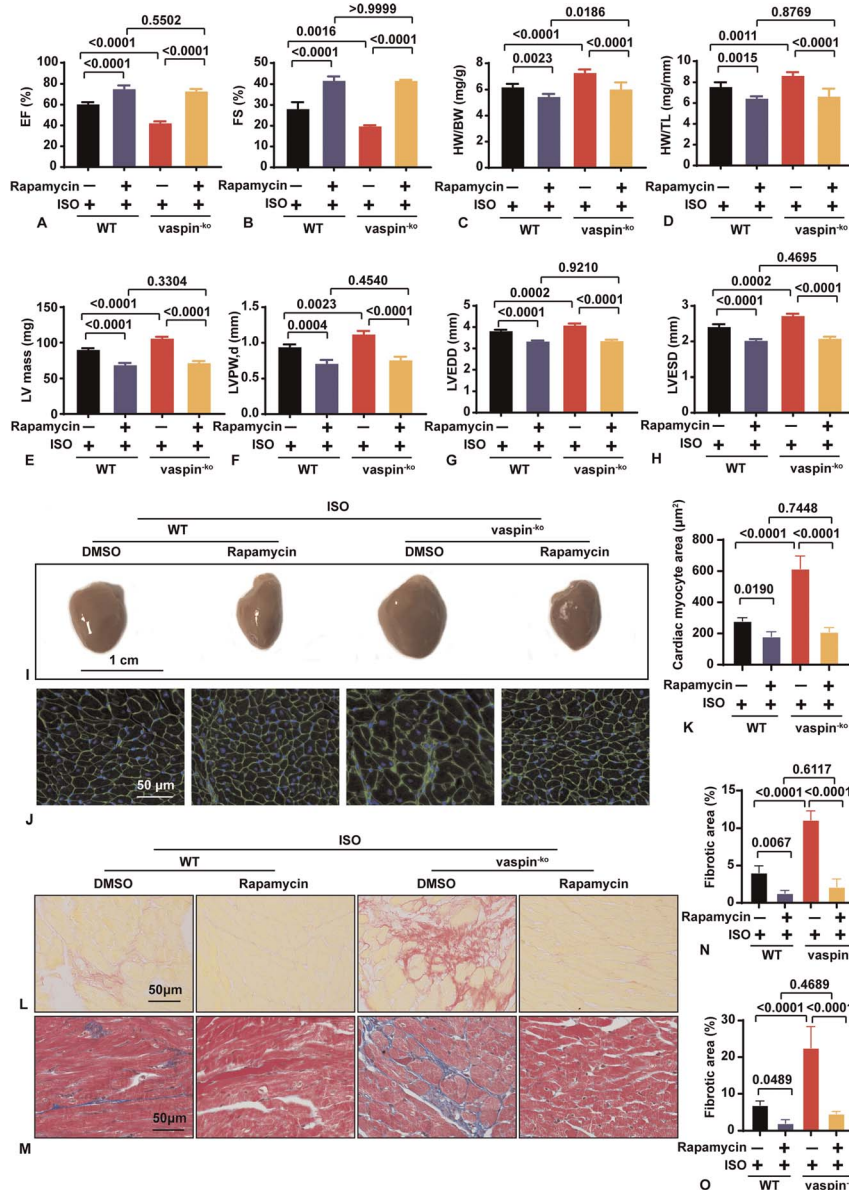
by Dunn post hoc test ( $\geq 3$  groups) were used.  $P < 0.05$  was considered significant.

