

# Autophagy regulates TGF- $\beta$ 2-induced epithelial-mesenchymal transition in human retinal pigment epithelium cells

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**Abstract.** Transforming growth factor (TGF)- $\beta$ 2-induced epithelial-mesenchymal transition (EMT) in human retinal pigment epithelium (RPE) cells has an important role in the pathophysiology of intraocular fibrotic disorders, which may cause vision impairment and blindness. Autophagy, an intracellular homeostatic pathway, contributes to the physiological and pathological processes of RPE. Furthermore, autophagy has previously been reported to function in the EMT process in numerous tissue and cell types. However, the association between autophagy and the EMT process in RPE cells has not yet been fully determined. The present study demonstrated that TGF- $\beta$ 2-treated human RPE cells (ARPE-19 cell line) exhibited a significantly increased autophagic flux compared with control cells, as determined by western blot analysis of the protein levels of microtubule-associated protein 1 light chain 3-II and p62 (also termed sequestosome 1). Furthermore, it was demonstrated that autophagy activation enhanced the TGF- $\beta$ 2-induced EMT process in ARPE-19 cells, and inhibition of autophagy by chloroquine administration attenuated TGF- $\beta$ 2-induced EMT, which was determined by analyzing the expression of mesenchymal and epithelial markers by reverse transcription-quantitative polymerase chain reaction and/or western blotting. A transwell migration and invasion assays was also performed that demonstrated that autophagy activation by rapamycin enhanced TGF- $\beta$ 2-stimulated RPE cell migration and invasion, and inhibition of autophagy reduced TGF- $\beta$ 2-stimulated RPE cell migration and invasion. These results also demonstrated that autophagy activation enhanced the TGF- $\beta$ 2-induced EMT process in ARPE-19 cells, and inhibition of autophagy attenuated TGF- $\beta$ 2-induced

EMT. Overall, the results of the present study demonstrated that TGF- $\beta$ 2-induced EMT may be regulated by autophagy, thus indicating that autophagy may serve as a potential therapeutic target for the attenuation of EMT in intraocular fibrotic disorders.

## Introduction

The retinal pigment epithelium (RPE) is composed of a simple layer of pigmented cells and is an important element of the blood-retina barrier. Dysfunction of the RPE may result in retinal degeneration, visual function loss and blindness (1). The epithelial-mesenchymal transition (EMT) of the RPE is implicated in various intraocular fibrotic disorders, including proliferative vitreoretinopathy (2-5), proliferative diabetic retinopathy (6) and wet age-related macular degeneration (AMD) (7). Fibrotic lesions induce retinal detachment and result in severe visual impairment. However, the complex underlying mechanisms of the EMT process in the RPE (RPE-EMT) are yet to be fully determined.

Numerous studies have reported that autophagy is involved in the EMT process in numerous tissue and cell types, including human malignant glioma cells, atrial myofibroblasts and annulus fibrosus cells (8-10). Furthermore, autophagy has been demonstrated to exert beneficial and non-beneficial effects on EMT, which may be dependent on the associated cell type (11-14). However, to the best of our knowledge, it has not previously been determined whether autophagy activity is involved in the process of RPE-EMT.

Autophagy is an intracellular homeostatic pathway that assists in the degradation and recycling of proteins and cellular organelles (15). Autophagy is activated in response to metabolic stress and other variations within the microenvironment (8). Autophagy is crucial for the maintenance of RPE homeostasis. Autophagy aids the degradation of cytotoxic protein aggregates in RPE cells that result from stimulation by photo-oxidative stress and inflammation (16). In RPE cells, autophagy is involved in the prevention of cytotoxic protein aggregates; however, this capacity may be decreased in senescent or stressed RPE cells, and lipofuscin accumulation into lysosomes may be induced by decreased autophagy. The formation of lipofuscin impairs the autophagic clearance of protein aggregates and induces further RPE damage, which may contribute to AMD progression or alternative pathological development (17-19).

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RPE-EMT is an important pathological process in intra-ocular fibrotic disorders that results in severe non-reversible retinal pathological development. Therefore, it is important to determine the role of autophagy in the process of RPE-EMT. The present study aimed to investigate whether autophagy deficiency may induce or modulate the EMT process in RPE cells. It is well established that transforming growth factor (TGF)- $\beta$ 2 is a potent inducer of EMT; TGF- $\beta$ 2-induced EMT has been reported by numerous studies using *in vitro* EMT models (20–22). In the present study, the role of autophagy in TGF- $\beta$ 2-induced EMT in human RPE (ARPE-19 cell line) cells was investigated. The results demonstrated that the administration of TGF- $\beta$ 2 induced autophagy in ARPE-19 cells. Furthermore, the activation of autophagy enhanced the RPE-EMT process, while inhibition of autophagy attenuated the RPE-EMT process, in ARPE-19 cells.

