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Suppressed nuclear envelope proteins activate autophagy of vascular smooth muscle cells during cyclic stretch application

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ABSTRACT

Dysfunctions of vascular smooth muscle cells (VSMCs) play crucial roles in vascular remodeling in hypertension, which correlates with pathologically elevated cyclic stretch due to increased arterial pressure. Recent researches reported that autophagy, a life-sustaining process, was increased in hypertension. However, the mechanobiological mechanism of VSMC autophagy and its potential roles in vascular remodeling are still unclear. Using renal hypertensive rats *in vivo* and FX5000 stretch application Unit *in vitro*, the autophagy of VSMCs was detected. The results showed that LC3II remarkably enhanced in hypertensive rats and 15% cyclic stretch (mimic the pathologically increased mechanical stretch in hypertension), and the activity of mammalian target of rapamycin (mTOR) was suppressed in 15% cyclic stretch. Administration of autophagy inhibitors, bafilomycin A1 and chloroquine, repressed VSMC proliferation efficiently, but did not affect the degradation of two important nuclear envelope (NE) proteins, lamin A/C and emerin. Using RNA interference to decline the expression of lamin A/C and emerin, respectively, we discovered that autophagy was upregulated under both static and 5% cyclic stretch conditions, accompanying with the decreased mTOR activity. During 15% cyclic stretch application, mTOR inhibition was responsible for autophagy elevation. Chloroquine administration *in vivo* inhibited the expression of PCNA (marker of proliferation) of abdominal aorta in hypertensive rats. Altogether, these results demonstrated that pathological cyclic stretch suppresses the expression of lamin A/C and emerin which subsequently represses mTOR pathway so as to induce autophagy activation. Blocking autophagic flux may be a practicable way to relieve the pathological vascular remodeling in hypertensive.

1. Introduction

Hypertension is a kind of widespread cardiovascular disease, which causes dysfunctions of vascular cells and destruction of vascular homeostasis [1]. During hypertension, elevated blood pressure results in pathologically increased cyclic stretch which was resulted from the rhythmical distention and relaxation of arterial wall following the pulsatile blood pressure [2]. According to clinical ultrasound data, large artery dilates as much as 15% in hypertension, while about 5% in normotension [3,4]. Recently, chronic elevated cyclic stretch in hypertension has been widely reported to act as an important factor of dysfunction of vascular smooth muscle cells (VSMCs) [5,6]. However, the regulation of VSMCs by cyclic stretch is not fully elucidated.

Macroautophagy (hereafter referred to as autophagy) is a major specific or unspecific degradation process in eukaryote [7]. From yeast to mammals, the autophagy process also correlates with a wide range of physiological events, such as embryonic development, metabolic dysfunctions, stress response, and disease progress [8,9]. Accordingly, autophagy has become a fascination field of biological and medical sciences, including the pathological vascular remodeling during hypertension. According to recent report, the mean and diastolic arterial pressures induced by angiotensin II are all significantly decreased in murine with smooth muscle cell-specific knockout of Atg7 [10]. Furthermore, in spontaneously hypertensive rats (SHR), autophagy is enhanced in the hearts [11]. By the administration of chloroquine, a kind of autophagy inhibitor, the mean pulmonary arterial pressure and the

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right ventricular pressure are all reduced in the rat model, which suggests that the development of hypoxic pulmonary hypertension is successfully prevented [12]. Moreover, the content of autophagy related proteins, including LC3BII and ATG5, is increased in lung from pulmonary arterial hypertension (PAH), and the proliferation of pulmonary artery smooth muscle cells (PASMCs) is inhibited by chloroquine administration *in vivo* [13]. Although researches have revealed the increased autophagy in cardiovascular system in hypertension, the role and mechanism of autophagy in hypertension are still elusive. Importantly, the mechanobiological roles of cyclic stretch in autophagy and its potential mechanism in vascular remodeling in hypertension, have not been thoroughly investigated.

Recent research reported that in primary human IMR90 cells, the autophagy protein LC3 can directly integrates with lamin B1, a nuclear envelope (NE) protein, and then mediates lamin B1 degradation in the manner of nucleus-to-cytoplasm transport which fuses with the lysosome at last [14]. Interestingly, our previous study revealed that lamin A/C and emerin, another two kinds of important NE proteins just like lamin B1, are mechano-responsive and contribute to the VSMC proliferation induced by pathologically increased cyclic stretch in hypertension [15].

Therefore, we detected the relationship between autophagy and nuclear envelope proteins. Using *in vivo* renal hypertensive rat model and *in vitro* cyclic stretch application system, the autophagy of VSMCs was firstly detected. Moreover, the regulation relationship between autophagy and NE proteins, *i.e.*, whether autophagy participated in the degradation of NE proteins, or NE proteins regulated the autophagy, in hypertension and cyclic stretch application, was studied.

2. Methods

2.1. Hypertensive rat model

The animal experiments were in line with the recommendations of the Animal Management Rules of China (Documentation 55, 2001, Ministry of Health, China), and the experiment was approved by the Animal Research Committee of Shanghai Jiao Tong University.

Hypertensive rat model was generated by constriction the abdominal aorta [16]. The body weight of Male Sprague–Dawley rats were 200 ± 20 g, which were randomly divided into sham-operated and abdominal aorta-constriction groups. For abdominal aortic coarctation, rats were anesthetized with isoflurane inhalation, and then the enterocoelia was scissored for separating the abdominal aorta. About 5 mm above the bifurcation to right kidney artery, the aorta and the paralleled 21-gauge needle were tied together by 3–0 surgical suture. Next the needle was retrieved immediately to create the aortic constriction with a diameter of about 0.9 mm. For sham-operated groups, the same procedure was operated but without constriction. Blood pressure of two groups was measured by introducing the catheter into the carotid common arteries [16].

2.2. VSMCs isolation and culture

Primary rat VSMCs were obtained from the media of thoracic aorta [17]. Through intraperitoneal injections of sodium pentobarbital (75 mg/kg), male Sprague–Dawley rats were narcotized, and then thoracic aorta was isolated and taken out. In super clean bench, the thoracic aorta was plunged in Dulbecco's modified eagle medium (DMEM) (Gibco) with 100 U/mL penicillin and 100 μg/mL streptomycin. The adventitia was stripped and endothelium were digested by collagenase, leaving the medial portions, which were moved into 25 cm^2 culture vessel and minced into small pieces about 1 mm^2. The explants were immersed in DMEM containing 10% heat-inactivated fetal calf serum (FCS) (Gibco) and were cultured at 37 °C in a humidified incubator (95% air and 5% CO_2). VSMCs were identified by immunofluorescence targeting to smooth muscle-specific α-actin. VSMCs monolayers were used at passages 4–7 for experiments.

2.3. Application of mechanical stretch

VSMCs were plated on flexible silicone-bottomed plates (Flexercell International) coated by type I collagen at a density of 2×10^5 cells per well. After 24 h attachment, the cells were synchronized by DMEM for another 24 h and then applied to cyclic stretch produced by FX-5000T Strain Unit (Flexcell International) [2]. The mechanical stretch parameters were as follows: the 5% stretch magnitude to mimic normotension mechanical cyclic stretch [18], and the 15% stretch magnitude to mimic hypertensive mechanical cyclic stretch [4,19], at a consistent frequency of 1.0 Hz [18], and durations of 6 h, 12 h, 18 h and 24 h, respectively.

