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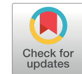
# Thrombospondin-2 is up-regulated by TGFβ2 and increases fibronectin expression in human trabecular meshwork cells



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ABSTRACT

Elevated intraocular pressure (IOP) is a major risk factor for the development of primary open-angle glaucoma (POAG). This is from an increased aqueous humour (AH) outflow resistance through the trabecular meshwork (TM). The pathogenic mechanisms leading to the increase in TM outflow resistance are poorly understood but are thought to be from a dysregulation of the TM extracellular matrix (ECM) environment. ECM modification and turnover are crucial in regulating the resistance to aqueous outflow. ECM turnover is influenced by a complex interplay of growth factors such as transforming growth factors (TGFβ) family and matrix metalloproteinases (MMPs). Elevated TGFβ2 levels result in an increase in ECM deposition such as fibronectin leading to increased resistance. Fibronectin is a major component of TM ECM and plays a key role in its maintenance. Thrombospondins (TSP)-1 and −2 are important regulators of the ECM environment. TSP-1 has been implicated in the pathogenesis of POAG through activation of TGFβ2 within the TM. TSP-2 does not contain the catalytic domain to activate latent TGFβ, but is able to mediate the activities of MMP 2 and 9, thereby influencing ECM turnover. TSP-2 knock out mice show lower IOP levels compared to their wild type counterparts, suggesting the involvement of TSP-2 in the pathogenesis of POAG but its role in the pathogenesis of POAG remains unclear. The purpose of this study was to investigate the role of TSP-2 in trabecular meshwork ECM regulation and hence the pathogenesis of POAG.

TSP-1 and TSP-2 expressions in immortalised glaucomatous TM cells (GTM3) and primary human non-glaucomatous (NTM) and glaucomatous cells (GTM) were determined by immunocytochemistry, immuno-blot analysis and qPCR following treatment with TGFβ2 and Dexamethasone. The level of ECM protein fibronectin was determined in TM cells using immuno-blot analysis following treatment with TSP-1 or -2.

TM cells secrete TSP-1 and -2 under basal conditions at the protein level and TSP-2 mRNA and protein levels were increased in response to TGFβ2 three days post treatment. Exogenous treatment with TSP-2 up-regulated the expression of fibronectin protein in GTM3 cells, primary NTM and GTM cells. TSP-1 did not affect fibronectin protein levels in GTM3 cells. This suggests that the role of TSP-2 might be distinct from that of TSP-1 in the regulation of the TM cell ECM environment. TSP-2 may be involved in the pathogenesis of POAG and contribute to increased IOP levels by increasing the deposition of fibronectin within the ECM in response to TGFβ2.

## 1. Introduction

The trabecular meshwork (TM) is the major site of aqueous humour (AH) outflow in the eye under normal physiological conditions (Bill and Phillips, 1971) and the principal regulator of intraocular pressure (IOP) levels (Llobet et al., 2003). Elevated IOP is a major risk factor in the development and progression of primary open angle glaucoma (POAG) and leads to damage at the optic nerve head with consequent visual field loss. There is increased AH outflow resistance through an impaired outflow drainage system in the TM. The pathogenic mechanisms of increased outflow resistance in glaucomatous TM are poorly understood, but are thought to include an increased deposition of

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extracellular matrix (ECM) components (Faralli et al., 2009; Lutjendrecoll et al., 1989; Tamm and Fuchshofer, 2007), loss of cells (Alvarado et al., 1984) and alterations in the cytoskeletal organisation in TM cells (O'reilly et al., 2011; Tan et al., 2006).

The TM ECM plays a critical role in regulating the AH outflow resistance (Haddadin et al., 2012; Tamm and Fuchshofer, 2007), whereby the amount and quality of ECM material is influenced by the dynamic re-structuring and turnover of proteins (Faralli et al., 2009; Tamm and Fuchshofer, 2007). Histological and morphological studies have shown an increase in ECM "plaque material" within the TM of glaucomatous eyes (Lutjendrecoll et al., 1989), which correlates with damage in the optic nerve (Faralli et al., 2009; Flugel-Koch et al., 2004; Maatta et al., 2005; Tamm and Fuchshofer, 2007; Wordinger et al., 2007). ECM components collagen type IV, collagen type VI, laminin and fibronectin are found in higher amounts in the TM of eyes with POAG (Faralli et al., 2009; Flugel-Koch et al., 2004). ECM modification and turnover are crucial in regulating the resistance to aqueous outflow. A shift in the balance of ECM formation and degradation may increase ECM deposition and restrict the outflow of AH across the TM, providing a physical resistance affecting the contractile properties of the TM (Faralli et al., 2009). The precise mechanisms of ECM turnover in the TM in POAG remain unclear.

