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Tissue plasminogen activator attenuates outflow facility reduction in mouse model of juvenile open angle glaucoma

Sofya Gindina [a,b,*], Yan Hu [a,b], Arturo O. Barron [a,b], Zain Qureshi [b], John Danias [a,b]

[a] Department of Cell Biology, SUNY Downstate Health Sciences University, Brooklyn, NY, USA
[b] Department of Ophthalmology, SUNY Downstate Health Sciences University, Brooklyn, NY, USA

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ABSTRACT

Tissue plasminogen activator (tPA) has been shown to prevent steroid-induced reduction in aqueous humor outflow facility via an upregulation in matrix metalloproteinase (*Mmp*) expression. The purpose of this study was to determine whether tPA can rescue outflow facility reduction in the *Tg-MYOC*^{Y437H} mouse model, which replicates human juvenile open angle glaucoma. Outflow facility was measured in *Tg-MYOC*^{Y437H} mice following: periocular steroid exposure and intraocular protein treatment with enzymatically active or enzymatically inactive tPA. Effects of tPA on outflow facility were compared to those of animals treated with topical sodium phenylbutarate (PBA), a modulator of endoplasmic reticulum stress. Gene expression of fibrinolytic pathway components (*Plat*, *Plau*, and *Pai-1*) and matrix metalloproteinases (*Mmp-2*, *-9*, and *-13*) was determined in angle ring tissues containing the trabecular meshwork. *Tg-MYOC*^{Y437H} mice did not display further outflow facility reduction following steroid exposure. Enzymatically active and enzymatically inactive tPA were equally effective in attenuating outflow facility reduction in *Tg-MYOC*^{Y437H} mice and caused enhanced expression of matrix metalloproteinases (*Mmp-9* and *Mmp-13*). tPA was equally effective to topical PBA treatment in ameliorating outflow facility reduction in *Tg-MYOC*^{Y437H} mice. Both treatments were associated with an upregulation in *Mmp-9* expression while tPA also upregulated *Mmp-13* expression. tPA increases the expression of matrix metalloproteinases and may cause extracellular matrix remodeling at the trabecular meshwork, which results in reversal of outflow facility reduction in *Tg-MYOC*^{Y437H} mice.

1. Introduction

Glaucoma is a group of optic neuropathies leading to progressive irreversible vision loss (Quigley, 2011; Weinreb et al., 2014; Manravadi and Vadhar, 2015). It is the second leading cause of blindness worldwide (Greco et al., 2016), with primary open angle glaucoma (POAG) comprising the majority of cases (Tamm, 2002). The major modifiable and specific risk factor associated with glaucoma is an elevation in intraocular pressure (IOP) (Weinreb et al., 2014; Greco et al., 2016). IOP is determined by the balance between the production of aqueous humor and its elimination. Since outflow through the classical outflow pathways (that include the trabecular meshwork, the juxtacanalicular tissue and the inner wall of Schlemm's canal) is pressure dependent, these pathways effectively determine IOP levels (Quigley, 2011; Abu-Hassan et al., 2014; Manravadi and Vadhar, 2015).

Tissue plasminogen activator (tPA) is a serine protease that catalyzes the conversion of plasminogen to plasmin (Vassalli et al., 1991). It has also been shown to regulate transcription, as well as the proteolytic activation, of matrix metalloproteinases (MMPs) to promote downstream extracellular matrix (ECM) turnover (Adibhatla and Hatcher, 2008; Geyeregetta et al., 2013). tPA reduces steroid-induced IOP elevation in sheep (Geyeregetta et al., 2013; Candia et al., 2014) and outflow facility reduction in mice (Kumar et al., 2015) via an upregulation in *Mmp* (*Mmp-2*, *Mmp-9* and *Mmp-13*) expression (Kumar et al., 2015).

More recently, we have shown that outflow facility is significantly reduced in mice deficient in tPA expression (*Plat*KO) (Hu et al., 2019). This finding suggests that tPA may play a larger role in the regulation of outflow facility. To investigate whether that is the case, we studied animals that carry a mutant human myocilin transgene.

Defects in the myocilin (*MYOC*) gene are the first described (Stone

Abbreviations: tPA, tissue plasminogen activator; NE-tPA, enzymatically inactive tPA; PBA, sodium phenylbutyrate; *Mmp*, matrix metalloproteinase gene; POAG, primary open angle glaucoma; TM, trabecular meshwork; IOP, intraocular pressure.

* Corresponding author. Department of Cell Biology, Box 5, SUNY Downstate Medical Center, 450 Clarkson Ave, Brooklyn, NY, 11203, USA.
E-mail address: Sofya.Gindina@downstate.edu (S. Gindina).

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et al., 1997) and most common (Swiderski et al., 2000; Tamm, 2002; Borras, 2014) genetic cause linked to glaucoma. They also account for approximately 8% of human juvenile glaucoma and are associated with high IOPs (Wiggs et al., 1998). While the function of the wildtype myocilin protein is unknown, mutant myocilin accumulates within the endoplasmic reticulum (ER) of TM cells (Jacobson et al., 2001; Borras, 2014), leading to their malfunction (Ringvold et al., 2002) and subsequent apoptosis (Joe et al., 2003; Yam et al., 2007). The final effect is a reduction in aqueous humor outflow facility and increase in IOP (Maddineni et al., 2018). The Tyr437His (Y437H) mutation in exon 3 of *MYOC* is associated with one of the most severe disease phenotypes (Stamnoros et al., 2006).

A transgenic mouse model having the human *MYOC* gene modified to contain the Y437H mutation (*Tg-MYOC*^{Y437H}) displays several glaucoma phenotype characteristics including IOP elevation and glaucomatous neurodegeneration (Zode et al., 2011). However, these mice do not display any structural abnormalities at the iridocorneal angle (Zode et al., 2011; Zode et al., 2012; Zhu et al., 2017). Alleviation of ER stress in these animals significantly improves aqueous humor outflow (Zode et al., 2011; Zode et al., 2012).

We utilized this animal model to determine whether tPA can improve outflow facility independent of its effect on steroid-induced outflow facility reduction and compared this effect to that of ER stress modulator sodium phenylbutarate (PBA) (Zode et al., 2012). We also tested whether steroids further reduce outflow facility in these animals. Finally, we determined whether tPA affects *Mmp* expression levels in *Tg-MYOC*^{Y437H} mice.

