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Epithelial-mesenchymal transition suppresses ERas to activate autophagy in retinal pigment epithelial cells in proliferative vitreoretinopathy

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Abstract. – OBJECTIVE: Proliferative vitreoretinopathy (PVR) is a complex ocular disease that leads to detached retinas and irreversible vision loss. The epithelial-mesenchymal transition (EMT) of retinal pigment epithelial (RPE) cells plays a critical role in PVR occurrence. However, the core targets driven by the EMT process that lead to the pathogenesis of PVR remain unclear. In our study, the relationship between embryonic stem cell-expressed Ras (ERas) and EMT in RPE cells was investigated.

PATIENTS AND METHODS: The subretinal and epiretinal membrane specimens of human PVR were examined for ERas and hallmarks of autophagy and EMT using Western blotting and immunofluorescence. EMT was induced by transforming growth factor (TGF)-β1 or epidermal growth factor (EGF) in ARPE-19 cells. Autophagy was inhibited by U0126 or bafilomycin A1 in ARPE-19 cells.

RESULTS: ERas was decreased and the classical autophagy biomarker microtubule associated protein 1 light chain 3 alpha (LC3) was upregulated in the subretinal and epiretinal membranes of PVR patients *in vivo*. Moreover, ERas was downregulated and autophagy was activated in RPE ARPE-19 cells in response to transforming growth factor (TGF)-β1 and epidermal growth factor (EGF) induction. Finally, overexpression of ERas in RPE cells inhibited autophagy *via* impaired formation of autophagosomes and lysosomes.

CONCLUSIONS: Our study revealed the role of ERas in the pathogenesis of PVR through EMT and provided a novel therapeutic target for PVR prevention and treatment.

Key Words:
Eras, PVR, RPE, EMT.

Introduction

Proliferative vitreoretinopathy (PVR) is the most common cause of failed repair of rhegmatogenous retinal detachment (RD), with an incidence ranging from 5.1 to 11.7%[1]. Despite advances in surgery, more than 25% of initially successful cases undergo redetachment due to vitreoretinal traction recurrence. To date, major efforts have been made towards the biochemical inhibition of cellular proliferation and membrane contraction in PVR to develop other forms of therapy to inhibit the pathological response that causes traction[2]. The major characteristic of PVR is the formation of fibrotic membranes within the vitreous cavity and subretinal and retinal surface, which is attributed to the epithelial-mesenchymal transition (EMT) occurring in retinal pigment epithelial (RPE) cells. Similar to other types of cells, RPE cells also lose polarity and adhesion upon EMT and turn into fibroblast-like cells with abundant expression of extracellular matrix (ECM) components, which can be triggered by multiple growth factors and cytokines, such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), epidermal growth factor and tumor necrosis factor-α[3].

Autophagy is a highly regulated catabolic pathway that is responsible for the degradation and recycling of cellular components in response to various cellular stresses[4]. Autophagy is an evolutionarily conserved process by which autophagosomes composed of double membrane vesicles engulf cellular proteins and organelles for delivery to the lysosome for degradation[5]. Autophagy-associated gene (ATG) 3/5/7/12, unc-51-like autophagy activating kinase 1 (ULK1), Beclin-1, and microtubule -associated protein 1 light chain 3 alpha (LC3) are widely acknowledged to contribute to autophagy through several signaling pathways, including the phosphatidylinositol 3-kinase/serine/threonine kinase 1/mechanistic target of rapamycin kinase (PI3K/

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Akt/mTOR)[6], protein kinase AMP-activated catalytic subunit alpha 1 (AMPK)[7], and the p53 pathways[8]. In RPE cells, autophagy not only governs energy stability and cellular quality but also induces cellular self-destruction and cell death in response to chronic stress conditions. Recent studies provide illuminating insight into the connection between autophagy and EMT in RPE cells. Autophagy is activated upon EMT initiation[9] and further enhances the EMT process[10], and these processes affect each other. Thus, we hypothesize that autophagy participates in the pathogenesis of PVR.

