

Overexpression of *ATG4a* promotes autophagy and proliferation, and inhibits apoptosis in lens epithelial cells via the AMPK and Akt pathways

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Abstract. Autophagy is a major intracellular degradation system that plays an important role in several biological processes. Although some studies indicate that autophagy may play a role in lens degradation and cataracts formation, its underlying mechanism remains to be elucidated. Autophagy-related gene 4a (*ATG4a*) cleaves autophagy-related protein 8 (*Atg8*) near the C terminus, allowing *Atg8* to conjugate with phosphatidylethanolamine via the exposed glycine; although this is pivotal in cancer development, no study has yet linked it to eye diseases. In the present study, the protein expression of *ATG4a* is significantly upregulated in hydrogen peroxide-treated lens epithelial cells (HLE-B3), indicating that *ATG4a* may play an important role in lens degradation. *ATG4a* was overexpressed using lentivirus in lens epithelial cells to observe the effect of *ATG4a* on various phenotypes by transmission electron microscopy, western blotting, EdU incorporation assay, flow cytometry and *in situ* cell death detection. The results demonstrated that the overexpression of *ATG4a* could promote autophagy by promoting the adenosine 5'-monophosphate-activated protein kinase pathway and inhibiting the Akt pathway. It also upregulated the proliferation and downregulated the apoptosis of lens epithelial cells. Overall, the present study showed that *ATG4a* plays a vital role in lens degradation and that it could be a potential target in cataract therapies.

Introduction

The lens is an avascular tissue located in front of the vitreous body in the eyes. Light passes through the lens and reaches the retina to form a clear image and therefore the transparency of the lens is important. The lens contains two types of cells, which are lens epithelial cells and lens fiber cells; the organelles in the epithelial cells degrade and differentiate into fiber cells (1). A cataract is one of the most common eye diseases and the leading cause of blindness worldwide (2). Age-related cataracts is the most common type of this disease and it can significantly lower quality of life (3). Perturbation of the lens redox status has been considered as the major cause of age-related cataracts (4). At present, surgical removal of the lens is the primary therapy for this disease. However, there is a lack of effective medical treatment; therefore, several researchers have tried to explain its underlying mechanism in order to delay or eliminate the occurrence of the disease and to identify a therapeutic target (5,6). Hydrogen peroxide (H₂O₂) elicits reactive oxygen species (ROS) production and ROS increases oxidative stress and crystal protein mutation in lens epithelial cells; therefore, H₂O₂ is widely used in building a cataract cell model to simulate the development of an aging lens and the formation of age-related cataract (7).

Autophagy is a major intracellular degradation system, which occurs in almost all cells (8). The dysfunctional cytoplasmic materials, such as mitochondria and endoplasmic reticulum, are sequestered by double-membrane autophagosomes and then delivered into the lysosomes for degradation and recycling (9). Normally, autophagy is divided into the following three types: Macroautophagy, microautophagy and chaperone-mediated autophagy; the first type is the most widely investigated (10). It has been demonstrated that autophagy, especially macroautophagy, may play an important role in the development of cataracts in the lens (11). p62, an autophagy substrate, is downregulated when autophagy is activated (12). By contrast, during autophagy, microtubule-associated protein light chain 3 (LC3)B changes from the soluble form (LC3BI) to the lipid-modified form (LC3BII). Beclin is a critical component of the class III

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Abbreviations: *ATG4a*, autophagy-related gene 4a; PE, phosphatidylethanolamine; TEM, transmission electron microscopy

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phosphatidylinositol 3-kinase complex, which can regulate the autophagy process (13). Therefore, beclin, LC3BII/I and p62 are used as biomarkers to demonstrate the occurrence of autophagy.

Thus far, >30 autophagy-related genes have been reported. Among them, autophagy-related gene 4 (*ATG4*) encodes a cysteine protease that contains four homologs (*ATG4A/ATG4B/ATG4C/ATG4D*) (14). *ATG4a* can cleave autophagy-related protein 8 (*Atg8*) near the C terminus, allowing *Atg8* to conjugate phosphatidylethanolamine (PE) via the exposed glycine. This *Atg8*-PE system plays an important role in autophagosome formation (15,16).

Although several studies have demonstrated that *ATG4* is pivotal in the development of some cancers, such as colorectal cancer, cervical cancer and breast cancer, and it is a promising therapeutic target to treat tumors (17-19), to the best of the authors' knowledge, no research has connected it to lens degradation and cataract formation. As autophagy is a crucial process in lens degradation, the present study explored the biological function of *ATG4a* in lens epithelial cells.

