

# Simultaneous application of bevacizumab and anti-CTGF antibody effectively suppresses proangiogenic and profibrotic factors in human RPE cells

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**Purpose:** Retinal pigment epithelial (RPE) cells play key roles in the development of choroidal neovascularization and subsequent fibrosis. We investigated the impact of bevacizumab, antihuman vascular endothelial growth factor (VEGF) antibody, and anticollagenase growth factor (anti-CTGF) neutralizing antibody, individually or in combination, on proangiogenic and profibrotic properties of RPE cells.

**Methods:** Primary cultures of human RPE cells were incubated with different concentrations of bevacizumab (0.25, 0.5, and 0.8 mg/ml) and/or anti-CTGF (10 µg/ml), and cell proliferation and apoptosis were determined. Expression and activity of proangiogenic and profibrotic genes including matrix metalloproteinases (MMP)-2 and 9, VEGFA, CTGF, vascular endothelial growth factor receptor-1 (VEGFR-1), cathepsin D, tissue inhibitor of metalloproteinases (TIMP) -1 and -2, and alpha smooth muscle actin (α-SMA) were assessed with slot blot, real-time RT-PCR, and zymography.

**Results:** Bevacizumab alone inhibited proliferation of RPE cells while anti-CTGF or bevacizumab and anti-CTGF combined had no inhibitory effect in this regard. Bevacizumab increased MMP-2, MMP-9, and cathepsin D but decreased VEGFA and VEGFR-1 expression. The CTGF level was increased by using 0.25 mg/ml bevacizumab but decreased at the 0.8 mg/ml concentration of bevacizumab. Treatment with anti-CTGF antibody decreased MMP-2 expression whereas combined treatment with bevacizumab and anti-CTGF resulted in decreased expression of MMP-2, TIMP-1, cathepsin D, VEGFA, CTGF, and α-SMA in the treated cultures.

**Conclusions:** Treatment of RPE cells with the combination of bevacizumab and anti-CTGF could effectively suppress the proangiogenic and profibrotic activity of RPE cells.

## BACKGROUND

Pathological angiogenesis is the main feature of the exudative form of age-related macular degeneration (AMD). Once these new abnormal blood vessels begin to grow, they often cause hemorrhages, leading to further wound-healing responses and subretinal fibrosis [1]. Application of antiangiogenic drugs against choroidal neovascularization (CNV) exacerbates pathological fibrogenesis, but the underlying mechanisms remain unclear [2,3].

RPE cells play a key role in the development of CNV by producing several angiogenic and fibrotic factors that localize to human choroidal neovascular membranes and participate

in paracrine signaling between the RPE and choriocapillaris [4,5]. These factors include vascular endothelial growth factors (VEGFs), VEGF receptors (VEGFRs), matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), connective tissue growth factor (CTGF), cathepsin D, and alpha smooth muscle actin (α-SMA) [5-8]. RPE cells are innately plastic, and their morphological and biochemical phenotypes change in response to various environmental stimuli. RPE cells lose their epithelial characteristics upon expression of α-SMA, a well-known marker of mesenchymal cells. Evidence of the epithelial-mesenchymal transition is generally found in fibrotic but not normal tissues [9-13].

RPE cells constitutively produce VEGF, a potent endothelial cell mitogen that stimulates proliferation, migration, and capillary morphogenesis of these cells [14-16]. VEGF enhances vascular permeability [16-18] and contributes to fibrogenesis [19]. One consequence of VEGF/VEGFR signaling is the secretion of factors such as CTGF and matrix-degrading proteinases (e.g., MMPs and cathepsins). Atypical

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expression of MMP-2 has been correlated with the progression of neovascular and fibrotic diseases [20-23].

Of interest, RPE cells from AMD donors secrete two- to threefold more MMP-2 than RPE cells from age-matched healthy donors [24]. TIMPs 1-4 repress angiogenesis and promote fibrosis by inhibiting the degradation and processing of extracellular matrix (ECM) proteins. The balance between MMPs and TIMPs regulates the progression of angiogenesis and fibrosis [25]. The main biologic function of cathepsins is to degrade cellular and extracellular proteins [26]; deregulation of cathepsin activity may be a contributing factor in various degenerative diseases of the retina including AMD [27].

CTGF plays a critical role in regulating the ECM turnover. CTGF is also a primary factor in the development of sight-threatening fibrosis in the eye [28,29]. Although there are conflicting data regarding the effect of CTGF on angiogenesis (i.e., CTGF has been shown to promote and inhibit angiogenesis under different treatment protocols), there is an established relationship between CTGF and CNV [30-34].

Bevacizumab, a pan anti-VEGF antibody, has recently been used as an intraocular drug for treating proliferative eye diseases, particularly neovascular AMD [35-37]. However, the side effects in terms of enhanced fibrosis following angiogenesis inhibition may be a concern [3,38]. In this study, the effects of bevacizumab and an anti-CTGF neutralizing antibody, alone or in combination, on the expression and activity of proangiogenic and profibrotic factors were evaluated in human RPE cell cultures.

## METHODS

*Cell culture and sample preparation:* The study was approved by the ethics committee of the Ophthalmic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Informed consents concerning the use of the posterior tissues of the donated eyes for research purposes were also obtained by the Central Eye Bank of Iran. RPE cells were isolated within 24 h of death from healthy neonatal human globes provided by the Central Eye Bank of Iran. RPE cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM):F12 (1:1; Sigma, Munich, Germany) supplemented with 10% fetal bovine serum (FBS). When the cultures reached 80% confluence, the medium was removed, and fresh serum-free media containing various concentrations of bevacizumab (0.25, 0.5, and 0.8 mg/ml; Roche, Berlin, Germany), 10 µg/ml of the anti-CTGF neutralizing antibody (PeproTech, London, UK), or both bevacizumab (0.8 mg/ml) and anti-CTGF (10 µg/ml) were applied. The medium was collected 48 h later, centrifuged, concentrated using Whatman centrifuge

tube filters, and assessed for MMP-2 and MMP-9 expression and activity with western blot, slot blot, and zymography analysis. RNA was extracted from the treated RPE cells, reverse-transcribed using a cDNA synthesis kit, and subjected to amplification with real-time PCR.