Embryonic stem cell-expressed Ras (ERas) is a novel member of the Ras protein family that was first found in mouse embryonic stem (ES) cells[11] and was recently reported in multiple cancers[12,13]. ERas bridges autophagy and EMT in gastric cancer[14], which indicates that ERas may also play a similar role in RPE cells. However, the molecular mechanism of ERas in the pathogenesis of PVR is still poorly understood. In this study, we focused on the autophagic response of RPE cells undergoing the EMT induced by TGF-β1 and EGF and investigated the underlying molecular mechanism of PVR occurrence.

Patients and Methods

Sample Collection

Three PVR patients (two males and one female) who received standard vitreoretinal surgery of both eyes from January to December 2018 and one normal male as a control were enrolled in this study. The donated eyes from donated remains were provided by the eye bank of the Shandong Red Cross. The average age of the participants was 56.14 ± 12.24 years. All the participants understood and signed the informed consent form. Signed informed consent and Ethics Committee documents of the Ethics Committee of ZiBo Central Hospital and Red Cross Society of China were all provided to approve this investigation. The isolated human subretinal and epiretinal membranes were used for Western blot or immunofluorescence assays, respectively.

Cell Culture

The RPE cell line (ARPE-19) was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbeco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). ARPE-19 cells were maintained in a 37°C incubator with 5% CO_2. For some experiments, the following treatments were administered to ARPE-19 cells: 10 ng/ml TGF-β1 (Sigma-Aldrich, St. Louis, MO, USA) for 48 h[15], 20 ng/ml epidermal growth factor (EGF) (R&D Systems, Minneapolis, MN, USA) for 48 h[16], the autophagy inhibitor U0126 (20 μM) (InvivoGen, San Diego, CA, USA) for 8 h and 2 μM bafilomycin A1 (InvivoGen)[17] for 4 h. The coding sequence of wild-type ERas was cloned and inserted into pcDNA3.1 (Thermo Fisher Scientific, Waltham, MA, USA) for ectopic ERas overexpression. ERas siRNA was synthesized (Genechemlab, Shanghai, China) for ERas RNA interference (Table I). pLVX-GFP-LC3II purchased from Vigene was used for autophagy activity detection. The plasmids or RNA oligonucleotides were transfected into ARPE-19 cells using Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. After 48 h of transfection, the cells were harvested for the next experiments.

Western Blot Assay

Ten milligrams of tissue were ground in liquid nitrogen, 500 μl of radio immunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientif-

Table I. All the primers and oligonucleotides used in this study were listed.

Symbol name	Sequence	Tm (°C)
ERas siRNA	5'-GUUUAUUAAUUAUAAAGGA-3'	
ERas for qPCR	5'-CTGAACCACCAGTGC-3'	58
	5'-ACAAGGGGCTGGGCGGGGTG-3'	
β-Actin for qPCR	5'-ACAGAGCCTCGCCTTTGC-3'	58
	5'-CCACCATCACGCCCTGG-3'	
ERas full length cloning	5'-ATGGAGCTGC CAACAAA-3'	52
	5'-TCAGGCCACAGAGCAGCC-3'	