## Materials and methods

**Cell culture and cell treatment.** The ARPE-19 human RPE cell line was obtained from State Key Laboratory of Ophthalmology (Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China). The cells were cultured in complete medium composed of Dulbecco's modified Eagle's medium (DMEM; GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 1% penicillin-streptomycin solution (GIBCO; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (GIBCO; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO<sub>2</sub> humidified atmosphere. The cells were routinely passaged at a confluence between 80–90% for ~3–4 days and dissociated with 0.25% trypsin-EDTA solution (GIBCO; Thermo Fisher Scientific, Inc.). The ARPE-19 cells were subsequently transferred to a 6-well plate. After reaching 70–80% confluence, the medium was replaced with fresh medium prior to drug treatment. Following this, recombinant human TGF- $\beta$ 2 (2, 5 or 10 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) was added to the cells with or without chloroquine (50  $\mu$ M; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 3-methyladenine (3-MA; 5 mM; Sigma-Aldrich; Merck KGaA) and rapamycin (200 nM; Sigma-Aldrich; Merck KGaA) for 0–36 h. The control cells were treated with medium only without drugs. All the cells were cultured at 37°C in 5% CO<sub>2</sub> humidified atmosphere.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from cultures using the RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), in accordance with the manufacturer's protocol. RNA concentration was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Following this, total RNA (2  $\mu$ g) was reverse transcribed using the Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). The reaction mixture was incubated for 10 min at 25°C followed by 30 min at 50°C, and the reaction was terminated by heating at 85°C for 5 min. qPCR was subsequently performed using the StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using the PowerUp SYBR Green Master Mix (Thermo Fisher

Scientific, Inc.), according to the manufacturer's protocol. The reaction is as follows: 50°C for 2 min for UDG activation and 95°C for 2 min for the activation of dual-lock DNA polymerase. The PCR amplification was then performed for 40 cycles by denaturing the cDNA template at 95°C for 3 sec and annealing/extension at 60°C for 30 sec. The dissociation curve includes 95°C for 15 sec, 60°C for 1 min and 95°C for 15 sec. The primers (Thermo Fisher Scientific, Inc.) used were as follows: Human neural (N)-cadherin, 5'-AGCCAACCTTAACTGAGGAGT-3' (forward) and 5'-GGCAAGTTGATTGGA GGGATG-3' (reverse); human vimentin, 5'-AGTCCACTG AGTACCGGAGAC-3' (forward) and 5'-CATTTTCACGCAT CTGGCGTTC-3' (reverse); human fibronectin, 5'-CGGTGG CTGTCAGTCAAAG-3' (forward) and 5'-AAACCTCGGCTT CCTCCATAA-3' (reverse); human GAPDH, 5'-CTGGGCTAC ACTGAGCACC-3' (forward) and 5'-AAGTGGTCGTTGAGG GCAATG-3' (reverse). All samples were tested and normalized to the reference gene GAPDH. All experiments were performed in triplicate. Results were normalized to GAPDH according to the  $2^{-\Delta\Delta C_q}$  relative quantification method (23).

**Western blot analysis.** Total protein was extracted from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) with 1 mM phenyl-methylsulfonyl fluoride (Beyotime Institute of Biotechnology) on ice for 20 min. Protein was subsequently quantified using a BCA Protein Assay kit (Beyotime Institute of Biotechnology). Following protein quantification, 20  $\mu$ g of each sample was run on a 12 or 8% SDS-PAGE gel and proteins were transferred to polyvinylidene difluoride membranes (Merck KGaA, Darmstadt). Following blocking with 5% bovine serum albumin (MP Biomedicals, LLC., Santa Ana, CA, USA) for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: Rabbit anti-microtubule-associated protein 1 light chain (LC3) 3 $\alpha$ / $\beta$  (1:1,000; cat. no. 12741S; Cell Signaling Technology, Inc., Danvers, MA, USA; differences in molecular weight was used to distinguish between the cytosolic and membrane bound forms), rabbit anti-beclin 1 (1:1,000; cat. no. 3495S, Cell Signaling Technology, Inc.), rabbit anti-p62 (also termed sequestosome 1; 1:1,000; cat. no. 8025S; Cell Signaling Technology, Inc.), rabbit anti-vimentin (1:1,000; cat. no. 5741; Cell Signaling Technology, Inc.), mouse anti- $\beta$ -actin (1:1,000; cat. no. 3700; Cell Signaling Technology, Inc.) and rabbit anti-fibronectin (1:1,000; cat. no. ab6328; Abcam, Cambridge, MA, USA); and rabbit anti-N-cadherin (1:1,000; cat. no. 04-1126; Merck KGaA, Darmstadt, Germany). Following washing with Tris-buffered saline solution containing 0.05% Tween-20 buffer, the membranes were incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2,000) for 1 h at room temperature (anti-mouse secondary antibody cat. no. sc-2005; anti-rabbit secondary antibody cat. no. sc-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Luminol reagent was used as the visualization reagent (Merck KGaA) and blots were analyzed using the FluorChem Q system (ProteinSimple, San Jose, CA, USA). ImageJ v10.2 (National Institutes of Health, Bethesda, MD, USA) was used for quantification of the bands relative to  $\beta$ -actin expression and normalization to total protein loaded in each lane.

