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MEG3 is involved in the development of glaucoma through promoting the autophagy of retinal ganglion cells

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Abstract. – OBJECTIVE: In this study, we aimed at investigating whether MEG3 may be involved in the pathogenesis of glaucoma by regulating the autophagy of retinal ganglion cells (RGCs).

MATERIALS AND METHODS: We used qRT-PCR to detect the expression of MEG3 in RGC-5s cell line under high hydrostatic pressure. RGC-5s were transfected with a lentiviral vector to achieve MEG3 overexpression or knockdown. The influence of overexpression or inhibition of MEG3 on cell proliferation and apoptosis was observed using CCK-8 test and flow cytometry. After overexpression of MEG3 and/or knockdown of MEG3 or Beclin-1, detection of the expressions of autophagy-related and apoptosis-related proteins was performed using Western blot.

RESULTS: MEG3 expression level increased in RGC-5 cells under high hydrostatic pressure, while exogenously decreased MEG3 expression can reverse the impact of the high pressure on RGC-5 cells. Additionally, overexpression of MEG3 can improve Atg3 expression, promote cell apoptosis, inhibit cell proliferation, and enhance autophagy levels. Meanwhile, knockdown of Beclin-1 up-regulated Bcl-2 level.

CONCLUSIONS: Upregulation of MEG3 is involved in the pathogenesis of glaucoma through promoting apoptosis of retinal ganglion cells, the mechanism of which may be related to the enhanced autophagy levels.

Key Words:
Glaucoma, Ocular hypertension, MEG3, Autophagy, Apoptosis.

Introduction

Glaucoma is a neurodegenerative disease characterized by progressive loss of retinal ganglion cells and optic nerve axons, accompanied by loss of visual field sensitivity, which seriously threatens human health and quality of life[1]. Regardless of acute or chronic glaucoma, the most common and important risk factor for glaucoma is an increase of intraocular pressure. High intraocular pressure (HIOP) is a major feature of glaucoma resulting in damage to visual function[2]. Currently, the main clinical treatment of glaucoma is to lower intraocular pressure of patients with HIOP by drugs or surgery. However, such treatment showed poor response and/or low tolerance among some patients. Additionally, in some cases, although the intraocular pressure was significantly reduced, there would be a relapse of the visual impairment and fundus lesion[3,4]. Therefore, it was urgently required to better elucidate the pathogenesis of glaucoma in order to provide strategies for clinical treatment.

Long chain non-coding RNA (lncRNA), a class of non-coding RNA with longer than 200 nucleotides in length, was demonstrated to be involved in many regulatory processes, such as transcriptional regulation, post-transcriptional regulation, chromatin modification, genomic imprinting, transcriptional interference, cell cycle, and epigenetics[5]. MEG3, located at the DLK1-MEG3 locus of human chromosome 14q32.3, was first identified as a homologous marker gene of mouse Gt12[6]. MEG3 exerts low or no expression in lots of human tumor cells, including non-small cell lung cancer (NSCLC), hepatocellular carcinoma (HCC), glioma and gastric cancer[7-9]. In NSCLC and gastric cancer, decreased MEG3 expression level was associated with poor prognosis, while

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MEG3 overexpression inhibits the proliferation and promotes apoptosis of NSCLC cells[10,11]. This shows that the level of MEG3 expression is closely associated with cell proliferation. However, the role of MEG3 in glaucoma is rarely reported, and still needs further studied.

Autophagy is an evolutionarily highly conserved process of self-consumption in non-prokaryotes. Autophagy is an intracellular stress response that causes the degradation of intracellular macromolecules and damaged organelles into small molecules in lysosomes for cell re-use. Autophagy level is enhanced in the process of embryo development, weaning, hunger, and aging. In addition, much evidence show that autophagy pathway is a potential pathway for target therapy in many diseases, because autophagy plays an essential role in many cellular processes including cell homeostasis, growth, development, and disease occurrence. Of note, autophagy can be both a cellular self-defense mechanism and a programmed cell death mechanism (PCD)[12,13]. It has been reported in the literature[14] that in the glaucoma model of rat, autophagy level is highly enhanced. Furthermore, it is also suggested[15] that MEG3 can regulate the level of autophagy in the research of oncology.