## Results

### Vaspin-ko aggravated ISO-induced pathological cardiac hypertrophy in vivo

To explore the effects of vaspin on cardiac hypertrophy in an ISO-induced mouse model, we randomly divided WT and vaspin-ko

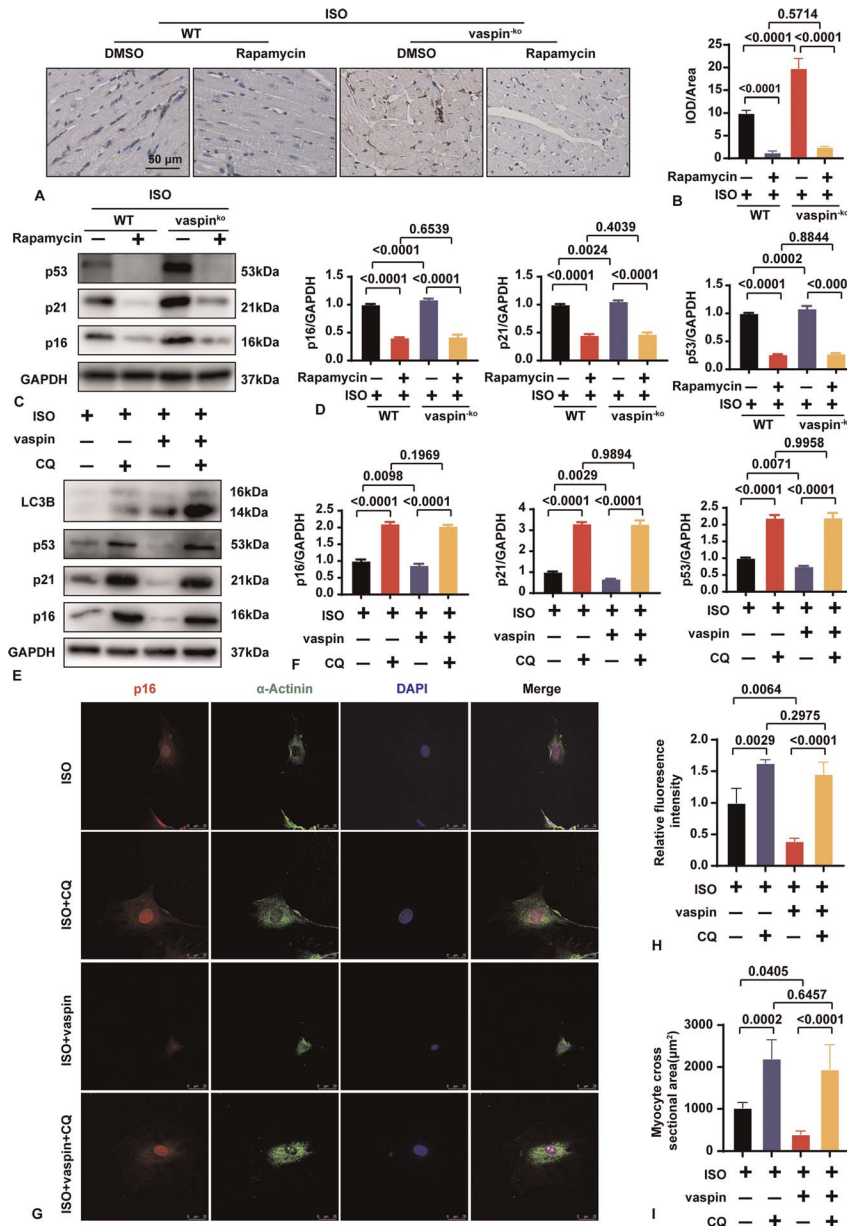
mice into 2 groups: the experimental group was subjected to subcutaneous ISO injection, and the same amount of saline was injected in the control group. Wild-type and vaspin-ko mice were subjected to functional cardiac phenotyping. Significantly, ISO caused worse echocardiography-detectable cardiac function in vaspin-ko mice than in ISO-treated WT mice, as evidenced by a decrease in the cardiac ejection fraction and fractional shortening (Figs. 1A, B). Wild-type mice injected with ISO had an increased heart weight-to-body weight ratio (HW/BW) compared with WT mice injected with saline, and