2.4. Cell proliferation

BrdU ELISA kit (Roche Diagnostics) was used to analyze cell proliferation *in vitro*. For drug treating study, cell was planted in 96 well plate at a density of 1×10^4 cells, and the next operation confirmed to the protocol. Six hours before the end of drug treating, BrdU was diluted into 1:1000 in the culture medium. As to the cell in Flexcell plate, BrdU was also diluted into 1:1000 in the culture medium in Flexcell plates 6 h before the experiment end. After the application of cyclic stretch, cell was digested from Flexcell plate and timely planted at a density of 1×10^4 per well in 96-well plates. After the cell adhering to the well, the operation procedure of ELISA was followed the manufacturer's instructions. The absorbance was measured in an ELISA plate reader (Bio-Rad 680).

2.5. Immunofluorescence

For the *in vivo* experiments, artery was isolated and immediately plunged into 4% paraformaldehyde overnight, dehydrated by 30% sucrose for 24 h, sectioned into 6 μm sections, and then stored in −80 °C. For cell in Flexcell plate, originally LC3 staining of VSMCs on Flexcell silicone was not ideal, so chloroquine (at a final concentration of 25 μM), a kind of autophagic flux repressor [20], was added into the medium, so the effect of cyclic stretch on autophagosome formation in VSMCs was demonstrated. After loading cyclic stretch ended, the silicone-bottomed was clipped into about 1 cm^2 and immersed into 4% paraformaldehyde for 15 min.

Next produce for artery and cell was the same, which were subsequently permeabilized with 0.3% Triton X-100 for 30 min, blocked by 10% goat serum for 1 h at room temperature, incubated with the primary antibody overnight at 4 °C, and next day incubated with a fluorescent secondary antibody and DAPI. The primary antibody was LC3 A/B (1:100) (Cell Signaling Technology). After mounting with cover glass, the specimen was visualized under a fluorescence microscope (Olympus DP75).

2.6. RNA interference

VSMCs were planted in 6-well plates or Flexercell plates (Flexcell International) at a density of 2×10^5 cells per well, and cultured in 10% FCS/DMEM for 24 h. The transfection medium was mixed followed the Lipofectamine 2000 (Invitrogen) manufacturer's instructions. Firstly, 5 μL small interfering RNA (siRNA) fragment (100 nmol) and 5 μL Lipofectamine 2000 were diluted in 200 μL Opti-MEM (Invitrogen) respectively, which were mixed together for 15 min at room temperature. Then the mixture was added into the well containing 400 μL DMEM, and then cultured at 37 °C in a humidified incubator. After 8 h, the DMEM with 2% FCS was added into the transfection mixture for a final volume of 2 mL per well. Nonsilencing siRNA was used as a negative control.

2.7. RT-qPCR assay

RT-qPCR assays were performed to detect the mRNA expressions of *LMNA* (gene name of lamin A/C), *EMD* (gene name of emerin), *LC3A*, *LC3B* and *GAPDH*. Total RNA of VSMCs was extracted using TRIzol reagent (Invitrogen), and then reversed transcribed into complementary DNA with the M-MLV RT system using random hexame primer (Thermo Scientific). TB Green® Fast qPCR Mix (TaKaRa) was used for qPCR amplification in Steponeplus instrument (Applied Biosystems). Relative expression of *LMNA*, *EMD*, *LC3A* and *LC3B* was normalized against *GAPDH* and calculated by the $2^{-\Delta\Delta C_T}$ method.

Specific primers for rat *LMNA* were: sense 5′-AGAG GTGT CCGG CATC AAGG-3′, and anti-sense 5′-CAGC CAAC AAAT CCCC CTCC-3′. Specific primers for rat *EMD* were: sense 5′-GGAG GACG CCTT ACTT TACC A-3′, and anti-sense 5′-CCAT AGAT GGGG CGTT CCC-3′. Specific primers for rat *LC3A* were: sense 5′-GCCT GTCC TGGA TAAG ACCA A-3′, and anti-sense 5′-ACCA TGCT GTGC TGGT TCAC-3′. Specific primers for rat *LC3B* were: sense 5′-CGCA CCTT CGAA CAAA GAG-3′, and anti-sense 5′-CTCA CCCT TGTA TCGT TCTA TTAT CA-3′. Specific primers for rat *GAPDH* were: sense 5′-TGGG AAGC TGGT CATC AAC-3′, and anti-sense 5′-GCAT CACC CCAT TTGA TGTT-3′.

2.8. Western blot analysis

The artery was grinded into powder in liquid nitrogen, mixed with RIPA lysis buffer (Beyotime), and vortexed for 10 min. Cells in plate were washed twice with PBS on ice, scraped into RIPA lysis buffer (Beyotime), and quiesced on ice for 10 min. Then the lysates were collected in tubes, centrifuged at 12,000 rpm for 3 min, and isolated the supernatants to boil for 5 min. The protein concentration of cell lysates was quantified using the Enhanced BCA Protein Assay Kit (Beyotime). Total Protein (30 μg) were electrophoretically separated by SDS-PAGE and then transferred to PVDF membranes (Millipore). The membranes were blocked for 1 h at room temperature with Tris-buffered saline Tween-20 buffer containing 5% nonfat milk, incubated overnight at 4 °C with primary antibody, and next day incubated for 1 h at room temperature with relevant secondary antibody (Jackson Immunoresearch). Lastly, the strips were displayed by the mixture of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) according to the manufacturer's instructions (KPL). The quantity assay was performed by using Quantity One imaging software (Bio-Rad).

The following primary antibodies were used in the present study: lamin A/C (1:500) (Abcam), lamin A (1:500) (Santa Cruz), emerin (1:500) (Abcam), LC3A/B (1:500) (Cell Signaling Technology), Atg5 (1:500) (Cell Signaling Technology), Atg7 (1:500) (Cell Signaling Technology), Atg12 (1:500) (Cell Signaling Technology), Atg3 (1:500) (Cell Signaling Technology), p-mTOR (1:500) (Cell Signaling Technology), mTOR (1:500) (Cell Signaling Technology), p-LKB1 (1:500) (Cell Signaling Technology), LKB1 (1:500) (Cell Signaling Technology), p62 (1:500) (Cell Signaling Technology) and GAPDH (1:1000) (Proteintech).

2.9. Drugs

There were three drugs in this experiment: bafilomycin A1 (Santa Cruz, a classic inhibitor of vacuolar-type H^+ ATPase), chloroquine (Sigma-Aldrich, a kind of lysosomal inhibitor), and rapamycin (Sigma-Aldrich, the first-generation mTOR inhibitor). After VSMCs seeded overnight and synchronized for 24 h, the drugs were added to the medium, respectively, with each suitable concentration according to the concentration gradient assay. For stretch application experiment, the drugs were added to medium 30 min before application of cyclic stretch. For rapamycin, the drug was directly added into the medium after VSMCs were seeded for 24 h.

The same volume of solvent was used as control. Bafilomycin A1 and rapamycin were dissolved in DMSO, and chloronquine was dissolved in H_2O.

Chloroquine was also applied to block the autophagic flux *in vivo* [13]. After abdominal aortic coarctation, animals received daily intraperitoneal injections of chloroquine (50 mg/kg), or vehicle, for a further 10 days.

