Metformin protects against abdominal aortic aneurysm by Atg7-induced autophagy

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Abstract

Background. Abdominal aortic aneurysm (AAA) is a pathological dilation of the abdominal aorta. It is often asymptomatic, yet it has a high susceptibility to rupture. Our previous study showed that metformin protected against the pathophysiology of AAA by reducing the activation of the PI3K/AKT/mTOR pathway.

Objectives. To investigate the potential involvement of the autophagy-related pathways in AAA and the ability of metformin to modulate these effects.

Materials and methods. The expression of autophagy-related proteins was detected with western blot in patients with AAA. Angiotensin II (Ang-II) was also used to construct an AAA model in mice and in vascular smooth muscle cells (VSMCs). The expression of Atg7 and Atg4 was determined using western blot assay. The Atg7 expression was regulated by overexpressed plasmid, siRNA (small interfering RNA), or metformin, and cell proliferation, migration, apoptosis and autophagy caused by Ang-II were examined.

Results. Autophagy-related proteins were increased in patients with AAA. The Ang-II also induced the expression of Atg7, and metformin reversed this effect both in vivo and in vitro. The suppression of Atg7 inhibited cell proliferation and cell migration, and reduced cell apoptosis and autophagy, while the overexpression of Atg7 enhanced cell proliferation and migration, and induced cell apoptosis and autophagy. Furthermore, Atg7 regulated the expression of the autophagy-related protein in Ang-II treated VSMCs. The Atg7-mediated autophagy was also attenuated by metformin.

Conclusions. Metformin reduced autophagy in AAA and this effect was mediated by Atg7, suggesting that Atg7 is a potential downstream effector of metformin in protecting against the pathophysiology of AAA.

Key words: metformin, abdominal aortic aneurysm, autophagy, Ang-II, Atg7

Cite as
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Background

Abdominal aortic aneurysm (AAA), a degenerative vascular disease, is a pathological dilation that can lead to a potentially fatal aortic rupture.[1] Although the incidence of AAA has recently decreased,[2] the worldwide mortality rate due to this condition has remained stable.[3] Aneurysms are characterized by a decrease in the number of medial smooth muscle cells (SMCs) in the aortic wall.[1] An increasing number of studies have reported that the development of AAA is associated with a reduction in the structural integrity of the vessel wall, which is caused by cell apoptosis and senescence in SMCs.[4,5] While the histopathological features of AAA are well documented, the cellular and molecular mechanisms underlying the pathogenesis of AAA remain unclear, and no effective pharmacological therapy has been identified to prevent AAA. Therefore, the search for a novel therapeutic approach remains a challenge.

Autophagy is a highly regulated process that includes the delivery of damaged organelles and cytoplasmic constituents to the lysosome for clearance.[6] Autophagy-related genes (Atgs) play a critical role in the regulation of this process.[7] The Atg7 is a central regulator of autophagy and serves as an E1-like enzyme for the ubiquitin-like proteins Atg12 and Atg8.[8] The *ApoE*$^{-/-}$ mice with SMC-specific Atg7 deficiency exhibit increased plaque sizes and accelerated SMC senescence in the fibrous cap.[9] Moreover, the SMC-specific deletion of Atg7 results in reduced serum-induced cell growth, increased cell death and a decreased cell proliferation rate.[10] Furthermore, the loss of Atg7 in SMCs exacerbates angiotensin II (Ang-II)-associated aortic remodeling.[11] The disruption of autophagic flux caused by vascular SMC (VSMC)-specific deletion of Atg7 results in defective mitophagy, an exacerbation of VSMC apoptosis and, in turn, plaque vulnerability.[12] This latter observation reveals the important role of autophagy in the pathogenesis of AAA. Others have reported that metformin suppresses Ang-II-induced AAA progression by inhibiting the activation of the NF-κB and STAT3 signaling pathways.[13–15] The levels of the autophagy factors Beclin and LC3 have also been shown to be elevated in human and mouse AAA tissues.[16] As the primary cellular constituent of the aorta, the loss of VSMCs through apoptosis or necroptosis is a significant distinguishing characteristic of AAA.[17] Moreover, a recent study showed that autophagy plays a key role in regulating VSMC death and aortic wall homeostasis and repair.[18]

Objectives

This study aimed to investigate the potential involvement of autophagy-related signaling pathways in AAA and the ability of metformin to modulate these effects.

Materials and methods

Clinical samples

Clinical samples were obtained from patients and controls at the Shandong Provincial Hospital (Jinan, China). Detailed information on patients and sample collection has been provided in our previous study.[15] The aneurysmal wall tissues of AAA and aortic tissues from the control group were lysed in RIPA buffer (ab156034; Abcam, Cambridge, UK) for western blot assays. This study was approved by the Human Research Committee of Shandong Provincial Hospital (Approval No. SZRJJ:NO.2021-160), affiliated with Shandong University. The procedures followed the Declaration of Helsinki and informed consent was obtained before conducting the experiments on human subjects.

Animal model

As previously described,[15] a mouse model of Ang-II-induced AAA was developed. All procedures with animals were approved by the Animal Care and Use Committee at Shandong Provincial Hospital and were conducted following institutional guidelines. A total of 30 mice (male, 8 weeks old) were randomly assigned to one of the 3 groups: AAA group (n = 10), sham group (n = 10) and AAA + metformin (Met) group (n = 10). In the AAA group, mice were infused with Ang-II (1.44 mg/kg/day, ab120183; Abcam, Cambridge, UK) for 7 or 28 days using implanted micro-osmotic pumps. Mice in the sham group received an equal volume of saline. In the AAA+Met group, mice were injected with Ang-II (1.44 mg/kg/day) by implanted micro-osmotic pumps, and were fed water containing metformin (100 mg/kg/day) for 7 or 28 days.