**Figure 4.** Promoting autophagy rescues ISO-induced cardiac hypertrophy and senescence in vaspin-ko mice. (A) and (B) Cardiac function of wild-type (WT) and vaspin-ko mice injected with isoproterenol (ISO) as shown by ejection fraction (EF) and fractional shortening (FS) obtained from echocardiography. (C) and (D) Heart weight-to-body weight ratio (HW/BW) and heart weight-to-tibia length (HW/TL) of WT and vaspin-ko mice. (E–H) Left ventricular mass (LV mass), left ventricular end-diastolic posterior wall thickness (LVPW,d), left ventricular end-diastolic diameter (LVEDD), and left ventricular end-systolic diameter (LVESD) of WT and vaspin-ko mice obtained from echocardiography. (I–K) Gross hearts and wheat germ agglutinin (WGA) staining (scale bar = 50 μm) were performed to determine the hypertrophic growth of the hearts in WT and vaspin-ko mice injected with isoproterenol (ISO). (L–O) Picrosirius red staining (scale bar = 50 μm) and Masson staining (scale bar = 50 μm) were performed to determine fibrosis of the hearts in WT and vaspin-ko mice injected with ISO. Means ± SDs are shown, with n = 6.  $P < 0.05$  was considered significant.

HW/BW was further enhanced in vaspin-ko mice injected with ISO (Fig. 1C). The ratio of heart weight to tibia length (HW/TL) in WT and vaspin-ko mice showed a similar trend (Fig. 1D). In addition, the echocardiographic parameters, such as left ventricular mass, left ventricular end-diastolic posterior wall thickness, left ventricular end-diastolic diameter, and left ventricular end-systolic diameter, were markedly elevated in ISO-treated vaspin-ko mice than in ISO-treated WT mice (Figs. 1E-H). Hypertrophic remodeling and

fibrosis of the myocardial tissues were significant in vaspin-ko mice injected with ISO compared with those in ISO-injected WT mice (Figs. 1I-O). Consistently, quantitative real-time PCR results showed that these hypertrophic pathological phenotypes were accompanied by the upregulation of hypertrophic and fibrotic genes (Figs. 1P-R). Furthermore, the change of brain natriuretic peptide in serum between vaspin-ko and WT mice stimulated by ISO showed a similar trend (Fig. 1S).



**Figure 5.** Promoting autophagy rescues ISO-induced cardiac senescence in vaspin-ko mice and inhibiting autophagy blocked the cardiomyocytes protection of vaspin. (A) and (B) P16 immunohistochemistry staining in the heart sections of wild-type (WT) and vaspin-ko mice injected with isoproterenol (ISO). (C) and (D) Representative Western blot bands and quantification of P16, P21, and P53 protein expression in WT and vaspin-ko mice injected with ISO. (E) and (F) Representative Western blot bands and quantification of P16, P21, and P53 protein expression in neonatal rat ventricular myocytes (NRVMs). (G-I) Representative immunofluorescence staining images and quantification for DAPI (blue), P16 (red), and  $\alpha$ -actinin (green) in NRVMs (scale bar = 25  $\mu$ m). Means  $\pm$  SDs are shown, with  $n = 6$ .  $P < 0.05$  was considered significant. CQ, chloroquine diphosphate; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DMSO, dimethyl sulfoxide.

**Vaspin-ko exacerbated ISO-induced premature myocardial senescence in vivo; vaspin overexpression attenuated premature senescence and hypertrophic remodeling in NRVMs**

To verify whether vaspin regulates ISO-induced premature myocardial senescence, key senescent markers were examined in a previously described cardiac hypertrophy model.<sup>[19]</sup> Western blot results demonstrated that the protein expression of P16, P53, and P21 was significantly increased in vaspin-ko mice treated with ISO compared with that in WT mice after ISO treatment (Figs. 2A, B). We also detected P16 levels in tissue sections using immunohistochemistry and observed positively stained cardiomyocytes in WT mice after ISO treatment. In contrast, the ratio of P16-positive cardiomyocytes further increased in ISO-treated vaspin-ko mice (Fig. 2C).

Consistent with the results of in vivo experiments, NRVMs were stimulated with human vaspin fusion protein and treated with ISO for 48 hours. We found that the vaspin protein levels in cardiomyocytes increased after vaspin stimulation, so we hypothesized that vaspin, as a secreted protein, might bind to the receptor protein on the surface of cardiomyocytes. Overexpression of vaspin attenuated ISO-induced cardiomyocyte premature senescence and hypertrophy (Figs. 2D–G).  $\alpha$ -Actinin staining was performed to determine the cell size. Western blotting for P16, P21, P53, SA- $\beta$ -gal staining, and P16 immunofluorescence staining represents senescence in cardiomyocytes.