Therefore, this study aimed at clarifying the effectiveness of MEG3 on the autophagy level and apoptosis of retinal ganglion cells, as well as the possible underlying mechanism in the pathogenesis of glaucoma.

Materials and Methods

Cell Culture, Transfection and Infection

Retinal ganglion cells (RGC-5 cells) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 1 g/L glucose, L-glutamic acid, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO_2. Lipofectamine 2000 reagent was used to transfect RGC-5 cells with Beclin1 siRNA (si-Becn1) and/or its negative control (si-Control). To achieve MEG3 overexpression or knockdown, cells were infected with lentivirus LV-MEG3 or LV-shMEG3, respectively.

Pressure System

To imitate the high intra-ocular pressure *in vitro*, cells of the experimental group were incubated in a pressure incubator of cell (PRINCE) heated to 36°C, while cells of the control group were cultured in a conventional incubator. After exposed to 30 mmHg pressure for 24 hours, cells were harvested for detection.

Western Blotting

Cells were collected to determine the protein concentration by Bradford. Then, the proteins were separated by 10% or 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose membrane. The immunoblots were blocked with 5% non-fat milk and incubated with primary antibodies diluted 1:l000 for 3 h at room temperature. After that, the immunoblots were washed in 1 x Tris-buffered saline-Tween (TBS-T) for 15 min then incubated with Horseradish Peroxidase (HRP)-labeled secondary antibody diluted 1:2000 for 1 h at room temperature for AEC staining. Images were scanned and quantified using Labworks software. The experiment was repeated 3 times.

Real-Time Quantitative Polymerase Chain Reaction (PCR)

Total RNA was isolated by TRIzol reagent and reverse transcribed to cDNA. The relative mRNA levels of genes were detected by Real-time quantitative PCR with the SYBR Green kit. Three replicates were set for each group. The reaction conditions were as follows: 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C and 10 min at 72°C, then extension at 72°C for 10 min. Primer sequences are listed below: MEG3 F: TCCTCACCTCCAATTTCCCCT R: GAGCGAGAGCCGTTCGATG; Bcl-2 F: GGTGGGGTCATGTGTGTGG R: CGGTTCAGGTACTCAGTCATCC; GAPDH: F: AGGTCGGTGTGAACGGATTTG R: GGGGTCGTTGATGGCAACA; Atg3 F: GACCCCGGTCCTCAAGGAAR:TGTAGCCCATTGCCATGTTGG; Atg8 F: TCCCCGGAACGAGGAACTC R: TTCGCTCCACAGCCCATTTC; Atg12 F: CTGCTGGCGACACCAAGAAA R: CGTGTTCGCTCTACTGCCC.

Cell Counting Kit-8 (CCK-8) Assay

Cells infected with lentivirus, as well as negative controls, were digested and seeded (5×10^4/well) into 96-well plates. After that, cells were cultured overnight in a 5% CO_2 incubator. A mixture of 10 μL CCK-8 reagent and 90 μL serum-free medium were added to each well at 0 h, 24 h, 48 h, and 72 h, respectively. Then, cells were cultured for 30 min, and the absorbance at 450 nm was detected with a microplate reader.

Flow Cytometry Analysis

The cell apoptosis rate and cell cycle were analyzed by flow cytometry respectively. Cell apoptosis rate was measured with the Annexin V-FITC (fluorescein isothiocyanate) kit. Generally, cells were harvested and resuspended in buffer solution. 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) staining solution were then added for further incubation for 15 min at room temperature in the dark. Stained cells were analyzed using a FACS flow cytometer.

