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RESEARCH ARTICLE

Proteotoxic Stress Desensitizes TGF-beta Signaling Through Receptor Downregulation in Retinal Pigment Epithelial Cells

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Abstract: ***Background*:** Proteotoxic stress and transforming growth factor (TGFβ)-induced epithelial-mesenchymal transition (EMT) are two main contributors of intraocular fibrotic disorders, including proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR). However, how these two factors communicate with each other is not well-characterized.

***Objective*:** The aim was to investigate the regulatory role of proteotoxic stress on TGFβ signaling in retinal pigment epithelium.

***Methods*:** ARPE-19 cells and primary human retinal pigment epithelial (RPE) cells were treated with proteasome inhibitor MG132 and TGFβ. Cell proliferation was analyzed by CCK-8 assay. The levels of mesenchymal markers α-SMA, fibronectin, and vimentin were analyzed by real-time polymerase chain reaction (PCR), western blot, and immunofluorescence. Cell migration was analyzed by scratch wound assay. The levels of p-Smad2, total Smad2, p-extracellular signal-regulated kinase 1/2 (ERK1/2), total ERK1/2, p-focal adhesion kinase (FAK), and total FAK were analyzed by western blot. The mRNA and protein levels of TGFβ receptor-II (TGFβR-II) were measured by real-time PCR and western blot, respectively.

***Results*:** MG132-induced proteotoxic stress resulted in reduced cell proliferation. MG132 significantly suppressed TGFβ-induced upregulation of α-SMA, fibronectin, and vimentin, as well as TGFβ-induced cell migration. The phosphorylation levels of Smad2, ERK1/2, and FAK were also suppressed by MG132. Additionally, the mRNA level and protein level of TGFβR-II decreased upon MG132 treatment.

***Conclusion*:** Proteotoxic stress suppressed TGFβ-induced EMT through downregulation of TGFβR-II and subsequent blockade of Smad2, ERK1/2, and FAK activation.

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1. INTRODUCTION

Intraocular fibrotic disorders, including proliferative vitreoretinopathy (PVR) and proliferative diabetic retinopathy (PDR), are the leading causes of visual impairment. PVR is a common complication of retinal detachment and ocular trauma. PVR occurs in 5%-10% of treated rhegmatogenous retinal detachment cases, and is currently the major challenge to a successful retinal reattachment surgery, accounting for approximately 75% of primary surgical failures [1]. PVR is characterized by the formation of fibrotic membranes in the inner surface of neural retina, the sub-retinal space, the vitreous base, and the ciliary body. These membranes compromise the retina flexibility, and the contraction of the membranes will result in macular pucker, retinal detachment, or ocular hypotony. On the other hand, PDR is presented as retinal neovascularization, vitreous hemorrhage, and fibrovascular proliferation in the vitreous retinal interface. Approximately 1.5% of patients with diabetes will develop PDR [2]. Despite the advances in pharmacological and surgical techniques, prevention and treatment strategies for PVR and PDR are still limited.

A large body of literature have suggested that epithelial-mesenchymal transition (EMT) of retinal pigment epithelial (RPE) cells is the major pathological change during the development and progression of

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PVR and PDR. Under physiological conditions, RPE cells are mitotically inactive. However, the dysfunction of blood-retinal barrier (BRB) in PVR and PDR exposes RPE cells to various growth factors in the vitreous body, and consequently simulates RPE cells to proliferate, migrate towards the vitreous body, and undergo EMT. During EMT, RPE cells rearrange cytoskeletal architecture, lose cell polarity, acquire mobility, and transdifferentiate into fibroblast-like cells. Of all the growth factors, transforming growth factor β (TGFβ) is the most potent inducer of EMT [3, 4]. During the development of PVR and PDR, TGFβ is significantly upregulated in aqueous humor and vitreous body [5, 6]. The canonical TGFβ pathway is mediated by the phosphorylation of Smad2 and Smad3, which then translocate into the nucleus and subsequently upregulate the expression of mesenchymal markers, including α-SMA, fibronectin (Fn), and vimentin (Vim) [7-9]. Additionally, TGFβ can also initiate EMT though non-canonical pathways. For example, TGFβ can activate mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases (JNKs), and regulate cell proliferation, migration, and differentiation [10, 11]. Also, TGFβ can promote phosphorylation of protein tyrosine kinases (PTKs), such as focal adhesion kinase (FAK), which also plays a functional role during EMT [4]. Therefore, inhibition of TGFβ-induced EMT is considered as a promising therapeutic intervention for PVR and PDR.

Another important contributor to the pathogenies of PVR and PDR is proteotoxicity triggered by oxidative stress. RPE cells are critical nursing cells of the photoreceptors, and are prone to damage caused by environmental stresses. During the development of PVR, retinal detachment and ocular trauma induce an inflammatory response, causing oxidative damage to the retina. Oxidative stress also contributes to the formation of PDR, as evidenced by an increased level of various oxidative stress markers in the vitreous body of diabetic retinopathy patients [12]. Prolonged oxidative stress significantly increases intracellular accumulation of unfolded or misfolded protein levels through direct damage to the protein structure [13, 14]. Notably, oxidative stress also impairs essential components in the ubiquitin-proteasome pathway (UPP) [14, 15], which is an essential protein quality control system that selectively degrades unfolded, misfolded, or damaged proteins [16]. For example, in Parkinson's disease, the vulnerability of dopaminergic neurons is exacerbated by oxidatively damaged and aggregated α-synuclein that compromises the UPP function [17]. Additionally, chronic oxidative stress leads to a marked decline in proteasome activity in trabecular meshwork cells, which is associated with glaucoma pathophysiology [18]. Our group and others have demonstrated that the proteasome in retinal pigment epithelium is susceptible to oxidative damage [15, 19, 20], and that inactivation of proteasome activity results in overproduction of proinflammatory cytokines, which may contribute to the development of age-related macular degeneration [20].

TGFβ-induced EMT and stress-induced proteotoxicity are closely interrelated; however, the relationship between the two is still controversial. Several studies suggested that EMT can be induced under proteotoxic stress, and that EMT can be considered as a cellular adaptive response to adverse environmental conditions. For example, heat shock factor 1 (HSF1), a transcription factor responsive to proteotoxic stress, is markedly elevated in various cancers as a potent EMT promoter [21]. In renal epithelial cells, chronic oxidative stress can induce EMT as evidenced by markedly upregulated expression of mesenchymal markers and increased cell motility, leading to carcinogenesis [22]. In liver cancer cells, inhibition of proteasome gene expression or inhibition of proteasome activity by MG132 causes EMT-like changes [23]. However, other studies reported that EMT can be suppressed under proteotoxic stress. For example, in lens epithelial cells, TGFβ-induced EMT and cell migration can be significantly inhibited through treatment of MG132, which is considered as a target of medical therapy for posterior capsular opacification after cataract surgery [24, 25]. Similarly, in human mammary epithelial cells, MG132 leads to reduced EMT as evidenced by elevated level of E-cadherin, an epithelial marker [26]. In human RPE cells, the crosstalk between TGFβ-induced EMT and proteotoxic stress is not clear. Understanding how proteotoxic stress affects TGFβ-induced EMT is essential to unlock the therapeutic potential of proteasome inhibitors in the treatment of PVR and PDR.