2.10. Statistical analysis

Each experiment was performed at least in triplicate, and all values were displayed as mean ± SD. Unpaired student's *t*-test was applied to analyze the difference between rats groups. The normalization was used to analyze the *in vitro* data, and paired student's *t*-test was performed to compare the results between different between control and treatment groups. The gradient experiments of drug concentration were analyzed by one-way ANOVA. Two-way ANOVA test was performed to analyze the autophagy change on different time phase during different cyclic stretch application. A value of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Autophagy is upregulated in VSMCs of hypertension rat thoracic aorta

After 1 w of coarctation operation, the mean arterial pressure of hypertension rat was 143.37 ± 14.73 mm Hg, which was significantly increased as compared with 93.63 ± 14.18 mm Hg of the sham-operated control rat (Supplementary Fig. 1). The expression of LC3, an autophagosomal marker protein [21,22], was investigated to detect autophagy change. Western blot showed that in thoracic aorta of hypertensive rats, the ratio of LC3II to GAPDH was markedly increased (Fig. 1A). Immunofluorescence was then used to examine autophagy level by assessing LC3 puncta labeling [23]. As shown in Fig. 1B, LC3 staining was systematically more abundant in media of hypertensive rat thoracic aorta as compared with those in sham-operated control, suggesting the increased autophagosome number in VSMCs of hypertensive rat thoracic aorta.

In mammals, a series of Atg proteins, including Atg3, Atg5, Atg7, Atg12, Atg12-Atg5, are essential for the formation of autophagosomes [24]. Hence, expressions of these Atg proteins were explored. The results revealed that Atg3, Atg5, Atg12 and Atg12-Atg5 were increased in thoracic aorta of hypertensive rats as compared with the sham-treated control, while there was no significant difference in Atg7 (Fig. 1C). In autophagy process, Atg7, which plays the similar role with ubiquitin-activating E1 enzymes, transfers phosphate group from ATP to the ubiquitin-like protein Atg12 and LC3I [25]. Maybe owing to its E1-like enzyme function, the content of Atg7 was not elevated with the increased LC3II.

Since mammalian p62/SQSTM1 is autophagic receptors, the degradation of p62 is available to measure autophagic flux [26]. In thoracic aorta hypertensive rat, the expression of p62 was decreased in comparison with the sham control (Fig. 1C). The result suggested that autophagosomes smoothly transformed to autolysosomes rather than blockade the autophagic flux in hypertension.

Taken together, these results demonstrated that autophagy and autophagic flux were increased in VSMCs of hypertensive thoracic aorta as compared with the sham-treated control.

3.2. Pathological cyclic stretch induces the autophagy of VSMCs in vitro

Compared with 5% cyclic stretch (mimics physiological mechanical stretch during normotension), 15% cyclic stretch (mimics pathologically increased mechanical stretch during hypertension) markedly enhanced the expression of LC3II in VSMCs (Fig. 1D), which had the resemblance change as that in hypertension (Fig. 1A). LC3 staining with immunofluorescence displayed more puncta formation in VSMCs subjected to 15% cyclic stretch than 5% control (Fig. 1E). Altogether, these

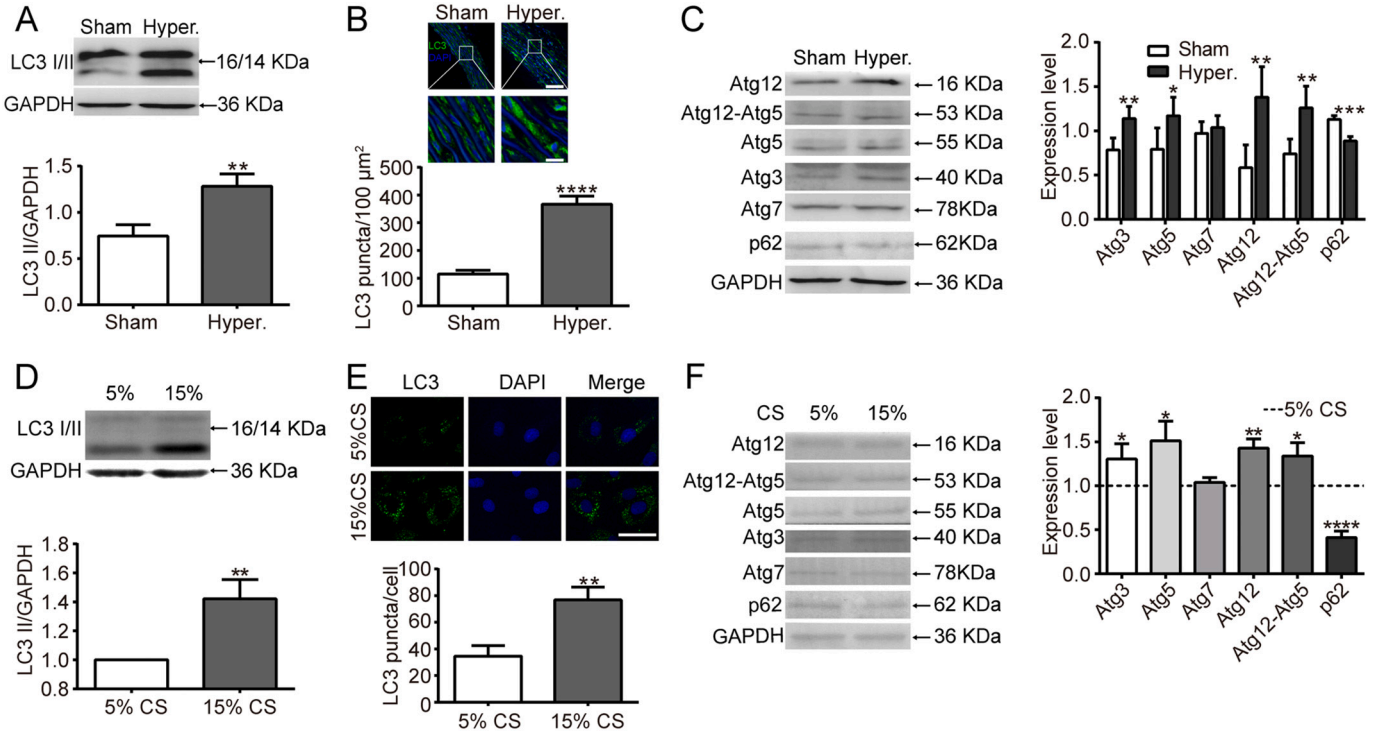


Fig. 1. Autophagy is activated in VSMCs under both *in vivo* hypertension and *in vitro* 15% cyclic stretch application. (A) Western blot analysis showed that the expression of LC3II was dramatically increased in thoracic aorta of the abdominal aorta-coarctation induced hypertensive rats (n = 5). (B) LC3 staining with immunofluorescence analysis showed that the puncta was more abundant in the media of thoracic aorta from hypertensive rat as compared with those in sham-operated control. (green, LC3; blue, DAPI; scale bar = 25 μm in upper image and 5 μm in lower image, respectively) (n = 4). (C) Western blot analyzed the key proteins of the autophagy pathway (Atg3, Atg5, Atg7, Atg12, Atg12-Atg5 and p62) in thoracic aorta of the abdominal aorta-coarctation induced hypertensive rats. Except Atg7 that was nearly constant and p62 was decreased, other autophagy molecules were all elevated (n = 5). (D) Western blot revealed that LC3 in VSMCs subjected to 15% cyclic stretch was dramatically increased as compared with 5% cyclic stretch (n = 5). (E) Immunofluorescence staining of LC3 showed that after 15% cyclic stretch the puncta was more abundant than 5% cyclic stretch (green, LC3; blue, DAPI; Scale bar = 20 μm) (n = 4). (F) Western blot showed the key proteins of the autophagy pathway, including Atg3, Atg5, Atg12, Atg12-Atg5 and p62, were elevated in VSMCs after 15% cyclic stretch application, but Atg7 was hardly changed and p62 was declined (n = 4). For western blot, GAPDH was used for normalization. Data are mean ± SD. *$P < 0.05$ and **$P < 0.01$ *vs.* the respective control.

results revealed that the autophagosome formation largely accelerated in VSMCs subjected to 15% cyclic stretch as compared with 5% cyclic stretch.

