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Involvement of the ubiquitin-proteasome system in the expression of extracellular matrix genes in retinal pigment epithelial cells

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ABSTRACT

Emerging evidence suggests that dysfunction of the ubiquitin-proteasome system is involved in the pathogenesis of numerous senile degenerative diseases including retinal disorders. The aim of this study was to assess whether there is a link between proteasome regulation and retinal pigment epithelium (RPE)-mediated expression of extracellular matrix genes. For this purpose, human retinal pigment epithelial cells (ARPE-19) were treated with different concentrations of transforming growth factor-β (TGFβ), connective tissue growth factor (CTGF), interferon-γ (IFNγ) and the irreversible proteasome inhibitor epoxomicin. First, cytotoxicity and proliferation assays were carried out. The expression of proteasome-related genes and proteins was assessed and proteasome activity was determined. Then, expression of fibrosis-associated factors fibronectin (FN), fibronectin EDA domain (FN EDA), metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinases-1 (TIMP-1) and peroxisome proliferator-associated receptor-γ (PPARγ) was assessed. The proteasome inhibitor epoxomicin strongly arrested cell cycle progression and down-regulated TGFβ gene expression, which in turn was shown to induce expression of pro-fibrogenic genes in ARPE-19 cells. Furthermore, epoxomicin induced a directional shift in the balance between MMP-2 and TIMP-1 and was associated with down-regulation of transcription of extracellular matrix genes FN and FN-EDA and up-regulation of the anti-fibrogenic factor PPARγ. In addition, both CTGF and TGFβ were shown to affect expression of proteasome-associated mRNA and protein levels. Our results suggest a link between proteasome activity and pro-fibrogenic mechanisms in the RPE, which could imply a role for proteasome-modulating agents in the treatment of retinal disorders characterized by RPE-mediated fibrogenic responses.

1. Introduction

Age-related macular degeneration (AMD) is a progressive disease of the central retina-choroid tissue complex and one of the leading causes of blindness worldwide [1]. The retinal pigment epithelium (RPE), a polarized monolayer of epithelial cells that separates the neural retina from the vascularized choroid, has been implied to play an important role in the pathogenesis of the disease. Early AMD is characterized by focal drusen deposits in the macula, mostly located between the basal lamina of the RPE and the inner collagenous layer of Bruch's membrane [2]. Drusen contain carbohydrates, zinc and nearly 150 proteins including vitronectin, apolipoproteins E and B, clusterin, connective tissue growth factor (CTGF) and complement system components [3,4]. Advanced AMD is divided into nonexudative or dry AMD which affects 8% of patients and is characterized by macular RPE atrophy and ensuing photoreceptor degeneration, and exudative or neovascular AMD (nAMD) which affects 5% of patients and is characterized by the development of choroidal neovascularization (CNV) [5]. CNV may ultimately lead to the development of a fibrous plaque or disciform scar that leads to secondary atrophy of the neurosensory retina and irreversible and untreatable loss of macular visual function [6–10]. The advent of anti-vascular endothelial growth factor (VEGF) therapy has

Abbreviations: AMD, age-related macular degeneration; ARPE-19, human retinal pigment epithelial cells; CNV, choroidal neovascularization; CTGF, connective tissue growth factor; EMT, epithelial-mesenchymal transition; ECM, extracellular matrix; FN, fibronectin; FN EDA, fibronectin EDA domain; IFNγ, interferon-γ; MMP-2, matrix metalloproteinase-2; nAMD, neovascular age-related macular degeneration; PPARγ, peroxisome proliferator-associated receptor-γ; RPE, retinal pigment epithelium; TIMP-1, tissue inhibitor of metalloproteinases-1; TGFβ, transforming growth factor-β; UPS, ubiquitin-proteasome system

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greatly improved the prognosis of nAMD patients, stabilizing or even improving visual function [11–13]. Subretinal fibrosis, however, is a common ensuing process of CNV membrane formation, occurring in approximately half of anti-VEGF treated eyes [7,10,14].

Fibrosis may be considered as a deregulated wound healing response to tissue damage [10,15,16]. Angiogenesis occurs in this process as an initial trigger for fibrin deposition, tissue repair, oxygen supply and recruitment of inflammatory cells to the wound [10,17]. In AMD, angiogenesis occurs in the subretinal or sub-RPE space, leading to exudation, hemorrhage and eventually fibrosis. During this process, various types of cells such as RPE cells, glial cells, fibroblasts, myofibroblast-like cells and macrophages infiltrate and/or proliferate, secreting pro-angiogenic and pro-fibrogenic factors that interact with inflammatory cytokines and growth factors. Prevention of visual loss in AMD may therefore depend on the development of successful therapeutic regimens that can halt subretinal fibrosis and preserve the RPE.

The fibrogenic response is stimulated by inflammatory-derived cytokines and growth factors, including transforming growth factor-β (TGFβ) [18], an ubiquitously expressed growth factor belonging to the large superfamily of activins/bone morphogenetic proteins [19] and connective tissue growth factor (CTGF), a member of the connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family of extracellular matrix (ECM) proteins, also known as CCN2 [20,21]. The expression of CTGF is regulated by TGFβ [22–24] and, likewise, CTGF has been shown to be an important mediator of TGFβ signaling and its effects in different cell types [24–32]. We and others have shown that both TGFβ and CTGF are major players in the fibrogenic response in the retina [4,16,21,33–40].

The ubiquitin-proteasome system (UPS), a multi-catalytic cytoplasmic and nuclear protein complex present in all eukaryotic cells, is responsible for non-lysosomal proteolysis and thus maintenance of a normal protein homeostasis in cells [41]. Mounting evidence suggests that UPS dysfunction is a major pathogenic mechanism in senile degenerative disorders [42], including AMD and other ophthalmic conditions [43–53]. Proteasomes diffuse rapidly in the cytoplasm and nucleus where they encounter intracellular proteins that are appropriately tagged or misfolded. Proteins are tagged by ubiquitination processes and as such recognized by the 19S regulatory particle of the proteasome [54]. Ubiquitin has been shown to be uniformly expressed in the RPE-Bruch's membrane complex of patients afflicted with AMD [53]. The 19S regulatory particle, combined with the 20S catalytic core, forms the standard proteasome. Within the proteasome core, specialized catalytic subunits are responsible for the cleavage of the carboxyl termini of proteins. There are 3 catalytic subunits in the standard proteasome: β1 for acidic amino acids, β2 for basic amino acids, and β5 for hydrophobic amino acids. The standard proteasome may in some instances undergo a change in configuration into the immunoproteasome. This is achieved upon replacement of the constitutive subunits in the standard proteasome by inducible subunits, β1i, β2i, and β5i [55,56]. Although uninjured RPE contains a baseline level of immunoproteasome subunits [52], cellular stress, such as retinal injury by cytotoxic T-lymphocytes [47], optic nerve trauma [57], aging mechanisms [44,48], complement overactivation [52], chronic oxidative stress [58] and exposure to pro-inflammatory cytokines [52,59] may increase the number of active immunoproteasome subunits. Therefore, the ratio between the nascent (β1, β2, and β5) and inducible subunits (β1i, β2i, and β5i) may be used as a marker of cellular stress [47,48,58].