ECM turnover is influenced by a complex interplay of growth factors such as transforming growth factors (TGFβ) family and matrix metalloproteinases (MMPs). TGFβ1 and β2 play an important role in the pathogenesis of POAG, where increased levels of TGFβ2 are present in the AH and TM of glaucomatous eyes (Flugel-Koch et al., 2004; Wordinger et al., 2007). Elevated TGFβ2 levels result in an increase in ECM deposition (such as fibronectin, collagen I, IV and VI) and cross-linking that impedes ECM degradation in cultured TM cells (Tamm and Fuchshofer, 2007) and also within organ culture models (Fleenor et al., 2006, Welge-Lussen and Lutjen-Drecoll, 2000). In vitro studies have shown that the continuous perfusion of human anterior segments with TGFβ2 generates a time dependent increase in IOP over 24 hours (h) (Fleenor et al., 2006). Likewise, the levels of tissue inhibitor of metalloproteinases (TIMP-2) are significantly increased over MMP-2 levels in POAG (Maatta et al., 2005), suggesting a reduction in ECM turnover in the TM. Perfusion of anterior segments with MMP's leads to a reversible increase in outflow facility (Bradley et al., 1998; Fuchshofer and Tamm, 2012).

TGFβ2 is secreted as an inactive cytokine that needs to be activated before it can bind to its receptors. Thrombospondin (TSP)-1, a secreted ECM protein, is a potent activator of latent TGFβ *in vivo* and *in vitro* (Crawford et al., 1998; Murphy-Ullrich and Poczatek, 2000; Schultzcherry et al., 1995) and is implicated in the pathogenesis of POAG (Flugel-Koch et al., 2004). TSPs belong to a family of matricellular proteins, of which the TSPs are categorized according to their trimeric (TSP-1 and -2) or pentameric (TSP 3–5) structure (Adams and Lawler, 2011). TSP-1 and-2 comprises the subgroup A TSPs and modulate cell-matrix interactions and cell behaviour. TSP-1 and -2 influence cell function by interacting with cell surface receptors, ECM, growth factors and proteases (Bornstein and Sage, 2002). TSP-1 and TSP-2 share some sequence homology, but differ in some specific regions (Bornstein, 1992). This is reflected by their differential expression within tissues where they potentially carry out specific functions. TSP-1 can activate TGFβ and bind to a number of ECM proteins such as collagens I–V, laminin and fibronectin (Murphy-Ullrich and Poczatek, 2000; Tan and Lawler, 2009). In contrast, TSP-2 does not contain the catalytic domain to activate latent TGFβ, but is able to mediate the activities of MMP2 and -9, influencing ECM turnover (Hiscott et al., 2006; Rhee et al., 2009). Moreover, TSP-2 is reported to inhibit TSP-1 activation of latent TGFβ by competitively binding with latent TGFβ (Schultzcherry et al., 1995).

TSP-1 and -2 are localised in normal TM, expressing different localisation patterns (Hiscott et al., 2006). TSP-2 is pre-dominantly expressed within the uveal meshwork, whilst TSP-1 is expressed in the juxta-canalicular (JCT) meshwork of the TM tissue. TSP-1 expression is up-regulated in glaucomatous eyes (Flugel-Koch et al., 2004) and TSP-1 mRNA has been detected in both fresh and cultured TM cells and is up-regulated by both TGFβ1 (Tamm et al., 2004) and dexamethasone (DEX) in glaucomatous TM (Flugel-Koch et al., 2004). The role of TSP-2 in the TM is less well defined. TSP-2 knock out mice show lower IOP levels, compared to their wild type counterparts, suggesting the involvement of TSP-2 in the pathogenesis of POAG (Haddadin et al., 2012). It is possible that TSP-2 plays a role in glaucoma via its regulation of MMP activity and, hence, ECM turnover.