ic, Waltham, MA, USA) with protease inhibitor cocktail (Beyotime, Shanghai, China) was added, and the concentration was quantified using bicinchoninic acid assay (BCA) methods. Aliquots of proteins (40 μg) were added to the lanes of 10% sodium dodecyl sulfate (SDS) polyacrylamide gels, and the proteins were separated through electrophoresis and transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked with 5% nonfat dry milk in 0.01 M phosphate-buffered saline buffer (pH 7.4) and 0.05% Tween-20 (PBST) for 1 h at room temperature (RT). The blocked membranes were then incubated with primary antibodies against ERas (1:2000, CST, Boston, MA, USA), Occludin (1:1500, Novus, Littleton, CO, USA), N-cadherin (1:2000, Novus), Vimentin (1:2000, Novus), α-SMA (1:2000, Novus), LC3II (1:2000, CST, Danvers, MA, USA), Atg5 (1:2000, Novus), Beclin1 (1:2000, Novus), p62 (1:2000, Novus) and β-Actin (1:5000, Beyotime) overnight at 4°C, followed by incubation with the appropriate secondary antibodies [horseradish peroxidase (HRP)-conjugated rabbit anti-mouse diluted with 1:10000 and donkey anti-rabbit diluted with 1:5000, Beyotime] for 30 min at RT. The expression was determined by the enhanced chemiluminescence (ECL) method using an Amersham Imager 600 system (GE Healthcare, Milwaukee, WI, USA), and the density of the immunoblots was measured with Quantity One 4.62 software (Bio-Rad, Hercules, CA, USA).

Immunofluorescence Staining Assay

Human PVR membrane specimens or cells were fixed in a 4% solution of paraformaldehyde, washed with phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and blocked with 0.5% horse serum in PBS. Immunostaining of samples was performed using the appropriate antibodies (1:200, refer to "Western blot assay" for antibody information) overnight at 4°C. After washing with PBS 4 times (5 min per wash), the appropriate secondary antibodies (1:20000; Jackson ImmunoResearch, West Grove, PA, USA) were added and incubated for 2 h at room temperature, followed by further incubation with 4',6-diamidino-2-phenylindole (DAPI) for 15 min and washing with PBS 4 times. After air drying, the slices were coated with mounting medium and cover glasses. The positive staining was statistically analyzed using ImageJ software.

Quantitative PCR

Total RNA was extracted from ARPE-19 cells using TRIzol and quantified using a Nanodrop. One hundred nanograms of RNA were used to conduct reverse transcription with a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). One microliter of product was used as the template for PCR preparation. The primers are listed in Table I. Analysis of DNA templates taken from RNA reverse transcription was conducted *via* qPCR using a QuantStudio 3 system (Thermo Fisher Scientific, Waltham, MA, USA). In accordance with the given instructions, the PCR conditions were 95°C for 30 s for initial denaturation, followed by 40 cycles at 95°C for 5 s, an appropriate annealing temperature of and 72°C for 10 s, and then 30 s. Ct values were determined and calculated using the delta-delta method. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as quality control. All primers used in this study are listed in Table I.

Statistical Analysis

The data are presented as the means ± SEM. All statistical analyses were performed using SPSS 20.0 statistics software (IBM Corp., Armonk, NY, USA). Group comparisons were analyzed with one-way ANOVA. A p-value less than 0.05 was considered a significant difference.

Results

Characterization of ERas Expression In PVR Membranes In Vivo

Initially, the expression of ERas was investigated in the subretinal and epiretinal membranes of PVR. We detected the transcription of ERas by qPCR and determined that the expression of ERas was reduced in the membranes of three PVR samples compared to that of the negative control (NC) (Figure 1A), which was consistent with Western blot determination of the protein level (Figure 1B). Moreover, the low expression of ERas in the cell membrane was observed in the subretinal and epiretinal membranes of PVR (PVR) compared to that of the negative control (NC), accompanied by upregulation of the autophagy protein LC3II in the cytoplasm, as measured by immunofluorescence staining (Figure 1C). Taken together, the aberrant downregulation of ERas and activated autophagy in PVR membranes was confirmed *in vivo*.