Cell culture and treatment

The VSMCs were isolated from the normal arteries of healthy rats as described previously[19] and cultured in Dulbecco's modified Eagle medium (DMEM) (10313039; Thermo Fisher Scientific, Shanghai, China), supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. These VSMCs were then treated with Ang-II (1 μM), metformin (10 mM), or a combination of siRNA or plasmid with Ang-II.

Transfection with plasmid or siRNA

The pcDNA3.1-Atg7 overexpression vector was constructed and validated using restriction enzyme digestion and DNA sequencing analysis. The polymerase chain reaction (PCR) primers for amplification of Atg7 (NM_006395) were Atg7-F (5′-CGCAAATGGGCGGTAGGCGTG-3′) and Atg7-R (5′-TAGAAGGCACAGTCGAGG-3′).

Verification of the correct cloning of the sequence in the pcDNA3.1-Atg7 overexpression vector is shown in Fig. 1. Negative control (NC) or Atg7 siRNA was purchased from GenePharma (Shanghai, China). The plasmid or siRNA was transfected into VSMCs using Lipofectamine 2000 (Invitrogen, Waltham, USA) according to the manufacturer's instructions.

Immunofluorescence

The Ang-II-treated VSMCs were transfected with NC siRNA, Atg7 siRNA, control plasmid, or overexpressed Atg7 plasmid for 48 h. Cells were fixed and blocked with 1% bovine serum albumin (BSA) for 30 min at room temperature. The cells were then incubated with anti-Atg7 antibodies overnight at 4°C. After washing with phosphate buffered saline (PBS) 3 times, fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were used. The DAPI was used to stain the cell nuclei. The images of these cells were taken with an Olympus CX71 microscope (Olympus Corp., Tokyo, Japan).

Cell apoptosis

Cells were harvested and stained with Annexin V and propidium iodide (PI), using an Annexin V-FITC apoptosis detection kit (Becton Dickinson Biosciences, Franklin Lakes, USA) according to the manufacturer's instructions. Cell samples were analyzed using CellQuest software on a FACSCalibur™ instrument (Becton Dickinson Biosciences).

5-ethynyl-2′-deoxyuridine (EdU) staining

Cell permeabilization and staining were performed using a Click-iT EdU Imaging Kit (Invitrogen). Cells were fixed in 4% formaldehyde for 15 min and then were incubated with 0.1% Triton X-100 for 10 min. Thereafter, cells were treated with 0.5 mL of Click-iT reaction cocktail in the dark for 30 min. After removing the reaction cocktail, cells were washed with 3% BSA in PBS, and their nuclei were stained with 1 μg/mL DAPI for 5 min in the dark. Cells were then washed with PBS, and Olympus CX71 microscope was used to capture the images.

Transwell assay

After overnight starvation, cells were reseeded in the upper chamber of a transwell plate (1×10^5 in 100 μL of medium) that was pretreated with 1% Matrigel (Becton Dickinson Biosciences) in PBS. The bottom chambers were also filled with 500 μL cell culture medium. After 24 h, the nonmigrated cells were removed and the migrated cells were fixed and stained with crystal violet. Images of these cells were captured using Olympus CX71 microscope.

Western blotting

Cells were lysed in ice-cold RIPA lysis buffer and protein concentrations were determined using a BCA protein kit (ab102536; Abcam). A total of 30 μg of protein was separated using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. After transfer to polyvinylidene difluoride (PVDF) membranes and blocking with 5% fat free milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies against Atg7, p62, Beclin 1, LC3B, or GAPDH. The membranes were washed 3 times with Tris-buffered saline with Tween (TBST) and incubated for 2 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibody. Enhanced chemiluminescence (ECL) was used to view the protein bands.

Transmission electron microscopy (TEM)

Cells were fixed with 2.5% glutaraldehyde and washed 4 times with PBS. The cells were then fixed with 1% osmic acid. After washing, the cells were dehydrated using 50% acetone, 70% acetone, 90% acetone, and 100% acetone. Cells were infiltrated, embedded, sliced, and stained using uranium acetate lead citrate. The cell morphology was examined using a Hitachi-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analyses

Data were analyzed using GraphPad Prism software v. 9.0.1 (GraphPad Software, San Diego, USA). All experiments were repeated at least 3 times. The corresponding data are expressed as mean ± standard deviation (SD). Unpaired Student's t-tests were used to determine statistical differences between 2 groups and one-way analysis of variance (ANOVA) was used to determine statistical differences between multiple groups, followed by Tukey's post hoc test. The Shapiro–Wilk test was used to assess normality. Statistical significance was defined as a p-value less than 0.05.