Here, we seek to address the relationship between TGFβ-induced EMT and proteotoxic stress in RPE cells. We have characterized the effect of proteasome inhibition on EMT and TGFβ signaling in both RPE cell line and primary human RPE cells isolated from human healthy donor eyes. Our results identify a negative regulatory role of proteotoxic stress on TGFβ signaling through receptor downregulation, and suggest a complex interplay between proteasome dysfunction and EMT during the pathogenesis of PVR and PDR.

2. MATERIALS AND METHODS

2.1. Isolation of Human RPE Cells

A pair of human eyes from a 2 years old donor was obtained from the eye bank of Zhongshan Ophthalmic Center. The research protocol was approved by the Institutional Review Board/Ethics Committee of the Sun Yat-sen University and the tenets of the Declaration of Helsinki were followed throughout the study. RPE cells were harvested according to a previously published procedure [27]. Briefly, the eyes were autopsied within 6 hours from death and shipped in sterile medium. The anterior segment from each eye was removed by cutting around the iris. The vitreous body and the retina were carefully peeled away from the retinal pigment

epithelium-choroid-sclera. Then, the retinal pigment epithelium-choroid was carefully separated from the sclera and placed face up in a small sterile Petri dish with 2 U/ml Dispase (Roche, Indianapolis, IN) prepared in Ca^{2+} and Mg^{2+} free Hank's balanced salt solution. After incubation at 37°C for 1 hour with occasional shaking, the supernatant was carefully aspirated and transferred into a sterile centrifuge tube. The tube was then centrifuged at 1000 rpm for 5 min and the pellet was resuspended gently in the Dulbecco's modified Eagle's medium (DMEM)/F12 (GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 1 mM NEAA (GIBCO), 2 mM Glutmax (GIBCO), and 100 U/mL penicillin and streptomycin. The suspended cells were transferred to T25 culture flasks coated with matrigel (1:100, BD Biosciences, San Jose, CA).

2.2. Cell Culture and Treatment

Human retinal pigment epithelial cell line (ARPE-19) and human primary retinal pigment epithelial cells were cultured in DMEM containing 10% FBS. Cells were grown in a humidified 37°C incubator with 5% CO_2 and dissociated with 0.25% trypsin-0.02% ethylenediaminetetraacetic acid (EDTA). Cells were seeded in six-well plates with the density of 1×10^5 cells/well, and treated with DMSO, MG132 (Sigma-Aldrich, St. Louis, MO, USA), or 10 ng/mL TGFβ2 (Cell Signaling Technology, Danvers, MA, USA) for indicated time. All treatments were carried out when the cells reached approximately 75% confluence (except the scratch wound assay).

2.3. Cell Viability Assay

Cell viability was evaluated using the Cell Counting Kit-8 (CCK-8) assay. 100 µL ARPE-19 suspension at a concentration of 2×10^4 cells /mL was seeded in a 96-well plate. After incubation for 24 hours, cells were treated with indicated reagents. Each group had five duplicate wells. After 0, 12, 24, 36, 48, and 60 hours of treatment, 100 µL culture medium with 10 µL CCK-8 reagent (Jinxin Company, Guanghzou, China) was added to each well. After incubation for 1 hour, the Optical density (OD) values at 450 nm were measured with a microplate reader. The assay was repeated 3 times.

2.4. Scratch Wound Assay

The cells were seeded on a 6-well plate with the concentration of 1.5×10^5 cells/well, and cultured for 24 hours to reach 90% confluence. The cell monolayer was wounded by a 200 µl micropipette tip and washed with PBS for four times to remove cell debris. Then the cells were incubated in 0.5% FBS medium with or without MG132 and TGFβ2 for 24 hours. Migration of cells into the wound area was examined by an inverted microscope and photographed in a digital format (×100). Cell migration area was analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc. Silver Spring, MD, USA). In each group, six randomly chosen microscopic fields were analyzed, and the average cell migration index was calculated. Cell migration index = (1-non-invading area/total wounded area) ×100%.

2.5. Western Blot

For total protein extraction, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor cocktail. After mixing with 5× SDS sample buffer, protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyl difluoride (PVDF) membranes (Bio-Rad). The membrane was blocked in 5% nonfat milk and incubated with primary antibody at 4°C overnight, and washed with PBST (0.1% Tween-20 in PBS). Then the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Protein bands were detected with chemiluminescence detection reagents. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin were used as loading controls. Quantitative analysis was done using Image J 1.41 (National Institutes of Health, Bethesda, MD, USA). The sources and dilutions of antibodies are: mouse anti-ubiquitin (1:4000, Cell Signaling Technology), rabbit anti-TGFβR II (1:200, Santa Cruz Biotechnology), rabbit anti-ERK (1:1000, Cell Signaling Technology), rabbit anti-p-ERK (1:1000, Thr 202/Tyr 204, Cell Signaling Technology), rabbit anti-Smad2 (1:1000, Cell Signaling Technology), rabbit anti-p-Smad2 (1:1000, Ser 465/467, Cell Signaling Technology), rabbit anti-FAK (1:500, Abcam), rabbit anti-p-FAK (1:500, Tyr 397, Abcam), mouse anti-α-SMA (1:200, Abcam), rabbit anti-fibronectin (1:200, Abcam), rabbit anti-Vimentin (1:1000, Cell Signaling Technology), rabbit anti-GAPDH (1:2000, Cell Signaling Technology), rabbit anti-β-actin (1:3000, Abcam), horseradish peroxidase (HRP)-conjugated horse anti-mouse IgG (1:2000, Cell Signaling Technology), and HRP-conjugated goat anti-rabbit IgG (1:2000, Cell Signaling Technology).

2.6. RNA Extraction and Quantitative PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA concentration was measured spectrophotometrically at 260 nm. To eliminate genomic DNA contamination, we also treated the samples with DNase I at 37°C for 20 min. A total amount of 2 µg RNA was used for reverse transcription to cDNA using a reverse transcription kit (Takara, Siga, Japan). SYBR PrimeScript RT-PCR kit (Takara) was used to amplify target genes by the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control. Primer sequences were listed in Table **1**. All the primers were synthesized by Beijing Genomics Institute (Beijing, China).

2.7. Immunofluorescence Staining

Cells were seeded on Millicell EZ 4-well glass slides (Millipore, MA, USA). After treatment for indicated time, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 1% bovine serum albumin (BSA). Cells were then incubated with primary antibodies at 4°C overnight, and

Table 1. Primers used for real-time quantitative PCR.

Gene	Forward primer	Reverse primer
TGFβR II	5'-ACCACCAGGGCATCCAGAT-3'	5'-TGAAGCGTTCTGCCACACA-3'
α-SMA	5'-CCGACCGAATGCAGAAGGA-3'	5'-ACAGAGTATTTGCGC-TCCGAA-3'
Fibronectin	5'-GAGCTGCACATGTCTTGGGAAC-3'	5'-GGAGCAAATGGCACCGAGATA-3'
Vimentin	5'-TGAGTACCGGAGACAGGTGCAG-3'	5'-TAGCAGCTTCAACGGCAAAGTTC-3'
GAPDH	5'-GAGTCAACGGATTTGGTCGT-3'	5'-AATGAAGGGGTCATTGATGG-3'

secondary antibody for 1 hour at room temperature. The sources and dilutions of antibodies are: mouse anti-α-SMA (1:50, Abcam), rabbit anti-fibronectin (1:200, Abcam), rabbit anti-Vimentin (1:100, Cell Signaling Technology), Alexa Fluor 555-conjugated anti-mouse IgG (1:1000, Cell Signaling Technology), Alexa Fluor 555-conjugated anti-rabbit IgG (1:1000, Cell Signaling Technology), and Alexa Fluor 488-conjugated anti-rabbit IgG (1:1000, Cell Signaling Technology). Cell nuclei were stained with 50 ng/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Slides were mounted with anti-fade fluorescent mounting medium (Applygen, #C1210). Images were acquired by a Zeiss LSM 510 confocal laser scanning microscope (CLSM, Carl Zeiss, Germany) and processed by Adobe Photoshop CS6.