The precise molecular mechanisms involving ECM turnover remain unclear and elucidating the mechanisms of ECM regulation may provide future targets for regulation of ECM in the TM. TSP-1 and -2 can influence ECM alterations and are well placed in contributing to the pathogenesis of glaucoma. TSP-1 has been shown to be involved in the pathogenesis of POAG through activation of TGFβ2 within the TM. We hypothesise that TSP-2 may be involved in the pathogenesis of POAG through the regulation of ECM turnover via the TGFβ2 signalling pathway. TGFβ2 has been implicated in the pathogenesis of POAG, while DEX is widely used as an inducer of glaucomatous changes in TM cells both by ourselves and others (Clark et al., 2005; Clark et al., 1995; Clark et al., 2013, O'reilly et al., 2011). Fibronectin fibrils are a major component of the ECM and play a key role in the maintenance of ECM in the TM under both normal and glaucomatous conditions (Filla et al., 2017). We investigated the effects of TGFβ2 and DEX upon TSP-2 expression and the effect of TSP-1 and -2 upon fibronectin expression in normal and glaucomatous primary and a immortalised glaucomatous cell line.

## 2. Materials and methods

### *2.1. TM cell culture*

Human transformed glaucomatous TM cells (GTM3) were obtained from Alcon Research Laboratories (Fort Worth, Texas, USA). Human primary TM cells (glaucomatous (GTM) and non-glaucomatous (NTM)) were either from either Alcon Research Laboratories (Fort Worth, Texas, USA) or derived from explants of TM tissue from corneal-scleral rims obtained from St Paul's Eye Unit, Royal Liverpool University Hospital; UK and Liverpool Research Eye Bank; UK, following previously described methods (Keller et al., 2018). All tissue adhered to the tenets of the Declaration of Helsinki and local ethics board ((01/066). TM cells were grown in Dulbecco's modified Eagle's medium (DMEM), low glucose supplemented with 2 mM L–glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin, 2.5 μg/ml amphotericin B (Sigma, St Louis, MO) and 10% v/v heat-inactivated fetal bovine serum (FBS; Biosera, East Sussex, UK). All cells were grown at 37 °C in controlled humidity and 5% $CO_2$ atmosphere. GTM3 have been characterised in other studies (O'reilly et al., 2011); primary TM cells were characterised with a panel of markers (chitinase-3 like-1 (1 μg/ml, Abcam, UK), tissue inhibitor of metalloproteinase −3 (1 μg/ml, Abcam, UK), and myocillin (1 μg/ml, R&D Systems, UK) (following 100 nM DEX treatment) Abcam, UK), as previously described (Keller et al., 2018; Stamer and Clark, 2017; Ding et al., 2014). All experiments using primary TM cells were used between passages 3-4. Primary cells were isolated from donors 65–80 years of age.

### *2.2. TM cell treatments and time course experiments*

Human primary TM and immortalised cells (GTM3) cells were grown to confluence and media containing 10% (v/v) FBS replaced every three days until 70–80% confluence was achieved. TM cells were serum starved for approximately 18 h prior to drug treatments in serum free medium. Recombinant human TGFβ2 (R&D Systems, UK) was made up in 4 mM HCL/bovine serum albumin (BSA). Recombinant human TSP-1 and TSP-2 proteins (R&D Systems, UK) were reconstituted

in phosphate buffered saline (PBS). DEX (Sigma UK) was reconstituted to a working solution of 100 nM in growth media. For TSP-2 and -1 expression experiments, primary TM cells were incubated with fresh serum free medium containing 2 ng/ml recombinant human TGFβ2 protein (Fuchshofer et al., 2009; Kasetti et al., 2018; Flugel-Koch et al., 2004) or 100 nM DEX for three days (Jain et al., 2013; Kasetti et al., 2017). To investigate the expression of fibronectin, TM cells were treated with 2 μg/ml TSP-2 or TSP-1 recombinant human proteins for three days (Armstrong et al., 2003). Control TM cells for all experiments were grown as described above and replaced with fresh serum free medium at time of treatment.