Results

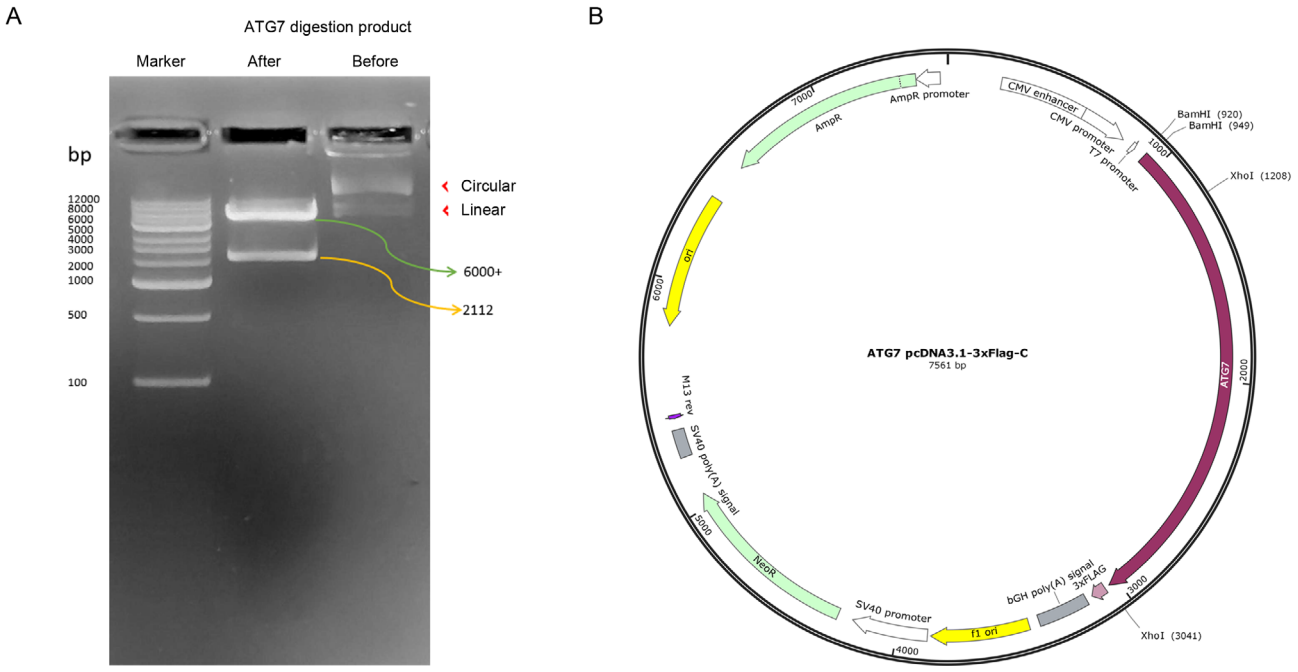
The expression of Atg7 is induced in patients with AAA and in an AAA mouse model

Our previous study found that metformin protected against the pathophysiology of AAA by inhibiting autophagy in vivo and in vitro. To understand the mechanism behind these effects, the expression of the autophagy-related proteins Atg4 and Agt7 was evaluated in patients with AAA. It was found that Atg7 expression was increased in patients with AAA compared to controls,

whereas the expression of Atg4 remained unchanged (Fig. 2A). Furthermore, the expression of Beclin 1 and LC3B II/I was increased and the expression of p62 was decreased (Fig. 2A), suggesting that the autophagy is triggered in AAA. The Ang-II was also used to construct a mouse AAA model, and it was discovered that Ang-II induced the expression levels of Atg7, Beclin 1 and LC3B II/I, and reduced the expression of p62 (Fig. 2B). On the other hand, metformin reversed the induction of Atg7, Beclin 1 and LC3B II/I (Fig. 2B). Given that Atg7 is an essential factor for the induction of autophagy, the increased level of Atg7 might contribute to the induction of autophagy in AAA.

Metformin attenuates Ang-II-induced Atg7 expression

The Ang-II was used to construct an AAA model in VSMCs and the expression of autophagy-related proteins was examined. It was found that Ang-II induced the protein expression level of Atg7 (Fig. 3A) but not of Atg4. Moreover, metformin reversed the induction of Atg7 (Fig. 3A). To assess the functional role of Atg7 in the AAA in vitro model (Ang-II treated VSMCs), Atg7 was overexpressed or suppressed using a plasmid or siRNA, and immunofluorescence (IF) was used to determine the transfection efficiency. As shown in Fig. 3B,C, the expression of Atg7 was increased or decreased by transfection.



C

>NM_006395.3:45-2156 Homo sapiens autophagy related 7 (ATG7), transcript variant 1

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Fig. 1. Cloning of Atg7. A. The electrophoresis results of Atg7 polymerase chain reaction (PCR) product before and after restriction endonuclease digestion; B. The vector map of pcDNA3.1-Atg7; C. The cDNA sequence of Atg7