2. Methods

2.1. Animals and treatments

8- to 12-week-old male and female mice were used for this study. The animals were housed and bred at the State University of New York (SUNY) Downstate Health Sciences University Division of Comparative Medicine (Brooklyn, NY) under a 12-h light/12-h dark cycle and were fed ad libitum. A *Tg-MYOC*^{Y437H} mouse colony was established from animals provided by Dr. Gulab Zode (Zode et al., 2011). These mice contain the transgenic human *MYOC* gene, with a Tyr437His mutation, on a B6SJL background (Zode et al., 2011). Prior to transfer to SUNY Downstate Health Sciences University, the mice had been backcrossed into the B6 background for at least 7 generations. Protocols were approved by the SUNY Downstate Institutional Animal Care and Use Committee, and experiments were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

To investigate whether corticosteroids affect outflow facility in *Tg-MYOC*^{Y437H} mice, animals received bilateral injections (20 μl) with either triamcinolone acetonide (TA) (40 mg/ml, Kenalog-40; Bristol-Myers Squibb, NY, USA) suspension or phosphate-buffered saline (PBS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) subconjunctivally using a 100 μl Hamilton syringe with a 26-gauge needle (Precision Glide, Becton Dickinson & CO, Franklin Lakes, NJ, USA). They were euthanized one week later for outflow facility measurement.

To investigate whether tPA can affect outflow facility in *Tg-MYOC*^{Y437H} mice, and determine whether tPA enzymatic action is necessary for such an effect, intravitreal injections were performed using a 10 μl Hamilton syringe with 36-gauge stainless steel needles (WPI Inc, Sarasota, FL, USA). Animals received unilateral intravitreal injections (2 μl, 5 μg/μl) of either tissue plasminogen activator (tPA) (Actilyse; Boehringer Ingelheim, Ingelheim am Rhein, Germany) or enzymatically inactive tissue plasminogen activator (NE-tPA/S478A-tPA) (Innovative Research, Novi, MI, USA) while the contralateral eye received bovine serum albumin (BSA) (2 μl of 5 μg/μl; Gold Biotechnology, St Louis, MO, USA). Animals were sacrificed either two or five days after intravitreal injections for outflow facility measurement. All injections were performed under isoflurane inhalation anesthesia and topical anesthesia with 0.5% proparacaine.

To compare the efficacy of tPA to that of sodium phenylbutarate (PBA) (MilliporeSigma, Burlington, MA, USA), PBA was dissolved in sterile PBS to make a 0.2% solution. Fresh solution was made weekly and stored at room temperature. *Tg-MYOC*^{Y437H} mice received bilateral topical ocular PBA twice daily for five days prior to euthanasia. A single drop of 50 μl PBA solution was instilled into each eye under isoflurane inhalation anesthesia for each drop administration.

2.2. IOP measurement

IOP was measured with a rebound tonometer after application of 0.5% proparacaine topical anesthesia while animals were restrained in a custom-made device (Dasias et al., 2008; Kumar et al., 2013). Five measurements were averaged per each eye. Measurements were made between 10 a.m. and 12 p.m., to minimize the effect of diurnal IOP variation.

2.3. Outflow facility determination

Mouse eyes were enucleated immediately after euthanasia. Outflow facility was determined using a microfluidic flow sensor (0.07–1.5 μL/min, MFS1; Elveflow, Paris, France) and constant pressure method, as previously described (Hu et al., 2018). Flow rates were plotted at unique pressure levels and the slope of the regression line was used to calculate the outflow facility for each eye. Any eyes that developed visible leaks or that had pressure-flow correlations with R^2 0.9 were excluded from analysis but were used for RNA quantification.

2.4. Tissue collection, RNA isolation and quantitative real time PCR

Eyes were flash frozen in liquid nitrogen after outflow facility determination and subsequently dissected on ice to obtain the angle ring tissues containing the TM, as previously described (Kumar et al., 2013). Dissected TM tissues were immersed in RNAlater solution (Invitrogen, Waltham, MA, USA) and then snap-frozen and stored at −80 °C until RNA extraction. Tissue collected was pooled (four eyes) and homogenized in TRIzol reagent for RNA isolation, per manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). RNA concentration was determined with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was synthesized by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA) according to manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed by using Green-2-Go qPCR Mastermix-ROX (Bio Basic, Amherst, NY, USA) on a QuantStudio 6 Flex thermal cycler (Applied Biosystems, Carlsbad, CA, USA).

mRNA expression of *Plat*, *Plau*, *Pai-1*, *Mmp-2*, *Mmp-9*, and *Mmp-13* in angle ring tissues was determined; sample size (n) values indicate the total number of eyes per treatment group. Primers were designed by using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome; in the public domain); primer sequences are listed in Table 1. Primer specificity was confirmed by agarose gel electrophoresis and multiple-sized amplification products were absent on inspection of melting curves. Target mRNA expression values were normalized to the expression levels of *Rps11* (ribosomal protein S11). The relative fold change was calculated by using the ΔΔCt method (Hu et al., 2018). Outliers identified via the Thompson Tau test were removed from analysis.

3. Results

3.1. Outflow facility and expression of fibrinolytic pathway components in *Tg-MYOC*^{Y437H} mice

The mean ± standard deviation outflow facility (μl/min/mmHg) was $63 \pm 17 \times 10^{-5}$ in *Tg-MYOC*^{Y437H} (n = 10) mice and $132 \pm 17.2 \times 10^{-5}$

Table 1
Primer sequences of genes analyzed by qRT-PCR.