2.8. Statistical Analysis

All data were presented as means ± S.D. SPSS 17.0 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used to compare means among three or more groups. All statistical tests were two tailed. $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Proteotoxic Stress Suppresses Proliferation, TGFβ-induced EMT and Migration of ARPE-19

To induce proteotoxic stress, we treated ARPE-19 with MG132, a potent and specific proteasome inhibitor that can lead to a dose-dependent and time-dependent accumulation of ubiquitinated proteins [28-30]. Upon treatment with 2.5 μM and 5.0 μM MG132 for 48 hours, we observed an accumulation of ubiquitinated proteins in ARPE-19 cells, indicating the blockage of protein degradation (Fig. **1A**). The proliferation of ARPE-19 cells was significantly inhibited upon treatment with MG132 for 36, 48, and 72 hours (Fig. **1B**). We next investigated the effect of proteasome inhibition on TGFβ-induced EMT. Treatment of ARPE-19 with TGFβ for 48 hours resulted in a significant increased expression of typical mesenchymal markers, including α-SMA, fibronectin, and vimentin, suggesting that the cells underwent EMT. Treatment with MG132 significantly suppressed TGFβ-induced EMT, as evidenced by a decrease in the protein levels of α-SMA, fibronectin, and vimentin (Fig. **1C-E**). Moreover, we examined the transcription of these mesenchymal markers by measuring the mRNA transcript levels. We found that TGFβ treatment significantly induced transcription of α-SMA, fibronectin, and vimentin, which could be blocked by co-treatment with MG132 (Fig. **1F**). These findings demonstrated that the reduction of these mesenchymal markers under proteotoxic stress directly resulted from transcriptional downregulation.

EMT facilitates cell migration by promoting the degradation of the underlying basement membrane [31]. During the formation of PVR and PDR, TGFβ contributes to the migration of RPE cells, glial cells, fibroblasts, and inflammatory cells [32], and our group and others have shown that blockade of TGFβ-induced EMT could significantly inhibit cell migration [3, 33, 34]. However, direct inhibition of TGFβ signaling may not be an ideal therapeutic target, as TGFβ is pivotal for the maintenance of normal retinal structure and function [35]. To assess whether proteotoxic stress also negatively regulate the migration of RPE cells, we performed a wound scratch assay. We found that the migration of RPE cells was significantly enhanced upon TGFβ treatment for 24 h, which could be suppressed by adding MG132 (Fig. **2A-B**).

3.2. Proteotoxic Stress Suppresses TGFβ-signaling Through Downregulation of TGFβ Receptor II in ARPE-19

To further dissect the mechanism of how proteotoxic stress regulates TGFβ-induced EMT and migration in RPE cells, we measured the phosphorylation level of Smad2, which was involved in the canonical TGFβ signaling pathway, as wells as the phosphorylation levels of ERK1/2 and FAK, which were involved in the non-canonical TGFβ signaling pathway [9, 10]. We found that upon treatment with TGFβ for 48 hours, the phosphorylation levels of Smad2, ERK1/2, and FAK were significantly increased, whereas the total expression level of these proteins remained constant (Fig. **3A-B**). The TGFβ-induced phosphorylation of Smad2, ERK1/2, and FAK could be suppressed by MG132 treatment (Fig. **3A-B**), indicating that both canonical and non-canonical TGFβ signaling were negatively regulated under proteotoxic stress. TGFβ signaling is initiated by TGFβ binding to TGFβ receptor type II (TGFβR-II), followed by its recruitment and phosphorylation of TGFβ receptor type I (TGFβR-I) [7, 8]. The expression levels of TGFβ receptors are tightly regulated to ensure appropriate physiological