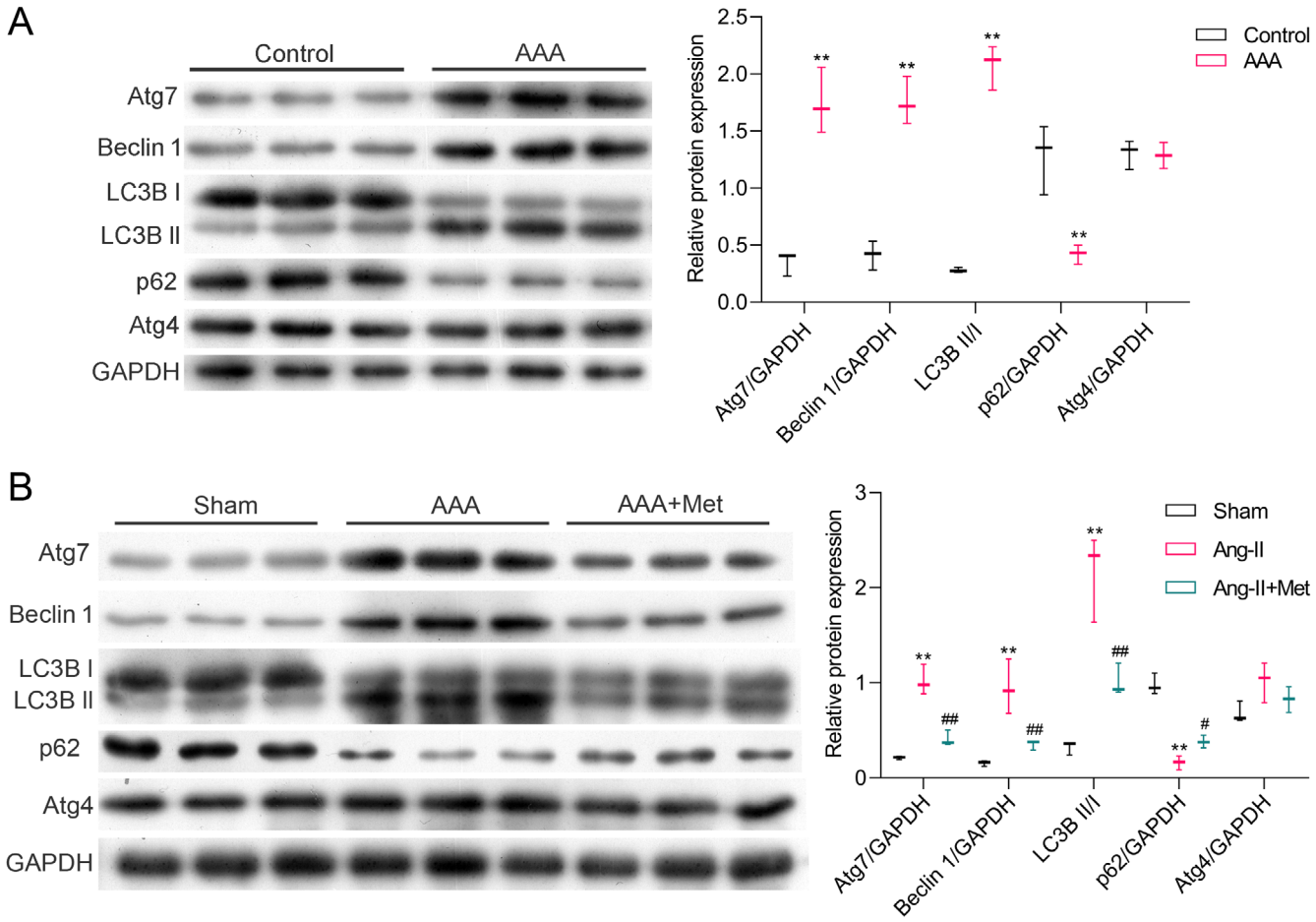

Fig. 2. The expression of Atg7 is induced in patients with abdominal aortic aneurysm (AAA) and in an AAA mouse model. A. Clinical samples from patients (n = 3) and controls (n = 3) were collected and lysed for western blot assays. The GAPDH was used as a loading control. Image J software was used to determine relative protein expression. **p < 0.01 compared to the control group. Results were statistically analyzed using an unpaired Student's t-test; B. Mice were infused with Ang-II (1.44 mg/kg/day) by implanting micro-osmotic pumps (AAA group) or infused with Ang-II and fed water containing metformin (AAA + metformin (Met) group). The aneurysmal wall tissues were collected and lysed for western blot assays. The GAPDH was used as a loading control. Relative protein expression was evaluated using Image J software. **p < 0.01 compared to the sham group, #p < 0.05, ##p < 0.01 compared to the Ang-II group. Results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test

Atg7 mediates the proliferation and migration of VSMCs under Ang-II treatment

To demonstrate the effects of Atg7 on the proliferation and migration of VSMCs, an Atg7-expressing vector was constructed (Fig. 1). The EdU staining was used to determine the cell proliferation rate and it was found that the overexpression of Atg7 significantly increased the rate of cell proliferation. The Atg7 overexpression occurred in an average of 82% of treated cells compared to 38% of controls. The reduction of Atg7 using siRNA reduced cell proliferation from 37% to 8% (Fig. 4A,C). Moreover, cell migration was induced by Atg7 and reduced by inhibiting Atg7 (Fig. 4B,D). These findings indicate that Atg7 regulates cell proliferation and migration in the AAA in vitro cell model.

Inhibition of Atg7 reduces cell apoptosis and autophagy in Ang-II-treated VSMCs

To determine the apoptotic effects of Atg7 on VSMCs, an Annexin V assay was performed to assess the early and late stages of cell apoptosis. As shown in Fig. 5A, silencing Atg7 reduced early cell apoptosis (22% compared to 13%), while the overexpression of Atg7 enhanced early cell apoptosis (19% compared to 30%). Moreover, the Atg7 overexpression increased autophagic vacuole formation, whereas the Atg7 suppression decreased autophagic vacuole formation (Fig. 5B). The expression of the autophagy-related proteins was further evaluated and it was found that Atg7 overexpression increased the expression of LC3B II/I and Beclin 1, whereas the level of p62 was decreased (Fig. 5C). Furthermore, the suppression of Atg7 by siRNA inhibited LC3B II/I and Beclin 1 but increased p62 expression (Fig. 5C). These results suggest that Atg7 mediates both, Ang-II-induced cell apoptosis and autophagy.