Number	Gene	Sequence (5′- 3′)
1	*Plat*	FP: CGAAAGCTGACGTGGGAATA
		RP: GTGTGAGGTGATGTCTGTGTAG
2	*Plau*	FP: GCGCCTTGGTGGTGAAAAAC
		RP: GACACGCATACACCTCCGTT
3	*Pai-1*	FP: ATGATGGCTCAGAGCAACAAG
		RP: CATTGTCTGATGAGTTCAGCATC
4	*Mmp-2*	FP: ACAGTGACACCACGTGACAA
		RP: GGTCAGTGGCTTGGGGTATC
5	*Mmp-9*	FP: GCGTCGTGATCCCCACTTAC
		RP: CAGGCCGAATAGGAGCGTC
6	*Mmp-13*	FP: TACCATCCTGCGACTCTTGC
		RP: TTCACCCACATCAGGCACTC
7	*Rps11*	FP: AAGACGCCTAAAGAGGCTATTG
		RP: GGTCCTCTGCATCTTCATCTTC

FP, forward primer; RP, reverse primer.

in wildtype (WT) littermate mice (n = 6). The *Tg-MYOC*^{Y437H} mouse eyes had a significantly (~52%) lower outflow facility compared to WT littermate eyes ($p < 0.0001$, T-test) (Fig. 1A). IOP was also significantly (~47%) elevated in *Tg-MYOC*^{Y437H} mouse eyes compared to WT eyes ($p < 0.05$, T-test; data not shown). There were no significant differences in the expression of *Plat* (Fig. 1B), *Plau* (Fig. 1C) and *Pai-1* (Fig. 1D) in angle ring tissue of eyes from *Tg-MYOC*^{Y437H} (n = 20) and WT littermate (n = 16) mice ($p > 0.05$, T-test). The mean ± standard deviation expression of *Plat, Plau* and *Pai-1* in angle ring tissues from *Tg-MYOC*^{Y437H} mice was 1.25 ± 0.43, 1.02 ± 0.28 and 0.69 ± 0.31, respectively. While the mean ± standard deviation expression of *Plat, Plau* and *Pai-1* in angle ring tissues from WT littermates was 0.96 ± 0.18, 1.12 ± 0.21 and 0.94 ± 0.36, respectively. However, *Tg-MYOC*^{Y437H} mice (n = 20) had significantly lower angle ring tissue expression of *Mmp-2* (Fig. 2A) and *Mmp-9* (Fig. 2B) compared to that of WT (n = 16) littermates ($p < 0.05$ and $p < 0.05$, T-test respectively). There was no significant difference in *Mmp-13* (Fig. 2C) expression ($p > 0.05$, T-test) between *Tg-MYOC*^{Y437H} mice and WT littermates. The mean ± standard deviation expression of *Mmp-2, Mmp-9* and *Mmp-13* in angle ring tissues from *Tg-MYOC*^{Y437H} mice was 0.71 ± 0.20, 0.72 ± 0.24 and 1.04 ± 0.63, respectively. While the mean ± standard deviation expression of *Mmp-2, Mmp-9* and *Mmp-13* in angle ring tissues from WT littermates was 0.96 ± 0.19, 1.08 ± 0.44 and 1.04 ± 0.46, respectively.

3.2. Effect of steroids on*Tg-MYOC*^{Y437H} mouse outflow facility

Outflow facility (mean ± standard deviation) in *Tg-MYOC*^{Y437H}mouse eyes treated with TA (n = 7) was 61 ± 20 × 10^{-5} µl/min/mmHg while that in *Tg-MYOC*^{Y437H} mouse eyes treated with PBS (n = 10) was 69 ± 17 × 10^{-5} µl/min/mmHg. Outflow facility was not significantly different between TA and PBS treated groups ($p > 0.05$, T-test) (Fig. 3).

3.3. Effect of enzymatically active and enzymatically inactive tissue plasminogen activator on outflow facility in *Tg-MYOC*^{Y437H} mice

In *Tg-MYOC*^{Y437H} eyes 2 days after treatment with the respective protein injection, the mean ± standard deviation outflow facility (µl/min/mmHg) was 57.4 ± 16.8 × 10^{-5} in BSA-treated (n = 17), 75 ± 13.7 × 10^{-5} in tPA-treated (n = 10) and 78.6 ± 15.6 × 10^{-5} in NE-tPA-treated (n = 8) groups ($p < 0.0001$, ANOVA). Treatment with tPA or NE-tPA significantly enhanced outflow facility compared to BSA treatment ($p < 0.05$ and $p < 0.01$, respectively, Tukey-Kramer post hoc analysis), but did not improve outflow facility to that of WT littermate mouse eyes (132 ± 17.2) (n = 6). There was no significant difference between tPA and NE-tPA treatments ($p > 0.05$, Tukey-Kramer post hoc analysis) (Fig. 4A).

Significant differences in the expression (mean ± standard deviation) of *Mmp-2, Mmp-9* and *Mmp-13* between WT, BSA, tPA and NE-tPA groups were detected (ANOVA, $p < 0.0001$, $p < 0.01$, and $p < 0.0001$, respectively). NE-tPA treated *Tg-MYOC*^{Y437H} eyes (0.95 ± 0.30)