The aim of the present study was to characterize the involvement of the proteasome pathway in TGFβ and CTGF-mediated expression of ECM genes in RPE cells. Likewise, potential anti-fibrogenic effects of the selective proteasome inhibitor epoxomicin were assessed in ARPE-19 cell cultures.

2. Materials and methods

2.1. Culture, maintenance and treatment of ARPE-19 cells

Experiments were conducted using ARPE-19 cells, a human RPE cell line that has structural and functional properties that are characteristic of RPE cells in vivo. Monolayers of cells cultured on transwell filters reached a transepithelial resistance of 30–40 omega cm^2 after 3 weeks of culture and expressed CRALBP, as detected by RT-PCR. Cells were cultured at 37 °C in 5% CO2 in gelatin-coated T75 cell culture flasks (Corning, Lowell, MA, USA) in Dulbecco Modified Eagle Medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA), low glucose, pyruvate in the presence of 1% penicillin/streptomycin and 10% fetal calf serum. Cell growth was monitored and medium was changed twice a week. For passaging of cells, TrypLE Express (Invitrogen, Carlsbad, CA, USA) was used and cell suspensions were diluted 3-fold. For experiments, cells were cultured in 6-well plates. Upon confluence, cells were washed once with phosphate-buffered saline (PBS), serum starved for 24 h and then treated with various concentrations of the selective and irreversible proteasome inhibitor epoxomicin (Sigma-Aldrich, St. Louis, MO, USA), rhCTGF (ProSpec-Tany TechnoGene, Rehovot, Israel), rhTGFβ1 (ProSpec) and interferon-γ (IFNγ) (PBL Biomedical, Piscataway, NJ, USA). All experiments were performed in triplicate and repeated at least twice.

2.2. Protein extraction

Cells were harvested using TrypLE Express (Invitrogen), collected in Eppendorf tubes and centrifuged for 10 min at 400*g*. Supernatant was removed and the pellet was suspended in TSDG buffer (10 mM Tris, pH 7.5, 25 mM KCl, 10 mM NaCl, 1.1 mM $MgCl_2$, 0.1 mM EDTA, and 8% glycerol), 5 mM ATP and 1x protease inhibitor (Roche Applied Science, Penzberg, Germany).

Cells were lysed with 3 cycles of freezing in liquid nitrogen and thawed at room temp. After centrifugation (15 min, 10,000*g*), the protein concentration was determined using a Bradford protein assay (Serva, Heidelberg, Germany). All experiments were performed in triplicate and repeated at least twice.

2.3. Cell cycle and cell viability assays

To assess the viability of healthy ARPE-19 cells and to assess the toxic effects of different stimulants used throughout assays, the PrestoBlue cytotoxicity assay (Invitrogen) was performed according to the manufacturer's instructions. The assays were carried out in 96-well plates (roughly 10,000–25,000 cells per well). After cells were conditioned and washed, PrestoBlue reagent was added to each well. The plates were subsequently incubated at 37 °C for the recommended time period (20–30 min). After incubation, the solution containing PrestoBlue reagent from the wells of the assay plates was transferred to new wells in a 96-well plate, and absorbance was read on a plate reader (Bio-Rad, Hercules, CA, USA) with the excitation/emission wavelengths set at 570/600 nm.

To evaluate the effects of different stimulants on cell proliferation, the Click-iT EdU Alexa Fluor 488 imaging kit (Invitrogen) was applied according to the protocol provided by the manufacturer. Briefly, ARPE-19 cells at 30–50% confluence were treated with EdU (10 μM). EdU was added 2 h prior to the addition of CTGF and TGFβ (both 24 h incubation) and epoxomicin (16 h incubation). Subsequently, cells were fixed, permeabilized, and click-labeled. As a negative control, untreated cells were used. Following incubation, fluorescence readout was determined using a FACS LSRII (Becton Dickinson, Breda, The Netherlands) to determine percentages of EdU-proliferative cells in S and M phase and EdU-negative quiescent cells in G0 and G1 phase. The experiment was performed in triplicate and repeated twice (N = 2).

Table 1
Primer details.

Gene	GenBank	Forward primer	Reverse primer	Size (bp)	T_m (°C)
PSME1	NM_006263	CAGCCCCATGTGGGTGATTATC	GCTTCTCGAAGTTCTTCAGGATGAT	139	82
PSMA7	NM_002792	CCTGGAAGGCCAATGCCATAG	TTTGCCACCTGACTGAACCACTTC	149	82
PSMB5	NM_002797	CCATGATCTGTGGCTGGGATAAG	GGTCATAGGAATAGCCCCGATC	144	83
PSMB8	NM_004159	CTGGAGGCGTTGTCAATATGTACC	GCAGCAGGTCACTGACATCTGTAC	81	76
VEGFA	NM_003375	GGCAGAAGGAGGAGGGCAGAAT	CACCAGGGTCTCGATTGGATGG	91	80
FN1	NM_002026	TGGGACCGTCAGGGAGAAAATG	CAGGAGCAAATGGCACCGAGAT	167	82
FN EDA	XM_005246414	GCAGTGACCAACATTGATCGC	ACCCTGTACCTGGAAACTTGCC	110	80
MMP2	NM_004530	GGAATGCCATCCCCGATAACC	CCAGCTTCAGGTAATAGGCACCCT	93	83
TIMP1	NM_003254	ACTTCCACAGGTCCCACAACCG	AGGGAAACACTGTGCATTCCTCAC	180	84
PPARG	NM_138712	CCTGCGAAAGCCTTTTGGTGAC	AAACCTGGGCGGTCTCCACT	135	79