Materials and methods

Cell culture. The lens epithelial cell line HLE-B3 was purchased from American Type Culture Collection. It was cultured in minimum essential medium (MEM; HyClone; Cytiva) supplemented with 10% fetal bovine serum (FBS; AusGeneX Pty, Ltd.) and 1% penicillin-streptomycin solution (HyClone; Cytiva) in a humidified environment of 5% CO₂ at 37°C. H₂O₂ (3%; Beijing Solarbio Science & Technology Co., Ltd.) was diluted with MEM to a concentration of ~200 µmol/l. HLE-B3 cells were treated with 200 µmol/l H₂O₂ and termed the H₂O₂ group. Untreated HLE-B3 cells were cultured as the control group.

Lentiviral transduction. To overexpress *ATG4a*, *ATG4a* sequence was cloned into a lentiviral vector, pLenti-GIII-CMV-GFP-2A-Puro vector (cat. no. LV082668; Applied Biological Materials, Inc.). An empty vector was used as a negative control (NC). The sequencing primers of the *ATG4a* lentiviral vector were as follows: CMV sequencing primer, 5'-CGC AAA TGG GCG GTA GGC GTG-3'; SV40 reverse sequencing primer, 5'-TAG TCA GCC ATG GGG CGG AGA-3'.

Cells were transferred to 6-well plates at a cell density of 50-60% and cultured in MEM with 10% FBS for 24 h. Then, the medium was replaced with Opti-MEM (Thermo Fisher Scientific, Inc.) reduced-serum medium, to which 4.0 µg plasmid DNA and 10 µl LipoGene™ 2000 Star Transfection Reagent (US Everbright, Inc.) with 0.5 ml Opti-MEM were added; the mixture was incubated for 20 min at room temperature. The mixture in each plate was mixed with another 2 ml Opti-MEM and incubated under 5% CO₂ in a 37°C incubator for 6 h; then, the medium was replaced with complete medium and cultured for another 48 h. Cells transfected with the *ATG4a* and empty vectors were termed the overexpression (OE)-*ATG4a* and NC groups, respectively. Total proteins were harvested 36 h after transfection, and other experiments were performed 24 h after transfection.

Transmission electron microscopy (TEM). Standard TEM was performed for the ultrastructural analysis of the H₂O₂, control, OE-*ATG4a* and NC groups. Cells were pretreated and fixed with electron microscope-fixing fluid (2.5% glutaric dialdehyde solution; Wuhan Servicebio Technology Co., Ltd.) for 2 h at room temperature and then stored at 4°C. The cells were then embedded in 1% agarose and fixed in 1% osmic acid for 2 h at room temperature, followed by dehydration with ethanol (50, 70, 80, 90, 95, 100%) and acetone (100%) at room temperature for 15 min each. Cells were then treated with 50% (2-4 h), 66% (overnight) and 100% (5-8 h) epoxy resin at room temperature, followed by 100% epoxy resin, and heated at 37°C overnight and then 60°C for 48 h. The agarose-embedded cells were cut into thin sections (60-80 nm) and stained with 2% uranyl acetate and 2% lead citrate for 15 min at room temperature. Then, the sections were observed at 80 kV by a Hitachi TEM system (HT7700, Hitachi High-Technologies Corporation). Cells and autophagosomes were defined as structures measuring 5.0 and 2.0 µm, respectively. The results were analyzed by ImageJ version 1.46r (National Institutes of Health) and GraphPad Prism 5 (GraphPad Software, Inc.) software.

Western blotting. Protein expression levels of autophagy biomarkers were determined in the H₂O₂, control, OE-*ATG4a* and NC groups. The total protein was extracted using the Total Protein Extraction kit for Animal Cultured Cells and Tissues (Invent Biotechnologies, Inc.) on ice and quantified using BCA (Beyotime Institute of Biotechnology). The extracted protein samples were stored at -80°C.

Equal amounts of protein (40 µg) were electrophoresed via SDS-PAGE on 12% gels (Beyotime Institute of Biotechnology) and blotted onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked with QuickBlock™ blocking buffer for western blotting (cat. no. P0252; Beyotime Institute of Biotechnology) for 15 min at room temperature and then incubated overnight at 4°C with the following primary antibodies: Anti-ATG4A (cat. no. ab223374; 1:1,000; Abcam), anti-p62 (cat. no. ab109012; 1:5,000; Abcam), anti-LC3 (cat. no. ab192890; 1:2,000; Abcam), anti-beclin (cat. no. ab207612; 1:2,000; Abcam), anti-Akt (cat. no. YT0178; 1:1,000; ImmunoWay Biotechnology Company), anti-phosphorylated (p-)Akt (cat. no. YP0006; 1:1,000; ImmunoWay Biotechnology Company), anti-adenosine 5'-monophosphate-activated protein kinase (AMPK; cat. no. YT0216; 1:1,000; ImmunoWay Biotechnology Company), anti-p-AMPK (cat. no. YP0575; 1:1,000; ImmunoWay Biotechnology Company), anti-GAPDH (cat. no. ab181602; 1:10,000; Abcam). After washing three times for 10 min each, the membranes were incubated with the secondary antibodies (cat. no. S0001; 1:2,000; Affinity Biosciences) for 2 h at room temperature and then washed again in TBS with 0.1% Tween-20 three times for 10 min each. The membranes were visualized using the Super ECL Plus kit (US Everbright, Inc.) and developed using a chemiluminescence system (FluorChem FC2 imaging system, ProteinSimple). Signal intensities were visualized by AlphaView software version 3.4.0.0 (ProteinSimple). Data were analyzed with ImageJ version 1.46r (National Institutes of Health) and GraphPad Prism 5 (GraphPad Software, Inc.) software. All experiments were performed in triplicate.