The expressions of Atg3, Atg5, Atg7, Atg12 and Atg12-Atg5, the key proteins of the autophagy pathway, were investigated. The protein expressions of Atg3, Atg5, Atg12 and Atg12-Atg5, but not Atg7, were notably raised in VSMCs subjected to 15% cyclic stretch (Fig. 1F), which was similar to the variation in hypertensive rat model (Fig. 1C). Meanwhile, compared with 5% cyclic stretch, the expression of p62 was dramatically declined in VSMCs subjected to 15% cyclic stretch (Fig. 1F).

Further study showed that the autophagy activation was increased by 15% cyclic stretch in a time-dependent way (Supplementary Fig. 2). The expression of LC3II was significantly increased, while the p62 markedly decline from 15% cyclic stretch application for 6 h; while the increase of LC3II and decrease of p62 by 15% cyclic stretch were persistent until 24 h.

Therefore, according to the expression changes of Atg proteins and p62 in VSMCs, the results suggested that the autophagy level and autophagic flux were all evidently enhanced under the application of high cyclic stretch.

3.3. Repressed autophagy decreases VSMC proliferation

Our previous studies demonstrated that pathologically increased cyclic stretch induces VSMC proliferation and contributes to vascular remodeling during hypertension [2,15]. Hence, the role of autophagy in VSMC proliferation was then detected. Bafilomycin A1 and chloroquine, two kinds of autophagy inhibitor, were used to evaluate the effect of autophagy on VSMC proliferation. Initially, the concentration gradient of bafilomycin A1 and chloroquine were explored. Bafilomycin A1 was diluted to final concentration gradient at 0, 1, 2.5, 5 and 10 nM (Supplementary Fig. 3A), while chloroquine at 0, 10, 25, 50 and 100 μM (Supplementary Fig. 3B). Bafilomycin A1 at a concentration of 2.5 nM, nearly full accumulated the expression of LC3II, which had no statistical difference as compared with 5 nM. For chloroquine, a concentration of 25 μM reached the saturated expression of LC3II. Together, bafilomycin A1 at 2.5 nM and chloroquine at 25 μM were selected for further analysis.

Then the proliferation change after autophagy inhibition under the basic static condition was fully explored. After bafilomycin A1 and chloroquine respectively treated 24 h under static condition, the proliferation of VSMCs was markedly inhibited, especially chloroquine (Fig. 2A). In order to identify whether autophagy is involved in the enhanced VSMC proliferation induced by high cyclic stretch, in which autophagy was markedly elevated as compared with physiological mechanical stretch, bafilomycin A1 and chloronquine were added to the medium before 15% cyclic stretch application. The result revealed that the proliferation was suppressed as well (Fig. 2B).

Taken together, these results suggested that the abnormal proliferation under high cyclic stretch could be decreased by autophagy inhibition. VSMC proliferation induced by autophagy which was up-regulated by pathologically increased cyclic stretch in arterial wall of hypertension might be contributed to the pathogenesis of vascular remodeling.