### 2.3. Protein extraction and immuno-blot analysis

Total protein cell lysates were prepared from confluent normal and glaucomatous TM cells three days following the appropriate drug treatment. Cultured cells were lysed in ice cold RIPA lysis buffer (50 mM Tris-HCL pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.1% (v/v) SDS), with cOmplete™ Protease Inhibitor Cocktail tablets (Roche, UK) for 5 min and cell lysates harvested by scraping. Prior to sample loading, lysates were loaded with 4X Laemmli sample buffer (Biorad, UK) and heated to 95 °C for 5 min. Proteins were resolved by SDS-PAGE on a 7.5% (w/v) gel under reducing and denaturing conditions before transfer to nitrocellulose membrane at 80 V for 2 h. Primary antibody solutions used were prepared in 5% (w/v) BSA dissolved in 0.1% (v/v) PBS Tween 20. Antibody concentrations were: anti-TSP-2 goat polyclonal (2 μg/ml, R&D Systems. UK), anti-TSP-1 goat polyclonal (2 μg/ml, R&D Systems, UK), anti-α tubulin mouse (1.2 μg/ml, Sigma, UK) and anti-fibronectin rabbit (1.2 μg/ml, Sigma, UK). Blots were re-probed with anti-α tubulin antibody to confirm equal loading of samples. Immuno-complexes were detected with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) by a chemi-luminescent method (SuperSignal®West Pico Chemiluminescent Substrate, ThermoScientific, UK), using a BioRad Geldoc system. Relative intensities of the protein bands (180 kDa band for TSP-2 and -1 (Streit et al., 1999) and 250 kDa band for fibronectin) were obtained by densitometry analysis using Biorad Image Lab analysis software (version 5.0 build 18) and normalised to endogenous α-tubulin or total protein using Ponceau staining.

### 2.4. Immunocytochemistry

TM cells were seeded into glass four well Lab-Tek Chamber Slides at 5000 cells/cm$^2$ (Nunc, Rochester, NY, USA) and grown to pre confluence and serum starved before drug treatments were performed as described in 2.2. Media was removed following treatment at indicated times and cells were washed in PBS. Cells were fixed with 4% (v/v) Neutral Buffered Formalin in PBS for 10 min followed by 3 × 5 min washes. Following permeabilization with 0.3% (w/v) Triton X-100 in PBS for 5 min, followed by a 5 min PBS wash, cells were incubated in blocking buffer (5% (w/v) normal rabbit or goat serum (Sigma, UK) in PBS) for 60 min at 37 °C before incubating with primary antibody diluted in antibody dilution buffer (1% (w/v) BSA in PBS) overnight at 4 °C. Cells were washed for 5 min in PBS before addition of diluted fluorochrome-conjugated secondary antibodies (Invitrogen, UK) for 60 min, followed by 3 × 5 min washes in PBS (0.05% v/v Tween). Nuclei were stained using propidium iodide (PI) 1 μg/ml or DAPI 1 μg/ml (Invitrogen, UK). Slides were mounted with Fluorescence Mounting Medium (Dako, Cambridgeshire, UK) and examined using a Zeiss LSM 510 confocal microscope for TSP-2 expression or a Nikon Eclipse Ti-E cell microscope for widefield fluorescence imaging.

### 2.5. RNA isolation and real-time quantitative PCR

Total RNA was isolated from TM cells, as described by the manufacturer (RNeasyKit, Qiagen Ltd. West Sussex, UK) with on-column DNase I treatment for removal of genomic DNA. RNA concentration and purity were assessed by spectrophotometry. Total RNA was reverse transcribed and first-strand cDNA was synthesized from 1 μg RNA using SuperscriptII Reverse Transcriptase (Invitrogen, UK). Primers for TSP-1 and-2 were designed using Primer3web version 4.1.0 software and were designed to span exon-exon junctions. Primer sequences used were *TSP-2* Forward *GGGTGATATTTGTAAAGATGAT, TSP-2* Reverse *GAAGTTC CTGAAGTCTGT, TSP-1* Forward *GGACTCGCTGTAGGTTATGATG, TSP-1* Reverse *CGGCTGCTGGACTGGTAG, GAPDH* Forward *AACAGCCTCA AGATCATCAG, GAPDH* Reverse *TGAGTCCTTCCACGATACC.*

Real-time qPCR was performed using LightCycler 480 Sybr Green 1 Master Kit (Roche, West Sussex, UK) as described by the manufacturer. The PCR conditions were 1 pre incubation cycle of 95 °C for 5 min, followed by 40 amplification cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C for 5 s. Incorporating 1 cycle of Melting curve of 95 °C for 5 s, 65 °C for 1 min and 97 °C continuous step. Cooling was at 40 °C 1 cycle for 10 s. Amplifications were performed in triplicate. Data were normalised to GAPDH housekeeping gene and expressed as relative fold changes in mRNA expression between control and experimental treatment groups, determined using the $2^{-\Delta\Delta CT}$ method.