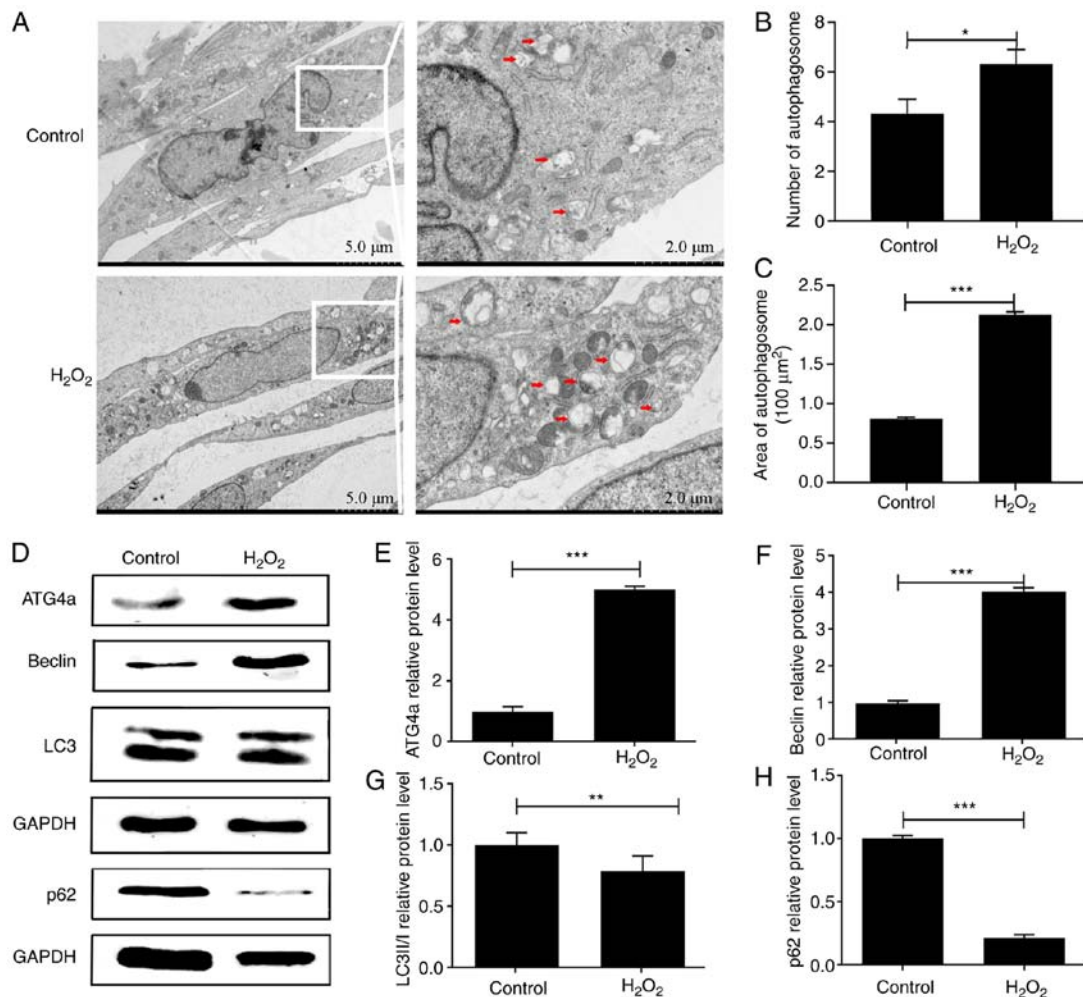


Figure 1. Autophagosomes in lens epithelial cells. (A) Transmission electron micrographs of the autophagosomes (red arrows) in the H₂O₂ and control groups. (B) Quantification of the number of autophagosomes in the H₂O₂ and control groups. (C) Quantification of the size of autophagosomes in the H₂O₂ and control groups. (D) Western blot analysis of the protein expression level of (E) ATG4a and (F) beclin in each group. (G) Quantification of the protein expression ratio of LC3II/I in each group. (H) Quantification of the protein expression level of p62 in each group. *P<0.05, **P<0.01, ***P<0.001. ATG4a, autophagy-related protein 4; LC3, microtubule-associated protein light chain 3; H₂O₂, hydrogen peroxide.