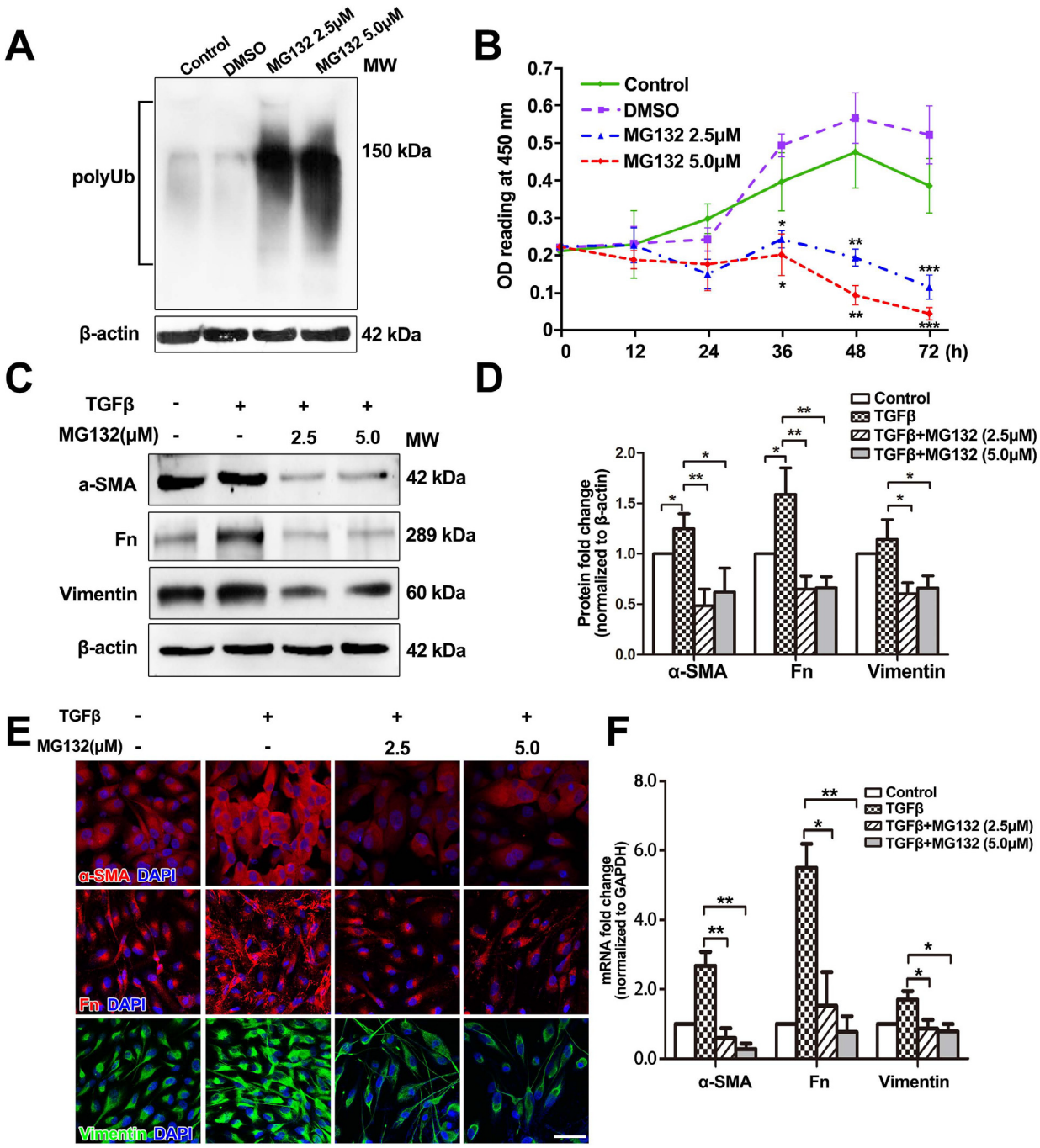

Fig. (1). Inhibition of proteasome results in proteotoxic stress, and suppresses TGFβ-induced EMT in ARPE-19. A. Cultured ARPE-19 cells were exposed to 2.5 μM or 5.0 μM MG132 for 48 hours. Untreated cells and cells treated with DMSO for 48 hours were used as controls. Proteins were extracted and probed for ubiquitin. β-actin was used as a loading control. MW: molecular weight. **B.** Cell proliferation was analyzed by CCK-8 assay after treatment with DMSO, 2.5 μM MG132, and 5.0 μM MG132 for 0, 12, 24, 36, 48, and 72 hours. OD readings at 450 nm of the MG132 treatment groups at each time point were compared to that of the DMSO group. **P*<0.05, ***P*<0.01, ****P*<0.001, n=3. **C.** Cultured ARPE-19 cells were treated with TGFβ, TGFβ+2.5 μM MG132, and TGFβ+5.0 μM MG132 for 48 hours. Proteins were extracted and probed for α-SMA, fibronectin (Fn), and vimentin (Vim). β-actin was used as a loading control. MW: molecular weight. **D.** Quantification of the protein expression levels in C. Fold change relative to the level of the untreated group (control) is displayed. **P*<0.05, ***P*<0.01, n=3. **E.** Cultured ARPE-19 cells were treated with TGFβ, TGFβ+2.5 μM MG132, and TGFβ+5.0 μM MG132 for 48 hours. Immunofluorescence was performed by probing α-SMA, Fn, and Vim. Scale bar: 100 μm. **F.** Cultured ARPE-19 cells were treated with TGFβ, TGFβ+2.5 μM MG132, and TGFβ+5.0 μM MG132 for 48 hours. The mRNA levels of α-SMA, Fn, and Vim were determined by real-time PCR and normalized to GAPDH. Fold change relative to the level of the untreated group (control) is displayed. **P*<0.05, ***P*<0.01, n=3.

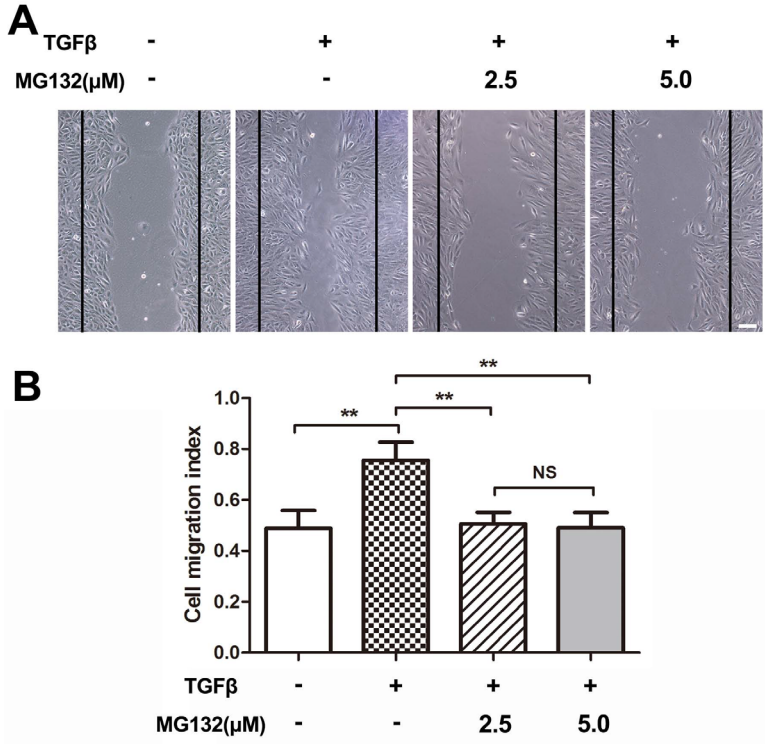

Fig. (2). Proteotoxic stress suppresses TGFβ-induced migration of ARPE-19. A. Cultured ARPE-19 cells were treated with TGFβ, TGFβ+2.5 μM MG132, and TGFβ+5.0 μM MG132 for 24 hours. Cell migration was observed by an inverted phase contrast microscope. Straight black lines indicate the wound edges. Scale bar: 100 μm. **B.** Cell migration index was calculated. ***P*<0.01, NS: not significant, n=6.

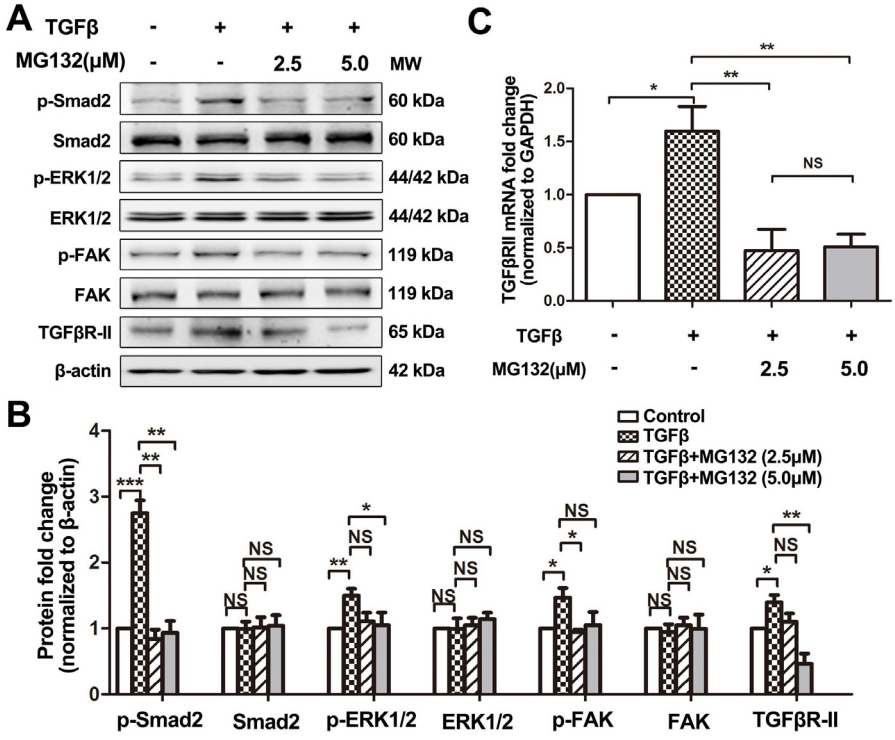

Fig. (3). Proteotoxic stress suppresses TGFβ signaling by downregulation of TGFβR-II in ARPE-19. A. Cultured ARPE-19 cells were treated with TGFβ, TGFβ+2.5 μM MG132, and TGFβ+5.0 μM MG132 for 48 hours. Proteins were extracted and probed for p-Smad2, total Smad2, p-ERK1/2, total ERK1/2, p-FAK, total FAK, and TGFβR-II. β-actin was used as a loading control. MW: molecular weight. **B.** Quantification of the protein expression levels in A. Fold change relative to the level of the untreated groups is displayed. **P*<0.05, ***P*<0.01, ****P*<0.001, NS: not significant, n=3. **C.** The mRNA level of TGFβR-II was determined by real-time PCR and normalized to GAPDH. Fold change relative to the level of the untreated group is displayed. **P*<0.05, ***P*<0.01, NS: not significant, n=3.

responses, and can be induced by the ligand TGFβ to potentiate ignaling [36]. We found an increased expression level of TGFβR-II upon TGFβ treatment in RPE cells (Fig. **3A-C**). Notably, the protein and mRNA levels of TGFβR-II were both significantly decreased upon MG132 treatment, suggesting that proteotoxic stress induced by MG132 negatively regulated TGFβ signaling through downregulation of TGFβR-II transcription in ARPE-19 (Fig. **3A-C**).