### 2.6. Statistical analysis

Data were analysed using Microsoft Excel and statistical analysis performed using Minitab ® 17.1.0. Experiments were performed in triplicate, with 3 independent experiments for TM cell lines. For experiments using primary NTM and GTM cells 6 donors and 3 donors were used, respectively unless stated otherwize. Comparisons between multiple groups were made using a one-way ANOVA, post hoc Tukey's HSD analysis. Differences were considered statistically significant at $p < 0.05$.

## 3. Results

### 3.1. Expression and localisation of TSP-2 in trabecular meshwork cells

Expression of TSP-2 was investigated in GTM3 cell line, primary NTM and GTM cells. It was observed that TSP-2 was localised throughout the cytoplasm in GTM3 cells in a diffuse pattern (Fig. 1 A), with punctate peri-nuclear staining following immunocytochemistry and imaged by confocal microscopy ( identified by the arrow heads Fig. 1 A (i, iv and v)). The cellular localisation of TSP-2 in primary human NTM cells was observed as areas of punctate staining localised to the cell cytoplasm (Fig. 1 B (i-iv)) and also within areas of ECM around TM cells (identified by the arrow head in Fig. 1 B (iii).

TSP-2 protein expression was also further confirmed in both primary NTM and GTM cells using immuno-blotting technique. TSP-2 ran as a ~180 kDa protein band (Fig. 1 C (i)) (n = 3 donors for NTM and n = 3 donors for GTM). Densitometry analysis showed that there was a significant increase of TSP-2 expression of 1.9 fold in the primary GTM cells relative to the primary NTM cells (Fig. 1 C (ii)).

### 3.2. TGFβ2 increases the expression of protein and mRNA levels of TSP-2 in TM cells

TSP-2 and -1 protein expression levels were investigated in response to TGFβ2 and DEX treatment in TM cells. TSP-1 and -2 protein expression levels from whole cell protein lysates of GTM3 cell line cells were detected via immuo-blot analysis. TSP-1 protein levels served as a positive control for positive TGFβ2 treatment upon TM cells. All TM cells were treated with either 2 ng/ml TGFβ2 or 100 nM DEX for three days and untreated cells were used as controls.

In the GTM3 cell line, immuno-blots revealed TSP-2 expression levels increased approximately 1.6 fold in response to TGFβ2 treatment, compared to untreated GTM3 cells (Fig. 2 A (i) and B (i)). TSP-1 protein expression increased in response to TGFβ2, showing approximately a