EdU incorporation assay. Cell proliferation rates were analyzed using the EdU incorporation assay in the OE-ATG4a and NC groups. HLE-B3 cells at a density of 80% were cultured in a 96-well plate, each group had three replicates. The analysis was performed using the Cell-Light EdU Apollo 488 *In Vitro* kit (cat. no. C10310-3; Guangzhou RiboBio Co., Ltd.) following the manufacturer's protocol. The percentage of EdU positive cells between the OE-ATG4a and NC groups was compared using ImageJ version 1.46r (National Institutes of Health) and SPSS 23.0 (IBM Corp.) software.

Flow cytometry analysis. Cell apoptosis (early and late apoptosis) in the OE-ATG4a and NC groups was analyzed using a flow cytometer. HLE-B3 cells were cultured in a 6-well plate and each group had three replicates. The cells were harvested using trypsin without EDTA and washed with PBS twice. The cells were resuspended in 1X binding buffer at a concentration of $>1 \times 10^6$ cells/ml. Thereafter, 100 μ l of the solution was transferred into a 5-ml culture tube, to which 5 μ l FITC Annexin V Apoptosis Detection kit I (BD Biosciences) and 5 μ l propidium iodide (PI) were added. The mixture was gently vortexed for 15 min at room temperature in the dark. Subsequently, 400 μ l

1X binding buffer was added to each tube and analyzed using BD LSRFortessa and FACSDiva version 6.2 (BD Biosciences) within 1 h. The results were analyzed by ImageJ version 1.46r (National Institutes of Health) and GraphPad Prism 5 (GraphPad Software, Inc.) software.

In situ cell death detection. Cell death was detected using the *In Situ* Cell Death Detection kit (Roche Diagnostics) in the OE-ATG4a and NC groups. HLE-B3 cells at a density of 80% were cultured in a 96-well plate at 37°C, each group had three replicates. In brief, the cells were washed with PBS three times and fixed with 4% paraformaldehyde in PBS at room temperature for 1 h. The cells were then incubated in permeabilization solution for 2 min on ice. The TUNEL reaction mixture was prepared and added into each well (50 μ l/well); the cells were incubated at 37°C in the dark for 1 h. DAPI (1:1,000) was used to stain the cell nucleus at room temperature for 5 min in the dark and mounting medium was applied to cells (cat. no. S2110; Beijing Solarbio Science & Technology Co., Ltd). Images were captured by an Olympus IX71 fluorescence microscope (Olympus Corporation) at magnification, x200. The percentage of dead