**Transwell migration and Matrigel invasion assays.** Transwell Chambers with 8.0- $\mu$ m pore inserts (Corning Incorporated, Corning, NY, USA) were used to investigate RPE cell migration and invasion. To perform Transwell migration assays,  $1 \times 10^5$  ARPE-19 cells were placed in the upper chamber with a volume of 200  $\mu$ l serum-free DMEM. Subsequently, 5 ng/ml TGF- $\beta$ 2, 50  $\mu$ M chloroquine, 5 ng/ml TGF- $\beta$ 2 + 50  $\mu$ M chloroquine, 200 nM rapamycin or 5 ng/ml TGF- $\beta$ 2 + 200 nM rapamycin was added to the upper chamber in each group. A total of 600  $\mu$ l DMEM with 10% fetal bovine serum was added to the lower chamber of each well. Following 24 h incubation at 37°C, the non-migrating cells were scraped off with a cotton swab, and the migrated cells on the lower surface were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.1% crystal violet for 1 h at room temperature. To assess the average number of migrating cells, images (magnification, x100) were captured with an inverted light microscope (Zeiss Observer A1, Oberkochen, Germany) and cells were counted in five randomly selected fields with Adobe Photoshop CC 2015 (Adobe Systems, Inc., San Jose, CA, USA). All experiments were repeated three times. In order to perform the Transwell invasion assays, Matrigel was melted at 4°C and diluted using serum-free DMEM (1:3). Subsequently, 40  $\mu$ l diluted Matrigel was added to the Transwell chamber inserts, which were then placed in the incubator at 37°C for 4 h to coagulate. The remainder of the assay was performed in accordance with the aforementioned migration assay protocol.

**Statistical analysis.** Data presented in the figures are representative of three repetitions. Data were analyzed by one-way analysis of variance with Tukey's post-hoc test using GraphPad Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean  $\pm$  standard deviation.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**TGF- $\beta$ 2 induces autophagy in cultured ARPE-19 cells.** To determine whether autophagy is modulated during EMT, the effects of TGF- $\beta$ 2 on autophagy were investigated by measuring the protein expression of LC3, beclin-1 and p62 using western blot analysis (Fig. 1). LC3-phosphatidylethanolamine conjugate (LC3-II) is the lipidated form of the cytosolic form of LC3 (LC3-I), and the conversion from LC3-I to LC3-II represents the formation of autophagosomes (24). p62 combines polyubiquitinated proteins and forms the completed autophagosomes, which are subsequently degraded into autolysosomes; therefore, the quantity of p62 may be considered an index of autophagic degradation (24). As demonstrated in Fig. 1A and B, the administration of TGF- $\beta$ 2 increased the expression of LC3-II at 12, 24 and 36 h post-treatment (Fig. 1B), compared with the control group. However, compared with the control group, the protein expression of p62 was significantly decreased following TGF- $\beta$ 2 administration in a time-dependent manner between 0 and 36 h (Fig. 1A and C). The protein expression of LC3-II also increased following treatment of cells with different concentrations of TGF- $\beta$ 2, compared with the control group (2, 5 and 10 ng/ml; Fig. 1D and E), while p62 expression was

significantly decreased following administration of TGF- $\beta$ 2 in a dose-dependent manner between 2 and 10 ng/ml, compared with the control group (Fig. 1D and F). No marked alterations in beclin-1 expression were observed across treatment groups (Fig. 1A and D). These results indicate that administration of TGF- $\beta$ 2 induced autophagy in ARPE-19 cells.

**Effects of autophagy inhibitors and inducers on autophagy-associated protein expression in cultured ARPE-19 cells.** To validate the effects of autophagy inhibitors and inducers on autophagy-associated protein expression in ARPE-19 cells, ARPE-19 cells were treated with TGF- $\beta$ 2 (5 ng/ml) with or without chloroquine (50  $\mu$ M), 3-MA (5 mM) or rapamycin (200 nM) for 24 h, and the protein expression levels of LC3 and p62 were analyzed by western blotting. As demonstrated in Fig. 2, administration of chloroquine resulted in the accumulation of LC3-II and the inhibition of p62 degradation, thus demonstrating an inhibitory effect with regards to autophagy. Although administration of chloroquine led to LC3-II accumulation rather than reduced LC3-II expression, accumulation of LC3-II occurs as the autophagosome-lysosome fusion at the post-sequestration step following LC3-II formation is inhibited by chloroquine, which represents inhibition of autophagy (24). 3-MA is a selective phosphatidylinositol 3-kinase inhibitor. The effect of 3-MA on autophagy is conditional and depends on the time and the environment of application (24). In the present study, administration of 3-MA decreased the expression levels of LC3-II and p62, therefore demonstrated the increase of autophagic flux and demonstrated the activation of autophagy (Fig. 2). Rapamycin is a mechanistic target of rapamycin inhibitor and predominantly functions as an autophagy inducer (24). Administration of rapamycin increased LC3-II expression and p62 degradation, therefore indicating that rapamycin activated autophagy (Fig. 2).