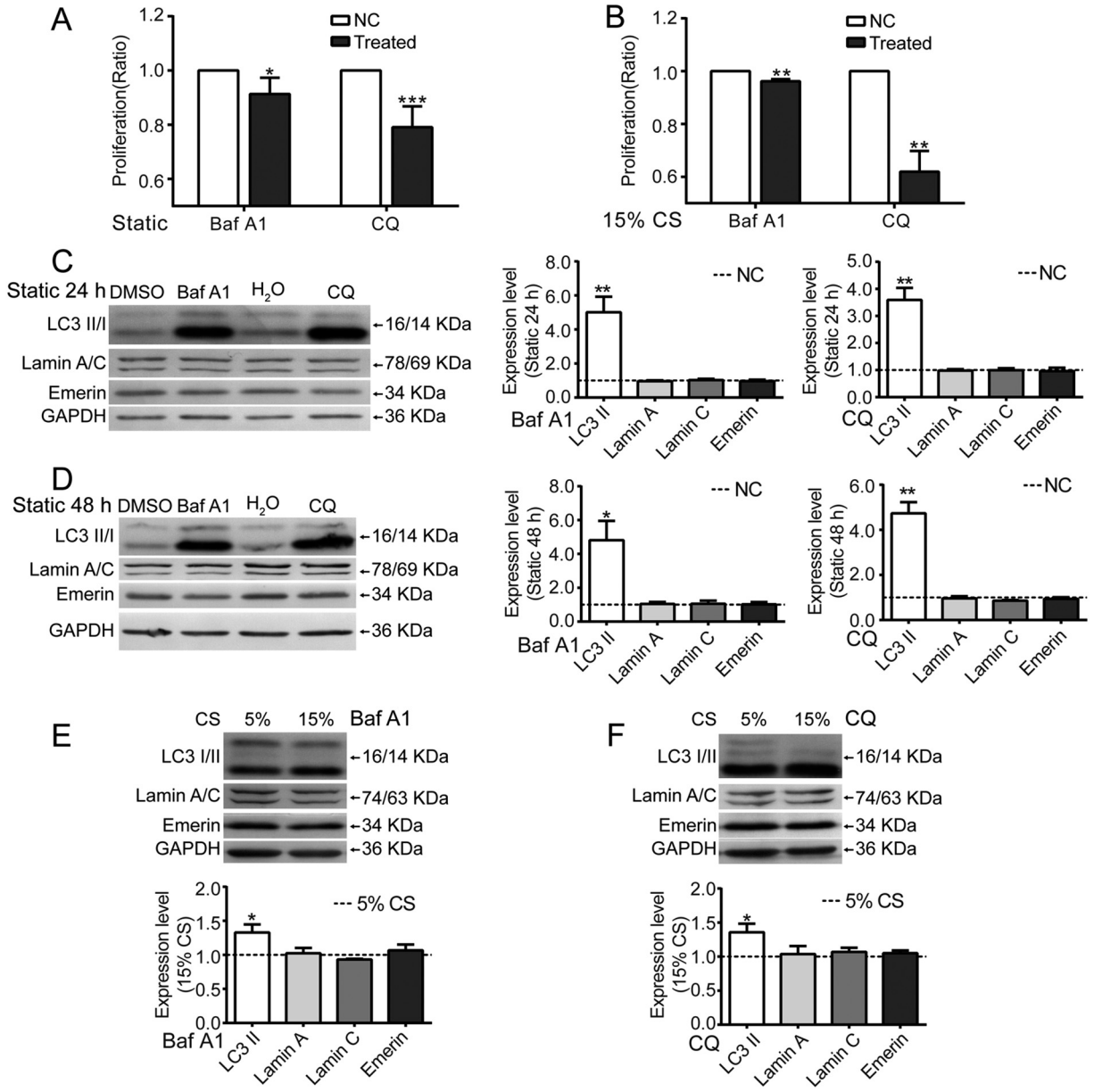


Fig. 2. Autophagy modulates VSMC proliferation, but had no significant effect on the degradation of lamin A/C and emerin under both static and stretch conditions. (A) Bafilomycin A1 (Baf A1, 2.5 nM) and chloronquine (CQ, 25 μM) suppressed VSMC proliferation significantly under static condition (n = 4). (B) Bafilomycin A1 and chloroquine applied during 15% cyclic stretch also decreased VSMC proliferation markedly (n = 4). (C) After bafilomycin A1 and chloronquine incubation respectively for 24 h, there were no obvious change in the expressions of lamin A/C and emerin as compared with respective solvent control (n = 4). (D) Extension bafilomycin A1 and chloroquine treating time to 48 h, the expressions of lamin A/C and emerin still displayed relative stable as compared with respective solvent control (n = 4). (E) After Bafilomycin A1 treatment, 15% cyclic stretch repressed the expressions of lamin A/C and emerin as compared with 5% cyclic stretch (n = 3). (F) After chloronquine treatment, 15% cyclic stretch application repressed the expressions of lamin A/C and emerin as compared with 5% cyclic stretch (n = 3). For Western blots, GAPDH was used for normalization. Values are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* the respective control.

3.4. Autophagy may not degrade NE proteins in VSMCs under both static and stretch conditions

Recent study reported that lamin B1, a kind of NE proteins, is degraded through autophagy *via* direct interaction with LC3 in primary human IMR90 cells [14]. Interestingly, our previous study revealed that the expression of lamin A/C and emerin, another two kinds of NE proteins, are suppressed in VSMCs by pathologically 15% cyclic stretch *in vitro* and hypertension *in vivo* [15]. Hence, we hypothesized that autophagy may mediate the degradation of lamin A/C and emerin in VSMCs during high cyclic stretch application.

After incubation with bafilomycin A1 and chloroquine, respectively, for 24 h in static, the content of LC3II was evidently accumulated, but the expressions of lamin A/C and emerin were nearly not changed as compared with the solvent control (Fig. 2C). In order to confirm the result, we extended the drugs incubation time to 48 h, and the content of lamin A/C and emerin still did not appear evidently change (Fig. 2D). Furthermore, 15% cyclic stretch still repressed the expressions of lamin A/C and emerin in comparison with 5% cyclic stretch under bafilomycin A1 (Fig. 2E) and chloroquine (Fig. 2F) treatment respectively, which revealed that bafilomycin A1 and chloroquine had hardly effect on the content of lamin A/C and emerin during cyclic stretch application.

Taken together, these data show that autophagy may not mediate the degradation of lamin A/C and emerin, and we further detected whether NE proteins participated in autophagy regulation.

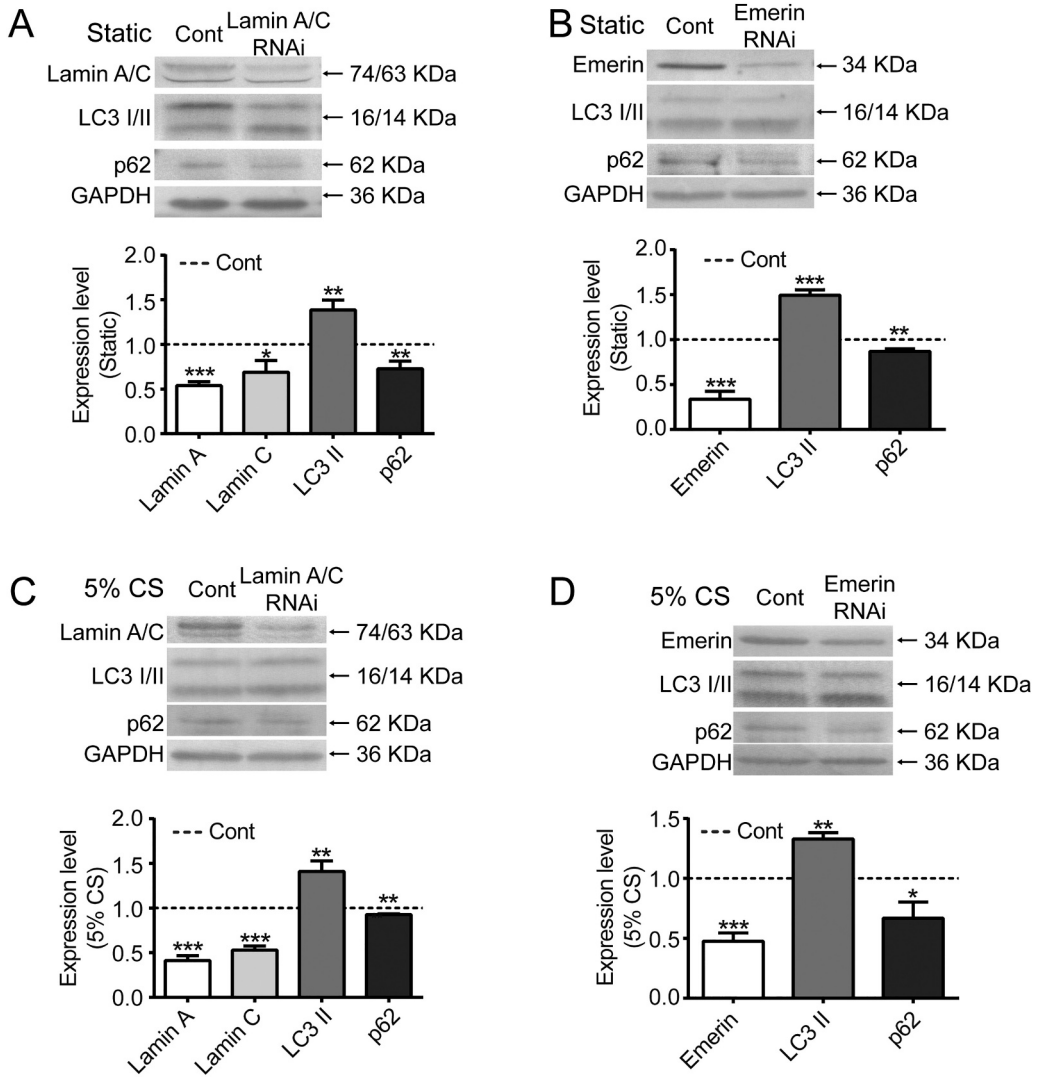


Fig. 3. Suppressed lamin A/C or emerin upregulates VSMC autophagy. (A) Under static condition, transfection of siRNA targeting lamin A/C dramatically decreased the expression of lamin A/C, elevated LC3II, and decreased p62 content; (B) under static condition, transfection of siRNA targeting emerin significantly decreased the expression of emerin, increased LC3II, and declined p62 content; (C) under 5% cyclic stretch application, lamin A/C-targeting siRNA increased the expression of LC3II and decreased p62 in VSMCs; (D) under 5% cyclic stretch, emerin-targeting siRNA increased the expression of LC3II, and decreased p62 in VSMCs. GAPDH was used for normalization. Values are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* the respective control (n = 4).

3.5. Suppressed NE proteins activates autophagy

Firstly, RNA interference was performed to specifically knockdown lamin A/C (Fig. 3A) or emerin (Fig. 3B) under static condition for 48 h. The LC3II expression was increased, meanwhile the p62 content was decreased. The data manifested that the autophagic flux was upregulated in VSMCs after knockdown of lamin A/C or emerin.