Overall, these results indicated that vaspin might play a critical role in the regulation of cardiac hypertrophy and is associated with premature myocardial senescence.

**Vaspin mediated autophagy activation in pathological cardiac hypertrophy**

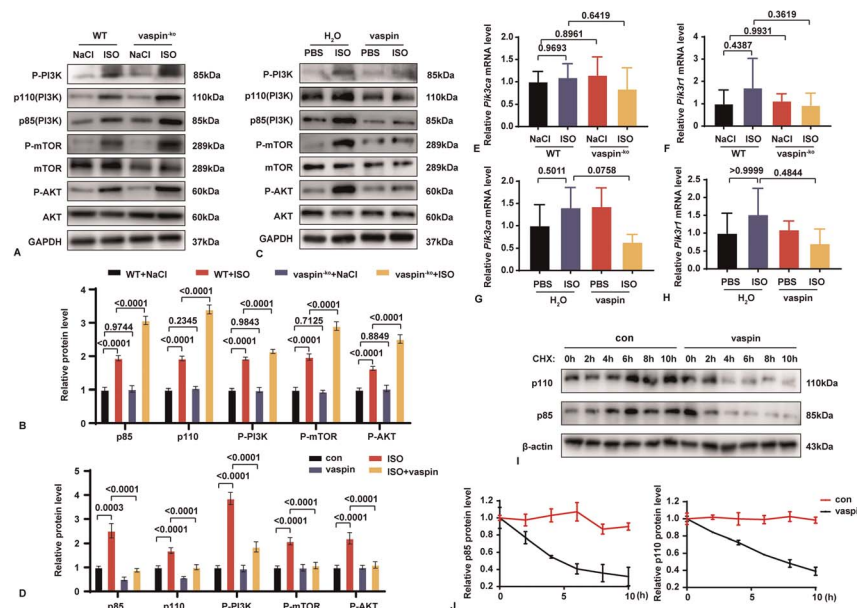
We evaluated the effect of vaspin on autophagy in the ISO-induced cardiac hypertrophy model. Western blot analysis indicated

that LC3B levels were significantly lower in the heart tissue of ISO-injected vaspin-ko mice compared with those in ISO-injected WT mice, whereas P62 levels were higher (Figs. 3A, B). Immunohistochemical staining and transmission electron microscope examination also showed that LC3B levels, autophagy vacuoles, and autolysosomes were significantly lower in cardiomyocytes from ISO-injected vaspin-ko mice than in those from ISO-injected WT mice (Figs. 3C, D).

We further examined the role of vaspin in the activation of autophagy and human vaspin fusion protein overexpression in NRVMs following ISO treatment. Similar to the changes observed in vivo, vaspin significantly increased LC3-II/LC3-I levels in NRVMs treated with ISO, whereas the P62 level was lower (Figs. 3E, F). Confocal immunofluorescence microscopy analyses also showed that recombinant human vaspin pretreatment increased the levels of LC3B in NRVMs treated with ISO as well as the numbers of both autophagosomes and autolysosomes in ISO-treated H9c2 cells (Figs. 3G, H). Collectively, these data suggest that vaspin exerts both promotive and reparative effects on autophagy in pathological cardiac hypertrophy.