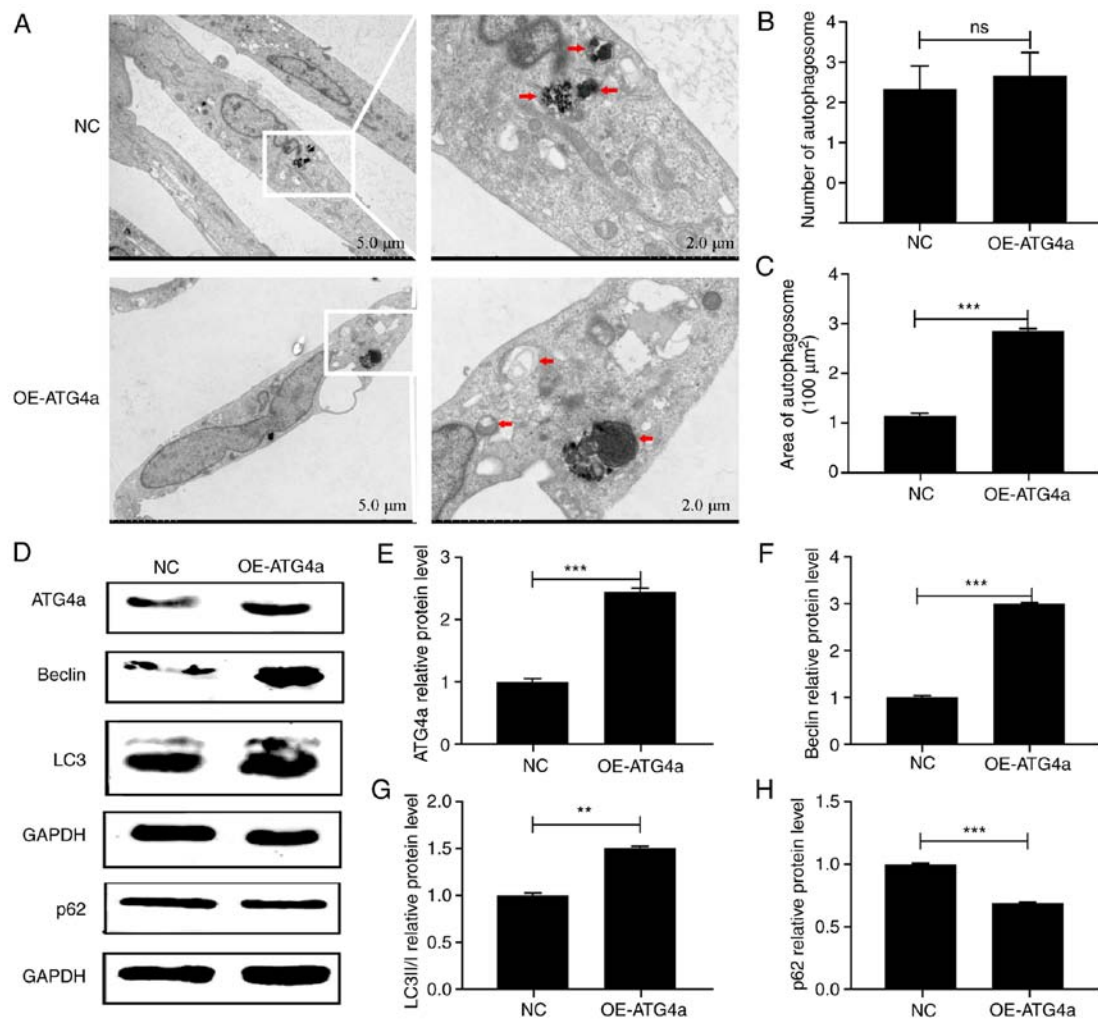


Figure 2. Protein expression level of autophagy biomarkers in lens epithelial cells. (A) Transmission electron micrographs of the autophagosomes (red arrows) in the OE-ATG4a and NC groups. (B) Quantification of the number of autophagosomes in the OE-ATG4a and NC groups. (C) Quantification of the size of autophagosomes in the OE-ATG4a and NC groups. (D) Western blot analysis of the protein expression level of ATG4a, beclin, LC3 and p62 in lens epithelial cells. (E) Quantification of the protein expression level of ATG4a and (F) beclin in each group. (G) Quantification of the protein expression ratio of LC3II/I in each group. (H) Quantification of the protein expression level of p62 in each group. ** $P < 0.01$, *** $P < 0.001$. NC, negative control; ns, no significance; ATG4a, autophagy-related protein 4; LC3, microtubule-associated protein light chain 3; OE, overexpression group.

cells between the OE-ATG4a and NC groups was compared using ImageJ version 1.46r (National Institutes of Health) and SPSS 23.0 (IBM Corp.).

Statistical analysis. All data were analyzed using SPSS version 23 (IBM Corp.) and GraphPad Prism 5 (GraphPad Software, Inc.). The values are presented as mean \pm SD of three independent experiments. The data were analyzed using the independent-samples t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

H₂O₂ can induce autophagy in lens epithelial cells. Autophagy plays an important role in lens degradation and cataract formation (11). To investigate whether H₂O₂ can induce autophagy in lens epithelial cells, TEM and western blotting were performed in both H₂O₂ and control groups. TEM showed that, compared with the control group, the number ($P < 0.05$) and size ($P < 0.001$) of autophagosomes in the H₂O₂ group were

significantly upregulated, indicating that the rate of autophagy was increased (Fig. 1A-C). The results of western blotting showed that the protein expression of ATG4a was significantly upregulated in the H₂O₂ group compared with the control group ($P < 0.001$; Fig. 1D and E), indicating that this gene might play an important role in autophagy in lens epithelial cells. The protein expression of beclin was significantly upregulated in the H₂O₂ group compared with the control group ($P < 0.001$; Fig. 1D and F). By contrast, the protein expression of p62 was significantly downregulated ($P < 0.001$; Fig. 1D and H). Although the expression of LC3BII/I ($P < 0.01$; Fig. 1D and G) did not indicate the upregulation of autophagy, the results of both TEM and western blotting demonstrated the existence of autophagy in H₂O₂-induced lens epithelial cells.