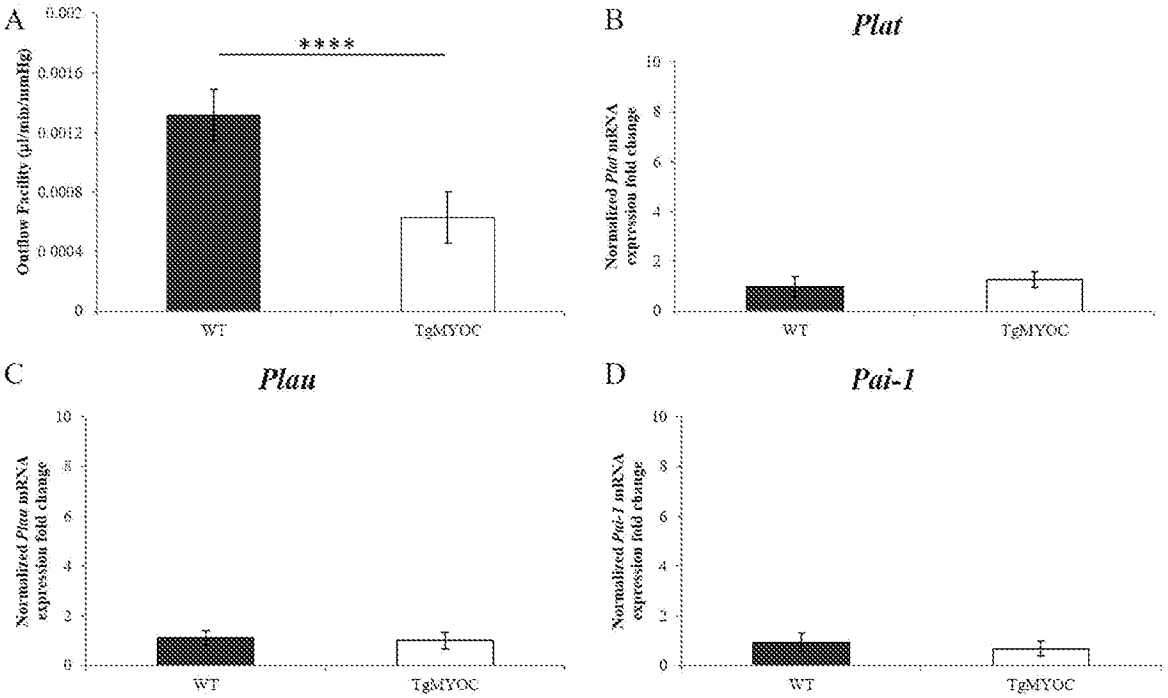

Fig. 1. (A) Outflow facility in *Tg-MYOC*^{Y437H} and wildtype littermate mouse eyes. Outflow facility (mean ± SD µl/min/mmHg) was significantly reduced in *Tg-MYOC*^{Y437H} eyes (n = 10) compared with wildtype (WT) littermate eyes (n = 6) (****$p < 0.0001$, T-test). Gene expression changes in *Plat* (B), *Plau* (C), and *Pai-1* (D) were normalized (mean ± SD) to values in WT littermate eyes. Expression was not significantly different between*Tg-MYOC*^{Y437H} angle ring tissues (n = 20) and WT angle ring tissues (n = 16) ($p > 0.05$, ANOVA).

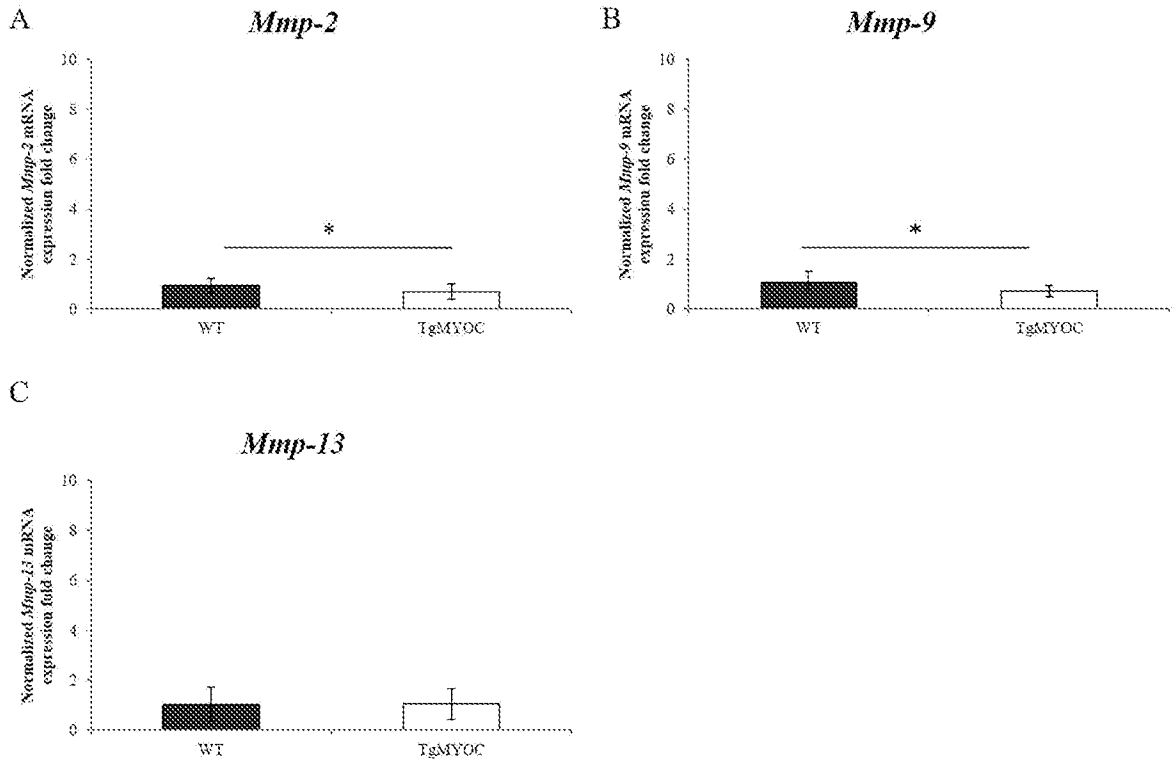

Fig. 2. *Mmp* gene expression changes in *Tg-MYOC*^{Y437H} angle ring tissue. Gene expression changes in *Mmp-2* (A), *Mmp-9* (B), and *Mmp-13* (C) were normalized (mean ± SD) to values in wildtype (WT) littermate eyes. Group means were significantly different in *Tg-MYOC*^{Y437H} angle ring tissues (n = 20) and WT angle ring tissues (n = 16) for *Mmp-2* expression and *Mmp-9* expression ($p < 0.05$ and $p < 0.05$, T-test). Group means were not significantly different for *Mmp-13* expression ($p > 0.05$, T-test).

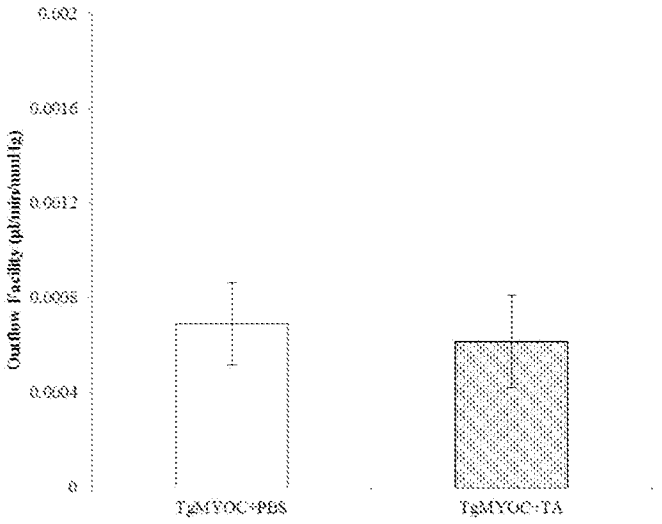

Fig. 3. *Tg-MYOC*^{Y437H} mice do not have steroid-induced outflow facility reduction. Outflow facility (mean ± SD µl/min/mmHg) was not further reduced in TA treated *Tg-MYOC*^{Y437H} eyes (n = 7) compared with PBS treated *Tg-MYOC*^{Y437H} eyes (n = 10) ($p > 0.05$, T-test).