**Promoting autophagy rescues ISO-induced cardiac hypertrophy in vaspin-ko mice and inhibiting autophagy blocked the cardiomyocytes' vaspin protection**

We tested our hypothesis that autophagy plays a protective role in myocardial senescence and hypertrophy remodeling in vaspin-ko mice. Rescue experiments using rapamycin administration showed that increased autophagic activity significantly protected vaspin-ko mice from hypertrophy, cardiac function, and myocardial fibrosis (Figs. 4A–O), as shown by wheat germ agglutinin staining, HW/BW, HW/TL, left ventricular mass, left ventricular end-diastolic posterior wall thickness, left ventricular end-diastolic diameter, left ventricular end-systolic diameter, cardiac ejection fraction



**Figure 6.** The protective effect of vaspin on cardiac hypertrophy and senescence depends on the PI3K-AKT-mTOR pathway-dependent activation of autophagy. (A) and (B) Representative Western blot bands and quantification in wild-type (WT) and vaspin-ko mice injected with NaCl or isoproterenol (ISO). (C) and (D) Representative Western blot bands and quantification in neonatal rat ventricular myocytes (NRVMs). (E–H) The mRNA expression levels of *Pik3ca* and *Pik3r1* were assessed by real-time PCR. (I–J) Protein expression of p85 and p110 in NRVMs treated with cycloheximide (100  $\mu$ g/mL) and vaspin detected by Western blot. Means  $\pm$  SDs are shown, with n = 6.  $P < 0.05$  was considered significant. PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

and fractional shortening, picrosirius red staining, and Masson staining. In addition, rapamycin-treated vaspin-ko mice showed reduced P16 levels detected by immunohistochemical staining (Figs. 5A, B). In addition, senescence was assessed by immunoblotting, and rapamycin administration decreased the P16, P21, and P53 levels in the heart tissue of vaspin-ko mice treated with ISO (Figs. 5C, D).

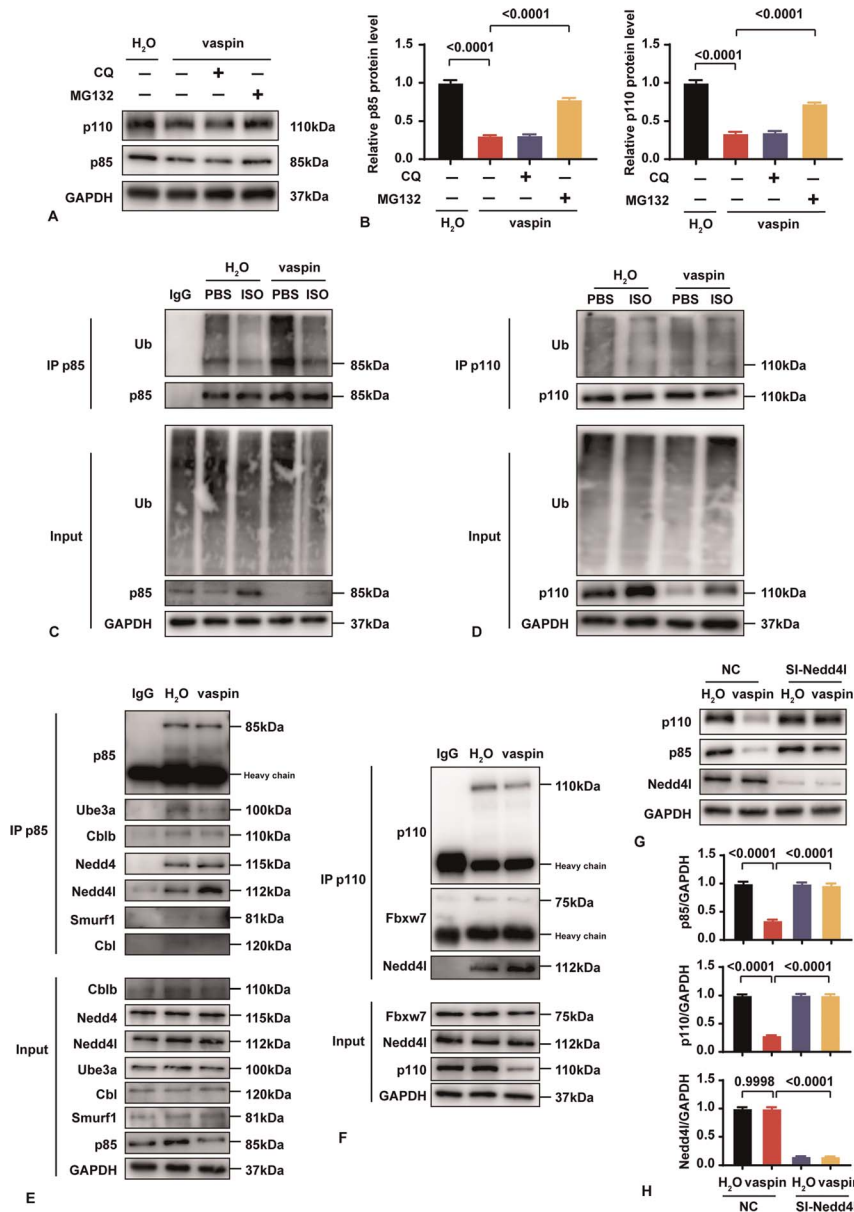
We investigated the role of vaspin-mediated autophagy in cardiomyocyte senescence and hypertrophy. Immunoblotting showed that vaspin reversed the cardiomyocyte senescence phenotype in ISO-stimulated NRVMs, which was blocked by CQ treatment. Importantly, vaspin decreased the expression levels of senescence markers compared with the vehicle group in NRVMs, whereas vaspin lacked this ability in CQ-pretreated NRVMs (Figs. 5E, F).