*Immunocytochemistry:* RPE cells were cultured on coverslips in 24-well plates and fixed in -10 °C methanol for 10 min. The cells were permeabilized with Tritonx-100 (0.25%) and blocked in 1% bovine serum albumin (BSA) in PBS (1X; was prepared by dissolving 8 g NaCl, 0.2 g KCl, 2.68 g Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O and 0.24 g KH<sub>2</sub>PO<sub>4</sub> in 800 ml ddH<sub>2</sub>O and then pH was adjusted to 7.4 and final volume was adjusted to 1 liter) for 1 h at room temperature. The epithelial/RPE identity of the isolated cells was confirmed by staining with a specific mouse anti-human cytokeratin 8/18 monoclonal antibody (1:1,000; Santa Cruz, Carlsbad, CA) that labels epithelial cells and a rabbit anti-human RPE65 polyclonal antibody (1:100; Santa Cruz) that specifically labels RPE microsomal membranes. Fluorescein isothiocyanate (FITC)-conjugated antibodies (goat anti-mouse immunoglobulin G [IgG] and goat anti-rabbit IgG, diluted 1:400; Santa Cruz) were used to detect the immunoreactivity of the cultures to the primary antibodies. After the final washes, the slides were incubated with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 1.5 mg/ml; Santa Cruz) for 10 min to stain nuclear DNA. The slides were examined by using a Zeiss Axiophot fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a 460 nm filter for DAPI and a 520 nm filter for FITC-conjugated antibodies.

*Cell proliferation ELISA:* RPE cells were plated in 96-well plates at 10,000 cells/well in 200 µl of complete medium and incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> until approximately 80% confluence was reached. The medium was then removed and replaced with 100 µl of fresh serum-free medium plus the indicated concentrations of bevacizumab and anti-CTGF. To determine whether the drugs changed cell proliferation, 5-bromo-2'-deoxyuridine (BrdU) was added after 24 h of treatment, and the proliferation assay was performed according to the manufacturer's instructions (Roche).

*Cell death ELISA:* RPE cells were plated in 96-well plates at 10,000 cells/well in 200 µl of complete medium and incubated at 37 °C until approximately 80% confluence was reached. The medium was removed and replaced with 100 µl of fresh serum-free medium containing the indicated concentrations of bevacizumab and anti-CTGF. The cytotoxicity was evaluated after 48 h of treatment by subjecting the RPE cells to the cell death assay kit according to the manufacturer's instructions (Roche).

**Western blot analysis:** Equivalent amounts of concentrated medium were denatured by boiling in sample buffer (reducing conditions) for 5 min and were then analyzed on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred to a preactivated polyvinylidene fluoride (PVDF) membrane overnight. The gel was stained with Coomassie brilliant blue to confirm that the proteins had been completely transferred. Non-specific binding was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) for 2 h at room temperature. MMP-2 and MMP-9 were detected by incubation with primary monoclonal mouse-anti-human MMP-2 and MMP-9 antibodies (1:1,000 and 1:500, respectively; Calbiochem, Darmstadt, Germany) at 4 °C overnight (MMP-2) or for 48 h (MMP-9). The membranes were then incubated with the secondary antibody (horseradish peroxidase-conjugated rabbit anti-mouse IgG, 1:60,000; Sigma) for 1 h at room temperature. Blots were washed three times in TBST, and proteins were detected using the enhanced chemiluminescence (ECL) chemiluminescence system (Amersham, Arlington Heights, IL).

**Slot blot assay:** Concentrated media were transferred to nitrocellulose membranes with a slot blotter and a vacuum pump. Blots were blocked for 2.5 h at room temperature with 5% skim milk in TBST, and then primary monoclonal antibodies were applied at room temperature for 2 h. The membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, and bands were detected using the ECL chemiluminescence system (Amersham). Results were quantified using the Quantity One software (Bio-Rad, Hercules, CA).

**Zymography:** Equivalent concentrated media from various samples were mixed with the SDS sample buffer without

reducing agents and separated on 10% polyacrylamide gel containing 20 mg/ml gelatin. After electrophoresis, the gel was washed in renaturation buffer (2.5% Triton X-100 in distilled water) three times. The gel was then incubated overnight (for approximately 22 h) at 37 °C in zymography incubation buffer (0.15 M NaCl, 10 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub> in 50 mM Tris–HCl, pH 7.5) and then stained for 1 h in Coomassie brilliant blue (0.05% Coomassie blue R-250, 25% ethanol and 10% acetic acid in distilled water), destained in 20% isopropanol and 10% acetic acid in distilled water, and photographed in digital format. Results were quantified using the Quantity One software (Bio-Rad).

**Real-time RT–PCR:** Total RNA was extracted from treated and control RPE cells using an RNA extraction kit (Qiagen, Hilden, Germany). The concentration and purity of the isolated RNA were determined with spectrophotometric analysis, and the integrity of the RNA was verified with agarose gel electrophoresis followed by ethidium bromide staining. The reverse transcription reaction was performed with oligo dT primers and a superscript reverse transcriptase kit (Invitrogen Corp., Carlsbad, CA). Quantitative real-time RT–PCR was performed using SYBR Green QPCR Master Mix (Roche). The PCR parameters were as follows: initial denaturation (one cycle at 95 °C for 10 min); 40 cycles of denaturation, amplification, and quantification (95 °C for 30 s, 52–60 °C for 17 s, and 72 °C for 25 s); and the melting curve (starting at 65 °C and gradually increasing to 95 °C). The mRNA expression was normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA, and expression differences were calculated according to the standard curve and efficiency (E) established for each primer set. Primer sequences used for real-time PCR are listed in Table 1.

TABLE 1. PRIMER SEQUENCES, SEQUENCE OF DESIGNED PRIMERS PRESENTED IN THE TABLE.