To further verify the effects of lamin A/C and emerin on autophagy during cyclic stretch application, specific siRNA was transfected, and then VSMCs were exposed to 5% cyclic stretch in which the expressions of lamin A/C and emerin were relatively high as compared with 15% cyclic stretch. The results displayed that, under 5% cyclic stretch, transfection of lamin A/C (Fig. 3C) or emerin (Fig. 3D) targeting siRNA significantly elevated LC3II expression and inhibited p62 expression as compared with negative control, which were similar as that under static condition.

Together, the decreased lamin A/C and emerin suffered from cyclic stretch may participate in the increased autophagy level, and the possible molecular mechanisms were further analyzed.

3.6. Suppressed NE proteins upregulates autophagy via mTOR

The mammalian target of rapamycin (mTOR) and the AMP-activated protein kinase (AMPK) pathways are master negative and positive regulators of autophagy respectively [7], and LKB1 is the upstream activator of AMPK [27]. Hence, we evaluated the effects of cyclic stretch on the activations of mTOR and LKB1. Compared with the 5% cyclic stretch, 15% cyclic stretch significantly declined the phosphorylation of mTOR, and ratio of phospho-mTOR/mTOR was also significantly depressed (Fig. 4A). Meanwhile, the phosphorylation of LKB1 and ratio of phospho-LKB1/LKB1 were decreased as well. Since LKB1/AMPK pathway is positive regulators of autophagy [7], these results revealed that mTOR rather than LKB1 was responsible for the increased autophagy in VSMCs under high cyclic stretch.

Then effects of lamin A/C and emerin on mTOR pathway under static and cyclic stretch application were then explored. The results showed that, compared with negative control, transfection of lamin A/C and emerin targeting siRNA significantly decreased the phosphorylation of mTOR under both static and 5% cyclic stretch conditions (Fig. 4B–E).

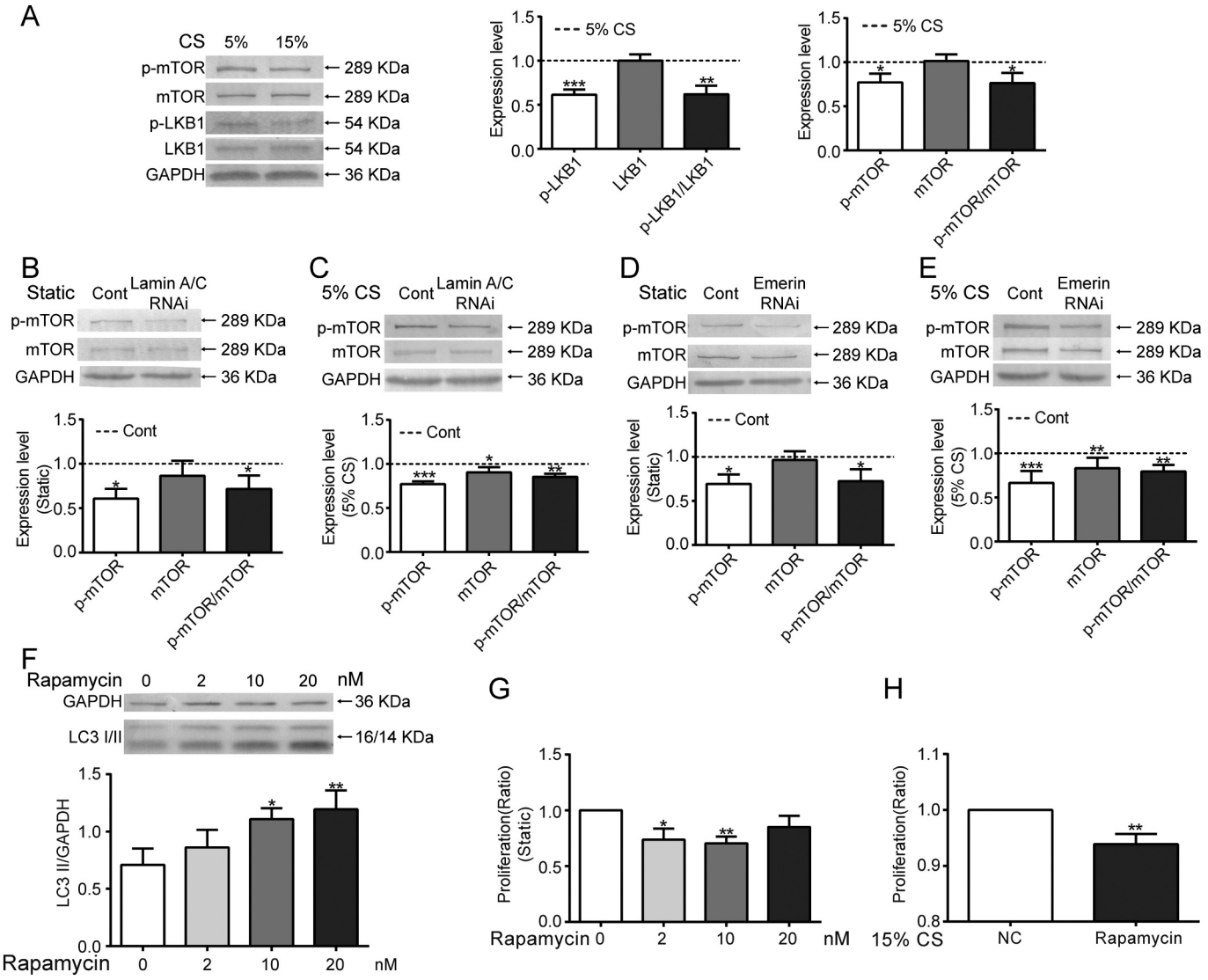


Fig. 4. Suppressed lamin A/C and emerin upregulates autophagy *via* mTOR. (A) Western blot revealed that the phosphorylations of mTOR and LKB1 were all declined by 15% cyclic stretch application as compared with 5% cyclic stretch. (B) Specific siRNA of lamin A/C resulted that the phosphorylation of mTOR was inhibited under static condition. (C) Specific siRNA of lamin A/C resulted that the phosphorylation of mTOR was suppressed during 5% cyclic stretch application. (D) Emerin knockdown resulted that the phosphorylation of mTOR was inhibited under static condition. (E) Emerin knockdown resulted that the phosphorylation of mTOR was declined during 5% cyclic stretch application. (F) Autophagy was elevated by rapamycin (10 and 20 nM respectively) under static condition. (G) The proliferation was significantly suppressed by rapamycin (2 and 10 μM respectively) under static condition. (H) The proliferation was declined by rapamycin (10 μM) during 15% cyclic stretch application. For Western blots, GAPDH was used for normalization. Data are mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* control (n = 4).

Rapamycin, a classic inhibitor of mTOR [28], was applied to further identify the mTOR function. The concentration gradient of rapamycin was explored by detecting the phosphorylation of mTOR. Rapamycin was diluted to final concentration gradient at 0, 2, 10, 20, 50 and 100 nM in the medium (Supplementary Fig. 4), which was incubated for 24 h. As a result, the phosphorylation of mTOR was greatly declined at 2 nM compared with negative control, while the expression of mTOR was nearly stable.

Then the expression of LC3 was detected to identify the autophagy effect of rapamycin. With the rapamycin concentration increasing, the LC3II was accumulated (Fig. 4F). VSMC proliferation was significantly suppressed by rapamycin at 2 and 10 nM in the medium compared with negative control (Fig. 4G). Furthermore, the VSMC proliferation was also significantly decreased by rapamycin (10 nM) under 15% cyclic stretch condition (Fig. 4H).