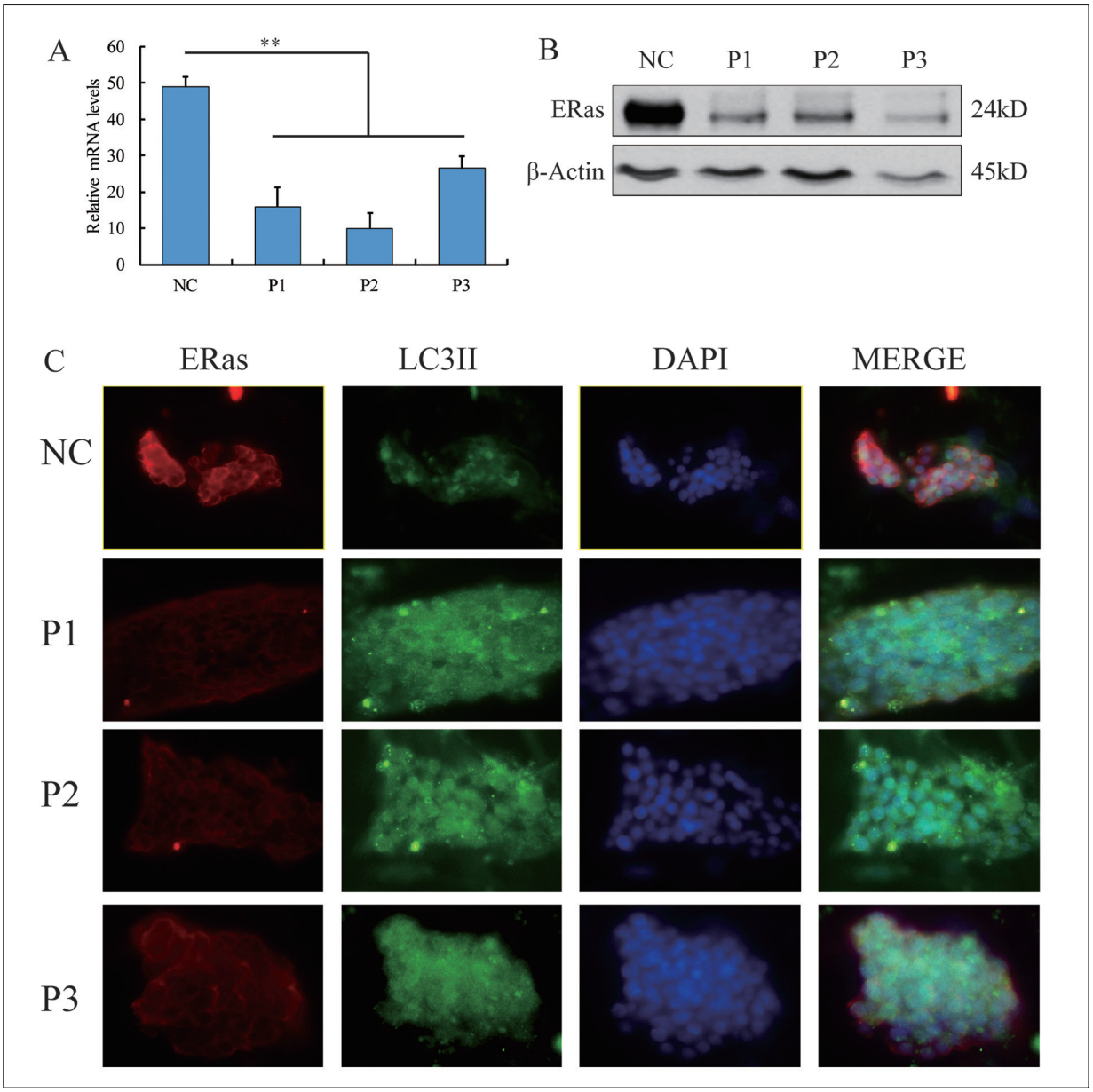

Figure 1. The ERas expression pattern in PVR membranes *in vivo*. qPCR (**A**) and western blot analysis (**B**) to detect ERas expression in PVR membranes. The distribution of ERas and LC3II in PVR membranes (**C**). The images are obtained at 200× magnification. NC, P1, P2 and P3 represent the negative control and three patients. The data are presented as the mean ± standard error of the mean of three individual repeated experiments. "**" represents a p-value < 0.01 by one-way ANOVA.