2.4. Proteasome activity labeling

Proteasome subunits were labeled in the lysate with 0.5 μM activity-based probe BODIPY-epoxomicin for 1 h at 37 °C (BodipyFl-Ahx3L3VS, MV121, provided by H. Overkleeft, Institute of Chemistry, Leiden, The Netherlands) [60] and sample buffer (350 mM Tris-HCl pH 6.8, 10% SDS, 30% glycerol, 6% β-mercaptoethanol, 0.02% bromophenol blue), added to 20 μg protein lysate. Samples were boiled for 5 min and loaded on a 12.5% SDS-PAGE gel. As a positive control, maximal proteasome activity was determined after treatment with IFNγ (50 U). After running the proteins on the gel, fluorescence imaging was performed on a Trio Typhoon (GE Medical Systems, Little Chalfont, UK) using the 580 bandpass filter to detect the probe directly on the gel. Proteasome total activity values were normalized according to the total proteasome content in cells as indicated by the levels of the α7 subunit of the 20S proteasome (1:1000; MCP72; Enzo Life Sciences, Zandhoven, Belgium). The experiment was performed in triplicate and repeated three times (N = 3).

2.5. Western blot analysis

Proteins were isolated using a 1% Triton X-100 cell lysis buffer (10 mM Hepes, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100 and 1x Complete Protease Inhibitors; Roche Biochemicals, Almere, The Netherlands). All samples were run on SDS-PAGE under denaturing conditions. Briefly, 20 μg of protein was loaded on a 12.5% SDS-PAGE gel, and after electrophoresis transferred to nitrocellulose membranes using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were incubated overnight or longer at 4 °C with a monoclonal antibody against the α7 subunit of the 20S proteasome (1:1000; BML-PW8110-0025; Enzo Life Sciences) and the following polyclonal antibodies: anti-β5 subunit (1:1000; BML-PW8895-0100; Enzo Life Sciences) and anti-β5i subunit (1:1000; ab3329; Abcam, Cambridge, UK). Anti β-actin (1:10,000; a5441; Sigma-Aldrich, St. Louis, MI, USA) was determined for the loading control. Intensity of bands was quantified by densitometric analysis using Odyssey (LI-COR Biosciences, Lincoln, NE, USA). Quantification was performed with Image studio Lite 4.0 (LI-COR). Values were normalized using β-actin (1:10,000; ab8227; Abcam). The experiment was performed in triplicate and repeated three times (N = 3).

2.6. RNA isolation and mRNA quantification

Total RNA was isolated from ARPE-19 cell cultures using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. ARPE-19 cells were stimulated with TGFβ (5 ng, 30 ng or 50 ng for 24 h), CTGF (50 ng or 200 ng for 24 h), epoxomicin (50 nM, 100 nM, 250 nM, 500 nM for 16 h) and IFNγ (50 U for 72 h) in 6-well plates. In addition, in order to assess whether epoxomicin treatment was able to counteract TGFβ-mediated responses, TGFβ-stimulated ARPE-19 cells were treated with 500 nM epoxomicin. Total RNA (1 μg) was treated with DNAse I (amplification grade; Life Technologies) and reverse transcribed into first strand cDNA using a Maxima® First Strand cDNA Synthesis Kit (Thermo Scientific, Roskilde, Denmark). Real-time qPCR was performed using a CFX96 system (Bio-Rad) as described previously [61]. Primer details are given in Table 1. Ct-values were converted to absolute amounts with the formula 2^{-Ct} and taken relative to the absolute amounts of control samples, that were set to 1. The experiment was performed in triplicate and repeated three times (N = 3).

2.7. Enzyme-linked immunosorbent assay (ELISA)

ARPE-19 cell samples treated with epoxomicin (50 nM, 100 nM, 250 nM, 500 nM for 16 h) and supernatant was collected. Concentrations of activated TGFβ2 were determined by Quantikine ELISA assays according to the manufacturer's protocol (R&D Systems, Minneapolis MS, USA). The experiment was performed in triplicate and repeated twice (N = 2).

2.8. Statistical analysis

Data are presented as fold change, with a fold change of 1.0 meaning the same level as control samples. Asterisks (*) indicate a significant change relative to the control samples. Differences between experimental conditions were calculated with one-way or two-way ANOVA with $P < 0.05$ indicating a statistical difference. Statistical analysis of data was performed using IBM SPSS 20 (SPSS, Chicago, IL, USA).

3. Results

3.1. TGFβ and epoxomicin arrest cell cycle progression

CTGF (50 and 200 ng for 24 h), TGFβ (5 and 50 ng for 24 h) and epoxomicin (50 and 500 nM for 16 h) did not induce any significant cytotoxicity in ARPE-19 cells (data not shown). TGFβ significantly reduced cell proliferation (35–37% cell cycle arrest in G0/G1 phase corresponding to a 20% increase when compared to control), whereas CTGF did not show an anti-proliferative effect (Fig. 1). On the other hand, epoxomicin, a cell-permeable potent and selective irreversible proteasome inhibitor [62], strongly arrested cell cycle progression (Fig. 1). These results suggest that epoxomicin has significant anti-proliferative effects in ARPE-19 cells.

3.2. CTGF and TGFβ affect proteasome subunit mRNA levels

As expected, treatment with high doses of epoxomicin significantly decreased mRNA levels of PA28α and β5i (data not shown), whereas IFNγ increased mRNA levels of α7 and β5 (Fig. 2). CTGF marginally increased mRNA expression of subunit β5 (Fig. 2). Upon TGFβ stimulation, mRNA levels of the immunoproteasome subunit β5i and proteasome regulatory subunit PA28α were decreased.

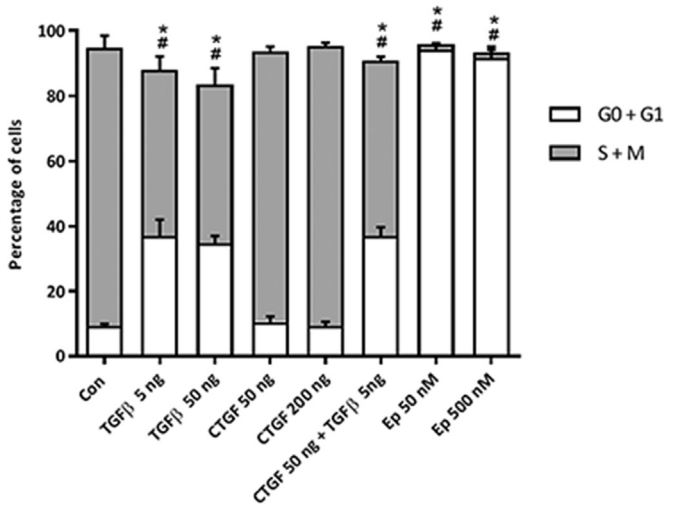

Fig. 1. Proliferation of ARPE-19 cells in the presence of TGFβ, CTGF, TGFβ and CTGF or epoxomicin expressed as percentage of cells in the S and M phase versus cells in the G0 and G1 phase after flow cytometric analysis of the percentage of cells that had incorporated EdU. *Significant difference from control of percentage of cells in G0 and G1 phase. #Significant difference from control of percentage of cells in the S and M phase. The experiment was performed in triplicate and repeated twice (N = 2).