Sequence definition	Sense primer	Anti-sense primer
GAPDH	ACAGTCAGCCGCATCTTC	CTCCGACCTTCACCTTCC
MMP-2	TGGCAAGTACGGCTTCTGTG	TTCTTGTCGCGGTCGTAGTC
VEGFA	ACTTCTGGGCTGTTCTCG	TCCTCTTCCTTCTCTTCTCC
CTGF	GCAGGCTAGAGAAGCAGAGC	ATGTCTTCATGCTGGTGCAG
Cathepsin D	TGTGGAGGACCTGATTGC	CGAAGACGACTGTGAAGC
TIMP-1	TGCGGATACTTCCACAGGTC	GCATTCTCACAGCCAACAG
TIMP-2	AAGAGCCTGAACCACAGGTA	GAGCCGTCACCTTCTTTGAT
α-SMA	TTGAGAAGAGTTACGAGTTG	GGACATTGTTAGCATAGAGG
	VEGFR-1 Qiagen, Germany, QT00073640	
	MMP-9 Qiagen, Germany, QT00040040	

VEGFR-1 and MMP-9 primers were pre-designed commercially supplied from Qiagen.

**Statistical analysis:** Data from the cell death, cell proliferation, slot blot, and zymography assays were expressed as the mean  $\pm$  standard deviation (SD) of three (in triplicate) and five (in duplicate) independent experiments, respectively. The real-time RT-PCR analysis was performed in duplicate as three independent experiments. Differences between the control and experimental groups were analyzed using the Student *t* test.  $p < 0.05$  was considered statistically significant.

## RESULTS

**Isolation and characterization of human RPE cells:** Primary human RPE cells were cultured in 1 ml of DMEM: F12 supplemented with FBS 10% (v/v) in 24-well plates on coverslips. When the cultures reached to 80% confluence culture medium was removed and slides were fixed by methanol and subjected to ICC protocol [39]. The isolated cells expressed cytokeratin 8/18 and RPE65, confirming their identity as RPE cells (Figure 1).

**Effect of bevacizumab and anti-CTGF on the RPE cell proliferation:** Cell proliferation in treated and control RPE cells was evaluated with an enzyme-linked immunosorbent assay (ELISA) assay. Bevacizumab reduced the rate of RPE

cell proliferation compared with control cells. However, the effect was not dose-dependent. Neither anti-CTGF nor the combined bevacizumab and anti-CTGF treatment changed the proliferation rate of the RPE cells compared with the control cells (Figure 2).

**Effect of bevacizumab and anti-CTGF on the RPE cell death:** Potential cytotoxic effects of bevacizumab and/or anti-CTGF were evaluated using ELISA. None of the treatments had a cytotoxic effect on the RPE cells compared with the positive control supplied with the cell death detection kit (Figure 3).

**Effect of bevacizumab, anti-CTGF, and combined bevacizumab and anti-CTGF on the expression of MMP-2 and MMP-9:** Western blot and slot blot analyses were performed to examine the effects of bevacizumab, anti-CTGF, and the combined bevacizumab and anti-CTGF treatment on MMP-2 and MMP-9 expression. Treatment with bevacizumab (0.25, 0.5, and 0.8 mg/ml) increased the secretion of MMP-2 and MMP-9, whereas the anti-CTGF and bevacizumab and anti-CTGF treatments decreased MMP-2 expression. MMP-9 expression was increased upon treatment with anti-CTGF but was not changed by the combined bevacizumab and anti-CTGF treatment (Figure 4).

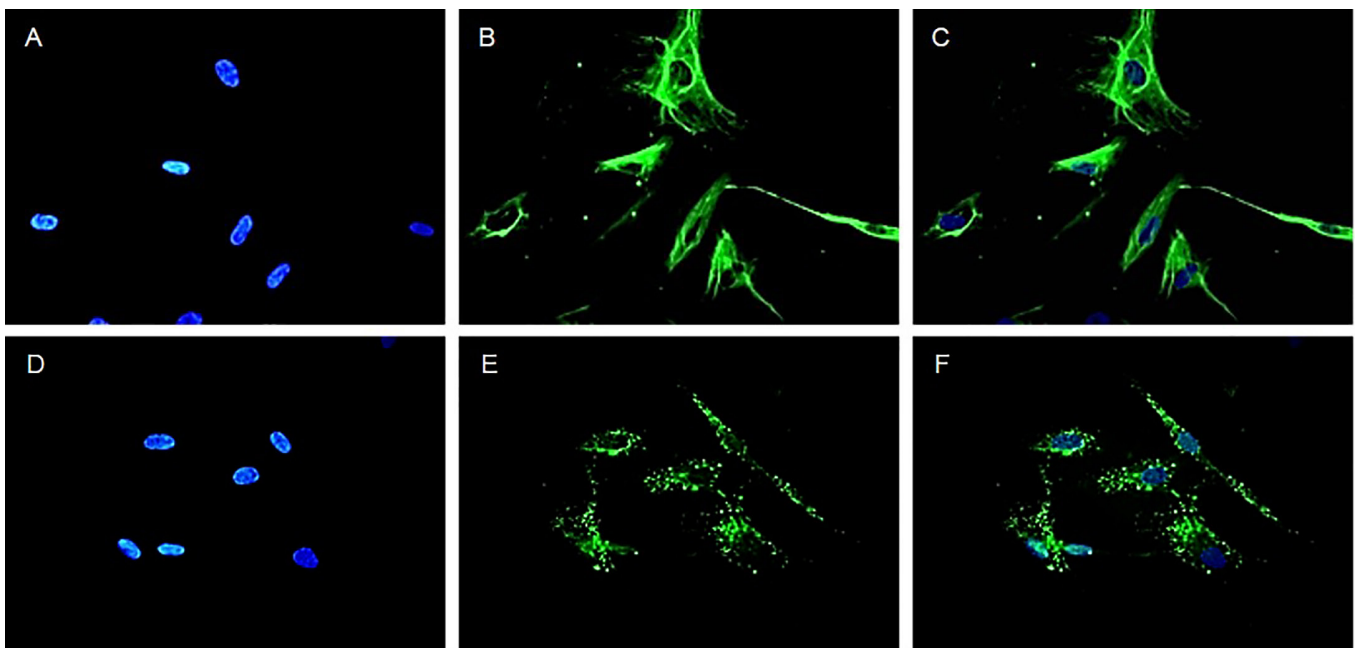


Figure 1. Immunocytochemistry of RPE cells indicating RPE cell identity and culture purity. The immunostaining procedure was performed as described in the Methods section. To confirm the epithelial origin of the cultures, cytokeratin 8/18 expression was assessed, and to confirm that isolated cells were RPE cells, RPE65, which is involved in converting all-trans retinol to 11-cis retinal during phototransduction, was surveyed. **A:** Nuclei stained blue with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). **B:** RPE cells stained positively for the fluorescein isothiocyanate (FITC)-conjugated cytokeratin antibody (green). **C:** Merged image (FITC-labeled cytokeratin and DAPI). **D:** DAPI-stained RPE cell nuclei (blue). **E:** RPE cells stained positively for the RPE65 antibody (green). **F:** Merged image (FITC-labeled RPE65 and DAPI; 400X).