ERas Suppression and Autophagy Activation Induced by EMT In RPE Cells

Additional TGF-β1 or EGF was added to induce EMT in ARPE-19 cells. Given the uses of TGF-β1 and EGF for treatment in previous studies (Refer to "Materials and Methods" for more details), the expression of biomarkers such as Occludin, N-cadherin, Vimentin and α-SMA was used to validate the effect of EMT in our system (Figure 2A). We observed that ARPE-19 cells underwent EMT upon TGF-β1 or EGF induction. Moreover, elevated LC3II, Atg5 and Beclin1 and downregulated p62 and ERas were also observed in ARPE-19 cells that were treated with TGF-β1 or EGF. Furthermore, the GFP-LC3II fluorescence assay results also showed activated autophagy upon EMT initiation (Figure 2B). In contrast, the addition of U0126 or bafilo-

mycin A1, which act as autophagy inhibitors by blocking MAPK kinases or autophagosome and lysosome fusion, failed to affect the EMT process or ERas expression in ARPE-19 cells that were treated with TGF-β1 or EGF (Figure 2A). Taken together, EMT arrests the expression of ERas and stimulates autophagy activation in RPE cells.

The Essential Role of ERas In Bridging EMT and Autophagy

To determine the relationship between ERas and autophagy induced by EMT, ERas was overexpressed in ARPE-19 cells that were treated with TGF-β1 or EGF and silenced in ARPE-19 cells that were treated with U0126 or bafilomycin A1. Our data showed that ectopic expression of ERas significantly reduced the expression of LC3II, Atg5 and Beclin1, prevented p62 degradation (Figure 3A) and compromised autophagy activation even though the EMT process was still ongoing. Moreover, ERas knockdown rescued LC3II, Atg5 and Beclin1 in ARPE-19 cells that were treated with low concentrations of U0126 (Figure 3B, C) and bafilomycin A1 (Figure 3D, E) and was beneficial for maintenance of autophagy activation. However, ERas knockdown alone was not enough to efficiently inhibit the effects of high working concentrations of U0126 (20 μM) and bafilomycin A1 (2 μM).

Overall, our data showed that ERas was affected by EMT, governed autophagy activity in RPE cells, and played a role in bridging EMT and autophagy in PVR RPE cells.

Discussion

In the present study, the expression patterns of ERas and LC3B in surgically excised subretinal and epiretinal membranes were characterized, which directly represent the pathogenesis of PVR *in vivo*. By exploring the EMT cell model in