**Inhibition of autophagy suppresses TGF- $\beta$ 2-induced EMT.** As autophagy is activated during TGF- $\beta$ 2-induced EMT in ARPE-19 cells, the association between the EMT process and autophagy level was investigated. Furthermore, whether the modulation of autophagy may influence the EMT process was also investigated. ARPE-19 cells were treated with TGF- $\beta$ 2 (5 ng/ml) with or without chloroquine (50  $\mu$ M) for 24 h, total RNA was collected and the mRNA expression levels of N-cadherin, vimentin and fibronectin mesenchymal markers were determined. As revealed in Fig. 3A, chloroquine administration decreased the mRNA expression levels of all three of these mesenchymal markers. Furthermore, the protein expression levels of mesenchymal markers (N-cadherin and vimentin) and the epithelial marker epithelial (E)-cadherin in ARPE-19 cells were investigated (Fig. 3B and C). It was demonstrated that chloroquine administration decreased the expression levels of N-cadherin and vimentin, and increased the expression level of E-cadherin. Additionally, the effect of chloroquine administration on RPE cell migration and invasion was also investigated (Fig. 3D-G). It was demonstrated that administration of TGF- $\beta$ 2 enhanced the migratory and invasive potential of the RPE cells compared with control cells, and treatment with chloroquine significantly reduced TGF- $\beta$ 2-induced RPE cell migration and invasion. These



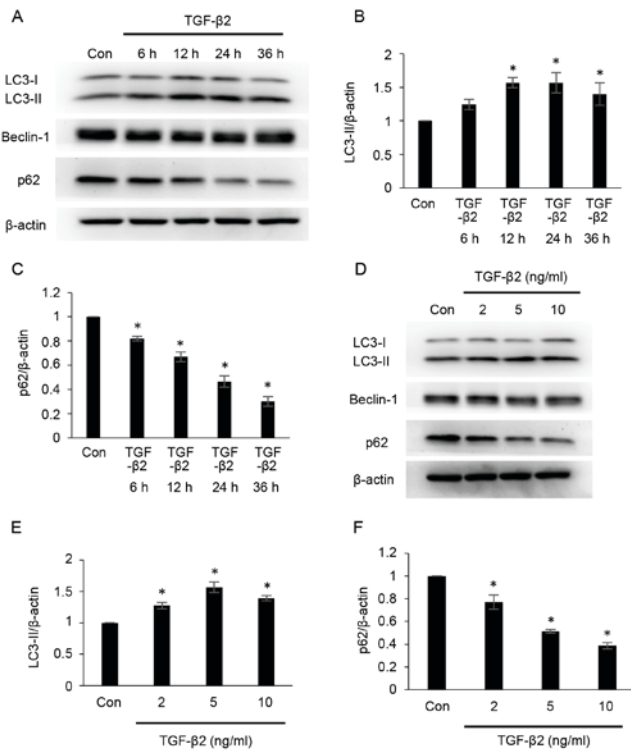


Figure 1. TGF- $\beta$ 2 induces autophagy in cultured ARPE-19 cells. (A) ARPE-19 cells were treated with TGF- $\beta$ 2 (5 ng/ml) for 0-36 h. Protein expression levels of LC3-II, beclin-1 and p62 were analyzed by western blotting. Protein loading was confirmed using  $\beta$ -actin. Densitometric analysis of (B) LC3-II and (C) p62 protein expression levels in ARPE-19 cells treated with TGF- $\beta$ 2 (5 ng/ml) for 0-36 h. (D) ARPE-19 cells were cultured with various concentrations of TGF- $\beta$ 2 (2, 5 and 10 ng/ml) for 12 h. LC3-II, beclin-1 and p62 protein expression levels were analyzed by western blotting. Densitometric analysis of (E) LC3-II and (F) p62 protein expression levels in ARPE-19 cells treated with TGF- $\beta$ 2 (2, 5 and 10 ng/ml) for 12 h. For each experiment, LC3-II and p62 expression levels were normalized to corresponding  $\beta$ -actin expression levels. Bars represent the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05 vs. Con. TGF- $\beta$ 2, transforming growth factor- $\beta$ 2; LC3, microtubule-associated protein 1 light chain; LC3-II, LC3-phosphatidylethanolamine conjugate; p62, sequestosome 1; LC3-I, cytosolic form of LC3; Con, control.

results indicated that inhibition of autophagy may suppress the EMT process that is induced by TGF- $\beta$ 2 in RPE cells.

**Increased levels of autophagy exacerbates TGF- $\beta$ 2-induced EMT.** To determine whether stimulation of autophagy affects EMT, the effects of TGF- $\beta$ 2 stimulation in ARPE-19 cells in the presence of autophagy inducers were investigated. ARPE-19 cells were treated with TGF- $\beta$ 2 (5 ng/ml) with or without 3-MA (5 mM), an autophagy stimulator, or rapamycin (200 nM), for 24 h. The results demonstrated that administration of 3-MA and rapamycin increased the mRNA expression levels of N-cadherin, vimentin and fibronectin mesenchymal markers (Fig. 4A and B). Furthermore, cells treated with rapamycin demonstrated increased protein expression levels of N-cadherin and vimentin mesenchymal markers, and decreased expression levels of the epithelial marker E-cadherin (Fig. 4C and D). In addition, the effect of rapamycin administration on RPE cell migration and invasion was investigated (Fig. 4E-H). It was revealed that treatment with rapamycin significantly enhanced TGF- $\beta$ 2-induced increases in RPE cell