(n = 8) showed significantly higher *Mmp-9* expression compared to BSA treated eyes (0.50 ± 0.24) (n = 16) ($p < 0.05$, Tukey-Kramer post hoc analysis) (Fig. 4C). tPA (3.33 ± 1.13) (n = 8) and NE-tPA (2.90 ± 0.78) (n = 8) treated *Tg-MYOC*^{Y437H} eyes showed significantly higher *Mmp-13* expression compared to BSA treated eyes (1.18 ± 0.41) (n = 16) ($p < 0.01$ and $p < 0.01$, respectively, Tukey-Kramer post hoc analysis). There was no significant difference in *Mmp-2* (Fig. 4B) expression when comparing NE-tPA (0.35 ± 0.11) (n = 8) and tPA (0.48 ± 0.19) (n = 8) treated eyes to BSA (0.43 ± 0.17) (n = 16) treated eyes ($p > 0.05$, Tukey-Kramer post hoc analysis). *Mmp-2* expression remained decreased in BSA, tPA and NE-tPA groups compared to that in WT littermate mice ($p < 0.0001$, $p < 0.01$, $p < 0.0001$, respectively, Tukey-Kramer post hoc analysis).

3.4. Comparison of the effectiveness of tissue plasminogen activator and sodium phenylbutarate in improving outflow facility in *Tg-MYOC*^{Y437H} mice

In *Tg-MYOC*^{Y437H} eyes 5 days after treatment with intravitreal protein, the mean ± standard deviation outflow facility (µl/min/mmHg) was 85.4 ± 28.8 × 10^{-5} in tPA-treated (n = 13) and 54 ± 8.3 × 10^{-5} in BSA-treated (n = 15) eyes. In *Tg-MYOC*^{Y437H} eyes treated with topical 0.2% PBA, the mean ± standard deviation outflow facility was 105 ± 37.8 × 10^{-5} (n = 14) ($p < 0.0001$, ANOVA). Treatment with either intravitreal tPA or topical PBA significantly enhanced outflow facility compared to BSA treated eyes ($p < 0.05$ and $p < 0.0001$, respectively; Tukey-Kramer post hoc analysis). There was no significant difference between tPA and PBA treated eyes ($p > 0.05$, Tukey-Kramer post hoc analysis) (Fig. 5A).

The (mean ± standard deviation) expression of *Mmp-2*, *Mmp-9* and *Mmp-13* was significantly different between WT, BSA, tPA and PBA groups (ANOVA, $p < 0.0001$, $p < 0.001$ and $p < 0.0001$, respectively). Although, *Mmp-2* expression was lower in BSA, tPA and PBA groups compared to WT littermates ($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, respectively, Tukey-Kramer post hoc analysis) there was no significant difference in *Mmp-2* expression when comparing tPA (0.16 ± 0.08) (n = 8) and PBA (0.11 ± 0.07) (n = 8) treated eyes to BSA (0.12 ± 0.08) (n = 8) treated eyes ($p > 0.05$, Tukey-Kramer post hoc analysis) (Fig. 5B). Both tPA (0.68 ± 0.38) (n = 8) and PBA (0.48 ± 0.19) (n = 8) treated