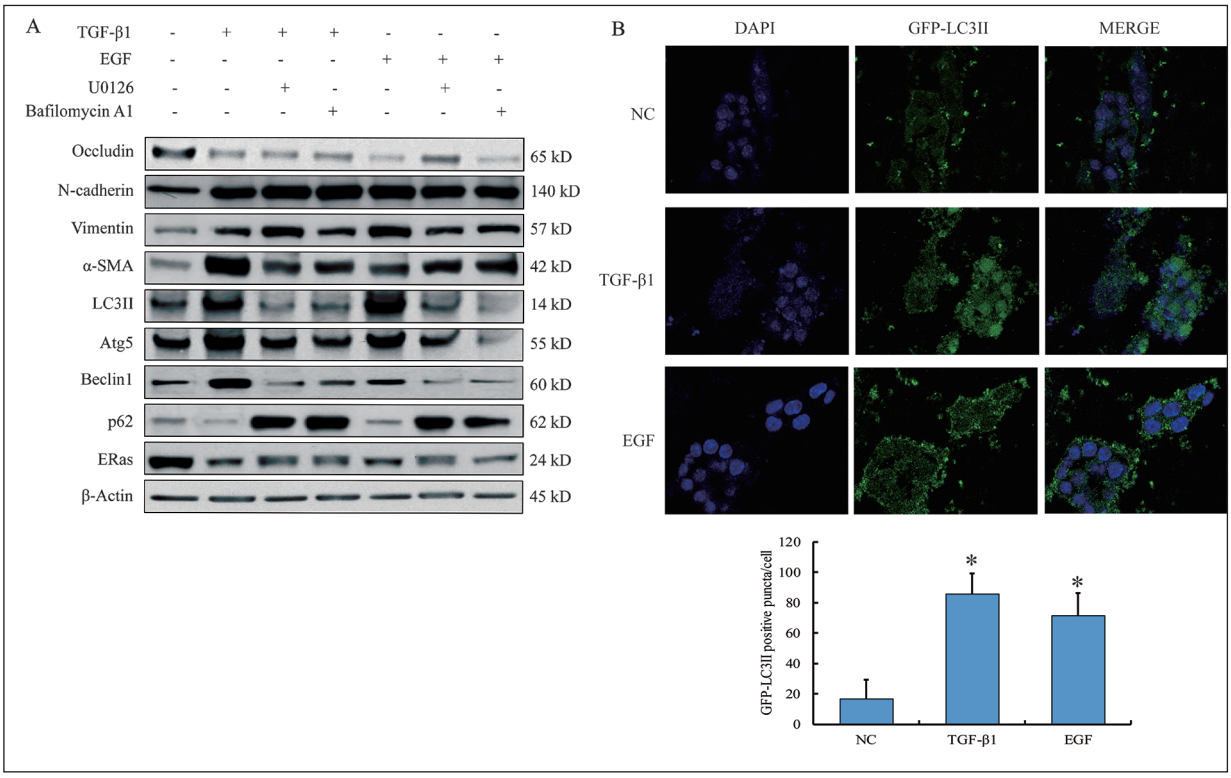

Figure 2. Autophagy activity is affected by EMT in ARPE-19 cells. Western blot analysis of EMT markers (Occludin, N-cadherin, Vimentin and α-SMA), autophagy hallmark proteins (LC3-II, Atg5, Beclin1 and p62) and ERas in ARPE-19 cells that were treated with the combination of TGF-β1 (10 ng/ml), EGF (20 ng/ml), U0126 (20 μM) and Bafilomycin A1 (2 μM) (**A**). The localization of GFP-LC3B puncta in ARPE-19 cells that were treated with TGF-β1 or EGF (**B**). Blue fluorescence represents nuclei stained with DAPI. The bar graph indicates the average number of LC3B puncta per cell obtained by fluorescence analysis. The images are obtained at 400× magnification. The data are presented as the mean ± standard error of the mean of three individual repeated experiments. "*" represents the comparison to NC with a p-value < 0.05 by one-way ANOVA.