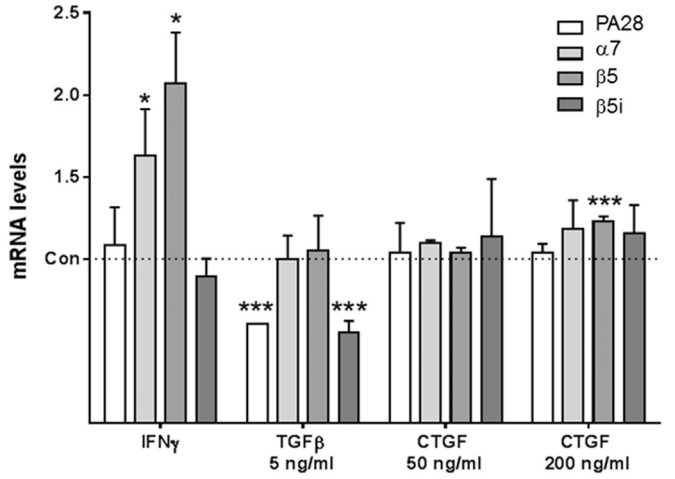

Fig. 2. Proteasome mRNA levels induced by IFNγ, TGFβ or CTGF. mRNA levels of PA28α, β5, β5i, and α7 subunits of the proteasome in ARPE-19 cells, after stimulation with IFNγ, TGFβ and low and high concentrations of CTGF. IFNγ upregulates mRNA expression of α7 and β5 subunits whereas TGFβ downregulates mRNA expression of PA28α and β5i subunits and CTGF upregulates mRNA expression of β5 subunit. Values represent mRNA expression levels (mean ± SD) relative to untreated control cells. *, Significant change ($P < 0.05$); ***, significant change ($P < 0.001$). The experiment was performed in triplicate (N = 3).

3.3. CTGF increases the β5i/β5 ratio

To assess whether the induced changes in expression of proteasome-associated genes was associated with increased protein expression levels of the respective subunits, we performed western blotting on ARPE-19 cells treated with CTGF (50 ng), TGFβ (50 ng), a combination of CTGF and TGFβ, and IFNγ as positive control (Fig. 3). The ratio of β5i and β5 (β5i/β5) was taken as a marker of immunoproteasome activation. As expected, IFNγ induced maximal immunoproteasome activation, as indicated by a 28-fold change in the β5i/β5 ratio (data not shown). Protein levels of β5i were slightly higher in the presence of CTGF (Fig. 3**A,B**) which translated in a 19% increased β5i/β5 ratio (Fig. 3**C**), whereas TGFβ stimulation down-regulated the expression level of the proteasome β5i subunit by 33% (Fig. 3**A,B**), whereas the β5i/β5 ratio was not significantly affected (Fig. 3**C**).

These results indicate that, to a limited extent, expression of immunoproteasome β5i subunit protein is up-regulated by CTGF and down-regulated by TGFβ.

3.4. CTGF upregulates proteasome activity in ARPE-19 cells

To assess whether proteasome gene expression and protein level changes induced by CTGF and TGFβ affect proteolytic activity of the proteasome, the activity of individual subunits after treatment of cells with CTGF, TGFβ or IFNγ was determined (Fig. 4). IFNγ, as expected, induced a substantial increase (4–5-fold change, $P = 0.049$) in activity of all subunits. CTGF (50 ng) significantly up-regulated the activity of the β1/β5i complex (2.6-fold change, $P = 0.005$) and β5/β1i complex (1.5-fold change, $P = 0.026$). TGFβ (30 ng) did not affect proteasomal activity, whereas a combination of CTGF (50 ng) and 6 h later TGFβ (5 ng) did not induce changes in the activity of the β1/β5i and β5/β1i complexes.

These results demonstrate that CTGF upregulates the activity of specific proteasome subunits, probably mediated by a change in the configuration of the standard proteasome into the immunoproteasome and up-regulation in mRNA and protein levels of proteasome β5 and β5i subunits.

3.5. TGFβ up-regulates mRNA levels of ECM-associated genes

In order to characterize the effects of TGFβ and CTGF on the transcription of ECM-associated genes, we assessed the mRNA levels of CTGF, TGFβ1 and TGFβ2, VEGF, fibronectin (FN), fibronectin EDA domain (FN EDA), metalloproteinase-2 (MMP-2), tissue inhibitor of metalloproteinases-1 (TIMP-1) and peroxisome proliferator-associated receptor- γ (PPARγ) upon stimulation with CTGF, TGFβ, and CTGF followed by TGFβ (Fig. 5).

TGFβ up-regulated mRNA levels of CTGF and VEGF (Fig. 5**A**). The same effect was observed when ARPE-19 cells were treated with CTGF followed by TGFβ (data not shown). This effect was dependent on the concentration of TGFβ, which implies that TGFβ may be the main mediator of this response in ARPE-19 cells.

With respect to fibrosis-related genes, TGFβ, but not CTGF, up-regulated mRNA levels of FN EDA, FN and MMP-2 (Fig. 5**B**). Transcript levels of the anti-fibrogenic factor PPARγ were down-regulated in the presence of TGFβ (Fig. 5**B**). Again, these effects were dependent on the concentration of TGFβ, irrespective of simultaneous treatment with different concentrations of CTGF (data not shown). These results confirm the role of TGFβ as a major pro-fibrogenic mediator in RPE cells.

3.6. Proteasome inhibition by epoxomicin down-regulates expression of ECM-associated genes

In order to test the effects of proteasome modulation on mRNA expression of CTGF, TGFβ1, TGFβ2, VEGF, FN, FN EDA, TIMP-1, MMP-2 and the anti-fibrogenic protein PPARγ, we assessed the effects of different concentrations of epoxomicin and TGFβ plus epoxomicin (Fig. 6).