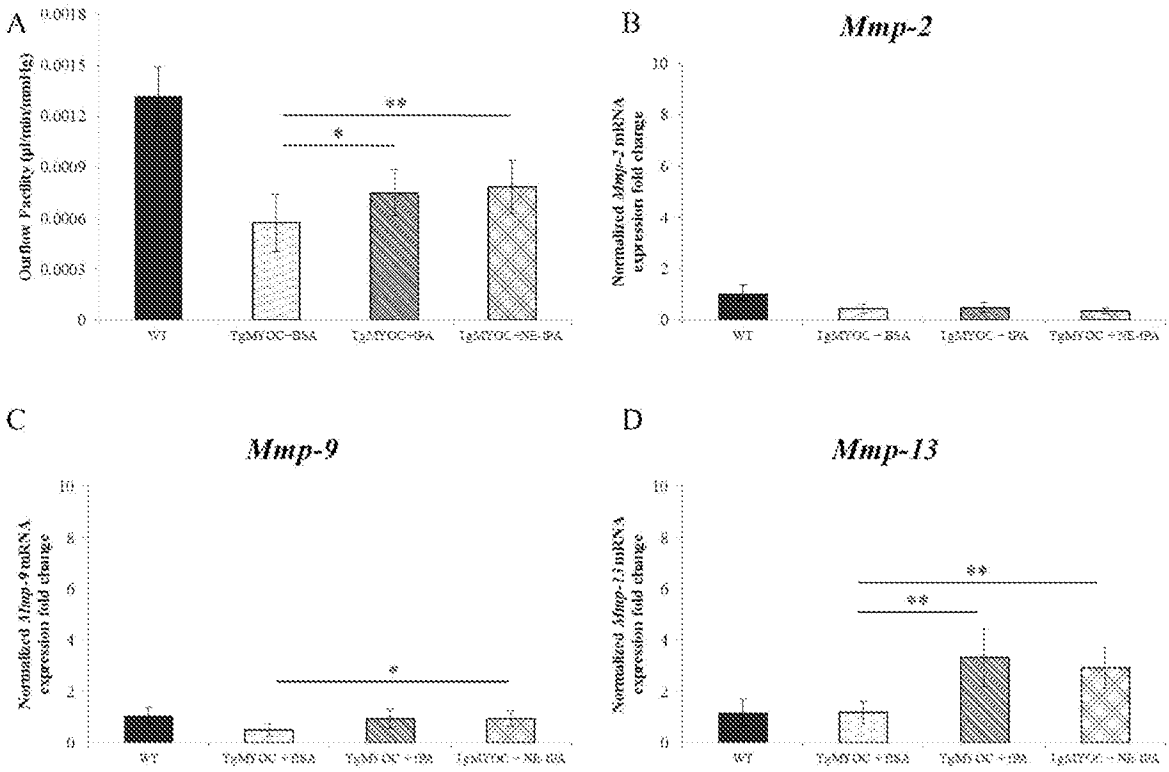

Fig. 4. (A) Outflow facility in protein treated *Tg-MYOC*^{Y437H} mouse eyes. Outflow facility (mean ± SD µl/min/mmHg) was significantly increased in eyes treated with tPA (n = 10) and NE-tPA (n = 8) compared with those treated with BSA (n = 17) (****p < 0.0001, ANOVA with Tukey-Kramer post hoc analysis). The outflow facility of naïve (not treated with protein) wildtype (WT) littermate animals (n = 6) are included for comparison purposes. The outflow facility in these eyes is different from the outflow facility in BSA, tPA and NE-tPA treated eyes. Gene expression changes in *Mmp-2* (B), *Mmp-9* (C), and *Mmp-13* (D) were normalized (mean ± SD) to values in WT littermate eyes. Group means were significantly different for *Mmp-2*, *Mmp-9* and *Mmp-13* expression in WT eyes (n = 16), BSA eyes (n = 16), tPA eyes (n = 8) and NE-tPA eyes (n = 8) (ANOVA, *Mmp-2*, p < 0.0001, *Mmp-9*, p < 0.01, *Mmp-13*, p < 0.0001). Asterisks indicate differences on Tukey-Kramer post hoc analysis, *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001.

eyes had higher *Mmp-9* expression (Fig. 3C) compared to BSA treated eyes (0.40 ± 0.35) (n = 8) (p < 0.05 and p < 0.001, respectively, Tukey-Kramer post hoc analysis). tPA-treated *Tg-MYOC*^{Y437H} eyes (6.37 ± 2.64) (n = 8) showed a significantly higher *Mmp-13* expression compared to BSA treated eyes (2.06 ± 0.61) (n = 8) and PBA treated eyes (1.40 ± 0.54) (n = 8) (p < 0.0001 and p < 0.0001, respectively, Tukey-Kramer post hoc analysis) (Fig. 3D). PBA treated eyes (n = 8) did not show any difference in *Mmp-13* expression from that of BSA-treated and WT eyes (p > 0.05, Tukey-Kramer post hoc analysis) (Fig. 3D).

4. Discussion

POAG has been associated with genetic polymorphisms in a large number of loci (Sears et al., 2019). However, despite an apparent strong genetic component, few *individual* genes have a direct causative role for the disease. One of these genes is *MYOC* which encodes for the myocilin protein. Mutations or polymorphisms in the myocilin gene account for ~4% of the cases of POAG and appear to often cause an aggressive disease that is characterized by IOP elevation early in life (Wiggs et al., 1998; Borras, 2014).

The myocilin protein was initially described in trabecular meshwork (TM) cell culture following glucocorticoid stimulation (Polansky et al., 1991) but is ubiquitously expressed in most body tissues (Fingert et al., 1998), as well as the eye (Kubota et al., 1997; Karali et al., 2006; Swiderski et al., 2010). Steroids not only cause an upregulation in myocilin expression, but also its secretion in the TM (Nguyen et al., 1998; Fingert et al., 2002; Borras, 2014). This increased expression, however, does not appear to be causative for steroid-induced IOP elevation (Patel et al., 2017).

Various mutations in the myocilin molecule cause disease of variable severity. The majority of glaucoma-associated *MYOC* mutations occur within exon 3, which encodes the olfactomedin-like domain (Fingert et al., 2002; Borras, 2014; Joe et al., 2017; Wang et al., 2019). Several of these mutations prevent proteolytic cleavage at this myocilin domain, within the endoplasmic reticulum (ER) (Caballero and Borras, 2001; Arroyo-Aguilar et al., 2016; Borras, 2014), which is necessary for its extracellular secretion into the aqueous humor (Caballero and Borras, 2001; Jacobson et al., 2001; Yam et al., 2007). This causes the accumulation of insoluble misfolded proteins within the ER (Caballero and Borras, 2001; Jacobson et al., 2001; Joe et al., 2003; Yam et al., 2007; Borras, 2014; Maddineni et al., 2018; Lieberman, 2019) leading to alterations in normal TM cell function and reducing phagocytic debris removal (Fingert et al., 2002), ultimately resulting in TM cell loss. One of the mutations associated with severe IOP elevation early in life is the Y437H mutation (Gobeil et al., 2006). This missense mutation has been shown to prevent proper protein folding within the ER, which hinders progression through the secretory pathway (Caballero and Borras, 2001; Gobeil et al., 2006).

Outflow resistance is highly dependent on the extracellular matrix (ECM) deposited by TM cells (Dautriche et al., 2014; Keller et al., 2018). Along with the intracellular ramifications of mutant myocilin, there are also changes to the extracellular environment of the outflow pathways. Since wildtype myocilin normally forms a dynamic network with several components of the TM ECM, such as fibrillin, laminin, collagen and fibronectin (Filla et al., 2002; Ueda et al., 2002; Fautsch et al., 2006), mutations in myocilin cause an imbalance in this network and promote TM structural abnormalities (Gobeil et al., 2006). *MYOC* mutations have also been linked to ultrastructural TM changes in human