Overexpression of ATG4a activates autophagy in lens epithelial cell. To confirm that ATG4a can specifically regulate autophagy, TEM and western blotting were performed in the OE-ATG4a and NC groups. TEM showed that in the OE-ATG4a group (Fig. 2A) the amount of autophagosomes

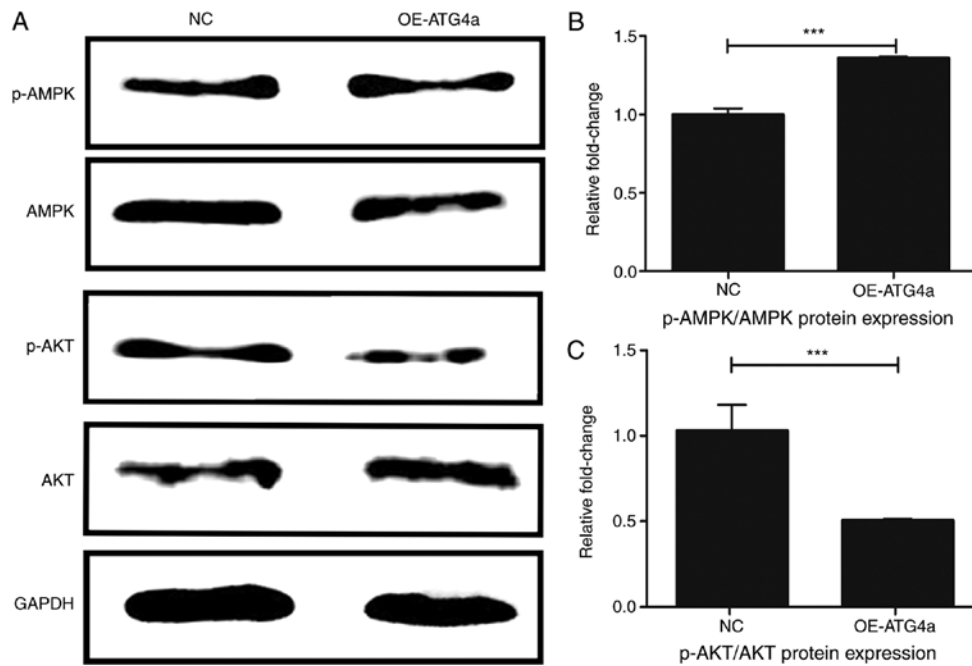


Figure 3. *ATG4a* promotes the AMPK pathway and inhibits the Akt pathway in lens epithelial cells. (A) Western blot analysis of the protein expression level of p-AMPK, AMPK, p-Akt and Akt in lens epithelial cells. Quantification of the protein expression ratio of (B) p-AMPK/AMPK and (C) p-Akt/Akt in each group. *** $P < 0.001$. ATG4a, autophagy-related protein 4; AMPK, AMP-activated protein kinase; p-, phosphorylated; OE, overexpression group.

was not significantly different from that in the control group ($P > 0.05$; Fig. 2B), but their size was significantly increased ($P < 0.001$; Fig. 2C). Western blotting confirmed that the protein expression of beclin ($P < 0.001$) and LC3BII/I ($P < 0.01$) was significantly upregulated in the OE-*ATG4a* group compared with the NC group (Fig. 2D, F and G). By contrast, the protein expression of p62 was significantly downregulated ($P < 0.001$; Fig. 2D and H), verifying that *ATG4a* can activate autophagy in lens epithelial cells.

ATG4a influences the AMPK and Akt pathways in lens epithelial cells. To investigate the autophagy pathways in which *ATG4a* is involved, western blotting was performed in the OE-*ATG4a* and NC groups (Fig. 3A). Compared with the NC group, the ratio of protein expression of p-AMPK and AMPK was significantly elevated in the OE-*ATG4a* group ($P < 0.001$; Fig. 3B), indicating that the overexpression of *ATG4a* significantly activated the phosphorylation of AMPK. By contrast, the trend was reversed for the ratio of p-Akt and Akt ($P < 0.001$; Fig. 3C), indicating that the overexpression of *ATG4a* can inhibit the activation of p-Akt. These results suggested that *ATG4a* may play a role in autophagy by promoting the AMPK pathway and inhibiting the Akt pathway.

ATG4a promotes proliferation and inhibits apoptosis. To investigate the relationship between *ATG4a* and cell proliferation and apoptosis, the EdU incorporation assay, FITC/PI double staining tested by flow cytometry and *in situ* cell death detection assay were performed in the OE-*ATG4a* and NC groups. The EdU incorporation assay showed that *ATG4a* could significantly promote cell proliferation ($P < 0.05$; Fig. 4A and D). Although the flow cytometry analysis results did not show a significant difference ($P > 0.05$), it was observed that in the OE-*ATG4a* group, the apoptosis rate of lens

epithelial cells was lower compared with that in the NC group, indicating that *ATG4a* may inhibit apoptosis (Fig. 4B and E). To further confirm this finding, a cell death detection assay was performed in the OE-*ATG4a* and NC groups. The results showed that, in the OE-*ATG4a* group, the percentage of TUNEL positive cells was lower compared with that in the NC group ($P < 0.001$) and the difference was significant, indicating that *ATG4a* can inhibit apoptosis (Fig. 4C and F).