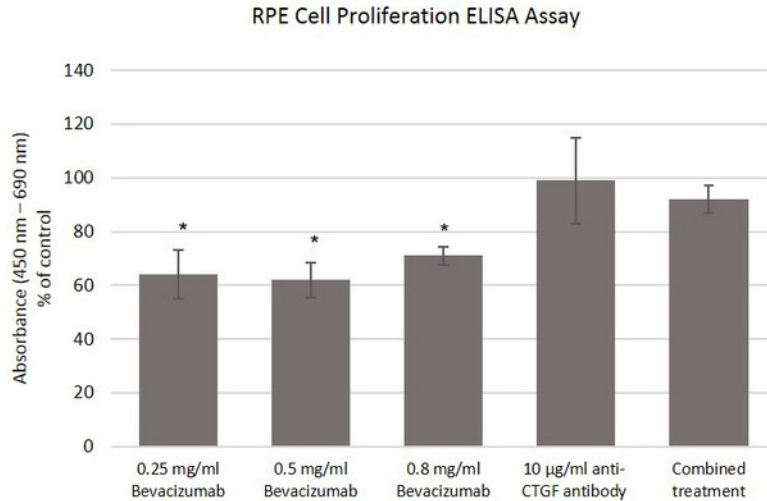


Figure 2. Effect of bevacizumab, anti-CTGF antibody and combined bevacizumab/anti-CTGF on the proliferation rate of human RPE cells. Cell proliferation ELISA assay was carried out as described in the methods. Briefly RPE cells treated by 0.25, 0.5 and 0.8 mg/ml of bevacizumab or 10 µg/ml of anti-CTGF or both of bevacizumab (0.8 mg/ml)/anti-CTGF (10 µg/ml). After 48 h cultures were harvested and proliferation assay was performed according to the manufacturer's instructions. Bevacizumab at 0.25, 0.5 and 0.8 mg/ml

concentrations reduced RPE cell proliferation rate compared to control but anti-CTGF alone or coincident application of both bevacizumab/anti-CTGF did not motivate any significant changes in treated cultures. Each column shows comparison of specified treated culture with control culture (% of control) and each bar represents the mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. \*p<0.05.

*Bevacizumab and anti-CTGF altered MMP gelatinolytic activity:* Zymography assessed MMP gelatinolytic activity in treated and control RPE cells. A dose-dependent increase in MMP-2 activity in the conditioned medium was observed following bevacizumab treatment. Anti-CTGF treatment decreased MMP-2 activity, whereas the treatment increased the activity of MMP-9. The combined bevacizumab and anti-CTGF treatment did not alter MMP-2 activity compared

with that of the control cells. It was not possible to measure MMP-9 activity in the bevacizumab-treated cells because MMP-9 and bevacizumab comigrated on the zymography gels, and the MMP-9 protein band was masked in the bevacizumab and bevacizumab and anti-CTGF cultures (Figure 5).

*Real-time RT-PCR analysis:* We next used real-time RT-PCR to investigate whether incubation of RPE cells with bevacizumab, anti-CTGF, or both bevacizumab and anti-CTGF

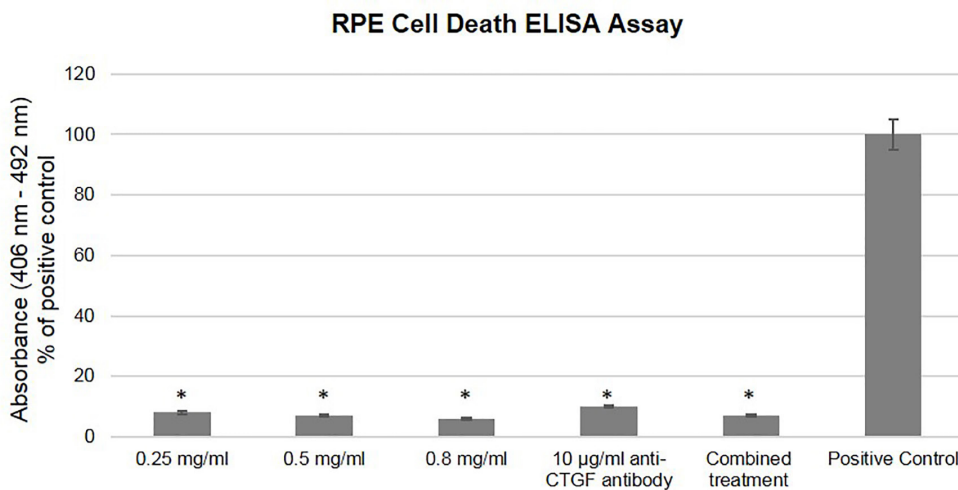


Figure 3. Determination of the cytotoxic effects of bevacizumab, anti-CTGF antibody and bevacizumab/anti-CTGF on human RPE cells. Cell death ELISA assay was performed as described in methods, seeking for cytotoxic effects of the drugs. RPE cells treated by 0.25, 0.5 and 0.8 mg/ml of bevacizumab or 10 µg/ml of anti-CTGF or both of bevacizumab (0.8 mg/ml)/anti-CTGF (10 µg/ml). After 48 hours cultures were harvested and subjected to cell death assay according to the manufacturer's instructions. Results indicated that

none of bevacizumab examined concentrations or anti-CTGF or simultaneous application of bevacizumab (0.8 mg/ml)/anti-CTGF (10 µg/ml) did not impose cytotoxic effects on treated cultures when compared to the positive control that provided by the kit. Each column shows comparison of specified treated culture with positive control (% of control) and each bar represents the mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. \*p<0.05.