Immunofluorescence staining for P16 presented similar results (Figs. 5G, H). The inhibition of autophagy with CQ promoted an ISO-induced increase in cardiomyocyte size and blocked the cardiomyocyte-protective effect of vaspin (Figs. 5G and I).

These findings confirmed that autophagy plays a role in the protective effects of vaspin against cardiac hypertrophy.

**The protective effect of vaspin on cardiac hypertrophy and senescence depends on the PI3K-AKT-mTOR pathway-dependent activation of autophagy**

To identify the pathways potentially mediated by vaspin, we examined autophagy-related pathways. The PI3K/AKT/mTOR pathway



**Figure 7.** Vaspin promotes ubiquitination-dependent protein degradation of p85 and p110. (A) and (B) Representative Western blot bands and quantification of p85 and p110 protein expression in neonatal rat ventricular myocytes (NRVMs). (C) and (D) Coimmunoprecipitation (Co-IP) performed to show ubiquitination of p85 and p110 proteins in NRVMs. (E) and (F) Co-IP using an anti-p85 and anti-p110 antibody to detect the interaction between PI3K subunits (p85 and p110 protein) and E3 ubiquitin ligase. (G–H) Representative Western blot bands and quantification of p85, p110, and NEDD4L protein expression in NRVMs. Means ± SDs are shown, with n = 6. P < 0.05 was considered significant. ISO, isoproterenol; PBS, phosphate-buffered saline.