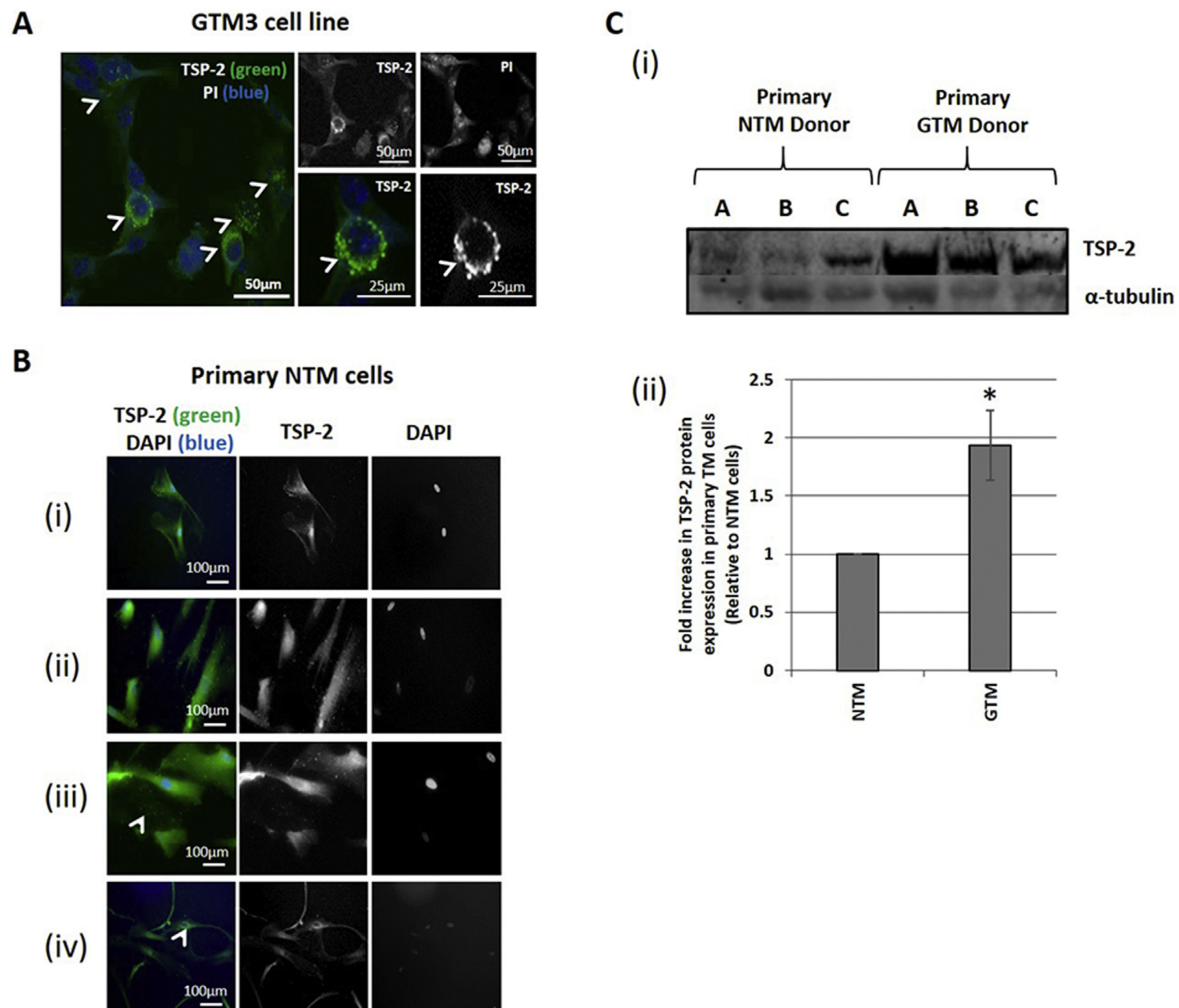


**Fig. 1. TSP-2 localisation and expression in human TM cells.** Immunocytochemistry of TSP-2 in GTM3 cultured cells. (A) (i) Merged image of GTM3 cells expressing TSP-2 (green channel) and nuclei stained with PI (blue channel) Scale bar 50 µm. (A) (ii) represents TSP-2 staining and A (iii) represents PI stained cells. Scale bar 50 µm. (A) (iv) Represents dual fluorescence TSP-2 (green channel) and nuclei stained with PI (blue channel). (A) (v) TSP-2 staining single black and white channel. Arrow heads indicate TSP-2 localisation. Scale bar 25 µm. (B) (i-iv) TSP-2 expression (green) and DAPI staining (blue) in primary NTM cells under basal conditions from three donors. Arrow heads indicates regions of TSP-2 matrix expression (iii) and perinuclear staining (iv). Scale bar represents 100 µm. (C) Immuno-blot analysis of TSP-2 protein expression in primary human TM cell lines under basal conditions. Cells were grown to confluence and whole cell lysates were separated by SDS PAGE, followed by immuno-blot analysis and probed for TSP-2 protein. (C) (i) TSP-2 expression in primary NTM (n = 3) and GTM (n = 3) cells as 180 kDa band and α-tubulin loading controls, showing increased levels of TSP-2 expression in GTM cells compared to the NTM cells. . (C) (ii) Densitometry analysis of TSP-2 expression in primary NTMand GTM cells (results shown as fold change relative to NTM cells). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article).)

2.8 fold increase in TSP-1 expression levels, compared to untreated cells (Fig. 2 A (ii) and B (ii)). DEX treatment did not significantly increase levels of TSP-1 and -2 (Fig. 2 A (i and ii) and B (i and ii)).