Meanwhile, cells were digested into a single cell suspension by trypsin. 1×10^6 cells each group were washed twice with phosphate-buffered saline and centrifuged at 800 r/min for 5 minutes. Then, we discarded the supernatant while the cells were fixed with 70% cold ethanol at 4°C overnight. In the next day, cells were washed with phosphate-buffered saline and centrifuged. The supernatant was discarded, and the cells were stained with PI for 10 min. The cell cycle was analyzed by a FACS flow cytometer.

Statistical Analysis

Statistical product and service solutions (SPSS13.0, SPSS Inc., Chicago, IL, USA) statistical software was used to analyze the results. Data were expressed as mean ± SD. The Student's *t*-test was applied to analyze the statistical significance of differences between groups. $p<0.05$ was considered as statistically significant.

Results

MEG3 Promotes Apoptosis in RGC-5

To investigate the specific role of MEG3 in RGC-5, cells were infected with lentivirus LV-MEG3 or LV-shRNA respectively to achieve MEG3 overexpression or knockdown (Figure 1A). The CCK8 assay showed that MEG3 overexpression significantly inhibited the proliferation of RGC-5, whereas MEG3 knockdown could promote the proliferation of RGC-5 (Figure 1B). In addition, flow cytometry analysis indicated that

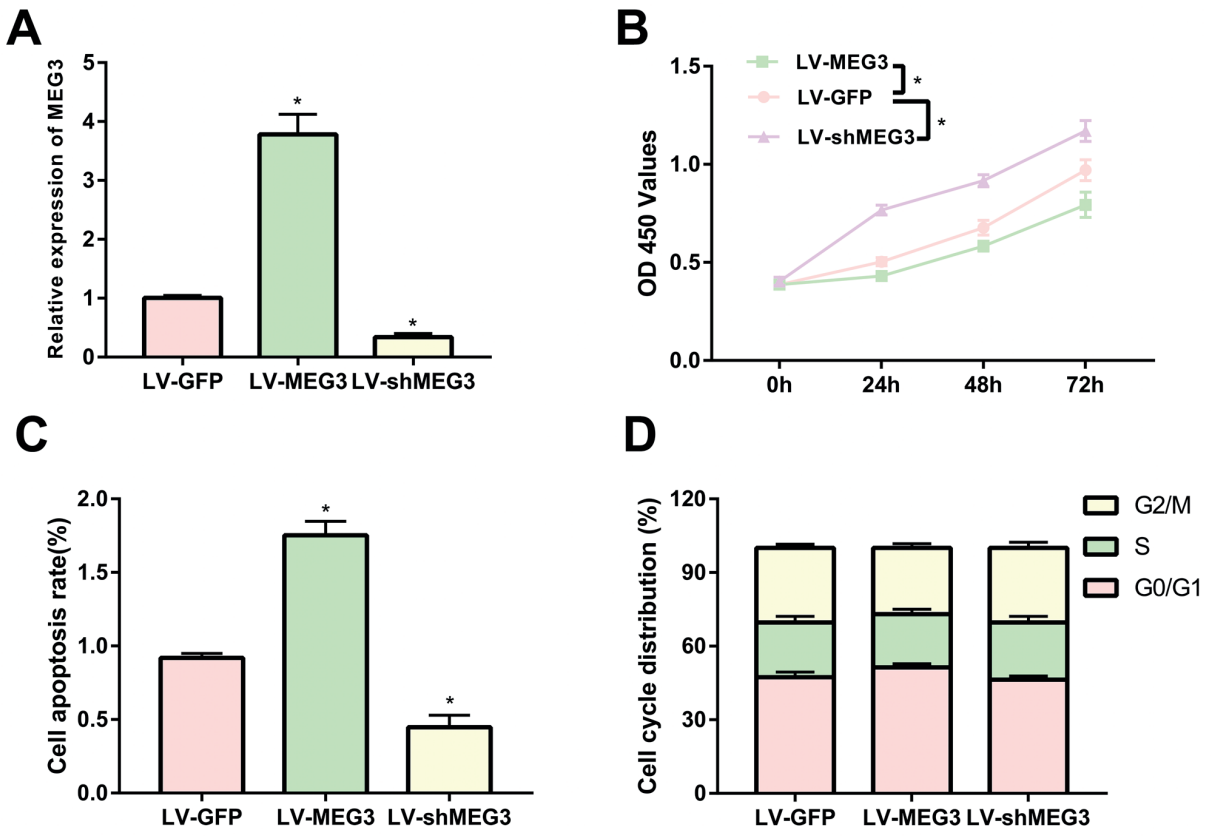

Figure 1. MEG3 promotes apoptosis in RGC-5. ***A,*** MEG3 expression was detected after infection of LV-MEG3 or LV-shMEG3 as well as their LV-GFP, respectively; ***B,*** The effect of MEG3 on cell proliferation was detected by CCK8 assay; ***C, D,*** The effect of MEG3 on apoptosis and cell cycle was detected by flow cytometry assay.