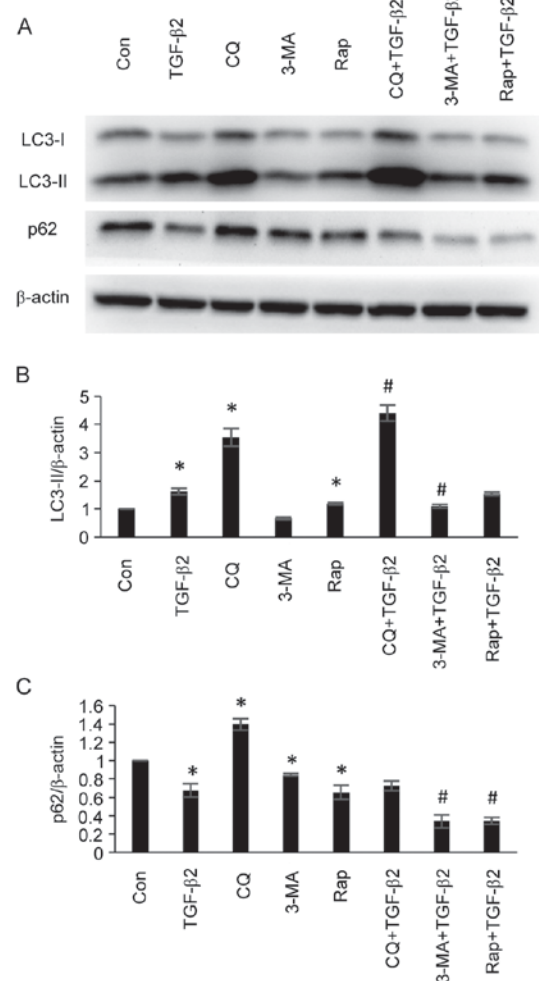


Figure 2. Effects of the administration of chloroquine, 3-MA and rapamycin on the expression levels of autophagy-associated proteins in ARPE-19 cells. (A) ARPE-19 cells were treated with TGF- $\beta$ 2 (5 ng/ml) with or without chloroquine (50  $\mu$ M), 3-MA (5 mM) and rapamycin (200 nM) for 24 h. The protein expression levels of LC3-II and p62 were analyzed by western blotting. Administration of chloroquine did not decrease LC3-II expression, however, accumulation of LC3-II was observed as chloroquine interrupted the autophagosome-lysosome fusion at the post-sequestration step in autophagy following LC3-II formation. Chloroquine inhibited the degradation of p62 and demonstrated an inhibitory effect with regards to autophagy. Administration of 3-MA decreased the expression levels of LC3-II and p62, which indicated that autophagy was induced. Administration of rapamycin increased LC3-II expression and p62 degradation, thus indicating that rapamycin administration induced the activation of autophagy. Densitometric analysis of (B) LC3-II and (C) p62 protein expression levels in ARPE-19 cells. Bars represent the mean  $\pm$  standard deviation of three independent experiments. \* $P$ <0.05 vs. Con; # $P$ <0.05 vs. TGF- $\beta$ 2 group. 3-MA, 3-methyladenine; TGF- $\beta$ 2, transforming growth factor- $\beta$ 2; LC3, microtubule-associated protein 1 light chain; LC3-II, LC3-phosphatidylethanolamine conjugate; p62, sequestosome 1; LC3-I, cytosolic form of LC3; Con, control; CQ, chloroquine, Rap, rapamycin.

migration and invasion. These results indicated that increased autophagy may induce the EMT process in ARPE-19 cells.

## Discussion

Autophagy has an important role in numerous biological processes, including cell differentiation and metabolism, and response to drug administration, radiation and mechanical stress. It is well established that EMT is an important mechanism in