The mRNA levels of TSP-2 and TSP-1 were investigated in GTM3 cells following treatment with either 2 ng/ml TGFβ2 or 100 nM DEX for three days. There was an approximately 4.9 fold increase in the expression of TSP-2 mRNA following treatment with TGFβ2 (Fig. 2 C (i)) for GTM3 cells, compared to control untreated cells. There was no significant increase in the expression of TSP-2 mRNA in GTM3 cells (Fig. 2 C (i)) following treatment with 100 nM DEX for 3 days. TSP-1 mRNA expression increased 3.1 fold in the GTM3 cells with TGFβ2 treatment compared to control untreated cells (Fig. 2 C (ii)). No significant difference in TSP-1 mRNA expression was observed with DEX treatment in the GTM3 cell line (Fig. 2 C (ii)).

In primary NTM cells TGFβ2 treatment increased TSP-2 protein expression with approximately a 2.5 fold increase compared to untreated controls (Fig. 3 A (i) and B (i)). No significant difference was observed in the expression of TSP-2 in response to DEX in the primary NTM cells (Fig. 3 A (i) and B (i)). Primary GTM cells showed approximately 1.7 fold increase in TSP-2 protein expression with both TGFβ2 and DEX treatments compared to untreated control cells (Fig. 3 A (ii) and B (ii)).

Primary NTM cells showed a significant increase in TSP-1 expression of approximately a 2 fold increase compared to untreated controls (Fig. 3 A (iii) and B (iii)). No significant difference was observed in TSP-1 protein expression levels with DEX treatment in NTM cells (Fig. 3 A (iii) and B (iii)). There was a distinct band for TSP-2 and TSP-1 at approximately 70 kDa that also showed an increase in response to TGFβ2 in both primary GTM and NTM cells (Fig. 3 A).

No significant difference was observed in the mRNA expression of TSP-2 in primary NTM or GTM cells with TGFβ2 or DEX treatment (Fig. 3 C (i and ii)). TSP-1 mRNA expression was increased significantly in NTM cells with a 11.7 fold increase compared to untreated controls, whilst DEX treatment showed no significant difference (Figure C (iii)).