3.3. Proteotoxic Stress Suppresses TGFβ-signaling Through Downregulation of TGFβ Receptor II in Primary Human RPE Cells

We next attempted to validate the above findings in isolated primary human RPE cells. The donor eyes were obtained from the eye bank of Zhongshan Ophthalmic Center. The pigment granules were preserved after the second passage (Fig. **4A,** red arrowheads), and we harvest these cells to perform the experiments. Consistent with our findings in ARPE-19, TGFβ-induced EMT was significantly suppressed by MG132 treatment (Fig. **4B-D**). This negative regulation of TGFβ signaling under proteotoxic stress was attributed to the downregulation of TGFβR-II, and followed by reduced phosphorylation of Smad2, ERK1/2, and FAK (Fig. **5**).

4. DISCUSSION

The innermost layer of the eye, the retina, originates as outgrowths of the brain during development; thus, like other tissues derived from the central nervous system, the retina remains very limited regenerative capacity after adulthood [37]. The retina consists of the neural retina and retinal pigment epithelium. The retinal pigment epithelium is a highly specialized monolayer of pigmented cells that interact with the light-sensitive outer segments of the photoreceptors on its apical side, and with Bruch's membrane and the choriocapillaris on its basal side. The retinal pigment epithelium is essential for visual function due to its key roles in light absorption, metabolic exchange, ion transportation, visual cycle maintenance, phagocytosis, secretion, and immune modulation [38]. Diverse environmental stresses can cause injury or death of RPE cells, leading to irreversible vision loss [37]. Therefore, it is essential to understand the pathophysiological roles of retinal pigment epithelium in retinal health and diseases. Identifying pharmaceutical targets to maintain RPE health may potentially be the key to cure refractory retinal diseases.

Cell proliferation, migration, and EMT are the main contributors of various intraocular fibrotic disorders, including PVR, PDR, and postoperative capsule opacification after cataract surgery [39-45]. It is intriguing to study how clinically distinct diseases occur through shared biological processes, pathways, or molecules, and thereby developing novel therapeutic methods. In this study, we demonstrated that proteotoxic stress induced by proteasome inhibitor MG132 potently inhibits the proliferation, migration, and EMT of RPE cells in both ARPE-19 and human primary RPE cells.

Proteotoxic stress can trigger a complex of response pathways in the cytosol [46, 47], endoplasmic reticulum [48], mitochondria [49], and nucleus [50] to restore or enhance proper protein folding and dampen cytotoxicity [51]. Proteotoxic stress has been extensively studied in neurodegeneration, cancer, aging, cardiovascular diseases. Our lab as well as others has shown that proteotoxic stress can be experimentally triggered by the proteasome inhibitor MG132 [52-55]. However, the role of proteotoxic stress varies among different cell types. For example, proteotoxic stress can either promote [21, 23] or inhibit [24-26] EMT. Awasthi *et al*. reported that proteotoxic stress induced by the proteasome inhibitor MG132 could inhibit the EMT as well as proliferation in lens epithelial cells [56, 57]. More recently, Kaarniranta *et al*. found that the heat shock protein 70, ubiquitin protein conjugates and SQSTM1/p62 were upregulated in the basal corneal epithelial cells from macular corneal dystrophy samples as well as in the human corneal epithelial cells upon MG132 inhibition, suggesting a novel proteotoxic stress associated mechanism for macular corneal dystrophy [58]. As with lens epithelial cells and corneal epithelial cells, the epithelial phenotype is also essential for the proper function of RPE cells. The close but uncertain relationship between proteotoxic stress and EMT process strongly drives us to study the role of proteasome pathway in the RPE cells. Intriguingly, Tang *et al*. reported that non-lethal doses of proteasome inhibitors promotes the resistance of human RPE cells to oxidative injury through the activation of autophagy pathway [59], the other main pathway working together with the proteasome pathway in the maintenance of proteostasis. In this study, we demonstrated that the proteotoxic stress induced by the proteasome inhibitor MG132 could suppress the EMT process in both human RPE cell line and primary human RPE cells. Moreover, our study found that the same proteotoxic stress inhibit the proliferation as well as migration in human RPE cells. Our findings collectively highlighted the potential of modulating proteotoxic stress levels in developing treatment against refractory retinal diseases, such as PVR and PDR, which share the common fibrotic pathologic changes during disease progress.

Another highlight of our study is that we, for the first time, showed the role of proteotoxic stress in decreasing the expression level of TGFβR-II and thus suppressing the TGF signaling pathway. This at least partially explain the molecular mechanism how proteotoxic stress induced by proteasome inhibition suppress the EMT process, especially upon TGF induction. EMT can be induced by various growth factors, including TGFβ, fibroblast growth factor (FGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF), which are essential regulators for cell proliferation, differentiation, and migration during development [60-63]. We showed that in human RPE