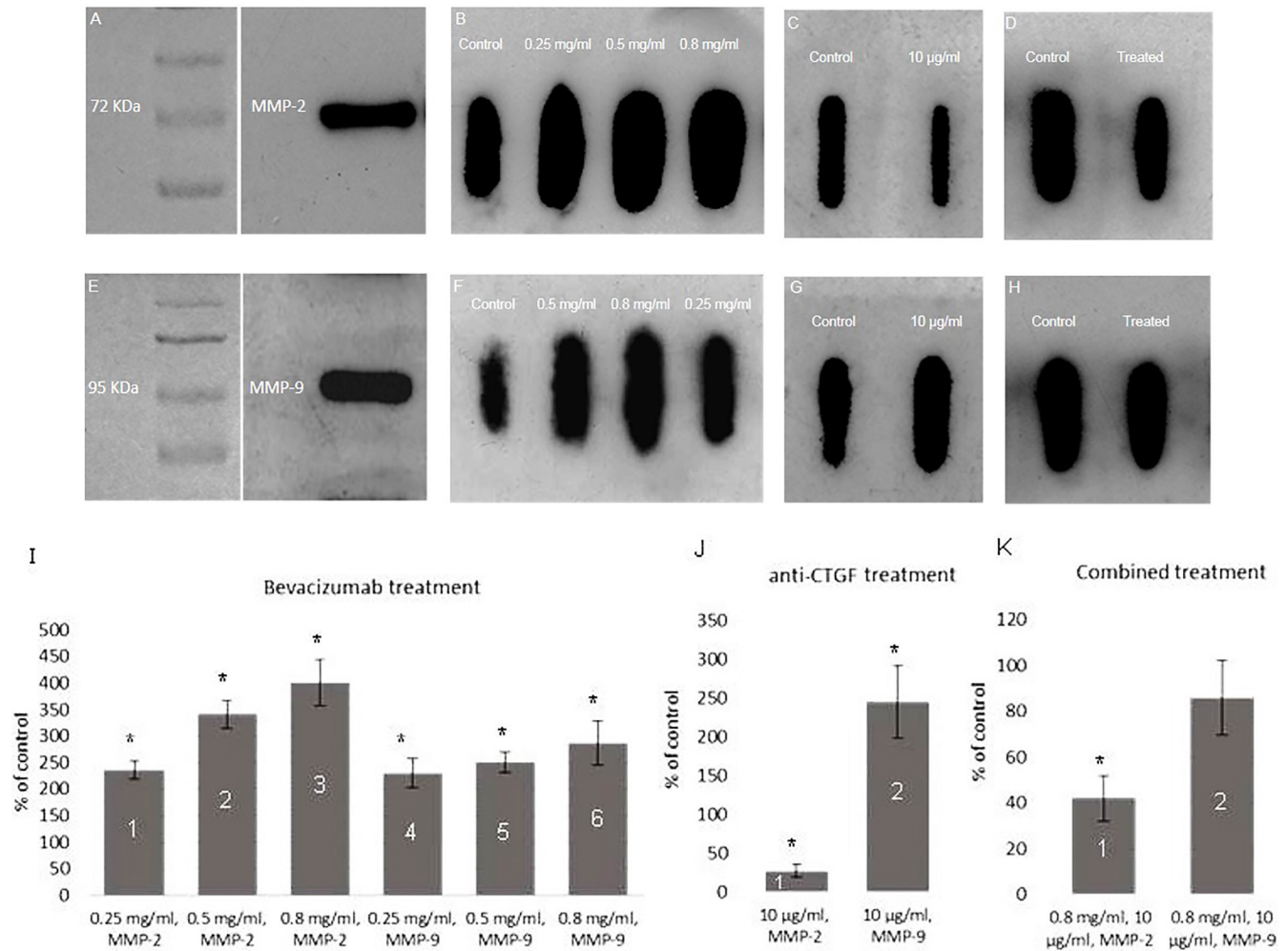


Figure 4. Effects of bevacizumab and anti-CTGF antibody on MMP-2 and MMP-9 secretion by treated cultures. (A) Matrix metalloproteinase-2 (MMP-2) and (E) MMP-9 were detected and confirmed with western blot analysis of conditioned medium collected from human RPE cell culture without any treatment. To unravel the amount of MMP-2 and MMP-9 secretion, slot blot analysis was performed on media collected from the control and treated cultures. Briefly, equivalent amounts of concentrated collected media were transferred to nitrocellulose membranes with a slot blotter, blots were blocked, and then specified primary monoclonal antibodies were applied. The membrane was then incubated with secondary antibody, and bands were detected using the enhanced chemiluminescence (ECL) chemiluminescence system. Conditioned media collected from cultures incubated with standard medium, DMEM:F12 (1:1), was used as the control in all of the presented panels. In the cultures treated with 0.25, 0.5, and 0.8 mg/ml concentrations of bevacizumab, (B) MMP-2 and (F) MMP-9 increased whereas anti-connective tissue growth factor (anti-CTGF) application imposed a meaningful decrease in (C) MMP-2 or an increase in (G) MMP-9 secretion. The effect of the combined treatment of bevacizumab and anti-CTGF decreased (D) MMP-2 but did not affect (H) MMP-9 secretion \* $p < 0.05$ . Results were quantified using the Quantity One software. Panel I; bars: 1, 2, and 3, Effect of cultures treated with 0.25, 0.5, and 0.8 mg/ml concentrations of bevacizumab on, MMP-2 secretion Panel I; bars: 4, 5, and 6, Effect of cultures treated with 0.25, 0.5, and 0.8 mg/ml concentrations of bevacizumab, on MMP-9 secretion Panel J (anti-CTGF treated culture compared to the control culture); bar: 1, anti-CTGF application imposed a meaningful decrease on MMP-2; Panel J; bar: 2, anti-CTGF application imposed a meaningful increase on MMP-9. Panel K; bar: 1, combined treatment of bevacizumab and anti-CTGF decreased MMP-2 Panel K; bar: 2, combined treatment of bevacizumab and anti-CTGF did not affect amount of MMP-9 in the culture media.