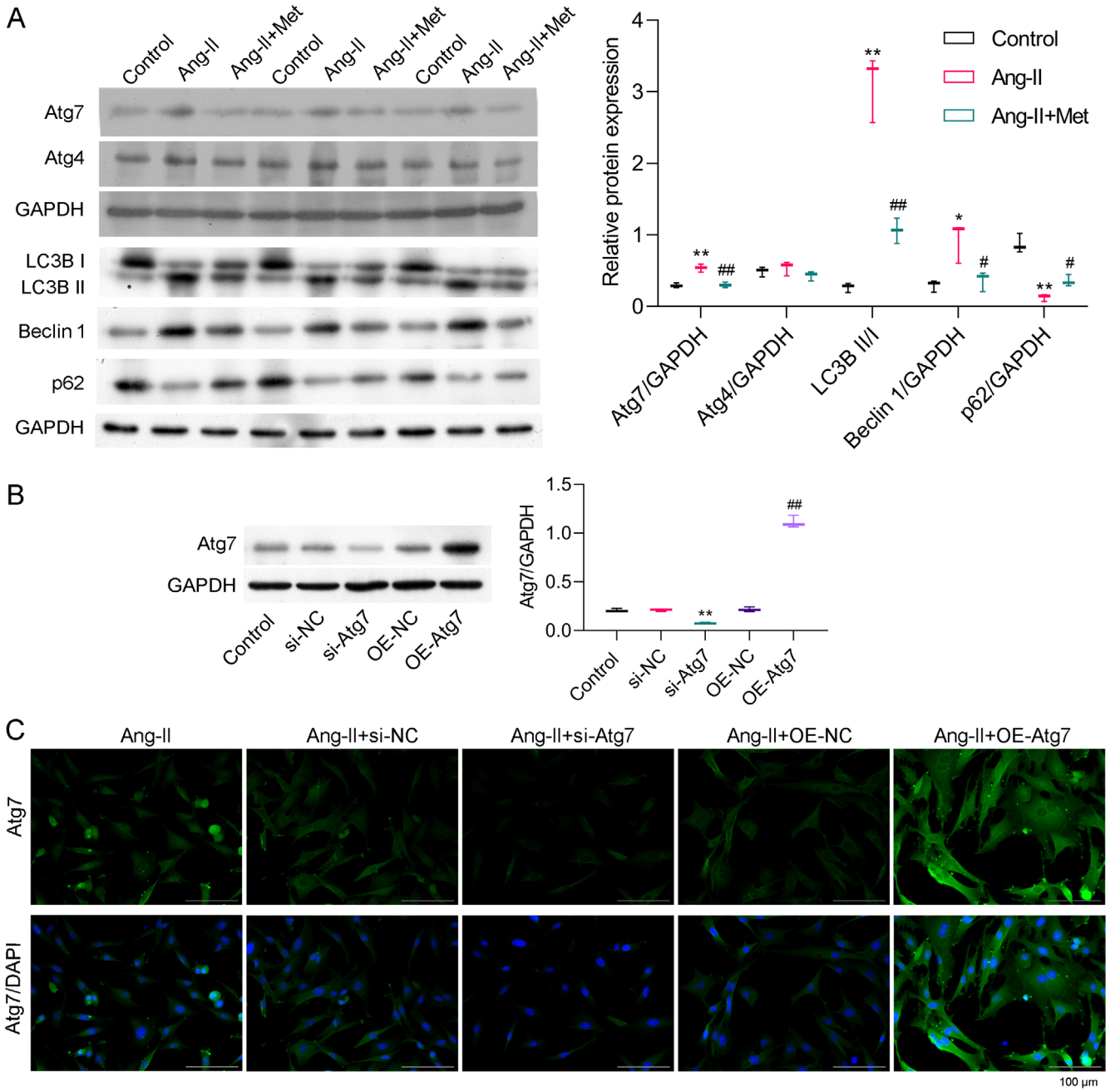

Fig. 3. Metformin attenuates Ang-II-induced Atg7 expression. A. The vascular smooth muscle cells (VSMCs) were treated with Ang-II alone or a combination of Ang-II (1 μM) and metformin (Met, 10 mM) for 48 h. Cells were collected and lysed for western blotting. The GAPDH was used as a loading control. Relative protein expression was assessed using Image J software. *p < 0.05, **p < 0.01 compared to control group, #p < 0.05, ##p < 0.01 compared to the Ang-II group. Results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test; B. The VSMCs were treated with Ang-II (1 μM) and transfected with negative control (NC) siRNA (si-NC), Atg7 siRNA (si-Atg7), a control plasmid (OE-NC) or an overexpressed Atg7 (OE-Atg7) plasmid for 48 h. Western blot analysis was performed to determine the expression of Atg7. **p < 0.01 compared to the si-NC group, ##p < 0.01 compared to the OE-NC group. Results were statistically analyzed using ANOVA followed by Tukey's post hoc test; C. Cells were fixed and stained using an Atg7 antibody

Reduction of autophagy by metformin in AAA is mediated by Atg7

To determine whether Atg7 regulated metformin-induced suppression of autophagy in Ang-II-treated VSMCs, the Atg7 was overexpressed in both metformin- and Ang-II-treated VSMCs (Fig. 6A), and it was found that metformin reversed the autophagic vacuole formation induced by Atg7 (Fig. 6A,B). Similarly, metformin reversed the increased expression of LC3B II/I and Beclin 1, and the decreased p62 levels induced by Atg7 overexpression (Fig. 6C). Therefore, our results suggest that metformin inhibits autophagy by suppressing Atg7.

Discussion

The AAA is a pathologic dilation of the abdominal aorta that is often asymptomatic, yet has a high susceptibility