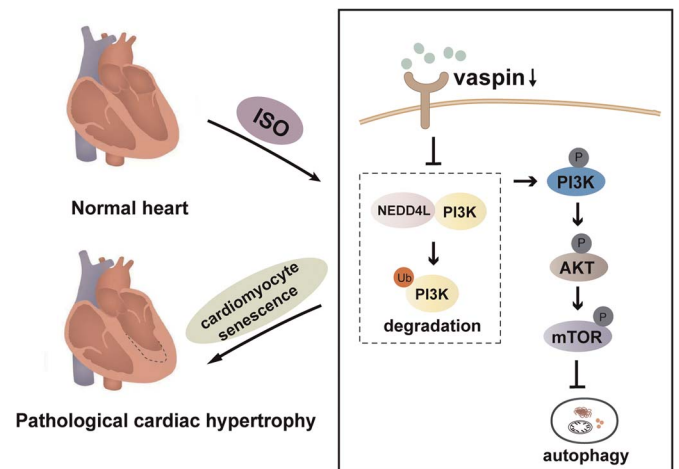
plays an important role in regulating the initiation of autophagy. Our data showed that ISO induced an increase in the expression of PI3K (p85 and p110 subunits), P-PI3K, P-AKT, and P-mTOR in the heart tissue of WT mice, and this phenomenon was more pronounced in vaspin-ko mice (Figs. 6A, B). Likewise, after ISO treatment, NRVMs displayed PI3K/AKT/mTOR pathway activation compared with the control group; however, this pathway was downregulated after treatment with human vaspin fusion protein (Figs. 6C, D). After observing the changes in the protein levels of the PI3K subunits, we tested whether vaspin affects their transcription levels. Interestingly, the abundance of *Pik3ca* and *Pik3r1* mRNAs did not change between the groups (Figs. 6E–H). These results prompted us to investigate the regulation of the PI3K subunit protein degradation by vaspin. To confirm that vaspin regulates PI3K subunit degradation, we first treated NRVMs with cycloheximide and vaspin at the indicated intervals and found that PI3K subunits were rapidly degraded in NRVMs treated with vaspin (Figs. 6I, J). In addition, pretreatment with the proteasome inhibitor MG132, but not the autophagy inhibitor CQ, efficiently protected p85 and p110 from vaspin-mediated degradation (Figs. 7A, B), implying that vaspin downregulates p85 and p110 proteins by promoting ubiquitination-dependent proteasome degradation. Moreover, p85 and p110 were ubiquitinated in the presence of vaspin (Figs. 7C, D). The potential E3 ubiquitin ligases of the p85 and p110 proteins were predicted using UbiBrowser (<http://ubibrowser.ncpsb.org>) and confirmed using a coimmunoprecipitation test. We found that endogenous PI3K subunits (p85 and p110 proteins) and NEDD4L formed a complex; notably, this interaction was enhanced after vaspin stimulation (Figs. 7E, F). NEDD4L is a specific E3 ligase for p85 and p110. NEDD4L knockdown attenuated the vaspin-mediated degradation of p85 and p110 (Figs. 7G, H).

Taken together, these results show that vaspin promotes the ubiquitination-dependent protein degradation of p85 and p110 by enhancing the PI3K subunit (p85 and p110 proteins) and NEDD4L interaction, which may be an important mechanism by which vaspin regulates the PI3K-AKT-mTOR signaling pathway.

## Discussion

In this study, we demonstrated that vaspin deficiency increased cardiac hypertrophy, fibrosis, and cardiomyocyte senescence in an ISO-induced pathological cardiac hypertrophy mouse model. Next, we demonstrated that exogenous vaspin supplementation prevented ISO-induced cardiomyocyte hypertrophy and senescence. Previous studies have indicated that autophagy is involved in the pathogenesis of cardiac hypertrophy and senescence. NEDD4L-PI3K-AKT-mTOR pathway-dependent autophagy activation is involved in the protective effects of vaspin against cardiac hypertrophy. Collectively, these data suggest that vaspin alleviates pathological cardiac hypertrophy by regulating autophagy-dependent myocardial senescence (Fig. 8). This study identified the translational potential of targeting the vaspin, NEDD4L, and PI3K-AKT-mTOR pathways to prevent pathological cardiac hypertrophy.

Although an increasing number of clinical trials have found that serum vaspin levels are closely related to diabetes, obesity, rheumatoid arthritis, polycystic ovary syndrome, and cardiovascular disease,<sup>[22,23]</sup> there is no significant evidence of a relationship between vaspin and cardiac hypertrophy. A previous study reported that male mouse cardiomyocytes showed a hypertrophic response to a high-fat diet, accompanied by increased expression of vaspin in the pericardial adipose tissue.<sup>[24]</sup> High-fat or high-sugar stimulation can increase vaspin mRNA and protein expression in brown adipose tissue.<sup>[25]</sup> However, lower levels of circulating vaspin positively correlate with the prevalence and severity of coronary artery disease.<sup>[8]</sup>



**Figure 8.** The proposed molecular mechanisms of the protective effect of vaspin on pathological cardiac hypertrophy. Vaspin promotes the ubiquitination-dependent protein degradation of p85 and p110 by enhancing the PI3K subunit (p85 and p110 proteins) and NEDD4L interaction, thereby activating PI3K-AKT-mTOR pathway-mediated autophagy and attenuating cardiomyocytes senescence and pathological myocardial hypertrophy. ISO, isoproterenol.