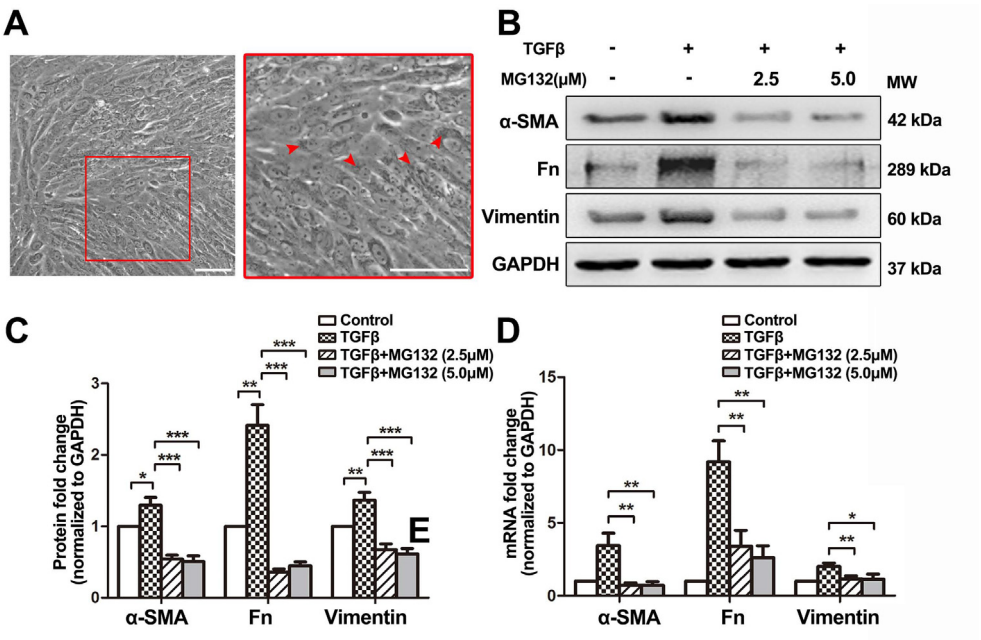

Fig. (4). Proteotoxic stress induces apoptosis and inhibits TGFβ-induced EMT in primary human RPE cells. A. Representative inverted microscope images show the pigment granules in the primary human RPE cells after the second passage (red arrowheads). Scale bar: 100 μm. **B.** Cultured human primary RPE cells (passage 3) were treated with 2.5 μM or 5.0 μM MG132 for 48 hours. Proteins were extracted and probed for α-SMA, Fn, Vim. GAPDH was used as a loading control. MW: molecular weight. **C.** Quantification of the protein expression levels in B. Fold change relative to the level of the untreated groups is displayed. **P*<0.05, ***P*<0.01, ****P*<0.001, n=3. **D.** Cultured human primary RPE cells (passage 3) were treated with 2.5 μM or 5.0 μM MG132 for 48 hours. The mRNA levels of α-SMA, Fn and Vim were determined by real-time PCR and normalized to GAPDH. Fold change relative to the level of the untreated groups is displayed. **P*<0.05, ***P*<0.01, n=3.

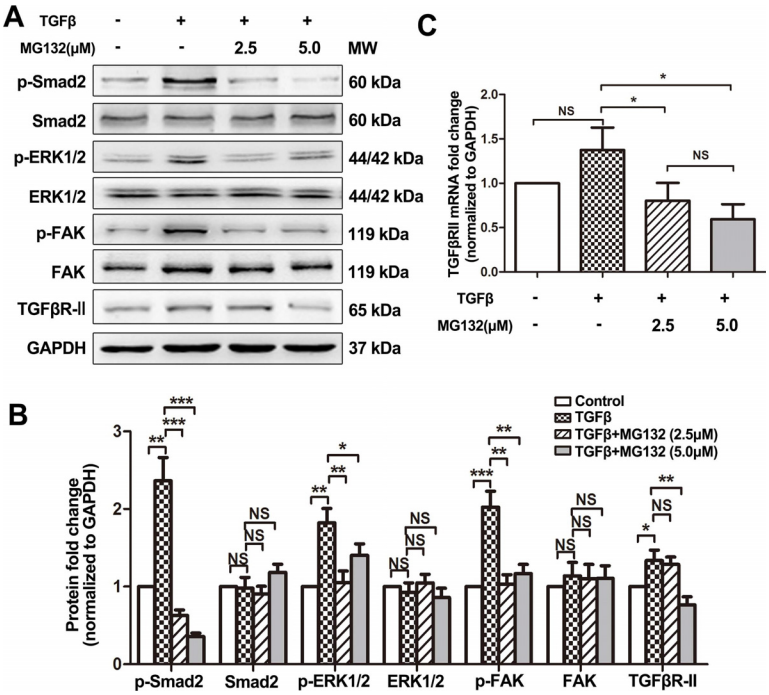

Fig. (5). Proteotoxic stress suppresses TGFβ signaling by downregulation of TGFβR-II in primary human RPE cells. A. Cultured human primary RPE cells (passage 3) were treated with 2.5 μM or 5.0 μM MG132 for 48 hours. Proteins were extracted and probed for p-Smad2, total Smad2, p-ERK1/2, total ERK1/2, p-FAK, total FAK and TGFβR-II. GAPDH was used as a loading control. MW: molecular weight. **B.** Quantification of the protein expression levels in A. Fold change relative to the level of the untreated group is displayed. **P*<0.05, ***P*<0.01, ****P*<0.001, NS: not significant, n=3. **C.** Cultured human primary RPE cells (passage 3) were treated with 2.5 μM or 5.0 μM MG132 for 48 hours. The mRNA level of TGFβR-II was determined by real-time PCR and normalized to GAPDH. Fold change relative to the level of the untreated group is displayed. **P*<0.05, NS: not significant, n=3.

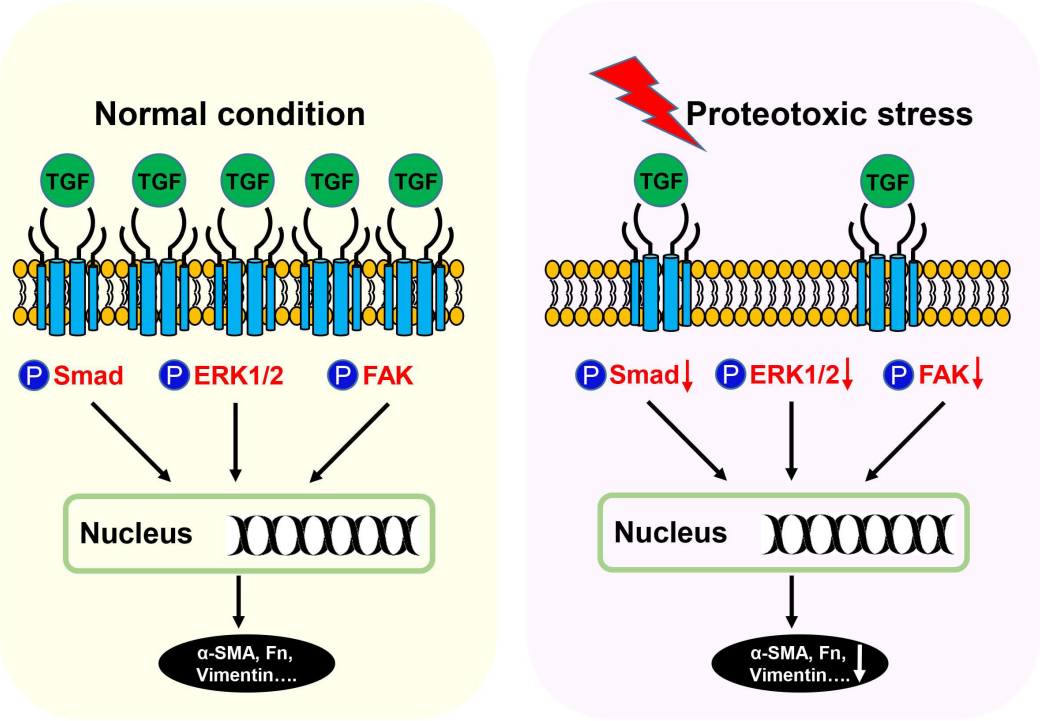

Fig. (6). A working model for regulation of TGFβ signaling pathway under proteotoxic stress. The binding of TGFβ ligand to the TGFβ receptor (TGFβR-II) initiates TGFβ signaling. The canonical TGFβ pathway needs phosphorylation of Smad2, whereas the non-canonical TGFβ pathway needs phosphorylation of ERK1/2 or FAK. Proteotoxic stress downregulates the expression of TGFβR-II and suppresses activation of the downstream effectors Smad2, ERK1/2, and FAK, thus inhibiting EMT.

cells, proteotoxic stress induced by proteasome inhibition could suppress both canonical and non-canonical TGFβ signaling pathways, by decreasing the phosphorylation level of Smad2, ERK1/2 and FAK. This finding suggested that proteotoxic stress may function at a more upstream level of Smad2 and ERK1/2, in other words, at the level of TGFβ receptor. Consistent with this idea, we found that upon proteotoxic stress induced by proteasome inhibition, the TGFβ receptor was downregulated at both mRNA level and protein level. This suggested the role of proteotoxic stress in the TGFβ receptor gene expression (Fig. **6**). This finding is consistent with the report that nucleus also has a role in responding to proteotoxic stress [50]. However, the specific mechanisms how TGFβ receptor gene is downregulated upon proteotoxic stress need to be addressed in future studies.