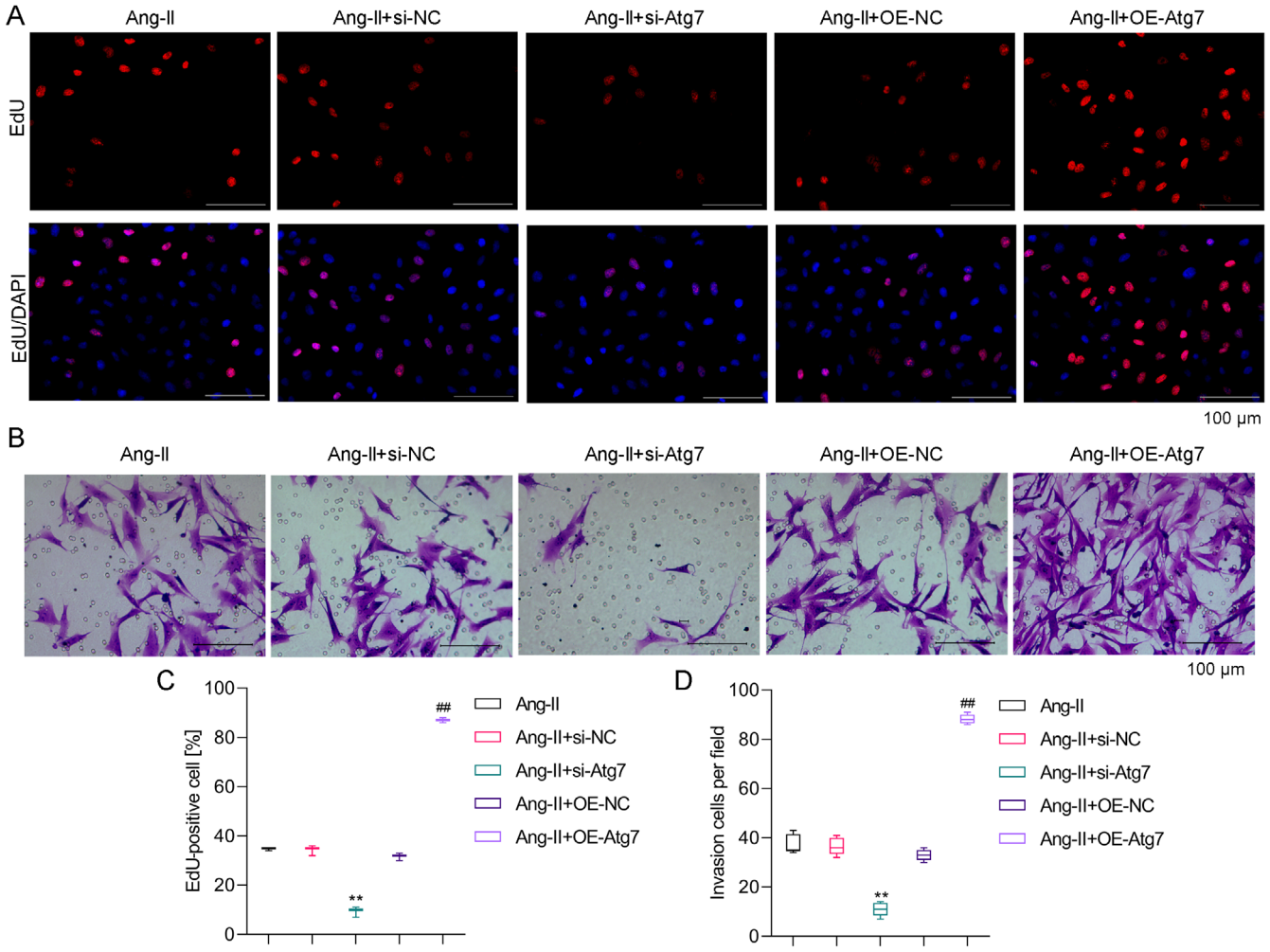

Fig. 4. The Atg7 mediates the cell proliferation and migration of vascular smooth muscle cells (VSMCs) following Ang-II treatment. The VSMCs were treated with Ang-II (1 μM) and transfected with NC siRNA (si-NC), Atg7 siRNA (si-Atg7), a control plasmid (OE-NC) or an overexpressed Atg7 (OE-Atg7) plasmid for 48 h. A. Cells were fixed and stained for EdU; B. Transwell assay was performed; C and D. A summary of the EdU and transwell assays. The respective images are shown. **p < 0.01 compared to the Ang-II+si-NC group, ##p < 0.01 compared to the Ang-II+OE-NC group. Results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test

to rupture. In this study, it was found that the expression of the autophagy-related protein Atg7 is increased in patients with AAA, and in vitro and in vivo models of the condition. More importantly, metformin reversed the induced expression of Atg7. Furthermore, cell proliferation, migration, apoptosis, and autophagy in an in vitro AAA cell model were regulated by Atg7. However, these Atg7-induced effects were reversed by metformin. Taken together, these findings suggest that Atg7 is a potential downstream effector of metformin that is involved in protecting against the pathophysiology of AAA.

The upregulation of LC3, Atg5 and Atg7 has been found in dissected AAA tissues from Ang-II-treated *ApoE*–/– mice, and Ang-II-challenged SMC Atg7-specific knockout mice exhibit severe cardiac dysfunction and larger suprarenal aortic diameters.[11] As a major component of the vessel wall, VSMCs control blood flow and arterial pressure by changing the lumen diameter in resistance vessels in response to Ang-II.[20] The Ang-II induces autophagy in cells by increasing Beclin 1, Vps34, Atg12-Atg5, Atg4 and Atg7 protein levels, Beclin 1 phosphorylation, and the number of autophagic vesicles.[21] In the current study, it was observed that Atg7 was upregulated not only in patients with AAA, but also in Ang-II-treated mice or VSMCs. Our previous study demonstrated that metformin suppressed the pathophysiology of AAA by inhibiting the autophagy pathway.[15] Furthermore, in this study, it was found that the induced expression of Atg7 in AAA was reversed by metformin. No previous studies have evaluated whether metformin regulates Atg7 in Ang-II-induced VSMCs. Our findings indicate that Atg7 might be involved in metformin-reduced autophagy in AAA, which is evidenced by a decrease in autophagy-related proteins.

The AAA is caused by changes in the aortic wall structure, including the loss of VSMCs and the degradation of the extracellular matrix, which results in thinning of the media and adventitia.[22] The proliferation and apoptosis of VSMCs are associated with the progression of AAA.[18] Furthermore, the loss of autophagy in VSMCs promotes VSMC death and endoplasmic reticulum stress-dependent