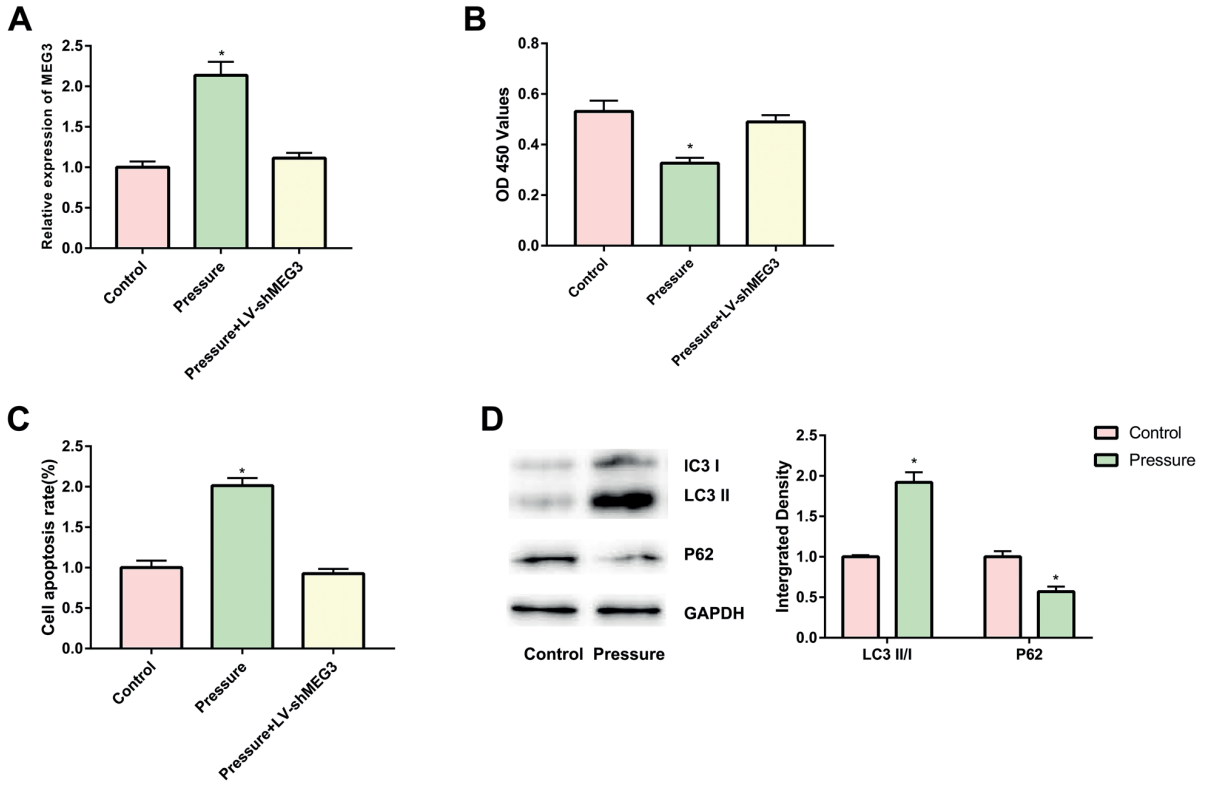

Figure 2. Elevated pressure induces apoptosis and autophagy in RGC-5. ***A,*** MEG3 expression was detected by qPCR after administration of pressure as well as infection of LV-shMEG3; ***B,*** Cell proliferation was detected by CCK8 assay after administration of pressure and infection of LV-shMEG3; ***C,*** Apoptosis was detected by flow cytometry after administration of pressure and infection of LV-shMEG3; ***D,*** Autophagy-related gene expression was detected by Western blot after administration of pressure and infection of LV-shMEG3.

high expression of MEG3 promoted apoptosis of RGC-5, while the knockdown of MEG3 could inhibit apoptosis of RGC-5 (Figure 1C). However, the cell cycle of RGC-5 was not affected by either overexpression or knockdown of MEG3 (Figure 1D). These *in vitro* results suggested that MEG3 promoted apoptosis in RGC-5 and may play an important role in glaucoma.

Elevated Pressure Induces Apoptosis and Autophagy in RGC-5

To imitate the high intra-ocular pressure *in vitro*, RGC-5 cells were incubated in a pressure incubator. Quantitative PCR results showed that the MEG3 expression was significantly increased with a pressure of 30 mmHg for 24 h. However, this elevation was inhibited by LV-shMEG3 infection (Figure 2A). Meanwhile, pressure treatment significantly inhibited cell proliferation and promoted apoptosis in RGC-5. In contrast, MEG3 knockdown by LV-shMEG3 could decrease apoptosis and restore proliferation in RGC-5 (Figure 2B-C). Besides, Western blot analysis indicated that pressure treatment significantly increased the expression of autophagy-related proteins, including LC3 II/LC3 I and P62, whereas MEG3 knockdown in RGC-5 reversed this elevation (Figure 2D). This suggested that autophagy plays an important role in glaucoma apoptosis.