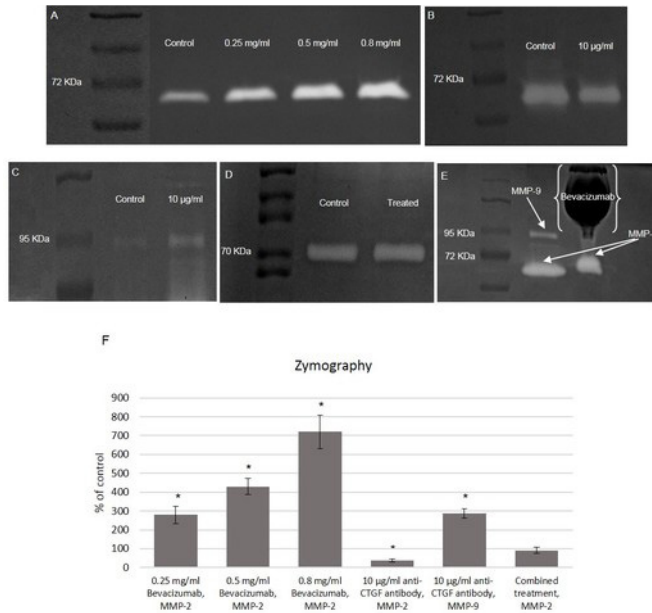


Figure 5. Representative zymogram for MMP-2 and MMP-9 activity in cultures incubated under the treatments of bevacizumab or anti-CTGF antibody or simultaneous application of both antibodies. Conditioned media of cultures treated with 0.25, 0.5, and 0.8 mg/ml concentrations of bevacizumab, or 10 µg/ml anti-connective tissue growth factor (anti-CTGF), or bevacizumab (0.8 mg/ml) and anti-CTGF (10 µg/ml) collected, centrifuged, concentrated, and assessed with zymography. **A** and bars 1, 2, and 3 from **F**: Bevacizumab at applied concentrations increased matrix metalloproteinase-2 (MMP-2) activity in treated cultures compared with the control.

**B** and bar 4 from **F**: Anti-CTGF decreased MMP-2 activity but (**C** and bar 5 from **F**) increased MMP-9 activity. **D** and bar 6 from **F**: MMP-2 activity remained unchanged with simultaneous application of bevacizumab and anti-CTGF. **E**: MMP-9 and bevacizumab comigrated on the zymography gel precluding comparisons of MMP-9 activity between the treated cells and the controls. Each column shows comparison of specified treated culture with control culture (% of control) and each bar represents the mean ± standard deviation (SD) of at least five independent experiments performed in duplicate (\* $p < 0.05$ ).

changed the gene expression of MMP-2, MMP-9, VEGFA, VEGFR-1, cathepsin D, TIMP-1, TIMP-2, CTGF, or  $\alpha$ -SMA. Relative mRNA expression levels in treated versus control RPE cells were calculated after the target mRNA expression levels were normalized against the housekeeping gene *GAPDH* (NG\_007073.2 GI:163954974). Bevacizumab treatment resulted in dose-dependent increases in the expression of MMP-2, MMP-9, and cathepsin D (Figure 6). Cathepsin D was increased in cells treated with 0.5 and 0.8 mg/ml bevacizumab, with a more pronounced effect observed in the latter group. MMP-2 and MMP-9 increased only in cells exposed to 0.8 mg/ml bevacizumab. Bevacizumab treatment decreased the expression of VEGFA and VEGFR-1 but did not alter the expression of TIMP-1, TIMP-2, or  $\alpha$ -SMA. The effect of bevacizumab on the expression of CTGF was interesting. The CTGF mRNA level increased after treatment with 0.25 mg/ml bevacizumab but was unaffected at the medium dose (0.5 mg/ml) and was reduced at the highest dose (0.8 mg/ml; Figure 6). Treatment with anti-CTGF alone decreased MMP-2 expression but did not have significant effects on the expression of MMP-9, VEGFR-1, CTGF, cathepsin D, TIMP-1, TIMP-2, VEGFA, and  $\alpha$ -SMA (Figure 7). The combined bevacizumab and anti-CTGF treatment led to decreased expression of MMP-2, TIMP-1, cathepsin D,

VEGFA, CTGF, and  $\alpha$ -SMA but did not alter the expression of MMP-9, VEGFR-1, and TIMP-2 (Figure 8).

## DISCUSSION

This study demonstrated that the combined bevacizumab and anti-CTGF treatment induced a prominent antifibrotic effect concomitant with a reduction in CTGF expression as well as decreased MMP-2 production and TIMP-1 expression. The remarkable decrease in CTGF expression might be attributed to the significant decrease in VEGFA gene expression. The reduction in  $\alpha$ -SMA expression further emphasizes the potential antifibrotic effect of bevacizumab and anti-CTGF combination therapy. These combined effects were not observed in cultures incubated with bevacizumab or anti-CTGF alone.

Development of CNV is the hallmark of exudative AMD and is associated with increased expression of VEGF, a potent proangiogenic and profibrotic factor [5,7,40-47]. Angiogenesis is a multistep process that requires coordinated activity of multiple pathways and gene products.

VEGF drives angiogenesis in the eye and upregulates CTGF in neovascular membranes; CTGF inactivates VEGF through inhibition of its production. Any shift in equilibrium of these two factors causes the angiofibrotic switch, and finally, excess CTGF drives fibrosis and leads to scarring