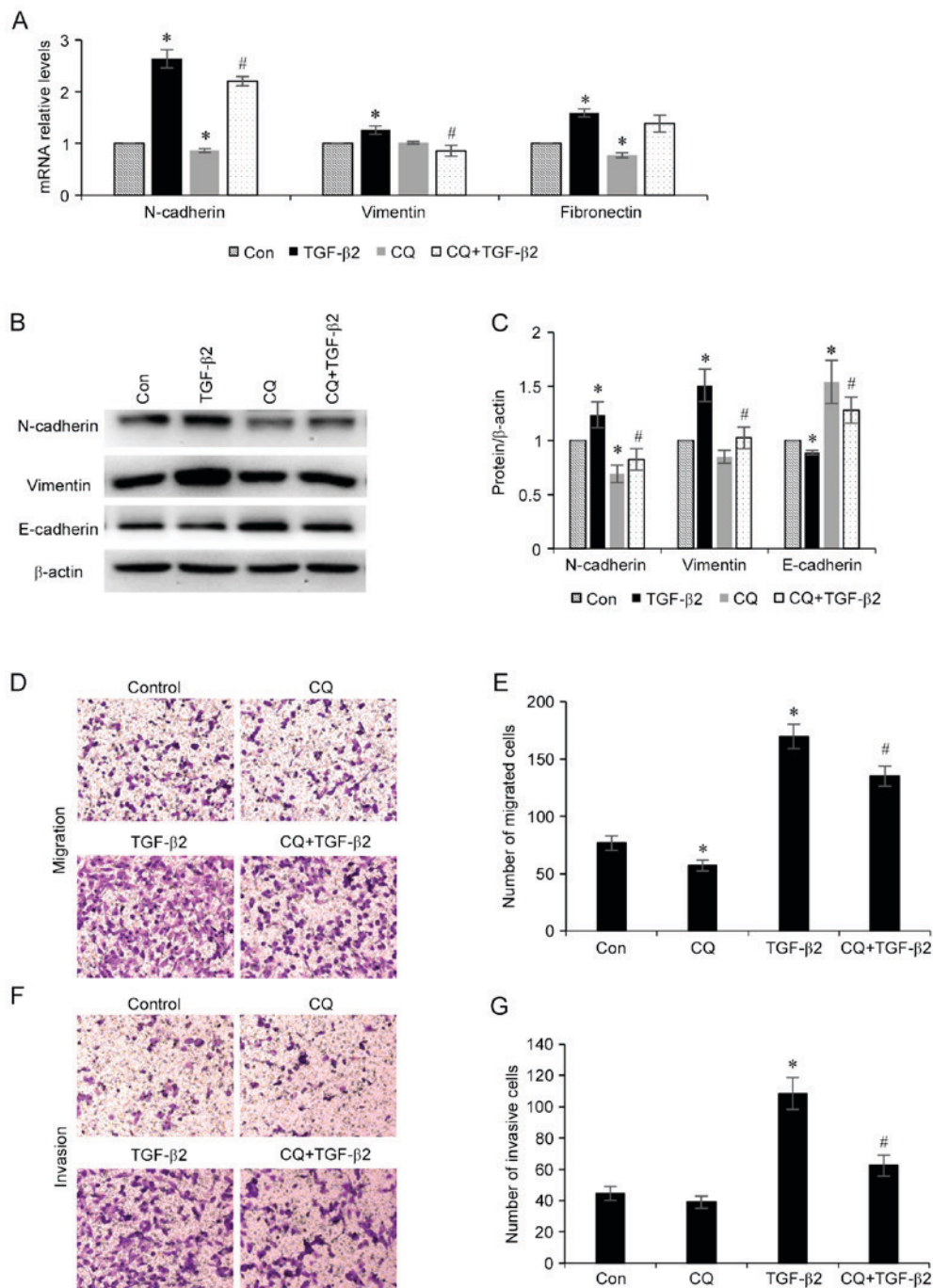


Figure 3. Suppression of autophagy inhibits TGF-β2-induced epithelial-mesenchymal transition in ARPE-19 cells. ARPE-19 cells were treated with TGF-β2 (5 ng/ml) with or without chloroquine (50 μM) for 24 h. (A) mRNA expression levels of N-cadherin, vimentin and fibronectin were determined by reverse transcription-quantitative polymerase chain reaction and analyzed by the  $2^{-\Delta\Delta C_q}$  method. GAPDH was used as a housekeeping gene. (B) Protein expression levels of N-cadherin, vimentin and E-cadherin were analyzed by western blotting. (C) Densitometric analysis of N-cadherin, vimentin and E-cadherin protein levels in ARPE-19 cells. (D) Representative images of lower chambers following Transwell migration assays in different treatment groups to determine the effect of chloroquine on the migratory ability of ARPE-19 cells (magnification, x100). (E) Quantified results of Transwell migration assays. (F) Representative images of lower chambers following Matrigel invasion assays in different treatment groups to determine the effect of chloroquine on the invasive potential of ARPE-19 cells (magnification, x100). (G) Quantified results of Matrigel invasion assays. Bars represent the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. Con; # $P < 0.05$  vs. TGF-β2 group. TGF-β2, transforming growth factor-β2; Con, control; CQ, chloroquine.

the pathogenesis associated with fibrotic disease. Furthermore, a number of previous studies have reported the occurrence of autophagy in cells undergoing EMT (8-12). Administration of TGF-β1 simultaneously induced fibrosis and autophagy in primary human atrial myofibroblasts, and autophagy markers were previously demonstrated to be elevated in scar tissue

isolated from post-myocardial infarction rats (9). It was also reported that autophagy may be induced by administration of TGF-β1 during the EMT process in malignant glioma cells, as demonstrated by the upregulated expression of LC3-II, beclin 1 and lysosomal-associated membrane protein 1 (8). In the present study, LC3-phosphatidylethanolamine conjugate (LC3-II) is