MEG3 Promotes Autophagy in RGC-5

To explore MEG3's involvement in autophagy, we examined the expression of autophagy-related genes after MEG3 overexpression or knockdown in RGC-5. Results showed that MEG3 overexpression increased the mRNA level of Atg3, while MEG3 knockdown inhibited autophagy-related gene expression (Figure 3A). Western blot analysis further confirmed this phenomenon (Figure 3B). Further analysis indicated that the expression of bcl-2, an inhibitor of apoptosis, was negative-

ly correlated to MEG3 expression (Figure 3C). These data implied that MEG3 may exert a great influence on autophagy of RGC-5. To further investigate the relationship between apoptosis and autophagy of RGC-5 cells, we detected the levels of bcl-2 by qPCR and Western blot after silencing Beclin-1 by siRNA, which is a key regulatory protein in autophagy. We found that bcl-2 expression was significantly increased after silencing of Beclin-1 (Figure 3D-E). These above results suggested that MEG3 could promote autophagy by regulating the expression of Atg3, which could further induce apoptosis in RGC-5.

Discussion

Glaucoma is an ocular disease caused by high intraocular pressure, which could lead to optic nerve damage and visual field defects. The basic pathology of optic nerve damage is the progressive death of retinal ganglion cells (RGCs). In China, approximately 5.3%-21% of blindness was caused by glaucoma among all the blind people[16]. It has been demonstrated[17] that acute or chronic HIOP can lead to visual impairment, apoptotic death of RGCs, and retinal pigment epithelium (RPE) cells, which ultimately leads to blindness.