Treatment of ARPE-19 cells with epoxomicin resulted in decreased mRNA levels of TGFβ1 and TGFβ2 (Fig. 6**A**). Mean levels of activated TGFβ2 protein were strongly reduced (to undetected levels when compared to untreated cells) after treatment of ARPE-19 cells with 50–500 nM epoxomicin (data not shown). At low concentrations (50 and 100 nM), epoxomicin down-regulated VEGF mRNA expression (Fig. 6**A**). Furthermore, treatment with epoxomicin resulted in decreased mRNA levels of FN, FN EDA, TIMP-1 and a corresponding increase in MMP-2 mRNA levels (Fig. 6**B**). Down-regulation of mRNA expression of TGFβ1, TGFβ2, FN EDA and VEGF with 500 nM epoxomicin was also observed in TGFβ-treated ARPE-19 cells. In addition, epoxomicin, alone or in the presence of TGFβ, down-regulated the expression of the anti-fibrogenic mediator PPARγ (Fig. 6**B**). These results

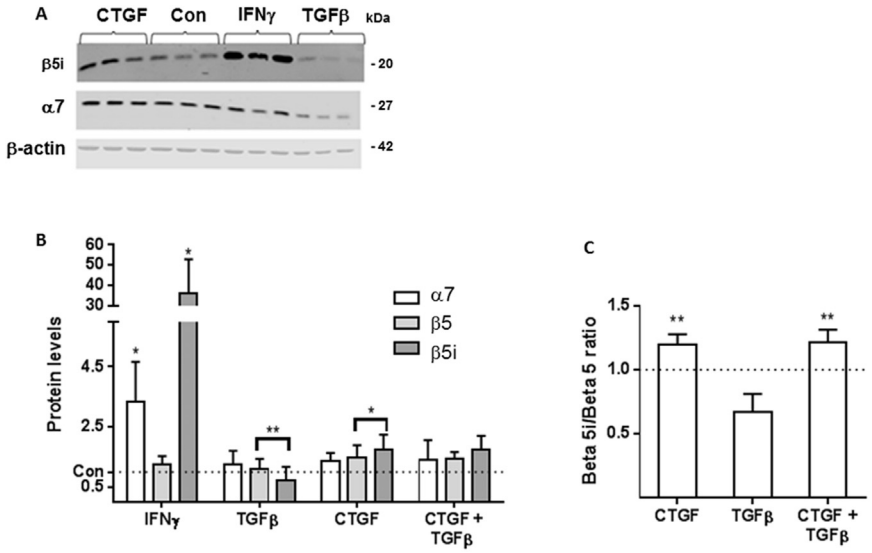

Fig. 3. Proteasome-specific subunit protein expression and β5i:β5 ratios upon IFNγ, CTGF, TGFβ or CTGF and TGFβ stimulation of ARPE-19 cells. (A) Western blot showing protein levels of β5i, α7 and actin (loading control) in cells that had been incubated with IFNγ, CTGF or TGFβ. (B) Protein levels of β5 and β5i subunits were assessed by western blot with actin as loading control and α7 subunit as proteasome content control. (C) Quantitative data of the average ratio of β5i and β5 relative to control samples after incubation in the presence or absence of CTGF, TGFβ or CTGF and TGFβ. Data are expressed as the mean ± SD. *, Significant change ($P < 0.05$): **, significant change ($P < 0.01$). The experiment was performed in triplicate (N = 3).

suggest that epoxomicin, even upon TGFβ secretion, counteracts the pro-fibrogenic transcription effects of TGFβ.

4. Discussion

This study attributes a role to the proteasome pathway in modulation of part of the fibrogenic response of RPE cells which is a multifactorial response dependent on activation and suppression of a myriad of growth factors and cytokines. For the purpose of this study, we selected TGFβ and CTGF as both are regarded as important mediators of pathological fibrosis in the eye and other organs [4,20–26,28,29,31,33,35–40,63–74]. Likewise, emerging evidence suggests a link between the fibrogenic response, proteasome modulation and TGFβ signaling in multiple systemic conditions [75–85].

Routine passaging of ARPE-19 cells was used as an in vitro wound response model to study the fibrogenic response in the retina. Epithelial cells (such as the RPE in the retina) are considered to be the major mediators of fibrogenic responses to tissue injury [10,71]. The RPE is a highly polarized monolayer of epithelial pigmented cells between the choroid and the neurosensory retina that plays a crucial role in the maintenance of visual function [86]. RPE cells proliferate and undergo epithelial-mesenchymal transition (EMT) when dissociated into single cells [87,88], whereas sheets of RPE cells in culture preserve their morphology for a longer period of time [89,90]. Disorganization and extensive damage to the RPE such as during subretinal CNV membrane formation, is a prerequisite for development of subretinal fibrosis [16]. Accordingly, subretinal fibrosis is frequently reported in late stages of nAMD [7,14]. Fibrosis in other organs such as lung, kidney, liver, skin and heart follows pathogenic pathways similar to subretinal fibrosis development in nAMD [10,91]. In all these tissues, an intact epithelium is considered protective against fibrosis development [92]. Although the ARPE-19 cell line was deemed appropriate for the purpose of this study, confirmation of the attained results in an in vivo model is warranted. Our study is focused on the fibrogenic response of RPE cells, however, we acknowledge that RPE cells are only one of many other cell types involved in RPE-mediated fibrosis.