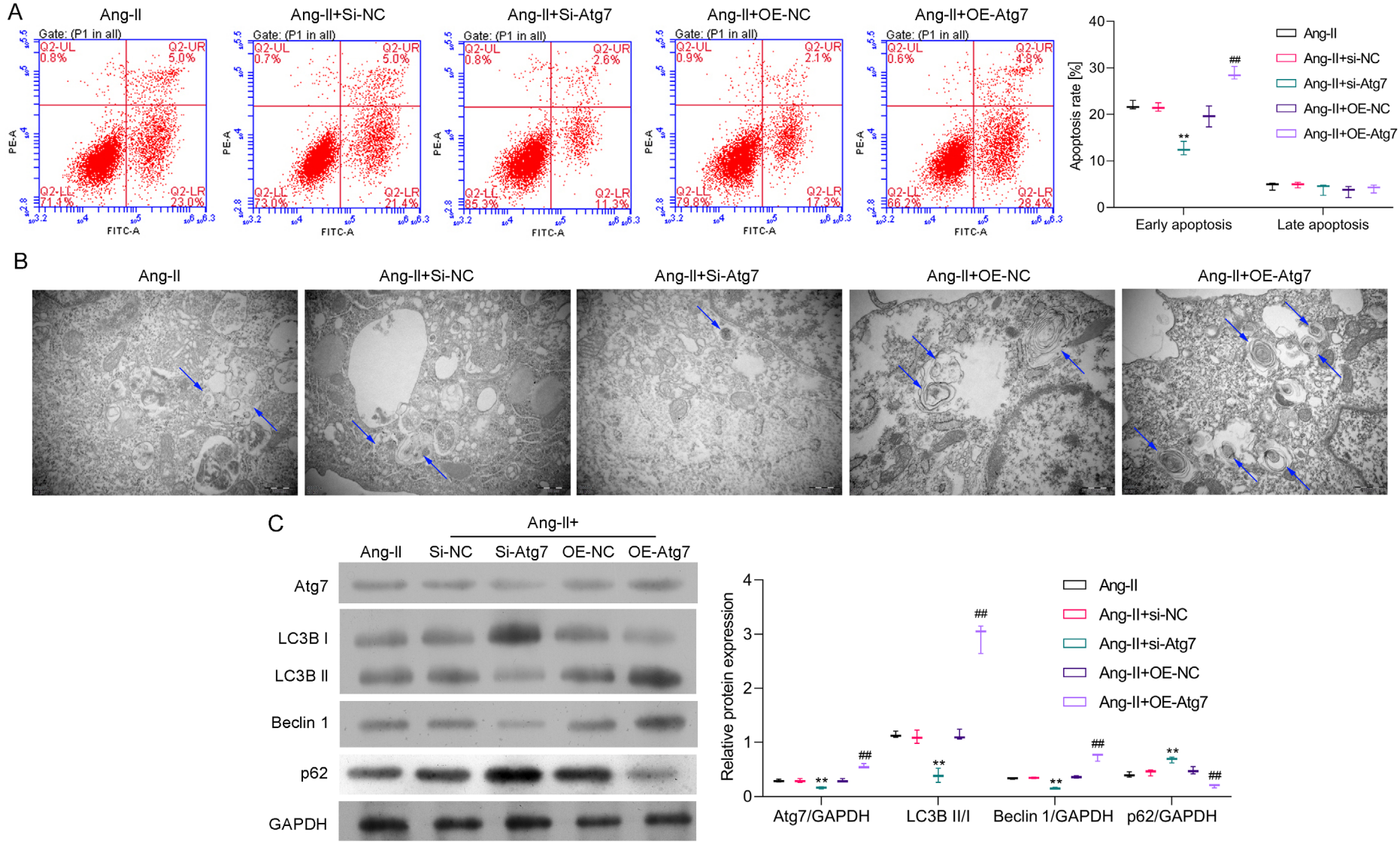

Fig. 5. Inhibition of Atg7 reduces cell apoptosis and autophagy in Ang-II treated vascular smooth muscle cells (VSMCs). The VSMCs were treated with Ang-II (1 μM) and transfected with negative control (NC) siRNA (si-NC), Atg7 siRNA (si-Atg7), a control plasmid (OE-NC), or an overexpressed Atg7 (OE-Atg7) plasmid for 48 h. A. Annexin V staining. **p < 0.01 compared to the Ang-II+si-NC group, ##p < 0.01 compared to the Ang-II+OE-NC group; B. Transmission electron microscopy (TEM) (the blue arrow indicates autophagic vacuoles); C. Western blot with the indicated antibodies. **p < 0.01 compared to the Ang-II+si-NC group, ##p < 0.01 compared to the Ang-II+OE-NC group. Results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test