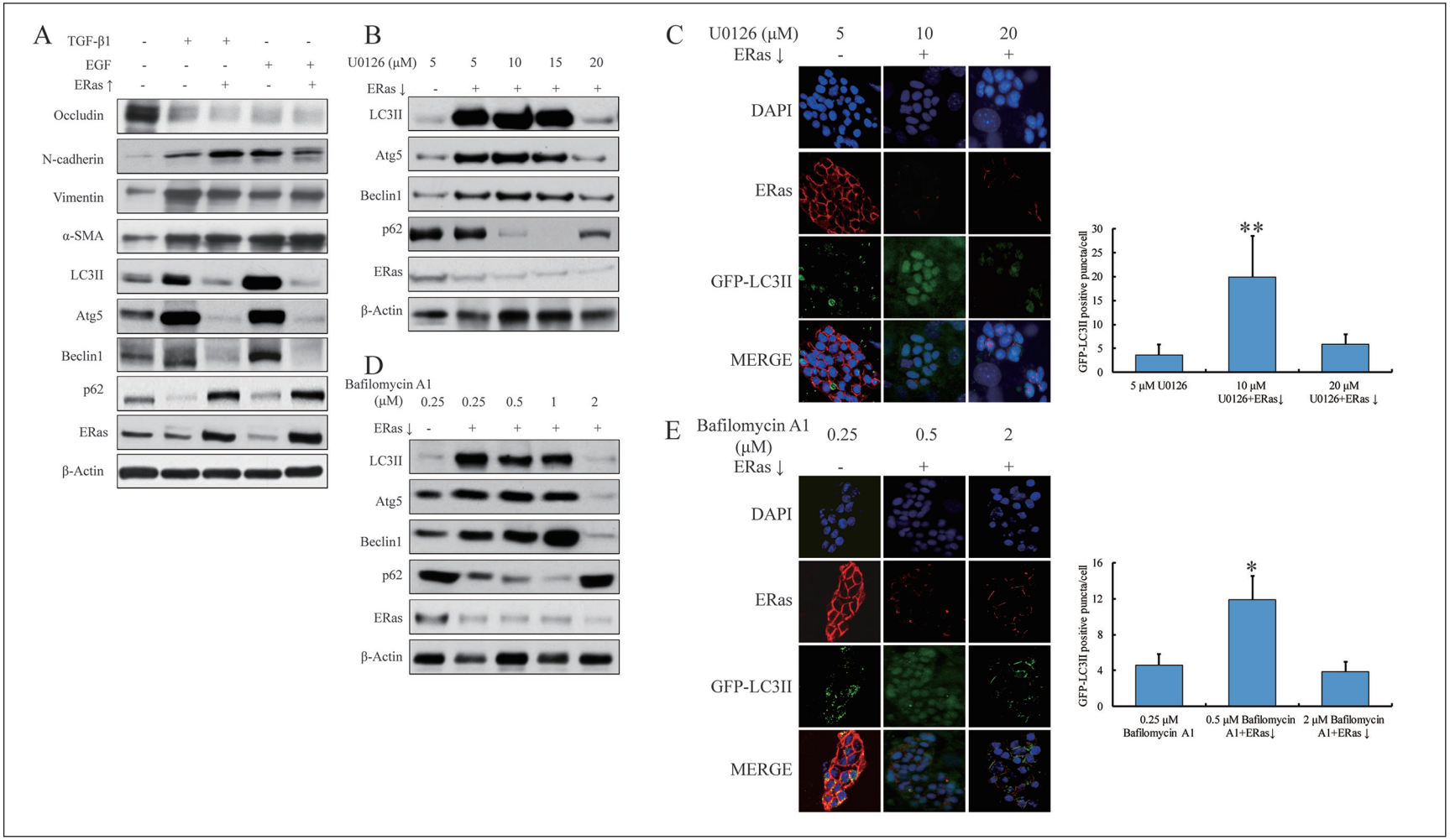

Figure 3. Autophagy activity was affected by ERas overexpression or knockdown in ARPE-19 cells. Western blot analysis of EMT markers (Occludin, N-cadherin, Vimentin and α-SMA), autophagy hallmark proteins (LC3-II, Atg5, Beclin1 and p62) and ERas in ARPE-19 cells that were treated with the combination of TGF-β1 (10 ng/ml), EGF (20 ng/ml) and ERas overexpression (**A**). Western blot analysis of the autophagy hallmark proteins (LC3-II, Atg5, Beclin1 and p62) and ERas in ARPE-19 cells that were treated with increasing concentrations of U0126 and ERas knockdown (**B**). The localization of GFP-LC3B puncta in ARPE-19 cells that were treated with increasing concentrations of U0126 and ERas knockdown (**C**). Western blot analysis of the autophagy hallmark proteins (LC3-II, Atg5, Beclin1 and p62) and ERas in ARPE-19 cells that were treated with increasing concentrations of bafilomycin A1 and ERas knockdown (**D**). The localization of GFP-LC3B puncta in ARPE-19 cells that were treated with increasing concentrations of bafilomycin A1 and ERas knockdown (**E**). The images are obtained at 400X magnification. The data are presented as the mean ± standard error of the mean of three individual repeated experiments. "*" and "**" represent the comparison to unsilenced ERas with p-values < 0.05 and 0.01 by one-way ANOVA.

which RPE cells were stimulated by TGF-β1 or EGF, we observed that the repressed expression of ERas and induced autophagy activation in RPE cells was stimulated during the EMT. These results suggest that the connection between ERas and autophagy was likely to be involved in the pathogenesis of PVR, which has not yet been illustrated.