CONCLUSION

In summary, our study demonstrated in both human RPE cell line and human primary RPE cells that proteotoxic stress induced by the proteasome inhibitor MG132 could suppress the proliferation, migration, and TGFβ-induced EMT. We also characterized that the suppression of TGFβ-induced EMT by proteotoxic stress is at least partially through the downregulation of TGFβ receptor gene expression. Our findings not only elucidated the role of proteotoxic stress in the cellular behaviors of human RPE cells, but also highlighted the potential of treating refractory retinal disorders such as PVR and PDR through the modulation of cellular proteotoxic stress level.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Pastor JC. Proliferative vitreoretinopathy: an overview. Surv Ophthalmol 1998; 43(1): 3-18.

[2] Zhang X, Saaddine JB, Chou C-F, *et al.* Prevalence of diabetic retinopathy in the United States, 2005-2008. JAMA 2010; 304(6): 649-56.

[3] Tan X, Zhu Y, Chen C, *et al.* Sprouty2 Suppresses Epithelial-Mesenchymal Transition of Human Lens Epithelial Cells

through Blockade of Smad2 and ERK1/2 Pathways. PLoS One 2016; 11(7): e0159275.
[4] Cicchini C, Laudadio I, Citarella F*, et al.* TGFβ-induced EMT requires focal adhesion kinase (FAK) signaling. Exp Cell Res 2008; 314(1): 143-52.
[5] Hoerster R, Muether PS, Vierkotten S*,* Hermann MM, Kirchhof B, Fauser S*.*. Upregulation of TGF-ss1 in experimental proliferative vitreoretinopathy is accompanied by epithelial to mesenchymal transition. Graefes Arch Clin Exp Ophthalmol 2014; 252(1): 11-16.
[6] Willard AL, Herman IM. Vascular complications and diabetes: current therapies and future challenges. J Ophthalmol 2012; 2012: 209538.
[7] Nakao A, Imamura T, Souchelnytskyi S*, et al.* TGF‐β receptor‐mediated signalling through Smad2, Smad3 and Smad4. EMBO J 1997; 16(17): 5353-62.
[8] Attisano L, Wrana JL. Signal transduction by the TGF-β superfamily. Science 2002; 296(5573): 1646-47.
[9] ten Dijke P, Hill CS. New insights into TGF-β–Smad signalling. Trends Biochem Sci 2004; 29(5): 265-73.
[10] Zhang YE. Non-Smad pathways in TGF-β signaling. Cell Res 2009; 19(1): 128-39.
[11] Parvani JG, Taylor MA, Schiemann WP. Noncanonical TGF-β signaling during mammary tumorigenesis. J Mammary Gland Biol Neoplasia 2011; 16(2): 127-46.
[12] Brzović-Šarić V, Landeka I, Šarić B*, et al.* Levels of selected oxidative stress markers in the vitreous and serum of diabetic retinopathy patients. Mol Vis 2015; 21: 649-64.
[13] Berlett BS, Stadtman ER. Protein oxidation in aging, disease, and oxidative stress. J Biol Chem 1997; 272(33): 20313-16.
[14] Grune T. Oxidative stress, aging and the proteasomal system. Biogerontology 2000; 1(1): 31-40.
[15] Zhang X, Zhou J, Fernandes AF*, et al.* The proteasome: a target of oxidative damage in cultured human retina pigment epithelial cells. Invest Ophthalmol Vis Sci 2008; 49(8): 3622-30.
[16] Goldberg AL. Protein degradation and protection against misfolded or damaged proteins. Nature 2003; 426(6968): 895-99.
[17] Dias V, Junn E, Mouradian MM. The role of oxidative stress in Parkinson's disease. J Parkinsons Dis 2013; 3(4): 461-91.
[18] Caballero M, Liton PB, Epstein DL*,* Gonzalez P. Proteasome inhibition by chronic oxidative stress in human trabecular meshwork cells. Biochem Biophys Res Commun 2003; 308(2): 346-52.
[19] Louie JL, Kapphahn RJ, Ferrington DA. Proteasome function and protein oxidation in the aged retina. Exp Eye Res 2002; 75(3): 271-84.
[20] Fernandes AF, Zhou J, Zhang X*, et al.* Oxidative inactivation of the proteasome in retinal pigment epithelial cells. A potential link between oxidative stress and up-regulation of interleukin-8. J Biol Chem 2008; 283(30): 20745-53.
[21] Powell CD, Paullin TR, Aoisa C, Menzie CJ, Ubaldini A, Westerheide SD. The Heat Shock Transcription Factor HSF1 Induces Ovarian Cancer Epithelial-Mesenchymal Transition in a 3D Spheroid Growth Model. PLoS One 2016; 11(12): e0168389.
[22] Mahalingaiah PKS, Ponnusamy L, Singh KP. Chronic oxidative stress leads to malignant transformation along with acquisition of stem cell characteristics, and epithelial to mesenchymal transition in human renal epithelial cells. J Cell Physiol 2015; 230(8): 1916-28.
[23] Kim JY, Nam JK, Lee S*, et al.* Proteasome inhibition causes epithelial–mesenchymal transition upon TM4SF5 expression. J Cell Biochem 2011; 112(3): 782-92.
[24] Awasthi N, Wang-Su ST, Wagner BJ. Downregulation of MMP-2 and-9 by proteasome inhibition: a possible mechanism to decrease LEC migration and prevent posterior capsular opacification. Invest Ophthalmol Vis Sci 2008; 49(5): 1998-2003.
[25] Hosler MR, Wang-Su S-T, Wagner B. Role of the proteasome in TGF-β signaling in lens epithelial cells. Invest Ophthalmol Vis Sci 2006; 47(5): 2045-52.
[26] Fu J, Ju D, Xie Y. Low Cell Density Induced Epithelial-Mesenchymal Transition in MCF10A Cell is Regulated by Proteasome Activity. J Oncobiomarkers 2013; 1(2): 4.
[27] Alge CS, Suppmann S, Priglinger SG*, et al.* Comparative proteome analysis of native differentiated and cultured dedifferentiated human RPE cells. Invest Ophthalmol Vis Sci 2003; 44(8): 3629-41.
[28] Chen G, Huang H, Fröhlich O*, et al.* MDM2 E3 ubiquitin ligase mediates UT-A1 urea transporter ubiquitination and degradation. Am J Physiol Renal Physiol 2008; 295(5): F1528-34.
[29] Felsenberg J, Dyck Y, Kloß A, Dahlmann B, Kloetzel PM, Eisenhardt D. Two inhibitors of the ubiquitin proteasome system enhance long-term memory formation upon olfactory conditioning in the honeybee (Apis mellifera). J Exp Biol 2014; 217(19): 3441-46. J Exp Biol 2014; 217(Pt 19): 3441-6.
[30] Zhan J, He J, Zhou Y*, et al.* Crosstalk Between the Autophagy-Lysosome Pathway and the Ubiquitin-Proteasome Pathway in Retinal Pigment Epithelial Cells. Curr Mol Med 2016; 16(5): 487-95.
[31] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial–mesenchymal transition. Nat Rev Mol Cell Biol 2014; 15(3): 178-96.
[32] Hiscott P, Sheridan C, Magee RM, Grierson I. Matrix and the retinal pigment epithelium in proliferative retinal disease. Prog Retin Eye Res 1999; 18(2): 167-90.
[33] Sun C-B, Teng W-Q, Cui J-T, Huang X-J, Yao K. The effect of anti-TGF-β 2 antibody functionalized intraocular lens on lens epithelial cell migration and epithelial–mesenchymal transition. Colloids and Surfaces B: Biointerfaces 2014; 113: 33-42.
[34] Zheng D, Song T, Zhongliu X, Wu M, Liang J, Liu Y. Downregulation of transforming growth factor-β type II receptor prohibit epithelial-to-mesenchymal transition in lens epithelium. Mol Vis 2012; 18: 1238-46.
[35] Walshe TE, Saint-Geniez M, Maharaj AS, Sekiyama E, Maldonado AE, D'Amore PA. TGF-β is required for vascular barrier function, endothelial survival and homeostasis of the adult microvasculature. PLoS One 2009; 4(4): e5149.
[36] Menke A, Geerling I, Giehl K, Vogelmann R, Reinshagen M, Adler G. Transforming growth factor-β-induced upregulation of transforming growth factor-β receptor expression in pancreatic regeneration. Biochim Biophys Acta 1999; 1449(2): 178-85.
[37] Zhao C, Wang Q, Temple S. Stem cell therapies for retinal diseases: recapitulating development to replace degenerated cells. Development 2017; 144(8): 1368-81.
[38] Strauss O. The retinal pigment epithelium in visual function. Physiol Rev 2005; 85(3): 845-81.
[39] Chen X, Ye S, Xiao W, Luo L, Liu Y. Differentially expressed microRNAs in TGFbeta2-induced epithelial-mesenchymal transition in retinal pigment epithelium cells. Int J Mol Med 2014; 33(5): 1195-200.
[40] Chan CM, Chang HH, Wang VC, Huang CL, Hung CF. Inhibitory effects of resveratrol on PDGF-BB-induced retinal pigment epithelial cell migration via PDGFRbeta, PI3K/Akt and MAPK pathways. PLoS One 2013; 8(2): e56819.
[41] Oberstein SY, Byun J, Herrera D, Chapin EA, Fisher SK, Lewis GP. Cell proliferation in human epiretinal membranes: characterization of cell types and correlation with disease condition and duration. Mol Vis 2011; 17: 1794-805.
[42] Zhu Y, Chen C, Lin Y*,* Liu Y, Wu M. Downregulation of Syndecan-4 by RNA Interference Inhibits Adhesion and bFGF-induced Proliferation of Lens Epithelial Cells. Invest Ophthalmol Vis Sci 2013; 54(15): 479-79.
[43] Xi L, Liu Y, Zhao F, Chen C, Cheng B. Analysis of glistenings in hydrophobic acrylic intraocular lenses on visual performance. Int J Ophthalmol 2014; 7(3): 446-51.
[44] Tan X, Lin H, Li Y*, et al.* Cataract screening in a rural area of Southern China: a retrospective cohort study. Lancet 2016; 388 Suppl 1: S53.
[45] Tan X, Zhan J, Zhu Y*, et al.* Improvement of Uveal and Capsular Biocompatibility of Hydrophobic Acrylic Intraocular Lens by Surface Grafting with 2-Methacryloyloxyethyl