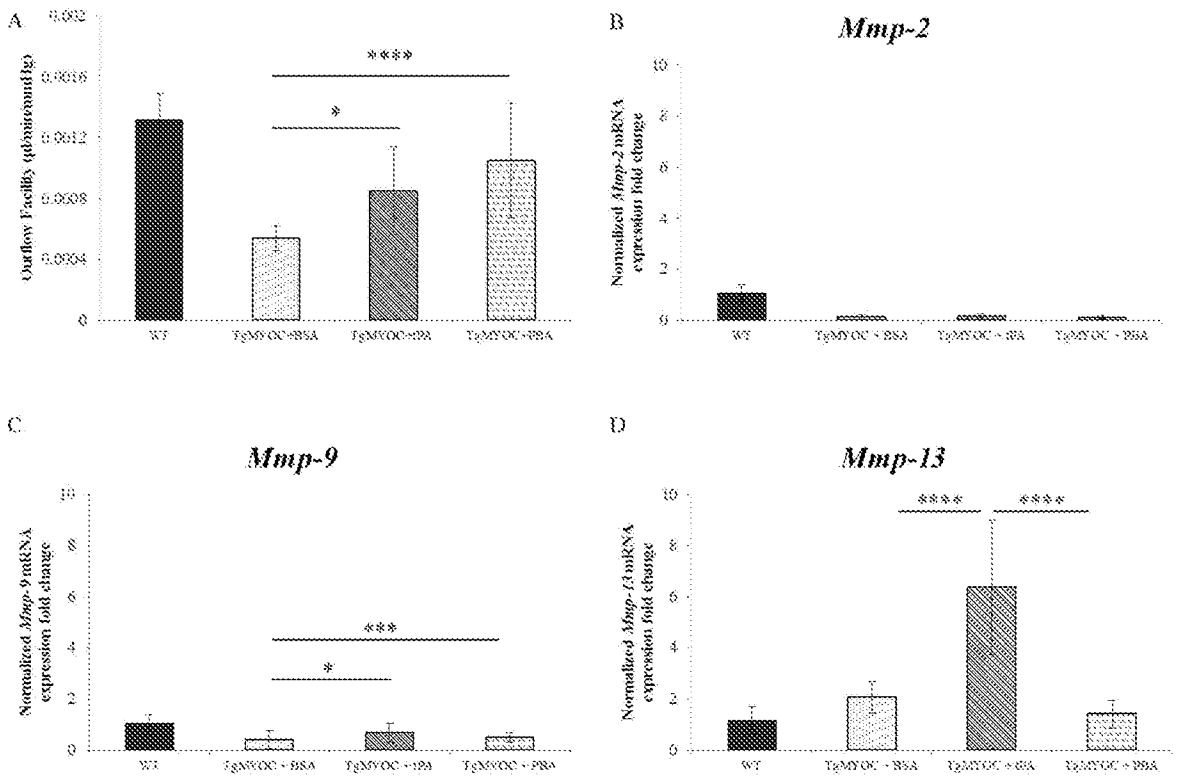

Fig. 5. (A) Outflow facility in protein and PBA treated *Tg-MYOC*^{Y437H} mouse eyes. Outflow facility (mean ± SD µl/min/mmHg) was significantly increased in eyes treated with tPA (n = 13) and PBA (n = 14) compared with those treated with BSA (n = 15) (****p < 0.0001, ANOVA with Tukey-Kramer post hoc analysis). The outflow facility of naïve (not treated with protein or PBA) wildtype (WT) animals (n = 6) are included for comparison purposes. The outflow facility in these eyes is different from the outflow facility in BSA and tPA treated eyes. Gene expression changes in *Mmp-2* (B), *Mmp-9* (C), and *Mmp-13* (D) were normalized (mean ± SD) to values in WT littermate eyes. Group means were significantly different for *Mmp-2*, *Mmp-9* and *Mmp-13* expression in WT eyes (n = 16), BSA eyes (n = 8), tPA eyes (n = 8) and PBA eyes (n = 8) (ANOVA, *Mmp-2*, p < 0.0001, *Mmp-9*, p < 0.001, *Mmp-13*, p < 0.0001). Asterisks indicate differences on Tukey-Kramer post hoc analysis, *p < 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001.

tissues, including thickened basement membranes and apoptotic TM cells (Hamanaka et al., 2017). In addition, mutant myocilin is associated with a reduction in the activity of matrix metalloproteinases (MMP-2 and MMP-9) in cultured TM cells as well as an accumulation of ECM components in the TM of *Tg-MYOC*^{Y437H} mice (Kasetti et al., 2016). The death of TM cells in the presence of over-accumulation of mutant myocilin in their ER likely exacerbates the ECM turnover deficiency.

Steroid-induced IOP elevation is another condition associated with changes in the ECM of the TM (Jane et al., 2007; Acott and Kelley, 2008; Clark and Wordinger, 2009; Overby et al., 2014; Mantravadi and Vadhar, 2015). We have previously studied some of the mechanisms involved in abnormal ECM deposition in steroid-induced glaucoma and have uncovered a critical role for tPA in ECM turnover regulation. Importantly, we have shown that absence of tPA leads to a decrease in outflow facility in mice *in the absence* of steroids (Hu et al., 2019). This implies that tPA (and potentially other fibrinolytic enzymes) may have a larger role in outflow facility regulation in glaucoma. To answer this question we utilized a mouse model of OAG that closely mirrors the well characterized human "myocilin" glaucoma. The transgenic mouse model containing the human *MYOC* gene modified with the Y437H mutation (*Tg-MYOC*^{Y437H}) (Zode et al., 2011) displays several glaucoma phenotypes, including: IOP elevation, retinal ganglion cell death and optic nerve axon degeneration (Zode et al., 2011). We confirmed that IOP is elevated in these animals early in life and that this is related to a significant reduction in outflow facility.

We initially explored whether fibrinolytic enzyme expression in *Tg-MYOC*^{Y437H} mice is reduced. Despite expectations to the contrary, expression of both tPA, uPA and their inhibitor (PAI-1) were not affected. This finding suggests that at least early in life, TM cells do not experience feedback inhibition on fibrinolytic enzyme transcription. However, since activation of both tPA and uPA (Jarvis and Summers, 1989; Allen et al., 1995; Agapitonov et al., 2003) requires processing within the ER it is possible that their activity is decreased. We did not check enzymatic activity of fibrinolytic enzymes in *Tg-MYOC*^{Y437H} mouse eyes.

Despite a seemingly unaffected fibrinolytic system in the outflow tissues of *Tg-MYOC*^{Y437H} mice, significantly lower expression of *Mmp-2* and *Mmp-9* was detected. Lower expression of *Mmps* could explain the reduction in aqueous humor outflow in these mice by disrupting ECM turnover (Singh and Tyagi, 2017).