Our results demonstrate that CTGF is associated with activation of the proteasome as demonstrated by the increased proteolytic activity of specific proteasome complexes, namely β1/β5i and β5/β1i. The proteasome activity probe assay is unable to discriminate between the activities of the various proteasome subunits. Nevertheless, it is likely that the observed changes in proteasome activity stem from β5 and β5i increased proteolytic activity since these are known to be the rate-limiting subunits at the level of the RPE [49,51]. Accordingly, these changes in proteasome activity are accompanied by a slight but significant up-regulation in β5 and β5i protein expression and β5 mRNA

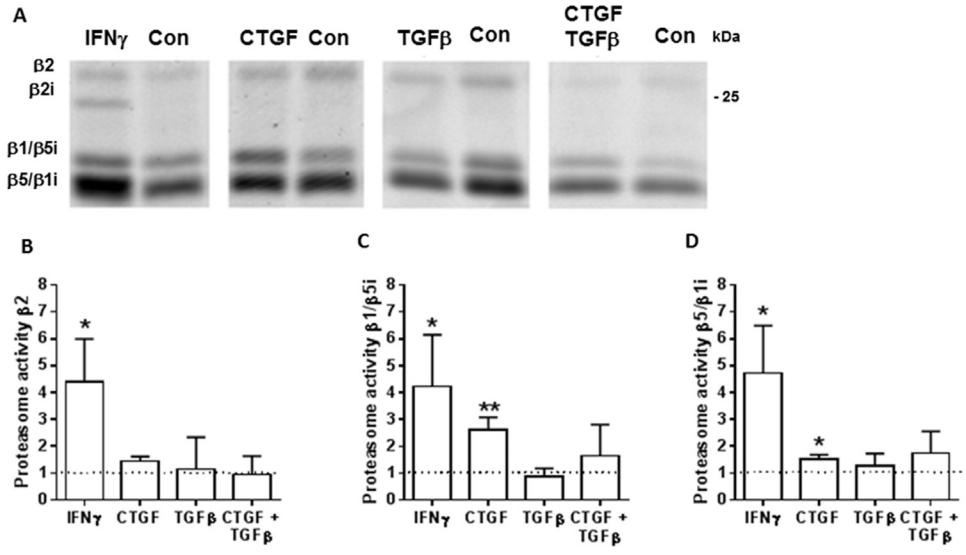

Fig. 4. Increased specific proteasome activity upon CTGF stimulation of ARPE-19 cells. (A) After treatment with IFNγ (50 U), TGFβ (50 ng), CTGF (50 ng) or CTGF (50 ng) followed 6 h later by TGFβ (5 ng), ARPE-19 cells were harvested and proteasomes were labeled with a Bodipy-Ep activity probe. Proteasome activities were assessed by western blotting. Quantitative data of the proteasome activity are presented for proteasome subunit β2 (B), β1/β5i complex (C), and β5/β1i complex (D). *, Significant change ($P < 0.05$); **, significant change ($P < 0.01$). The experiment was performed in triplicate (N = 3).

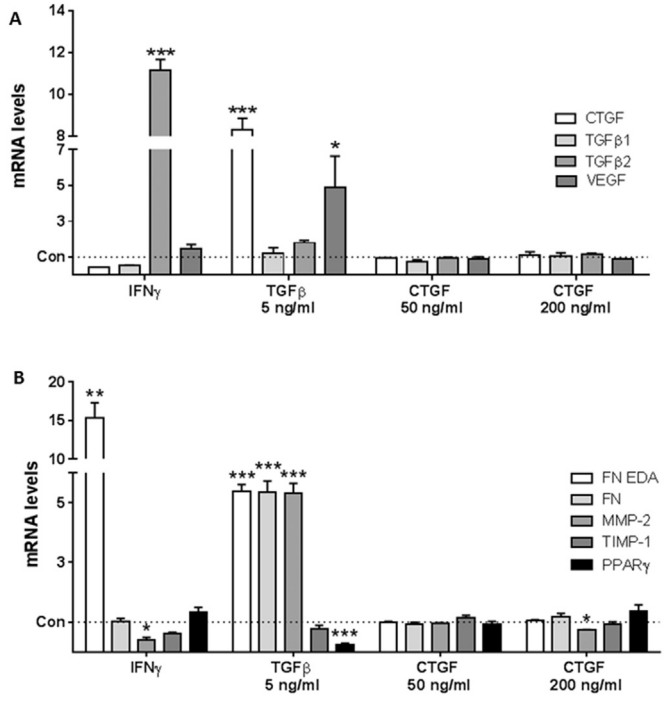

Fig. 5. TGFβ upregulates mRNA expression of CTGF, VEGF and pro-fibrogenic genes and downregulates mRNA expression of the anti-fibrogenic factor PPARγ. After stimulation with TGFβ, CTGF and IFNγ, mRNA levels of (A) CTGF, TGFβ1, TGFβ2, VEGF and (B) FN, FN EDA, MMP-2, TIMP-1 and PPARγ were assessed in ARPE-19 cells. Values represent mRNA expression levels (mean ± SD) relative to untreated control cells. *, Significant change ($P < 0.05$); **, significant change ($P < 0.01$); ***, significant change ($P < 0.001$). The experiment was performed in triplicate ($N = 3$).

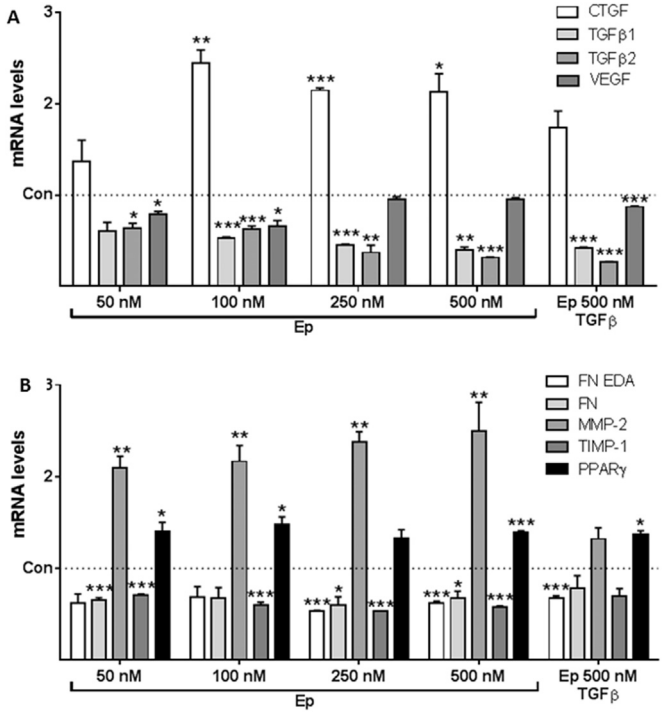

Fig. 6. Epoxomicin (Ep) downregulates mRNA expression of TGFβ1, TGFβ2, VEGF, FN, FN EDA, TIMP-1 and upregulates mRNA expression of CTGF and PPARγ. In the presence of TGFβ, epoxomicin dowregulates mRNA expression of TGFβ1, TGFβ2, VEGF and FN EDA and expression of PPARγ is up-regulated. After treatment with increasing concentrations of epoxomicin in untreated and TGFβ-treated ARPE-19 cells, mRNA levels of (A) CTGF, TGFβ1, TGFβ2, VEGF, (B) FN, FN EDA, MMP-2, TIMP-1 and PPARγ were assessed. Values represent mRNA expression levels (mean ± SD) relative to untreated control cells. *, Significant change ($P < 0.05$); **, significant change ($P < 0.01$); ***, significant change ($P < 0.001$). The experiment was performed in triplicate ($N = 3$).