Therefore, these results demonstrated that the autophagy activation in VSMCs may be caused by the inhibited mTOR pathway which was induced by the repression of lamin A/C and emerin during high cyclic stretch.

3.7. VSMC proliferation is declined after repressing autophagic flux during hypertension

In order to explore the effect of autophagy in hypertension *in vivo*, chloroquine was administered at 50 mg/kg daily for 10 days after rat abdominal aortic coarctation. Chloroquine significantly enhanced the expression of LC3II and p62, which manifested that the autophagic flux induced by hypertension was blocked by chloroquine (Fig. 5A).

The effect of chloroquine on the proliferation of VSMCs was further evaluated. The expression of the proliferation marker PCNA in thoracic aortic samples was determined by western blot. Compared with sham-treated rats, the PCNA content was increased in aorta from hypertensive rats, and the ascending trend was reversed by chloroquine treatment (Fig. 5B).

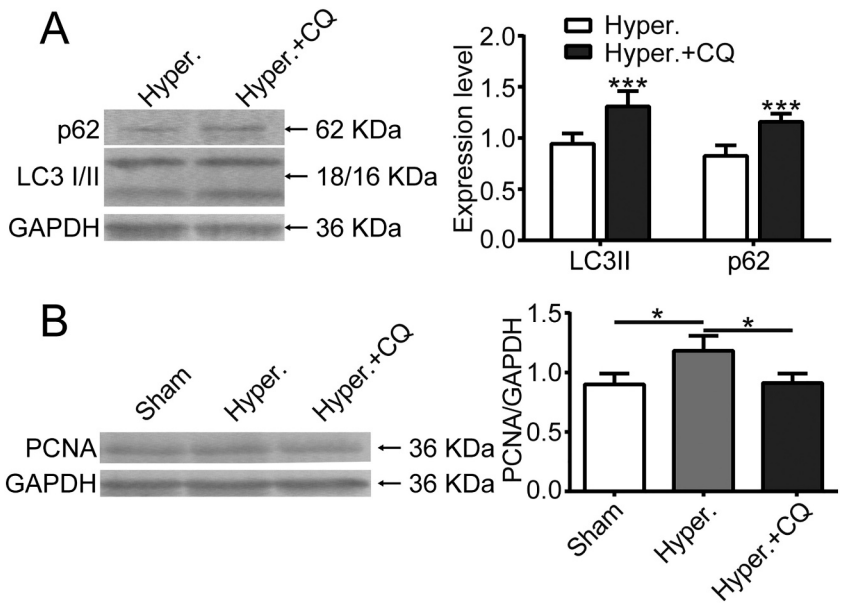


Fig. 5. VSMC proliferation is inhibited by chloronquine during hypertension. (A) Western blot analysis revealed that the expression of the autophagy markers, *i.e.*, LC3 and p62, in VSMCs of hypertensive rat thoracic aorta were elevated by chloroquine (CQ) as compared with saline solvent control (n = 6). (B) Western blot analysis showed that the expression of PCNA in hypertensive rat thoracic aorta was significantly increased as compared with the sham-treated control, and the increase was nearly reversed by chloroquine. GAPDH was used for normalization. Data are mean ± SD. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs.* control (n = 4).

Therefore, these findings suggested that the proliferation of VSMCs elevated in hypertension could be repressed by autophagy inhibition.

4. Discussion

Mechanical forces affect virtually biological processes, and cells have evolved plenty of ways to sense and transform mechanical stimuli into biochemical signals known as mechano-transduction [29]. During recent years, NE proteins are reported as the members of mechano-transduction networks. Using SILAC cross-linking mass spectrometry, Makarov et al. revealed that the coiled coils structure of lamin A rod can slide onto each other, giving rise to contract rod length [30]. So this result may partly explain the mechanical stretch adaption of nuclear lamina. In *Zmpste24*$^{-/-}$ mice that knockout a protease of lamin A maturation, the alveolar nuclear membrane compliance is ideally preserved and alleviated the mechanical load from ventilation abnormally increase in the relevant alveolar of lungs [31]. Moreover, emerin acts as an anchor of NE to inner nuclear membrane and binds to lamin A/C crossing the nucleoplasm lamina [32]. The expression of lamin A/C and emerin has certain cooperativity. After knockdown emerin, the expression of lamin A/C was decreased; conversely, lamin A/C siRNA also decreased the expression of emerin. Emerin overexpression increased the expression of lamin A/C; but lamin A overexpression had no significant effect on the expression of emerin [15]. Our previous study also demonstrated that lamin A and emerin are two important kinds of mechano-responsive molecules, and the expression are markedly decreased during high cyclic stretch application, which implicated in the regulation of elevated proliferation of VSMCs during hypertension [15].

In our previous study, lamin A/C and emerin were decreased during high cyclic stretch application, while the mechanisms involved in this process were not clear. The present study detected whether autophagy degraded the nuclear envelope proteins, but revealed lamin A/C and emerin were not remarkably change after repressing autophagy, suggesting that autophagy did not participate in the degradation of nuclear envelope proteins. Meanwhile, our previous study showed that during 15% cyclic stretch application for 24 h, the expression of lamin A/C and emerin were increased by MG-132(R), a proteasome inhibitor, as compared with non-treated groups [15]. Furthermore, miR-124-3p was also revealed to reduce the protein expression of lamin A/C [33]. Therefore, miRs and ubiquitine-proteasome pathway were at least partially responsible for the nuclear envelope proteins degradation under stretch application.

Lamins bind to numerous nuclear protein formation complexes and are contributed to nuclear stability, chromatin structure, and gene expression [34]. Mice carrying *Lmna*$^{H222P/H222P}$, which successfully reappear the characters of human Emery-Dreifuss muscular dystrophy, display defective autophagy in hearts, and heart function can be improved by enhanced autophagy with temsirolimus [35]. *Trametes robiniophila* Murr. (Huaier), a type of traditional Chinese medicine, displays typical characteristics of autophagosomes and autolysosomes in cytoplasm of human prostate cancer PC3 cells, accompanying with repressed mRNA and protein levels of Lamin B1 [36]. Moreover, C16-ceramide, a bioactive sphingolipid, mediates PRKACA to phosphorylate the LEM domain of emerin, and then the number of autophagosome gradually increases owing to the uniting between phosphor-emerin and LC3B [37]. In cardiac cells, mutant Lamin C (R205W and G489V) causes dominant defective cardiac function and elevates the expression of p62, while the cardiac function can be nearly restored, probably resulting from the elimination of aggregated cytoplasmic protein through overexpression Atg1 [38]. Our present study revealed that suppressed lamin A/C and emerin, under both static and stretch conditions, induce the expression of LC3II and represses the expression of p62. Taken together, NE proteins could regulate or influence the autophagy process.

Autophagy is characterized by the double-membrane vesicles named autophagosome, which next fuses with lysosome formation autolysosome [39]. Using electron microscopy, the autophagosome was firstly observed and then the process was termed autophagy by de Duve in 1963 [25]. In general, autophagy is mainly considered as a cell process of degradation the specific or unspecific substrates. Recently, the regulation field of autophagy has been expanded to a wide range, including stress response, immunity protection, cell senescence and organ lesions [40]. According to recent study, the autophagic degradation of CDKN1B, which is a kind of cell-cycle inhibitor, can regulate the proliferation of T lymphocyte [41]. In our present study, through blockage the autophagic flux, the proliferation of VSMCs was inhibited. Vascular remodeling, which is a predominant result from the proliferation of VSMCs, plays highlight role in cardiovascular diseases, especially hypertension [42].