Phosphorylcholine-Methacrylic Acid Copolymer. Sci Rep 2017; 7: 40462.
[46] Ritossa FM, Vonborstel RC. Chromosome puffs in Drosophila induced by ribonuclease. Science 1964; 145(3631): 513-4.
[47] Lindquist S, Craig EA. The heat-shock proteins. Annu Rev Genet 1988; 22: 631-77.
[48] Patil C, Walter P. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr Opin Cell Biol 2001; 13(3): 349-55.
[49] Haynes CM, Ron D. The mitochondrial UPR - protecting organelle protein homeostasis. J Cell Sci 2010; 123(Pt 22): 3849-55.
[50] Shibata Y, Morimoto RI. How the nucleus copes with proteotoxic stress. Curr Biol 2014; 24(10): R463-74.
[51] Arneaud SL, Douglas PM. The stress response paradox: fighting degeneration at the cost of cancer. FEBS J 2016; 283(22): 4047-55.
[52] Ohashi A, Ohori M, Iwai K*, et al.* Aneuploidy generates proteotoxic stress and DNA damage concurrently with p53-mediated post-mitotic apoptosis in SAC-impaired cells. Nat Commun 2015; 6: 7668.
[53] Liu Z, Taylor A, Liu Y, Wu M, Gong X, Shang F. Enhancement of ubiquitin conjugation activity reduces intracellular aggregation of V76D mutant gammaD-crystallin. Invest Ophthalmol Vis Sci 2012; 53(10): 6655-65.
[54] Zhang X, Dudek EJ, Liu B*, et al.* Degradation of C-terminal truncated alpha A-crystallins by the ubiquitin-proteasome pathway. Invest Ophthalmol Vis Sci 2007; 48(9): 4200-8.
[55] Bao X, Hou M, Qin Y, Luo F, Shang F, Wu M. Effect of an MG132-Sustained Drug Delivery Capsular Ring on the Inhibition of Posterior Capsule Opacification in a Rabbit Model. J Ocul Pharmacol Ther 2017; 33(2): 103-10.
[56] Awasthi N, Wang-Su ST, Wagner BJ. Downregulation of MMP-2 and -9 by proteasome inhibition: a possible mechanism to decrease LEC migration and prevent posterior capsular opacification. Invest Ophthalmol Vis Sci 2008; 49(5): 1998-2003.
[57] Awasthi N, Wagner BJ. Suppression of human lens epithelial cell proliferation by proteasome inhibition, a potential defense against posterior capsular opacification. Invest Ophthalmol Vis Sci 2006; 47(10): 4482-9.
[58] Kaarniranta K, Szalai E, Smedowski A*, et al.* A novel proteotoxic stress associated mechanism for macular corneal dystrophy. Histol Histopathol 2015; 30(8): 921-30.
[59] Tang B, Cai J, Sun L*, et al.* Proteasome inhibitors activate autophagy involving inhibition of PI3K-Akt-mTOR pathway as an anti-oxidation defense in human RPE cells. PLoS One 2014; 9(7): e103364.
[60] Lin Y, Ai S, Chen C*, et al.* Ala344Pro mutation in the FGFR2 gene and related clinical findings in one Chinese family with Crouzon syndrome. Mol Vis 2012; 18: 1278-82.
[61] Lin Y, Liang X, Ai S*, et al.* FGFR2 molecular analysis and related clinical findings in one Chinese family with Crouzon syndrome. Mol Vis 2012; 18: 449-54.
[62] Lin Y, Liu X, Yu S, *et al.* PAX6 analysis of two sporadic patients from southern China with classic aniridia. Mol Vis 2012; 18: 2190-4.
[63] Zhang Y, Morgan R, Chen C, *et al.* Mammary-tumor-educated B cells acquire LAP/TGF-beta and PD-L1 expression and suppress anti-tumor immune responses. Int Immunol 2016; 28(9): 423-33.