levels. Conversely, TGFβ was associated with a down-regulation of β5i and proteasome regulatory subunit PA28α mRNA levels. Association of PA28α with the 20S catalytic core has been shown to increase proteasome activity [93–95]. Studies have shown that expression of PA28α tends to decline in aged retina [44]. Unlike the effects of CTGF, proteasome activity assays in the presence of TGFβ demonstrated no changes in the proteolytic activity of specific proteasome subunit complexes. Recent evidence has suggested that the immunoproteasome, besides its role in immune surveillance, may be considered as a rescue mechanism in response to cellular stress [44,47,48,52,57–59,93,96–98]. Correspondingly, immunoproteasome activation has been demonstrated in the RPE of a mouse model of age-related atrophic degeneration of the RPE [52] as well as in the retina of AMD human donors [44].

There is converging evidence for TGFβ as an important pro-fibrogenic factor in the RPE. RPE cells from CNV membranes are strongly immunoreactive for TGFβ [99] and the RPE has been shown to be an intraocular secretion site of TGFβ [100]. Elevated mRNA levels of TGFβ (TGFβ2 isoform) have been demonstrated in the RPE-choroid complex and retina of AMD patients [101] and in the vitreous of patients with proliferative vitreoretinopathy and proliferative diabetic retinopathy [70,102]. TGFβ can induce EMT of RPE cells in suspension, but fails to do so when RPE cells have well-established cell-cell contacts [88]. Earlier studies have confirmed that TGFβ is an inducer of a number of growth factors such as CTGF, platelet-derived growth factor, fibroblast growth factors, and VEGF, as well as TGFβ itself [103,104]. Likewise, in the presence of a TGFβ signaling inhibitor (A-83-01), RPE cells were more tolerant to continuous wound response triggers (such as routine passaging of cell cultures) and retained the capacity to acquire a pigmented epithelial morphology [72]. In addition, inhibition of TGFβ signaling did not prevent RPE differentiation or productive RPE-mediated wound repair [72]. Semaphorin 3A, a TGFβ inhibitor, suppressed laser-induced CNV formation in mice by inhibition of the Smad2/3 signaling pathway [105]. These and our results suggest that TGFβ may be regarded as a fibrogenic marker in disrupted RPE cells and that targeting of TGFβ-mediated effects may improve wound repair mechanisms. Our results demonstrate that inhibition of the proteasome by epoxomicin is associated with down-regulation of the expression of TGFβ (both isoforms TGFβ1 and TGFβ2) and a complete blockage of TGFβ2 activity. These two isoforms were studied because in vivo roles and expression of the different TGFβ isoforms may not be uniform, although in vitro experiments often elicit similar responses [106].

CTGF expression is regulated by several signaling mechanisms including pathways of TGFβ/Smad [107]. TGFβ is a major inducer of CTGF [20,28] whereas CTGF has been shown to synergistically enhance the effects of TGFβ. Our results attribute an independent role for CTGF and TGFβ regarding modulation of the proteasome and fibrogenic response in RPE cells. When given consecutively, the effects of CTGF and TGFβ on proteasome expression and activity remained unchanged. Similarly, the pro-fibrogenic effects of TGFβ were not synergized by CTGF treatment. In other cell types, such as in hepatocytes, CTGF has been demonstrated to affect TGFβ signaling by facilitating binding of TGFβ to its receptor, down-regulation of the negative feedback loop via Smad7 and inhibition of receptor binding and signaling of the physiological TGFβ antagonist BMP-7 [28]. Likewise, CTGF is considered to be a downstream mediator of certain effects attributed to TGFβ such as cell proliferation, migration, adhesion, ECM production and EMT [20]. In the eye, CTGF has been shown to accumulate in basal deposits and in Bruch's membrane of early AMD specimens [4]. In accordance with our results, CTGF secretion has been shown to be linked to up-regulation of the expression of the ECM components FN, laminin and MMP-2 in ARPE-19 cells by mechanisms involving activation of ERK and p38 MAPK signaling pathways [4]. Furthermore, vitreous levels of CTGF correlated strongly with degree of fibrosis in vitreoretinal disorders

such as proliferative vitreoretinopathy, proliferative diabetic retinopathy and macular hole [33]. Although expression of CTGF may occur independently of TGFβ in other retinal cell types [32], our results suggest CTGF expression may be regulated by TGFβ-mediated pathways in RPE cells. In the presence of TGFβ, epoxomicin suppressed the strong up-regulation induced by TGFβ on CTGF mRNA levels. Since the pro-fibrogenic effects of TGFβ were more significant than those of CTGF, targeting of the TGFβ pathway instead of CTGF may have a more substantial anti-fibrogenic effect in subretinal fibrosis. Potential anti-fibrogenic effects were demonstrated after simultaneous treatment of RPE cell cultures with anti-VEGF (bevacizumab) and a CTGF inhibitor, but not when the CTGF inhibitor was administered alone [63]. Targeting the TGFβ pathway, however, could be less attractive due to concomitant inhibition of anti-inflammatory properties attributed to TGFβ alongside other important cellular effects [21].

Epoxomicin is one of the most selective inhibitors of the proteasome. Indeed, proteasomal subunits are the only cellular proteins covalently modified by the biotinylated derivatives of epoxomicin with no other proteolytic enzymes inhibited along this process [108]. Treatment of RPE cells with epoxomicin down-regulated the expression of the pro-fibrogenic ECM mRNA levels of FN and FN-EDA. In the presence of TGFβ, epoxomicin still down-regulated mRNA expression of FN EDA, VEGF, TGFβ1 and TGFβ2. FN, a glycoprotein that mediates cellular adhesion and migration of RPE cells, is one of the components of the ECM that is expressed in early phases of fibrosis [109]. FN is composed of two cross-linked subunits. Alternative splicing of the FN gene transcript results in several variants. One isoform (FN-EDA) has an extra domain in cellular FN [110]. Expression of FN-EDA is significantly increased in specific stages of embryonic development, during wound healing processes in the adult and in several fibrogenic diseases [69].