In hypertension, the stress increase due to an increase of arterial pressure causes VSMC dysfunction and remodeling of extracellular matrix [43]. Currently, autophagy as an emerging regulation manner has attracted more and more attention in hypertension. Autophagy is increased in PAH, but proliferation inhibition and apoptosis increase are all

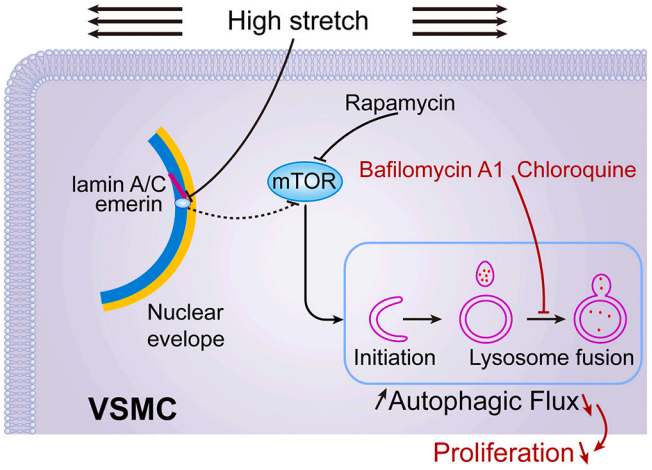


Fig. 6. Schematic illustration depicts the relationships among high stretch, NE proteins, autophagy, and proliferation. Pathologically high cyclic stretch represses the expressions of lamin A/C and emerin, which inhibits the mTOR pathway so as to enhance the autophagic flux. Using rapamycin to inhibit the activation of mTOR, the autophagy level is upregulated. Furthermore, after blocking the autophagic flux by bafilomycin A1 and chloroquine, the proliferation of VSMCs is markedly decreased.

appeared in PASMCs after chloroquine treatment *in vivo* [13]. Similarly, PAH induces an increase of LC3B in rat lung sections, and an elevated autophagic flux is also detected in pulmonary microvascular endothelial cells of PAH patients [44]. Interestingly, studies revealed that under different kinds of hypertension, the autophagy level and definite function maybe diverse. Take SHR for example, autophagic level is surprising declined in resistance vasculature, but the progress of premature vascular aging can be inhibited and even partly reversed through activation of autophagy in artery [45]. In our present study, autophagy is elevated in VSMCs of renal hypertensive rat, and suppressed NE proteins can activate autophagy of VSMCs. We also concerned the transcription level of *LC3*, after lamin A/C or emerin specific RNAi, and the mRNA expressions of *LC3A* and *LC3B* did not accordingly and markedly increased in line with the proteins, neither static nor 5% cyclic stretch condition (Supplementary Fig. 5). The results revealed that lamin A/C and emerin activate the autophagy not at transcriptional level.

The whole and long processes of autophagy, initiated from cell sensing and responding the autophagy incentive signals to fusion and formation of autophagolysosomes where the capture cargo is degraded, needs a series of related protein orchestrate, such as mTOR, AMPK, ATGs, BECN1, PIK3C3, p62 and so on [25]. The mTOR pathway is the master negative while AMPK pathway is the positive regulator of autophagy activation respectively [7], which are two well-studied and classic pathways. It had been reported that autophagy is upregulated by shear stress *via* the mTOR and AMPK pathway [7]; inhibition of mTOR results autophagy enhancement during 40% strain application [46]; long-term intermittent cyclic mechanical tension increases autophagy activity by rapamycin, a specific mTOR inhibitor [47]; whereas agarose compression increases autophagy independent on the mTOR pathway [48]. Besides classic mTOR or AMPK pathway, several novelty pathways have also been reported, *i.e.* blocking Prostaglandin2 production decreases stretch-induced autophagy [49]; AT1R-p38MAPK upregulates autophagy activation [50]; and YAP/TAZ-Armus promotes autophagosome turnover [51]. All these pathways respond to mechanical stresses. In this study, the repressed expressions of lamin A/C and emerin, mediates autophagy upregulation in rat VSMCs. Nuclear envelope proteins in responsive to cyclic stretch modulate autophagy activation *via* mTOR pathway. However, the relationship between mTOR and NE proteins is still elusive. According to recent report, lamin A/C and emerin can all link with chromatin [52,53], which implies that lamin A/C or emerin may reorganize the chromatin and then regulate certain genes which are upstream of mTOR activation. Furthermore, using CHIP-on-chip technology, we had successfully explored a series of transcription factors regulated by lamin A/C or emerin during stretch application, including E2F1, STAT1, STAT4, E2F3 and E2F4, which may regulate the molecules involved in autophagy process [15,54]. The definite mechanism in which NE proteins regulate the autophagy still needs further exploration in the future.

Especially, we detected that VSMC autophagy could be enhanced by rapamycin with culture medium with serum. Interestingly, rapamycin at the concentration of 20 nM increased the autophagy (Fig. 4F), but had no effect on the VSMC proliferation (Fig. 4G). After rapamycin treatment, the repressed mTOR may increase autophagy through decreasing phosphorylation of ULK1 and ATG13 in ULK1 complex [55–57], and the activated ULK1 complex which promotes the translocation of PI3K C III complex from dynein motor complex to endoplasmic reticulum by phosphorylation of AMBRA1 [58]. In addition, the association of RACK1-ATG5, which promotes the autophagy process, is required for mTOR inhibition [59]. Therefore, autophagy can be upregulated through suppressing mTOR by rapamycin. Meanwhile, mTOR is a multifunctional regulation protein, which regulates varied important biological process, such as adipogenesis, immune and aging, as well as proliferation [60]. In human B-cell, soluble BAFF stimulates the proliferation *via* mTOR-mediated PP2A-Erk1/2 signaling pathway [61]. The different regulation mechanism of mTOR may participate in the different effect of rapamycin on autophagy and proliferation. In addition, the autophagy enhanced effect was greatly weakened without serum as compared with serum, and so was the VSMC proliferation (Supplementary Fig. 6). As we all known, autophagy can be induced by starvation, so we boldly concluded that the difference autophagy effect of rapamycin with and without serum may be due to starvation incentive. The starvation induced autophagy partly covered up the autophagy effect difference without serum.

In conclusion, we demonstrate that high cyclic stretch suppresses the expressions of NE proteins, lamin A/C and emerin, which induces autophagy in VSMCs *via* mTOR pathway, and the activated autophagy may promote VSMC proliferation and subsequently contribute to vascular remodeling in hypertension (Fig. 6). This study provides an evidence that inhibition of autophagic flux may be a practicable approach to alleviate the abnormal proliferation of VSMCs during hypertension.

CRediT authorship contribution statement

Hai-Peng Li: Conceptualization, Methodology, Investigation, Writing - original draft. **Ji-Ting Liu:** Investigation, Data curation. **Yuan-Xiu Chen:** Investigation, Data curation, Software. **Wen-Bin Wang:** Investigation. **Yue Han:** Validation. **Qing-Ping Yao:** Software, Validation, Data curation. **Ying-Xin Qi:** Conceptualization, Supervision, Writing - review & editing.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

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