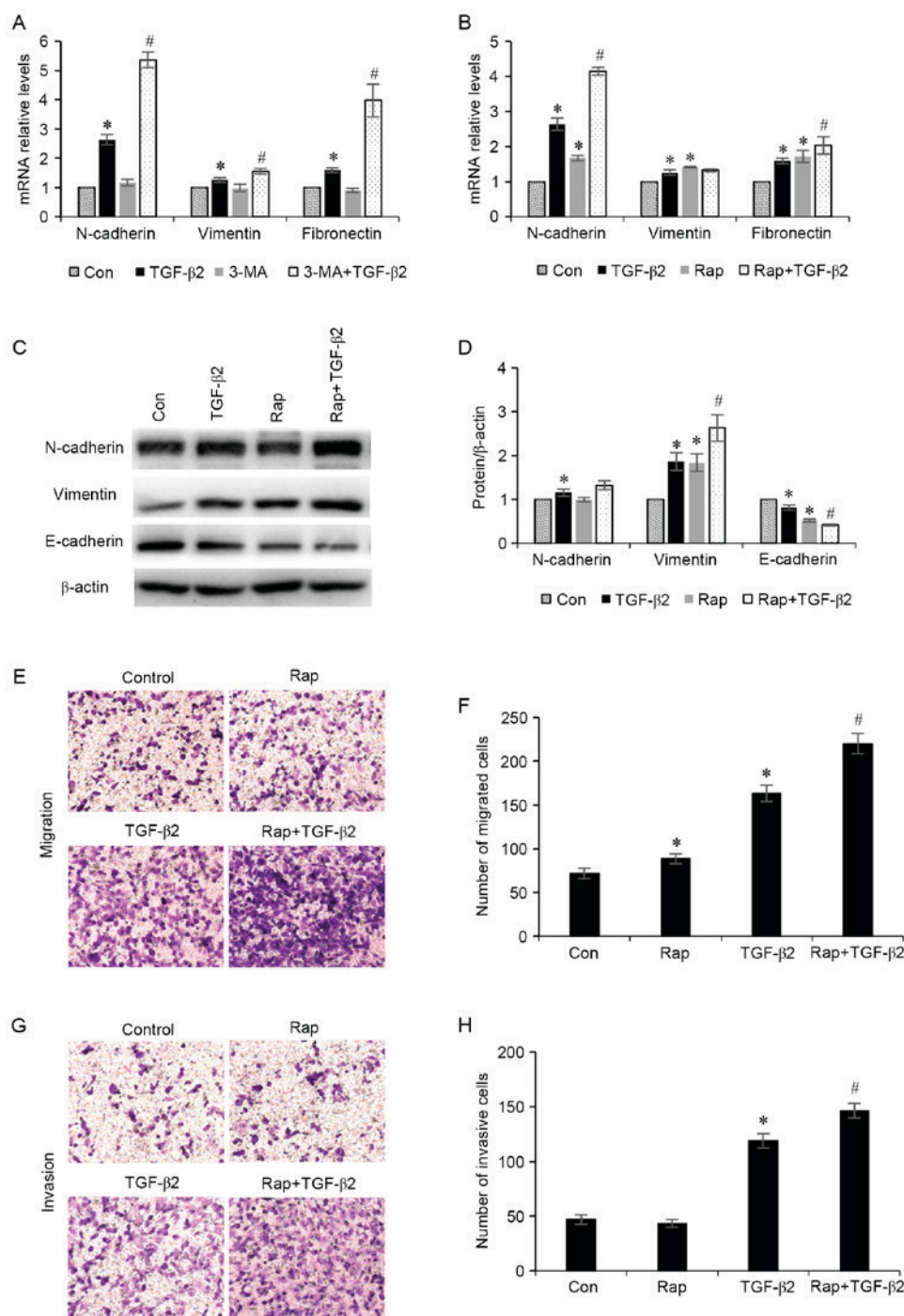


Figure 4. Activation of autophagy exacerbates TGF-β2-induced epithelial-mesenchymal transition in ARPE-19 cells. ARPE-19 cells were treated with TGF-β2 (5 ng/ml) with or without (A) 3-MA (5 mM) and (B) rapamycin (200 nM) for 24 h. mRNA expression levels of N-cadherin, vimentin and fibronectin were determined by reverse transcription-quantitative polymerase chain reaction and the data were analyzed using the  $2^{-\Delta\Delta C_q}$  method. GAPDH was used as a house-keeping gene. (C) ARPE-19 cells were treated with TGF-β2 (5 ng/ml) with or without rapamycin (200 nM) for 24 h. Protein expression levels of N-cadherin, vimentin and E-cadherin were analyzed using western blotting. (D) Densitometric analysis of N-cadherin, vimentin and E-cadherin expression levels in ARPE-19 cells. (E) Representative images of lower chambers following Transwell migration assays in different treatment groups to determine the effect of rapamycin administration on TGF-β2-induced cell migration (magnification, x100). (F) Quantified results of Transwell migration assays. (G) Representative images of lower chambers following Matrigel invasion assays in different treatment groups to determine the effect of rapamycin administration on the invasive ability of TGF-β2-treated cells (magnification, x100). (H) Quantified results of the Matrigel invasion assays. Bars represent the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$  vs. Con; # $P < 0.05$  vs. TGF-β2 group. TGF-β2, transforming growth factor-β2; 3-MA, 3-methyladenine; Con, control; Rap, rapamycin.

the lipidated form of the cystolic form of LC3 (LC3-I), and the conversion from LC3-I to LC3-II represents the formation of autophagosomes (24). p62 combines polyubiquitinated proteins and forms the completed autophagosomes, which

are subsequently degraded into autolysosomes; therefore, the quantity of p62 may be considered an index of autophagic degradation (24). Although beclin-1 is a regulatory protein of autophagy, its expression was not markedly altered when



autophagy was induced by TGF- $\beta$ 2 administration. Following induction of autophagy by various stimuli, beclin-1 is released from B-cell lymphoma 2 and combines with phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) to generate multiple complexes (24). Thus, it is possible that the expression of beclin-1 expression did not demonstrate a marked alteration in the present study due to binding to PIK3C3. Similar results have been reported by Lee *et al* (25). It is well established that TGF- $\beta$ 2 has been well established is a potent inducer of EMT in RPE cells. In the present study, the TGF- $\beta$ 2-induced EMT model was established in ARPE-19 cells *in vitro*, and an increased autophagic flux during the process of EMT in RPE cells was demonstrated, thus indicating that autophagy may participate in the EMT process of RPE cells.