Furthermore, epoxomicin led to a shift in the balance between MMP-2 and TIMP-1, as mRNA levels of MMP-2 were up-regulated and TIMP-1 levels were down-regulated. As demonstrated in other cell lines [111], TGFβ increased MMP-2 levels without affecting mRNA levels of TIMP-1. RPE cells are known to express MMP-2 and TIMP-1 [112,113]. MMPs are involved in a number of normal and physiological responses such as degradation of the basal lamina, remodeling of ECM, connective tissue turnover, angiogenesis and wound repair mechanisms [111]. TIMPs are the natural inhibitors of the functional effects of MMPs [114]. TIMPs have been shown to suppress angiogenesis and promote fibrosis by inhibiting the degradation and processing of ECM proteins [115]. TIMP-1, in particular, has a pivotal role in the fibrogenic response [115]. In vitreous samples of patients afflicted with proliferative diabetic retinopathy, TIMP-1and MMP-2 were shown to be involved in angiogenesis, with TIMP-1 possibly acting as a natural anti-angiogenic factor [38]. Moreover, the balance between MMPs and TIMPs may be important for the integrity of ECM components, including, amongst others, collagens, vitronectin, fibronectin, laminin, elastin, and proteoglycans, and as such it may be regarded as indicative of the initiation and progression of the fibrogenic response [16,116]. The observed changes in the MMP/TIMP-1 balance by epoxomicin may result in improved breakdown of ECM components and subsequent attenuation of RPE cells' migration and fibrogenic responses. On the other hand, these effects on the MMP/TIMP ratio might be transitory, as evidenced in a previous study in which TGFβ inhibition had a late inhibitory effect on MMP-2 mRNA levels [75].

The mechanism(s) by which proteasome inhibition protects against fibrosis remain unknown. Our results suggest that PPARγ modulation, alongside inhibition of TGFβ expression, may explain the anti-fibrogenic properties of epoxomicin. PPARγ, a member of the nuclear receptor superfamily, is a ligand-activated transcription factor known to be involved in various distinct physiological processes including fat cell differentiation, glucose homeostasis, lipid metabolism, aging and inflammatory and immune responses [117,118]. Furthermore, PPARγ possesses important anti-angiogenic and anti-fibrogenic properties and is involved in the oxidative stress response [119]. In the retina, PPARγ has been shown to be involved in multiple molecular processes, including VEGF-induced choroidal angiogenesis response [120], photoreceptor renewal process [121], retinal neuroprotection [122] and protection from oxidative stress [123,124]. Degradation of PPARγ has been reported to occur via the proteasome [125]. Inhibition of TGFβ signaling by PPARγ has been attributed to restriction of Smad 2,3 binding to TGFβ-responsive promoters [76]. After phosphorylation, Smad2,3 forms a complex with other Smad proteins, which in turn facilitate translocation to the nucleus. In the nucleus, coactivators or repressors (such as PPARγ) regulate the binding of the Smad complex with DNA [82]. Our results demonstrate inhibition of the proteasome was able to counteract TGFβ-mediated down-regulation of PPARγ, an effect also demonstrated in other cell types [125]. Proteasome inhibitors may also impair late TGFβ-mediated responses by up-regulation of transcriptional corepressors such as Ski novel gene N and cellular Ski [82].

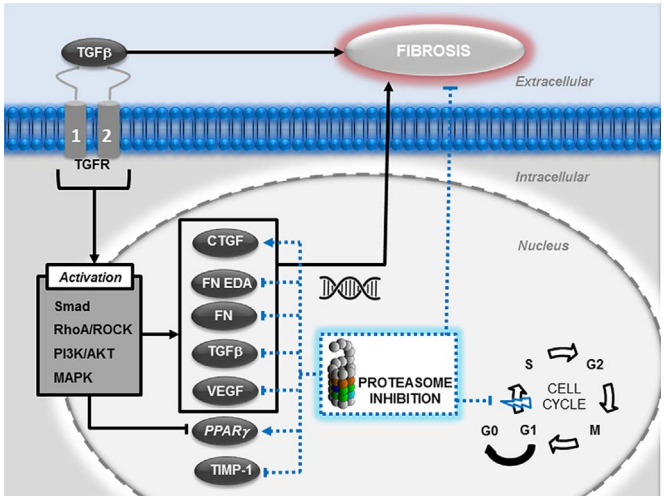

Fig. 7. A role for proteasome inhibition in the modulation of fibrogenic mechanisms mediated by RPE cells. TGFβ activates multiple pathways, including the Smad, Rho-like GTPase, PI3K/AKT and MAPK pathways, resulting in the transcription of several pro-fibrogenic genes such as CTGF, FN, FN EDA, VEGF and down-regulation of PPARγ transcription. These effects contribute to epithelial-mesenchymal transition processes in RPE cells and initiation of fibrosis. Proteasome inhibition halts cell cycle progression and downregulates transcription of FN, FN EDA, TGFβ, VEGF and TIMP-1 whilst transcription of the anti-fibrogenic factor PPARγ is up-regulated, also upon exposure to TGFβ. Effects of proteasome inhibition are depicted as dotted lines. Abbreviations: connective tissue growth factor (CTGF); fibronectin (FN); fibronectin EDA (FN EDA); MAP kinase pathway (MAPK); phosphatidylinositol-3-kinase pathway (PI3K/AKT); retinal pigment epithelium cells (RPE); Rho-like GTPase pathway (RhoA/ROCK); tissue inhibitor metalloproteinase-1 (TIMP-1); transforming growth factor β (TGFβ); transforming growth factor receptor (TGFR); vascular endothelial growth factor (VEGF).

Proteasome inhibition as a means to suppress pathological fibrogenic and proliferative responses has been proposed in various experimental studies. Proteasome inhibitors have been found to inhibit proliferation and induce apoptosis in renal interstitial fibroblasts [85], prevent development of experimental dermal fibrosis [79], attenuate diabetic nephropathy [78] and prevent hepatic fibrosis [126]. In Fig. 7 we propose a RPE-mediated fibrosis model and the signaling pathways affected by proteasome inhibition in the RPE.

5. Conclusion

In this study, we highlight specific fibrogenic and proliferative responses of RPE cells to proteasomal inhibition and propose mechanisms by which proteasomal inhibition may regulate TGFβ expression and signaling. Accordingly, the anti-fibrogenic properties of proteasome inhibitors may have a therapeutic role in RPE-mediated fibrosis. Further in vivo studies are required to elucidate the clinical value of these findings.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.01.005

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