Discussion

The present study for the first time, to the best of the authors' knowledge, demonstrated that *ATG4a*-mediated autophagy played an important role in the proliferation and apoptosis of lens epithelial cells, and that this might occur via the AMPK and Akt pathways.

H_2O_2 -induced lens epithelial cells have been widely used as a model for lens degradation and cataract formation, according to previous studies, 200 $\mu\text{mol/l}$ was chosen as the concentration (20,21). In the present study, this model was used to simulate the degradation of the lens in the eyes. TEM showed that the number and size of autophagosomes in the H_2O_2 group were significantly higher compared with those in the control group. The western blot analysis further validated the results; the protein expression of beclin was significantly increased and the protein expression of p62 was decreased after H_2O_2 treatment, suggesting that autophagy was significantly upregulated in H_2O_2 -treated lens epithelial cells. Furthermore, western blotting showed that the protein expression of *ATG4a* was upregulated in H_2O_2 -treated lens epithelial cells.

Briefly, *ATG4a* is an enzyme that can cleave LC3I to produce LC3II; It plays an important role in the formation of autophagic vesicle membranes (12). *ATG4a* is also the only protease encoded by the autophagy-related genes (22). The

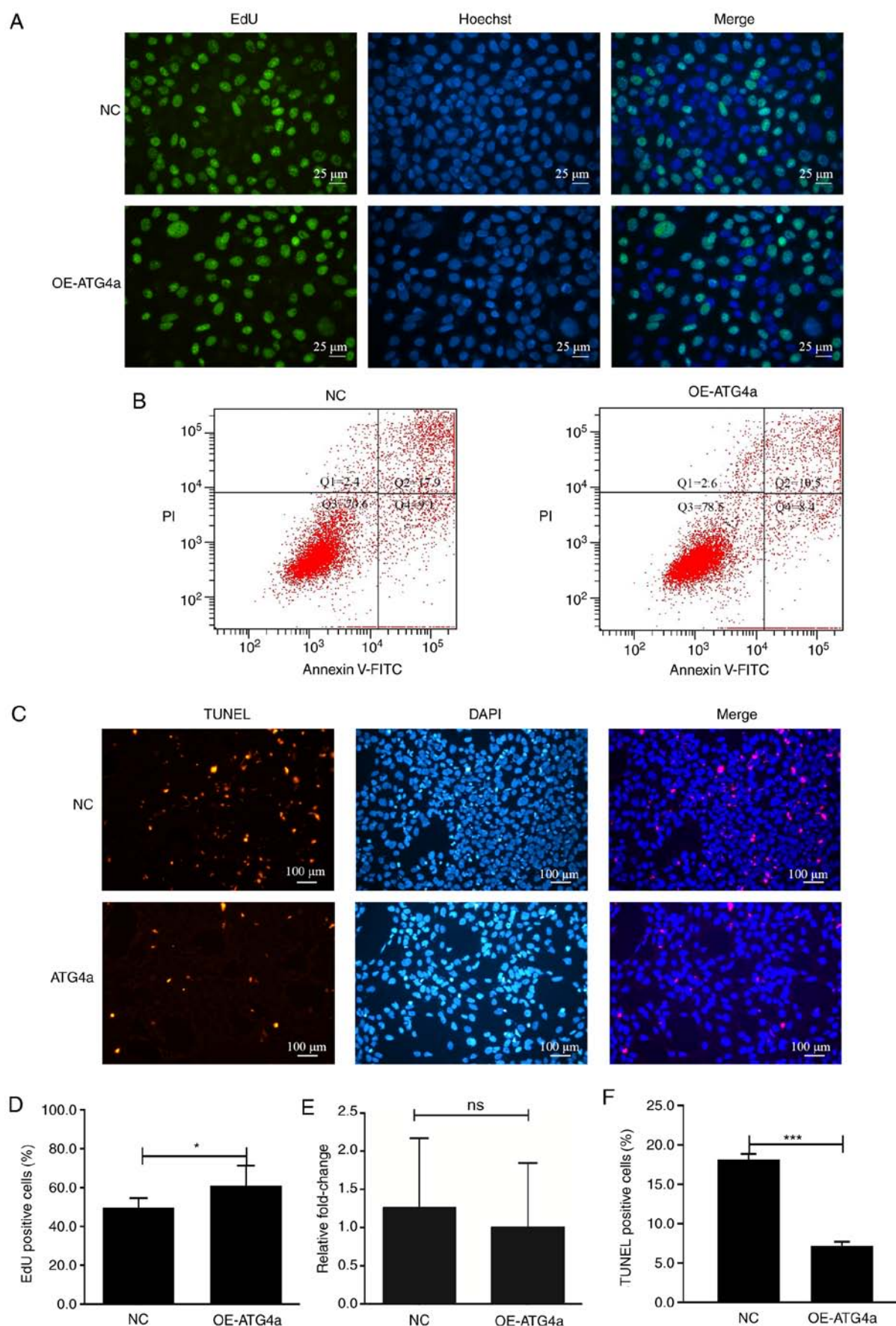


Figure 4. *ATG4a* promotes proliferation and inhibits the apoptosis of lens epithelial cells. (A) EdU incorporation assay in the OE-*ATG4a* and NC groups. (B) Flow cytometry results of Annexin V-FITC/PI staining in the OE-*ATG4a* and NC groups. (C) Cell death assay results of TUNEL staining in the OE-*ATG4a* and NC groups. (D) Quantification of EdU-positive cells between the OE-*ATG4a* and NC groups. (E) Quantification of Annexin V-FITC/PI staining between the OE-*ATG4a* and NC groups. (F) Quantification of TUNEL positive cells between the OE-*ATG4a* and NC groups. * $P < 0.05$, *** $P < 0.001$. ATG4a, autophagy-related protein 4; ns, no significance; NC, negative control; OE, overexpression group; PI, propidium iodide.

upregulation of *ATG4a* in H₂O₂-treated lens epithelial cells indicated that *ATG4a* may play a key role in lens degradation.

Previous studies have demonstrated that *ATG4a* can promote metastasis of tumor cells (23) and can be used as a target in chemotherapy of cancer (24). To explore the biological functions of *ATG4a* in lens epithelial cells, a model of *ATG4a* overexpression was constructed. TEM demonstrated that, compared with the NC group, the size of autophagosomes increased in the OE-*ATG4a* group. Several techniques including western blotting, immunofluorescence assay and a dual-fluorescence mRFP-eGFP-LC3 system can evaluate the function of effector proteins and autophagy flux (25,26). In the present study, western blotting demonstrated that the protein expression of beclin and LC3II/I was upregulated and that of p62 was downregulated in the OE-*ATG4a* group. TEM is considered one of the main and most important methods for the detection of autophagy (27,28). Furthermore, beclin, LC3II/I and p62 are routinely used as biomarkers to measure the rate and occurrence of autophagy (29-32). The results indicated that *ATG4a* could activate autophagy in lens epithelial cells.