The effect of autophagy on the EMT process in RPE cells was investigated further in the present study. Chloroquine, 3-MA and rapamycin pharmacological modulators, with the ability to regulate autophagy activity in ARPE-19 cells, were employed. Chloroquine, which possesses the ability to raise lysosomal pH, is generally used as an autophagy inhibitor, and rapamycin is commonly used as an autophagy inducer. 3-MA is a selective phosphoinositide 3-kinase inhibitor and its effect on autophagy was reported to be conditional. Rapamycin is an autophagy inducer by inhibiting mammalian target of rapamycin (mTOR) (24). Consistent with previous studies (26-28), the present study demonstrated that administration of chloroquine inhibited autophagy activity, and administration of rapamycin increased autophagy activity in ARPE-19 cells. Furthermore, it was also demonstrated that administration of 3-MA promoted the induction of autophagy. Therefore, future studies aiming to investigate the role of autophagy activity associated with RPE-EMT may use the aforementioned pharmacological modulators. The expression of E-cadherin, N-cadherin, fibronectin and vimentin was examined. E-cadherin and N-cadherin are classical members of the cadherin superfamily, which are a type of cell adhesion molecule that is important in the formation of adherens junctions to bind cells with each other (29). Fibronectin is a high molecular weight glycoprotein of the extracellular matrix that binds to integrins (30). Vimentin is a type III intermediate filament protein that is expressed in mesenchymal cells (31). Epithelial cells express high levels of E-cadherin, whereas mesenchymal cells express those of N-cadherin, fibronectin and vimentin (32). The results in the present study indicated that the inhibition of autophagy following chloroquine administration may significantly attenuate TGF- $\beta$ 2-induced EMT in ARPE-19 cells. Furthermore, induction of autophagy following administration of either 3-MA or rapamycin in ARPE-19 cells resulted in enhanced expression of mesenchymal markers.

However, the effect of autophagy on the EMT process is complex. The diverse effects of autophagy on EMT have been reported at different experimental conditions. Researchers demonstrated that induced autophagy increased EMT in some cells or tissues and inhibited EMT in others (8-14). Kim *et al* (11) investigated the role of autophagy in primary mouse mesangial cells and revealed that reduced levels of autophagy, via gene knockdown using specific small interfering RNA, led to enhanced expression of type I collagen, indicating that autophagy may exacerbate EMT. Furthermore,

Kim *et al* (11) also demonstrated that, following treatment with trifluoperazine, which is an inducer of autophagy, the expression level of type I collagen was decreased (11). However, numerous previous studies (8,9,14) have also demonstrated that autophagy was able to activate EMT, including a study by Li *et al* (14), which demonstrated that starvation-induced autophagy promoted the activation of EMT in hepatocellular carcinoma cells (14). Therefore, the exact involvement of autophagy activity in RPE-EMT remains to be determined and requires further investigation, such as through use of alternative EMT models and performing alternative experiments in order to further investigate autophagy activity. The complex effects of autophagy on fibrosis also highlights the importance for further investigation to determine the role autophagy in intraocular fibrotic disorders.

Numerous studies have reported the roles of autophagy in RPE, demonstrating that autophagy may aid the elimination of cytotoxic protein aggregates in RPE cells. Mitter *et al* (18) investigated RPE cells, retina samples from patients suffering from AMD and rodent models with an AMD phenotype, and demonstrated that autophagy was dysregulated. RPE cell damage resulting from autophagy dysregulation may induce more severe pathophysiological alterations, such as EMT and choroidal neovascularization (18). EMT in RPE cells is reported to exhibit an important role in retinal fibrotic diseases, which are predominantly irreversible and result in severe damage to vision (4,5). To the best of our knowledge, the present study demonstrated for the first time that autophagy may function as a regulator of EMT in RPE cells, thus indicating that autophagy may have an important function in the process of EMT in RPE cells. A potential mechanism underlying this process is that autophagy may provide adenosine triphosphate for the biosynthesis of profibrotic proteins (9). Future studies should investigate the potential underlying mechanisms associated with the effects of autophagy on EMT formation, which may provide new perspectives for the determination of the function of autophagy associated with the EMT process and the development of novel therapeutic agents for the treatment of retinal fibrotic pathogenesis.

In the present study, it was revealed that autophagy activity was enhanced in ARPE-19 cells treated with TGF- $\beta$ 2. Furthermore, it was demonstrated that autophagy activation exacerbated RPE-EMT, and inhibition of autophagy attenuated EMT. In addition, the results of the present study revealed that autophagy may be a regulator of EMT in RPE cells. The present study may contribute to an enhanced understanding of the role of autophagy in the pathophysiology of intraocular fibrotic disorders, which frequently result from EMT. Furthermore, these results indicate that autophagy may be a potential novel therapeutic target for the attenuation of EMT in fibro-proliferative disease.

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