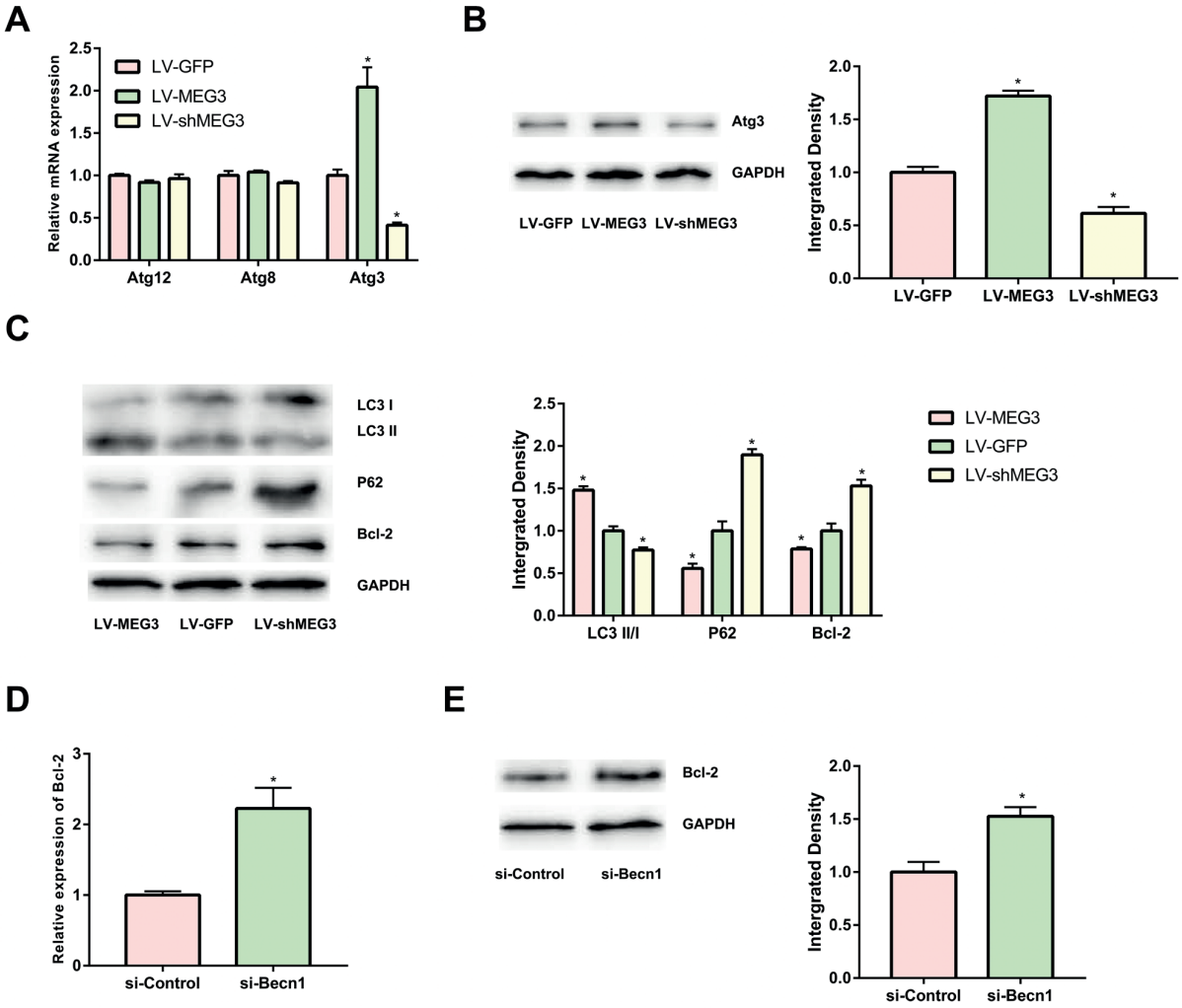

Figure 3. MEG3 promotes autophagy in RGC-5. ***A,*** mRNA expressions of Atg3, Atg8 and Atg12 were detected after infection of LV-MEG3, LV-shMEG3 and LV-GFP, respectively; ***B,*** Protein level of Atg3 was detected by Western blot after infection of LV-MEG3, LV-shMEG3 and LV-GFP, respectively; ***C,*** Autophagy-related protein levels were detected by Western blot after infection of LV-MEG3, LV-shMEG3 and LV-GFP respectively; ***D,*** The mRNA expression of Bcl-2 was detected by qPCR after transfection of si-Beclin-1; ***E,*** The protein level of Bcl-2 was detected by Western blot after transfection of si-Beclin-1.