The Akt and AMPK signaling pathways are important pathways in the autophagy process. Normally, Akt activates mammalian target of rapamycin complex 1, which is a negative regulator of autophagy, by inhibiting the tuberous sclerosis complex 1/2 (TSC1/TSC2) protein complex and thus inhibiting autophagy (33). In a previous study, the ratio of p-Akt/Akt in H9C2 cells was significantly reduced following exposure to H₂O₂ and triggered autophagy, indicating that the Akt pathway at least partly modulates autophagy (34). AMPK activation leads to the phosphorylation and activation of TSC1/2 and inhibition of mTOR; thus indicating that it is a positive regulator of autophagy (35). In the present study, the ratio of p-AMPK/AMPK was significantly increased and the ratio of p-Akt/Akt was decreased in the OE-*ATG4a* group compared with the NC group, indicating that these two pathways might be modulated by *ATG4a* in lens epithelial cells, but the exact mechanism needs further research. Nevertheless, the activation and inhibition of these two autophagy pathways further demonstrated that *ATG4a* can promote autophagy in lens epithelial cells.

Increasing evidence has demonstrated that cell proliferation and apoptosis are affected by autophagy (8,36,37). Studies have demonstrated that autophagy can promote cell growth (38,39), but the relationship between apoptosis and autophagy is complex and often appears contradictory (36,40). Normally, autophagy can maintain cell homeostasis under stressful conditions and prevent cell death (8), but in some neurodegenerative diseases, such as Alzheimer's disease, it can also be a pathogenic factor (37). Therefore, elucidating the exact functions of autophagy genes will improve our knowledge on how to protect or induce cell death. The present study demonstrated that *ATG4a* had a positive effect on cell proliferation, using the EdU incorporation assay. As for apoptosis, although the FITC/PI staining assay did not show a statistical difference between the two groups, the *in situ* cell death detection assay showed that the apoptosis rate in the OE-*ATG4a* group was significantly downregulated compared with the NC group, suggesting that *ATG4A* could inhibit apoptosis in lens epithelial cells.

Taken together, the present study demonstrated that *ATG4a* could induce autophagy in lens epithelial cells and that this might activate the AMPK and inhibit Akt pathways. Furthermore, *ATG4a* increased cell proliferation and decreased apoptosis, indicating that *ATG4a* plays an important role in lens degradation and cataract formation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CY performed all experiments and wrote the manuscript. JZ, YQ, FZ and LJ analyzed the experimental data. JZ designed the present study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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