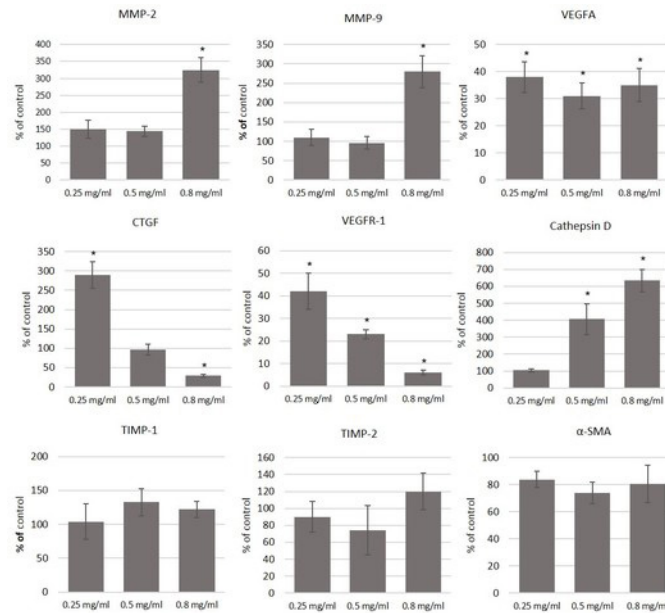


Figure 6. Real-time RT-PCR analysis of MMP-2, MMP-9, VEGFA, CTGF, VEGFR-1, cathepsin D, TIMP-1, TIMP-2, and  $\alpha$ -SMA expression in RPE cell cultures treated with bevacizumab. Cultures incubated with DMEM:F12 (1:1) supplemented with bevacizumab (0.25, 0.5, and 0.8 mg/ml concentrations) and without bevacizumab as the control. After 48 h, RNA was extracted, and gene expression analysis was performed with quantitative real-time RT-PCR as described in the Methods section. mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and presented as folds of the control values. Bevacizumab increased matrix metalloproteinase-2 (MMP-2) and MMP-9 gene expression when applied at 0.8 mg/ml. Cathepsin D was increased in cultures incubated with 0.5 and 0.8 mg/ml of the drug. Expression of vascular endothelial growth factor A (VEGFA) and VEGF receptor-1 (VEGFR-1) was decreased in the 0.25, 0.5, and 0.8 mg/ml concentrations. CTGF expression was increased at 0.25 mg/ml unaffected at 0.5 mg/ml and reduced at 0.8 mg/ml of bevacizumab compared to the control. Each column shows comparison of specified treated culture with control culture (% of control) and each bar represents the mean  $\pm$  standard deviation (SD) of at least three independent experiments performed in duplicate (\* $p < 0.05$ ).

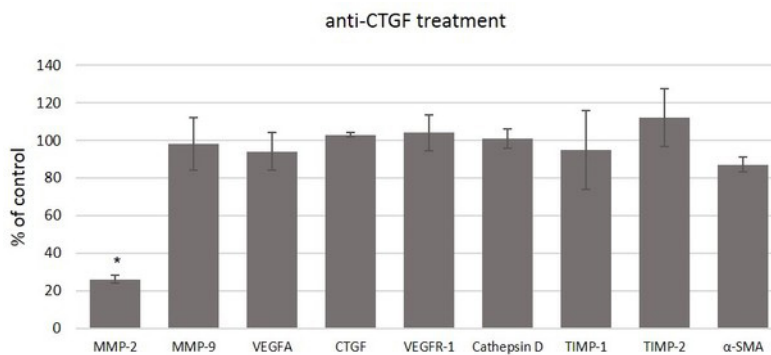


Figure 7. MMP-2, MMP-9, VEGFA, CTGF, VEGFR-1, cathepsin D, TIMP-1, TIMP-2, and  $\alpha$ -SMA gene expression levels in RPE cell cultures treated with anti-CTGF antibody. RPE cell cultures treated for 48 h with anti-connective tissue growth factor (anti-CTGF) 10  $\mu$ g/ml and without anti-CTGF as control, and then RNA extraction and real-time RT-PCR were performed as described in the Methods section.

mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and presented as folds of the control values. Anti-CTGF decreased gene expression of matrix metalloproteinase-2 (MMP-2) compared to the control. \* $p < 0.05$ .



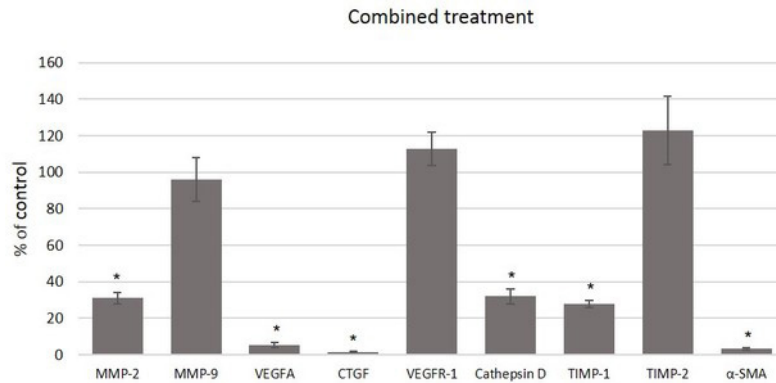


Figure 8. Relative gene expression analysis of MMP-2, MMP-9, VEGFA, CTGF, VEGFR-1, cathepsin D, TIMP-1, TIMP-2, and  $\alpha$ -SMA in the bevacizumab and anti-CTGF-treated RPE cell cultures. RPE cells treated with combined bevacizumab (0.8 mg/ml) and anti-connective tissue growth factor (anti-CTGF) (10  $\mu$ g/ml) for 48 h. RNA extraction and real-time RT-PCR were performed for the treated cultures and the controls.

mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and presented as folds of the control values. The combined bevacizumab and anti-CTGF treatment decreased gene expression of matrix metalloproteinase-2 (MMP-2), vascular endothelial growth factor A (VEGFA), CTGF, tissue inhibitors of metalloproteinase-1 (TIMP-1), cathepsin D, and alpha smooth muscle actin ( $\alpha$ -SMA) compared to the control. Each column shows comparison of specified treated culture with control culture (% of control) and each bar represents the mean  $\pm$  standard deviation (SD) of at least three independent experiments performed in duplicate (\* $p < 0.05$ ).

and blindness. This concept suggests CTGF is a potential therapeutic target in treatment of ocular fibrosis particularly in combination with anti-VEGF agents [48]. Results from anti-CTGF RNAi therapy for treatment of proliferative vitreoretinopathy (PVR) support the potential use of anti-CTGF treatment for PVR and other retinopathies including those resulting from neovascularization [49].

The role of CTGF as a profibrotic factor in the skin, kidney, and retina [50] has been shown. Moreover, VEGF upregulates CTGF in retinal endothelial cells [51]. In patients with AMD, CTGF colocalizes with VEGF in neovascular subretinal membranes in RPE cells that transdifferentiate into the major cell type that drives fibrosis [52].