Cell lines cultured with elevated hydrostatic pressure (EHP) are commonly used as *in vitro* cellular models of glaucoma[18]. Previous research[19] has demonstrated that a pressure of 30 and 100 mmHg could lead to apoptosis of cultured RGCs and many other neural cells. RGC-5 cell line has been widely used as a kind of transformed RGCs in scientific research. It has many characteristics of RGCs, including expression of Brn-3C, NMDA receptor, GABA-B receptor, etc., as well as no expression of GFAP, HPC-1 and 8A-1[20].

Deng et al[21] observed an elevation of LC3 II expression as well as LC3 II/LC3 ratio in chronic HIOP glaucoma model of the rhesus monkey, while Park et al[14] found the same elevation of autophagy-related proteins in retinal ganglion cells (RGC) of glaucoma model in SD rats. The autophagy in SD rats maintained at high levels for at least 8 weeks, indicating that RGC apoptosis in chronic glaucoma may be closely related to autophagy activation[21]. Transmission electron microscope showed that the autophagy was first activated in the dendritic part of RGC, then in the cytoplasm, which confirmed that autophagy occurred in a certain order in RGC neurons. In this study, we found that MEG3 could activate autophagy, and this sustained high level of autophagy may eventually promote cell apoptosis. Bcl-2 family is a type of protein which plays an important role in apoptosis. Bcl-2 was first found in follicular B-cell lymphoma, which could be activated by the chromosomal [t (14:18) (q32: q21)] translocation[22,23]. Bcl-2 expression decreases during apoptosis while overexpression of bcl-2 inhibits many apoptotic events induced by various factors. During apoptosis, driven by apoptosis-related factors, the pro-apoptotic proteins of bcl-2 family translocate to the mitochondrial membrane, which destroys the integrity of mitochondria, promotes the release of mitochondrial pro-apoptotic factors, eventually leading to apoptosis[24]. Autophagy is a metabolic mechanism that occurs in eukaryotic cells to maintain the homeostasis of the cell structure, which is a highly conserved catabolism of eukaryotes during evolution[25]. During autophagy, intracellularly damaged organelles and macromolecular substances are delivered to lysosomes for biodegradation *via* a bilayer membrane structure formed by two ubiquitin-like binding systems (Atg5-Atgl2 and Atg8/LC3)[26].

Our results indicated that MEG3 was involved in the regulation of autophagy by regulating the expression of Atg3. Meanwhile, we found that MEG3 overexpression could inhibit the expression of bcl-2, thereby promoting cell apoptosis. Both apoptosis and autophagy are the basic physiological mechanisms essential for the maintenance of homeostasis. However, the relationship between them has not been fully elucidated. A current research suggests that apoptosis and autophagy could mutually promote each other. Gorski et al[27] used a steroid to induce cell death in Drosophila and found that the expressions of both apoptotic and autophagy genes were up-regulated. In addition, it was found that common regulatory molecules were shared between apoptosis and autophagy. It is suggested that autophagy might promote cell apoptosis due to the degradation of survivin and other anti-apoptotic factors by the autophagy lysosomal pathway. Besides, it was demonstrated that autophagy might play a dual role on the cell apoptosis. In this study, we found that bcl-2 expression was upregulated after inhibiting autophagy by silencing Beclin-1, suggesting that over-activated autophagy may promote the occurrence of apoptosis in retinal ganglion cells through inhibition bcl-2.

Conclusions

MEG3 might be involved in the occurrence of glaucoma. Overexpression of MEG3 could promote the expression of Atg3, thereby activating autophagy in retinal ganglion cells and eventually leading to apoptosis.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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