We then attempted to determine whether *Tg-MYOC*^{Y437H} mice develop steroid-induced outflow facility reduction. Steroid-induced IOP elevation occurs in a very high percentage of patients with OAG (Pinsky et al., 2017). The model TA administration via subconjunctival injection has been shown to cause significant outflow facility attenuation in wildtype animals (Kumar et al., 2013). Similarly, dexamethasone subconjunctival administration has been shown to be effective in decreasing outflow facility and increasing IOP (Patel et al., 2017). Thus administration of periocular corticosteroids allows effects at the eye to be maximized while minimizing toxicity, which is seen in models using systemic steroid administration that requires doses close to the median lethal dose (Whitlock et al., 2010). Contrary to our expectations, we did not detect any further outflow facility reduction in *Tg-MYOC*^{Y437H} mice exposed to steroids. It is unclear why this is the case as mice show a robust steroid-induced effect on outflow facility (Kumar et al., 2013). A possible explanation may involve the already reduced baseline *Mmp* expression in the transgenic mice as prior studies have shown steroid-induced TM changes occur due to *MMP* expression

downregulation (el-Shabrawi et al., 2000).

tPA has been shown to prevent and reverse steroid-induced outflow facility changes in mice and to affect sheep in a similar way (Geromonta et al., 2013; Kumar et al., 2013). We have also shown that tPA reverses *Mmp* changes induced by steroids (Kumar et al., 2013). We therefore hypothesized that an upregulation in *Mmp* expression could enhance outflow facility in *Tg-MYOC*^{Y437H} mice. Our results demonstrate that intraocular administration of tPA can partially reverse outflow facility reduction in *Tg-MYOC*^{Y437H}mice. To determine whether this effect is dependent on enzymatic activity or receptor-mediated transcriptional upregulation, we also tested the ability of enzymatically inactive tPA to improve outflow facility in *Tg-MYOC*^{Y437H} mice. Enzymatically inactive tPA (NE-tPA/S478A-tPA) maintains the cytokine functions of tPA while completely abolishing its enzymatic activity (Werner et al., 1999; Yi et al., 2004; Pu et al., 2019). We have previously shown that both enzymatically active and enzymatically inactive forms of tPA are equally effective in reversing steroid-induced outflow facility reduction in C57BL/6J mice (submitted) and that this effect is mediated through transcriptional control of *Mmp-9*. This effect was replicated in *Tg-MYOC*^{Y437H} mice, where both tPA and NE-tPA significantly improved outflow facility by ~31% and ~37%, respectively. The fact that outflow facility did not improve to levels seen in wildtype animals is understandable given the short duration of these experiments as well as the fact that *Mmp-9* dysregulation may not be the only change that affects the outflow of the *Tg-MYOC*^{Y437H} mice.

It is however telling that enzymatically inactive tPA (NE-tPA) caused upregulation of both *Mmp-9* and *Mmp-13* expression, while enzymatically active tPA treatment caused *Mmp-13* expression upregulation following a 2 day treatment regimen. Although we were unable to detect a significant increase in *Mmp-9* expression 2 days after tPA administration, we were able to detect it 5 days post administration, suggesting that such expression increase may also be present earlier albeit at a lower level.

Though tPA was successful in enhancing outflow facility, it is of course unlikely that it significantly affects the underlying pathologic events in the *Tg-MYOC*^{Y437H} mouse eyes. Since myocilin mutations ultimately lead to glaucoma through a significant increase in IOP (and concomitant reduction in outflow facility) it is important to compare the efficacy in improving outflow facility by targeting a downstream event vs. targeting a key pathophysiologic event.

ER stress modulation has been previously shown to result in lower IOP and prevent optic nerve degeneration and retinal ganglion cell death in this mouse model of "myocilin" glaucoma (Zode et al., 2011; Zode et al., 2012). Phenylbutyric acid (PBA) has been used as it acts as a chemical chaperone to reduce ER stress (Ozcan et al., 2006). Both systemic (Zode et al., 2011) and topical (Zode et al., 2012) administration were effective in reducing IOP in *Tg-MYOC*^{Y437H} mice. Topical application twice daily resulted in measurable IOP changes within a period as short as 1 week. We utilized whole eye outflow facility as our metric because it has been shown to be more sensitive for detection of changes at the TM (Kumar et al., 2013) and provides a more reliable view of AH dynamics in human eyes (Overby and Clark, 2015).

To compare the efficacy of tPA administered by intraocular injection with that of topical PBA, we elected to measure outflow facility 5 days after initiation of treatment. We reasoned that tPA effectiveness would progressively decrease as medication concentration declines following bolus intraocular administration (Kimpela et al., 2018). Conversely, the effect from topical PBA would slowly increase as cells function better under a reduced ER stress. Thus, this time-point for measuring OF appeared to be a reasonable compromise in obtaining an effect from both tPA and PBA.

As expected, both PBA treatment and tPA treatment resulted in an improvement of outflow facility. It appears that both treatments were comparable in their effectiveness at attenuating outflow facility reduction in *Tg-MYOC*^{Y437H} mice. It is interesting that while PBA primarily functions by reducing ER stress, it also caused an upregulation in *Mmp-9* expression in treated eyes. Such an effect has been previously reported when PBA was used in osteoarthritis (Tang et al., 2018). tPA caused a similar increase in *Mmp-9* expression. Furthermore tPA-treatment resulted in a significant increase in *Mmp-13* expression that surpassed expression levels in both PBA treated eyes and naïve wildtype eyes.

In summary, tPA expression is not reduced in *Tg-MYOC*^{Y437H} mice, and these mice do not show any appreciable steroid-induced outflow facility reduction. However, tPA is effective in improving outflow facility in this genetic POAG model. Furthermore, the tPA effect is accomplished in a receptor-mediated fashion and does not rely on tPA enzymatic activity. Finally, tPA appears to be equally effective with PBA in improving outflow facility in these mice suggesting that it can have a therapeutic potential in the treatment of OAG. This action seems to involve upregulation of *Mmps*.

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Author contributions statement

S.G., Y.H. and J.D. designed research; S.G., Y.H., A.O.B., and Z.Q. performed research; S.G. and Y.H. analyzed data; and S.G. wrote the paper; all authors reviewed the manuscript.

Declaration of competing interest

None.

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