Human RPE cells culture provides a substantial prospect as an *in vitro* model system for studying the mechanisms responsible for changes in RPE cells and the *in vivo* manifestation and regulation at the cell and molecular levels. Depending on the *in vitro* conditions, the differentiation status of the RPE cells switches; they can lose specific features or again differentiate into epithelial characteristics. In other words, RPE cells undergo epithelial mesenchymal transition (EMT) and acquire certain properties of mesenchymal cells, which in culture is derived from human newborns' RPE; it is possible to obtain a polarized cell monolayer [53]. Accordingly, human RPE cells can be cultured to obtain either polarized, functional RPE cells or an adhesive monolayer of trans- or dedifferentiated cells. *In vitro* morphological and functional transitions in RPE cell culture correspond to those

observed in RPE from patients with different kinds of retinal degenerative or proliferative diseases.

To confirm the RPE cell identity, we used freshly isolated human RPE cells in low passages and recognized that our cultures saved characteristic expression of RPE65 and cytokeratin 8/18; therefore, it seems that we were successful in retaining important features of RPE cells in culture.

Because of the important similarities between RPE cells in cultures and in retinal disorders, our study is privileged for priority and consideration to be effectively translated for the laboratory model animals. We practiced these appreciable features of RPE cells culture to study the effects of bevacizumab and an anti-CTGF antibody, alone or in combination, on the expression and activity of pro-angiogenic and profibrotic factors in an *in vitro* culture.

Disintegration of Bruch's membrane and the RPE-interphotoreceptor matrix are important pathological mechanisms in ocular disease processes such as neovascularization and proliferative retinopathies. Variations in MMP expression and/or activity in these diseases suggest that these enzymes significantly contribute to the pathogenesis of ocular diseases [54]. Degradation of the extracellular matrix proteins is one of the earliest events during angiogenesis, and is predominantly performed by MMPs and cathepsin proteases [55].

Expression of MMP-2 and cathepsin D increased in RPE cells incubated with bevacizumab. TIMP-1 binds to the active form of MMPs forming noncovalent complexes, whereas TIMP-2 stabilizes the inactive form of MMPs, inhibiting formation of the active proteolytic complex [56]. However,

increased MMP-2 and MMP-9 activity in the current study was not the consequence of decreased expression of TIMP-1 and TIMP-2. VEGF induces MMP secretion in RPE cells and promotes MMP activity in microvascular endothelial cells; therefore, a decrease in VEGF expression may inversely impact MMP-2 and MMP-9 expression and/or activity [57,58].

Bevacizumab was expected to decrease the expression of proangiogenic factors, including MMP-2, MMP-9, CTGF, and cathepsin D, and increase the expression of antiangiogenic factors, including TIMP-1 and TIMP-2. The findings of this study, however, were in contrast to that expectation and corresponded to the findings of a study by Vary et al. that reported unpredictable responses in treatment of metastatic colorectal carcinoma (CRC) with bevacizumab [59]. They hypothesized that the balance between antiangiogenic and proangiogenic VEGF isoforms could affect cellular sensitivity to bevacizumab. Other studies have also suggested that VEGF<sub>xxx</sub> as an antiangiogenic isoform of VEGF family binds to bevacizumab with a similar affinity as VEGF<sub>xxx</sub> and inhibits the effect of bevacizumab on angiogenesis [59-62].

The impact of CTGF on angiogenesis is controversial, since pro- and antiangiogenic effects have been reported. A complex relationship exists between VEGF and CTGF; CTGF is upregulated by VEGF but inhibits VEGF-induced angiogenesis by forming VEGF-CTGF complexes [8,32,51,63,64]. CTGF is also reported to be a proangiogenic factor [30,31]. Our results indicated that suppressing CTGF activity may inhibit angiogenesis by decreasing the expression and activity of MMP-2. Anti-CTGF treatment revealed a reducing effect on MMP-2 as a critical factor in pathological angiogenesis and fibrosis; therefore, anti-CTGF antibody might assumingly be a promising agent for treating neovascular diseases. However, the stimulatory effect of anti-CTGF on MMP-9 can contradict its application as an antiangiogenic or antifibrotic factor.

In the bevacizumab and anti-CTGF treatments, although MMP-2 expression was downregulated, its activity remained unchanged. The lack of correlation between MMP-2 expression and activity is likely because MMP's activity and expression are regulated at multiple different levels, including gene transcription, mRNA stability, translational efficiency, zymogen activation, enzyme secretion, and inhibition by endogenous inhibitors such as TIMPs [65,66].

Neither bevacizumab nor anti-CTGF, applied individually or in combination, had a cytotoxic effect on RPE cells. Bevacizumab reduced RPE cell proliferation when applied alone. Thus, VEGF may act as an autocrine factor for RPE cells, and its suppression inhibits their proliferation. In contrast, RPE cell proliferation was not affected by

incubation with bevacizumab and anti-CTGF; this may be attributed to the ability of CTGF to inactivate VEGF by reducing its production and/or by forming a complex with VEGF [32,48,63]. Thus, anti-CTGF neutralizing antibody in combined treatment could suppress free CTGF levels, and thus, free VEGF levels would increase. This augmentation may compensate for the decreased VEGF levels in the presence of bevacizumab.

In summary, our experiments demonstrated the expression of MMP-2, TIMP-1, cathepsin D, VEGFA, CTGF, and  $\alpha$ -SMA in RPE cells and examined modulation of their relative levels in response to bevacizumab and anti-CTGF antibody individually or in combination. Although the contact of RPE cells with ECM components, as a condition that is more relevant to in vivo conditions, was not considered in our experiments, it provided insight into the anti-VEGF-mediated changes of MMP, TIMP, cathepsin D, and  $\alpha$ -SMA expression in RPE cells, which are well-known hallmarks of fibrosis, ECM degradation, cell motility, and EMT. It also revealed that to control mesenchymal differentiation of RPE cells and prevent fibrosis in eyes that receive multiple doses of anti-VEGF drugs, combined therapy with bevacizumab and anti-CTGF is a promising strategy.

These results revealed simultaneous application of bevacizumab and anti-CTGF neutralizing antibody had antiangiogenic and antifibrotic effects compared to the use of each drug individually. Prospective in vivo studies will follow these results to investigate the mechanisms beyond bevacizumab and anti-CTGF combined therapy in animal models. It would be also interesting to stimulate neuronal differentiation of RPE cells to recover the retinal damage following antiangiogenic and antifibrotic therapy. To repair injuries efficiently in ocular pathologies, the search for factors regulating RPE differentiation is important.

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