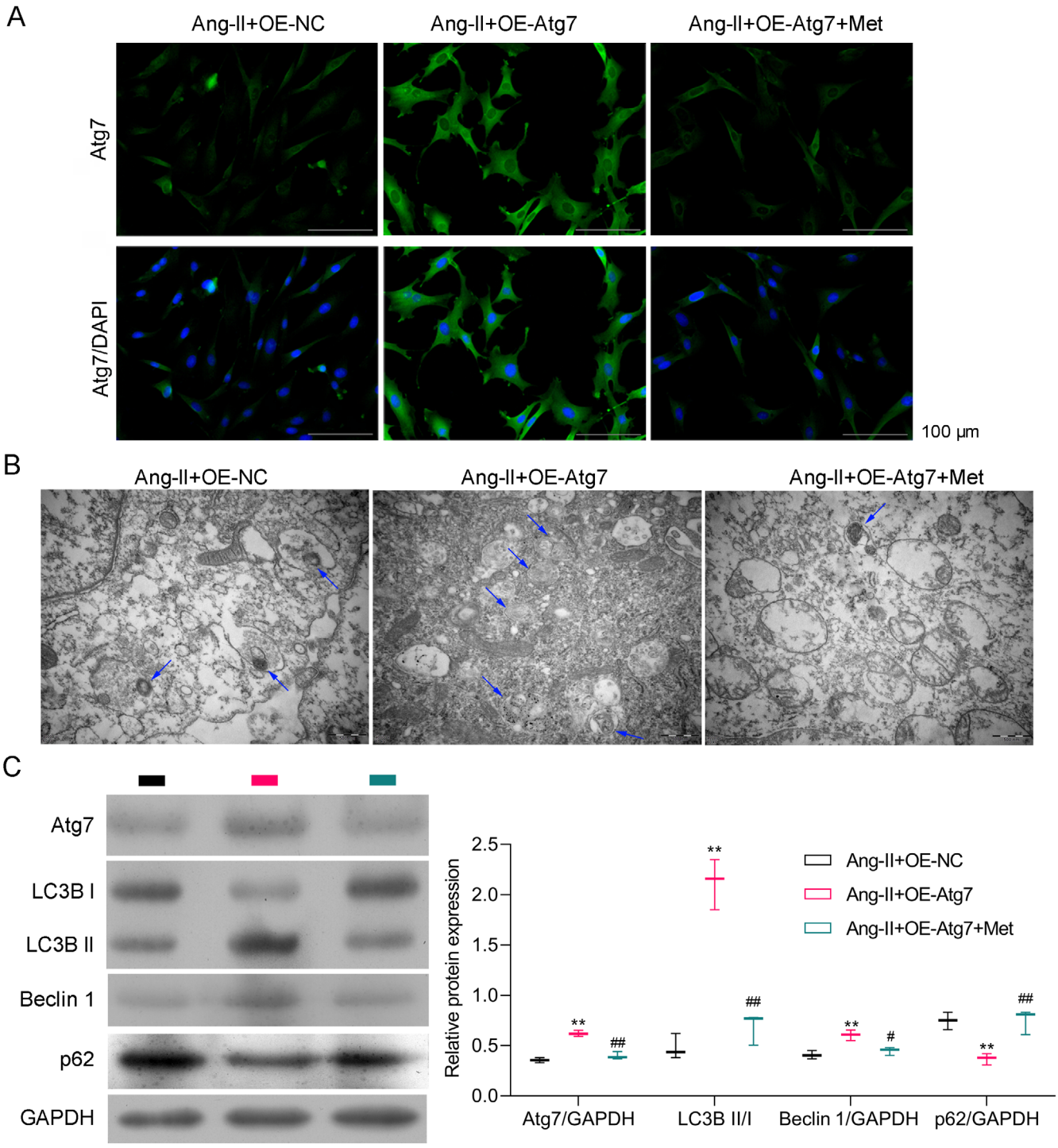

Fig. 6. The Atg7 mediates the inhibition of autophagy by metformin in abdominal aortic aneurysm (AAA). The vascular smooth muscle cells (VSMCs) were treated with a combination of Ang-II and metformin, and transfected with control (OE-NC) or Atg7 (OE-Atg7) overexpressed plasmids. A. The expression of Atg7 was evaluated using immunofluorescence (IF); B. Autophagic bodies and vesicles were identified using transmission electron microscopy (TEM) (the blue arrow indicates autophagic vacuoles); C. The expression of autophagy-related proteins was determined using western blotting. **p < 0.01 compared to the Ang-II+OE-NC group, #p < 0.05, ##p < 0.01 compared to the Ang-II+OE-Atg7 group. Results were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test

vascular inflammation, thus aggravating AAA.[11] Autophagy is a multistep process that is mediated by Beclin 1, Vps34 and 2 ubiquitin-like systems, LC3 and the autophagy-related proteins.[23] The Atg7 functions as the E1 enzyme of the ubiquitin-proteasome system to mediate the formation of an Atg12-Atg5-Atg16 complex, which is required for the elongation of the autophagosome.[24] Recent studies have revealed that atherosclerotic lesions in *ApoE−/−* mice with deleted Atg7 in VSMCs display enhanced apoptosis.[12] The current study provides evidence that inhibition of Atg7 suppresses cell proliferation, migration, apoptosis, and autophagy, whereas the overexpression

of Atg7 increases cell proliferation, migration, apoptosis, and autophagy. Therefore, Atg7 is an important regulator of proliferation, migration, apoptosis, and autophagy in Ang-II-treated VSMCs.

Metformin is a well-known drug that affects both metabolism and the inflammatory response, which makes it a potential therapeutic drug for several cardiovascular diseases.[25] Multiple studies have found that metformin significantly slows the growth of AAA[13,14,26] and inhibits cell growth and proliferation, leading to cell apoptosis.[27] Furthermore, metformin administration may be associated with a lower risk of AAA.[28] Recently, we demonstrated that metformin protects against Ang-II-induced AAA by activating the PI3K/AKT/mTOR/autophagy pathway.[15] However, the molecular mechanisms involved in these effects remained unclear. In this study, using an in vitro AAA model, it was shown that metformin reverses autophagy induced by Atg7, suggesting that metformin reduces autophagy by suppressing Atg7.

Limitations

A relatively low number of clinical samples were examined in the current study. Clinical samples continue to be collected and will be examined for further verification. In addition, we plan to identify the molecular mechanisms of Atg7-induced autophagy in in vivo rat models and in clinical samples in future studies.

Conclusions

In this study, it was found that Ang-II induced the expression of Atg7 and that metformin reversed this effect. The expression of Atg7 regulated cell proliferation, migration, apoptosis, and autophagy. Furthermore, Atg7 mediated the expression of autophagy-related proteins in Ang-II treated VSMCs. It was also found that metformin inhibited Atg7-induced autophagy. In conclusion, the current results revealed that the suppression of autophagy in AAA by metformin was mediated by an Atg7 central autophagy regulator. This suggests that Atg7 is a potential downstream effector of metformin in protecting against the pathophysiology of AAA.

Metformin reduces autophagy in AAA and Atg7 mediates this effect. These findings suggest that Atg7 acts as a downstream effector of metformin in suppressing the pathophysiology of AAA (Fig. 7).

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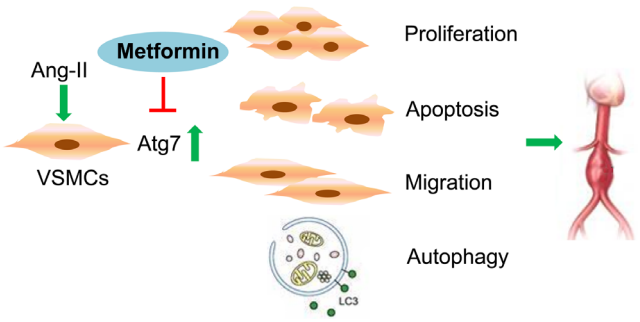

Fig. 7. Schematic diagram of the suppression of Atg7-induced cell proliferation, cell migration, cell apoptosis, and autophagy by metformin in an in vitro abdominal aortic aneurysm (AAA) model

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