Autophagy is a catabolic process that defends against sustained oxidative stress, thereby maintaining the homeostasis of RPE cells[18]. Impairment of autophagy causes accumulative damage and toxic substances in organelles, which further leads to serious dysfunction or death in RPE cells[19]. In contrast, uncontrolled and excessive autophagy activation results in deterioration, as observed in ocular diseases such as age-related macular degeneration, cataracts and diabetic retinopathy[20]. Since autophagy is critical to the integrity and fate of RPE cells, the role of autophagy in the EMT is also of concern. Although autophagy was activated in the EMT process of RPE cells, two previous studies provided contradictory statements on the feedback of autophagy to EMT. Atg7 knockdown RPE cells displayed a phenotype of suppressed epithelial and increased mesenchymal markers, with robust cell migration and contractility[9], while pharmacological inhibitors of autophagy, 3-methyladenine (3-MA) and bafilomycin A1, abrogated mesenchymal-specific proteins such as α-SMA, fibronectin and collagen IV in ARPE-19 cells and primary RPE cells induced by TGF-β2[3]. We hypothesize that pharmacological inhibitors may inhibit EMT by affecting other signaling pathways. These observations define a complex interaction between autophagy and EMT, which is influenced by several aspects. In this study, we demonstrated that inhibition of autophagy by U0126 or bafilomycin A1 failed to attenuate or enhance TGF-β1- or EGF-induced EMT in RPE cells, which is different from the results of the other study mentioned above. We hypothesize that the concentrations of pharmacological reagents or the background of co-called ARPE-19 cells in different labs may both impact the final outcomes.

Recent studies on ERas have primarily focused on tumorigenicity, especially in gastric cancer. ERas is closely linked with the activation of multiple signaling pathways, such as PI3K/Akt, JAK/Stat3, HIPPO and NF-κB[21], which contributes to chemotherapy resistance in gastric cancer[22]. Activation of the PI3K/Akt signaling pathway is beneficial for autophagy induction through mTOR kinases[23]. In our study, the performance of ERas in RPE cells is consistent with other studies on cancers showing that ERas suppresses autophagy activity.

The current research progress on the relationship between ERas and EMT is still confined to gastric cancer[14]. As one member of Ras proteins that is particularly expressed in embryonic stem cells, the regulatory mechanism of ERas is usually referred to as the Ras protein family. Both the Ras/PI3K/Akt and Ras/RAF/MEK/ERK pathways stimulate EMT through the activation of Snail2 expression and the reduction in E-cadherin in multiple cell lines, including colorectal carcinoma cells, rat parotid gland epithelial cells, and endometrial cancer cell lines[8]. Ras-responsive element binding protein 1 (RREB1), which was identified as a Ras transcriptional effector, can be activated by MAPK and facilitates the interaction between Smad and Snail to coordinately trigger EMT[24]. However, our results are seemingly inconsistent with the current statements. First, ERas is a downstream target that is affected by EMT *via* one-way modulation, while Ras promotes EMT initiation. Second, the expression of ERas seems to be negatively correlated with EMT, while oncogenic RAS positively promotes EMT in collaboration with other pathways. Thus, further studies will be needed to determine the difference in the underlying molecular mechanism of ERas and Ras in the EMT response.

Conclusions

This study provides evidence that ERas and autophagy are involved in the EMT of RPE cells. ERas contributes to the progression of autophagy activation that is regulated by EMT. Our findings provide a novel network for understanding the pathogenesis of PVR and highlight ERas as a putative novel therapeutic target for the prevention and treatment of human PVR.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

Jie Pan performed all the experiments and analyzed the data. Luxin Zhao designed the overall project and drafted the manuscript. This project is not supported by any funds.

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