Therefore, we hypothesized that vaspin levels are determined by the effect of different stimuli on vaspin expression in adipose tissue and that during the development of pathological myocardial hypertrophy vaspin expression levels in adipose tissue are altered, which in turn affects cardiomyocyte hypertrophy and senescence.

Vaspin plays a role in complex signaling networks of cardiovascular diseases including autophagy abnormalities, apoptosis, inflammation, and endoplasmic reticulum stress in macrophages and endothelial, smooth muscle, and myocardial cells. Vaspin exerts its antiatherosclerotic effects by reducing endothelial apoptosis<sup>[26]</sup> and activating the insulin signaling pathway (PI3K/AKT)-dependent endothelial nitric oxide synthase activity.<sup>[27]</sup> Our previous findings suggest that vaspin protects against myocardial IRI by upregulating AMPK-mTOR-dependent autophagic flux and restoring lysosomal function.<sup>[11]</sup> Another study demonstrated that vaspin could improve autophagy levels in rats with diabetic cardiomyopathy and alleviate the degree of myocardial apoptosis and fibrosis.<sup>[10]</sup> Therefore, improvements in vaspin-associated autophagy may contribute to cardiac damage repair. Furthermore, in the present study, vaspin alleviated pathological cardiomyocyte hypertrophy by upregulating NEDD4L-PI3K-AKT-mTOR-dependent autophagy, suggesting that vaspin may regulate autophagy through various pathways to protect the heart under different stimuli.

It has been shown that senescence progression might be associated with multiple physiological and pathological processes of cardiovascular diseases. Myocardial senescence is a critical risk factor for cardiac hypertrophy. Recent evidence suggests that the cellular senescence phenotype participates in heart remodeling caused by diabetes, obesity, chemotherapeutic drugs, and dilated cardiomyopathy.<sup>[28–30]</sup> The central finding of this study was that senescent cells can potentially be eliminated for the treatment of aging-related diseases.<sup>[31]</sup> Moreover, non-aging-related pathological cardiac hypertrophy caused by angiotensin II and ISO can be inhibited by antiaging factors and compounds such as resveratrol, metformin, and senescence marker protein 30.<sup>[32,33]</sup> Therefore, these research results have opened the door for the development of agents and strategies to target senescent cells, specifically for the prevention and treatment of heart failure. Our study found that the senescence phenotype

of cardiomyocytes was further aggravated in the hearts of vaspin-ko mice, and exogenous administration of vaspin reduced the senescence phenotype of cardiomyocytes. These data further verify the pathogenic role of myocardial senescence in cardiac hypertrophy and indicate that exogenous vaspin supplementation may play a protective role as a critical senescence regulator in future clinical trials of cardiac hypertrophy and heart failure.

### Limitations

Currently, only mice with vaspin gene deletion and mice with over-expression gene lacking vaspin can only be verified by exogenous supplementation of human vaspin protein in vitro. The specific molecular mechanism of vaspin regulating PI3K-AKT-mTOR pathway needs to be further explored. The effects of vaspin on fibroblast proliferation in ISO-induced pathological myocardial hypertrophy models need to be further examined.

### Conclusion

Vaspin alleviated pathological cardiac hypertrophy by regulating autophagy-dependent myocardial senescence. These findings suggest that vaspin, NEDD4L, and PI3K-AKT-mTOR pathways may act as possible therapeutic targets for pathological cardiac hypertrophy.

### Conflict of interest statement

The authors declare no conflict of interest.

### Author contributions

Yu H, Rui H, and Xue L participated in research design. Yu H, Rui H, and Zou D participated in the writing of the article. Yu H, Rui H, Chi K, and Xu P participated in the performance of the research. Yu H, Rui H, Liu L, and Song X contributed new reagents or analytic tools. Yu H, Rui H, Wu X, and Wang J participated in data analysis.

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### Ethical approval of studies and informed consent

This study was approved by the Ethics Committee of Qilu Hospital of Shandong University (KYLL-2022(ZM)-507, February 21, 2022).

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