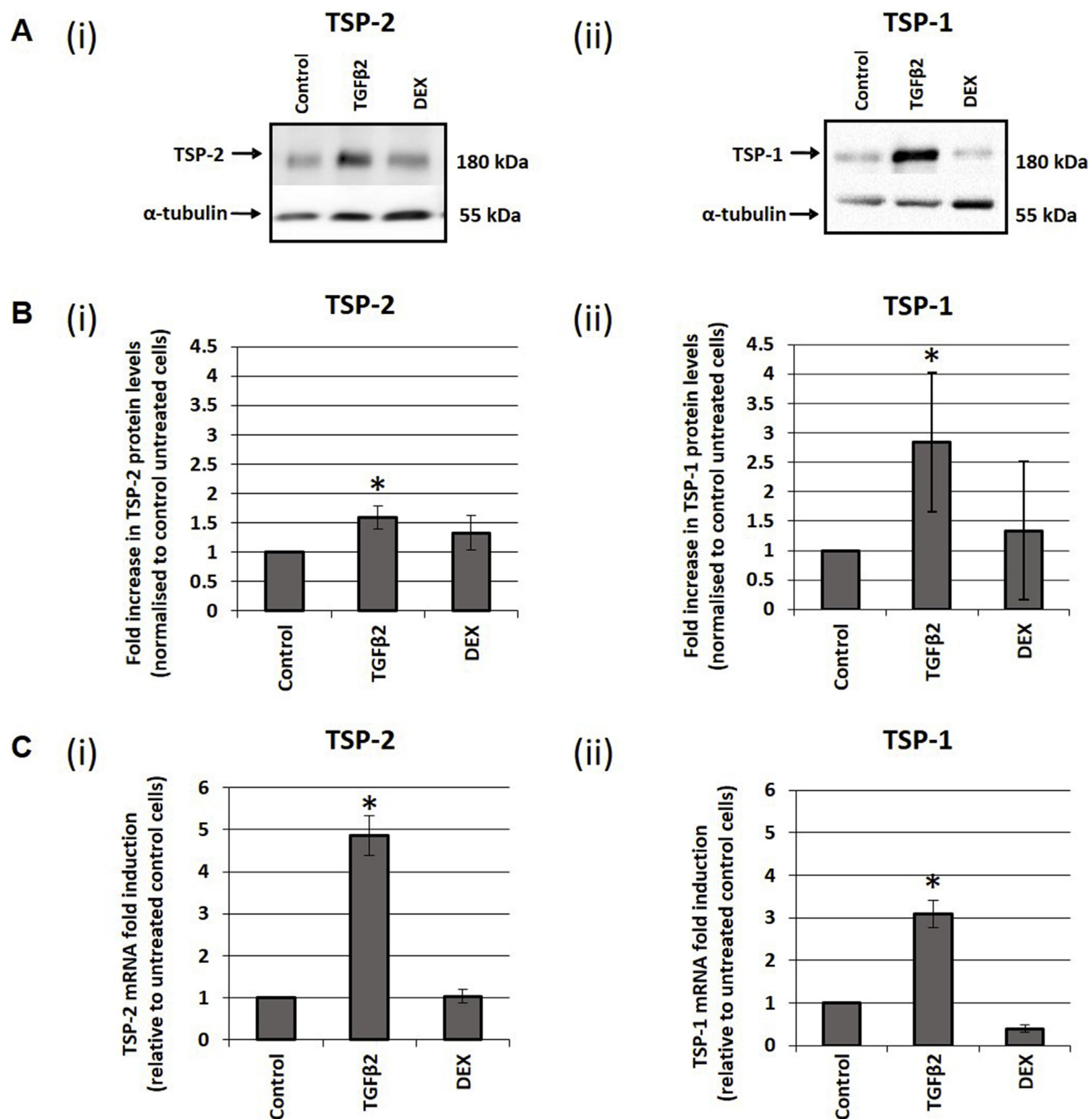


**Fig. 2. TSP-2 and -1 protein and mRNA expression in glaucomatous human GTM3 cells in response to TGFβ2 and DEX.** GTM3 cells were exposed to 2 ng/ml TGFβ2 or 100 nM DEX for 3 days. TSP-2 protein was measured by immuno-blot analysis. (A) (i) Representative immuno-blot showing levels of TSP-2 protein expression in GTM3 cells, following treatment with TGFβ2 and DEX. A (ii) Representative immuno-blot showing increased levels of TSP-1 protein expression in GTM3 cells, following treatment with TGFβ2 and DEX. (B) (i and ii) Histograms of densitometry analysis showing TSP-2 and TSP-1 protein fold increases in GTM3 cells following treatment with TGFβ2 and DEX, relative to untreated control cells. Error bars represent standard deviation, $n = 3$, $p < 0.05$. (C) GTM3 cells were exposed to 2 ng/ml TGFβ2 or 100 nM DEX for 3 days. TSP-2 and TSP-1 mRNA levels were measured using qPCR and mRNA levels normalised to housekeeping gene (GAPDH). Results shown as fold change compared to control GTM3 untreated cells. (C) (i) Represents TSP-2 and (ii) represents TSP-1 mRNA expressions. Error bars represent SD ($n = 3$), * represents $p < 0.05$ (One way ANOVA and post hoc Tukey's analysis).

### *3.3. TSP-2 alters the expression of fibronectin levels in TM cells*

The effects of either TSP-2 or TSP-1 were investigated upon their ability to modulate the expression of fibronectin. GTM3 cell line, primary NTM and GTM cells were treated with exogenous 2 μg/ml TSP-2 or -1 recombinant protein for three days. Whole cell lysates were collected to determine the levels of fibronectin and analysed via western immuno-blot analysis.

Fibronectin is expressed at the protein level in control untreated GTM3 cells and primary NTM and GTM cells (Fig. 4 A and B). Fibronectin protein levels were further increased following treatment with 2 μg/ml TSP-2 peptide for three days in the GTM3 cells, which showed approximately 2.3 fold increase in fibronectin levels, compared to the control untreated GTM3 cells, (Fig. 4 A (i) and B (i)). Fibronectin levels in TSP-1 treated GTM3 cells (0.8 fold) were comparable to control untreated cells (Fig. 4 A (i) and B (i)).

Primary NTM cells showed a significant increase in fibronectin protein expression following either TSP-2 or -1 treatments, with approximately 1.6 and 1.7 fold increase, respectively (Fig. 4 A (ii) and B (ii)). Primary GTM cells also showed approximately 2 and 2.5 fold increase in fibronectin expression with TSP-2 or TSP-1 treatment, respectively (Fig. 4 A (iii) and B (iii)).

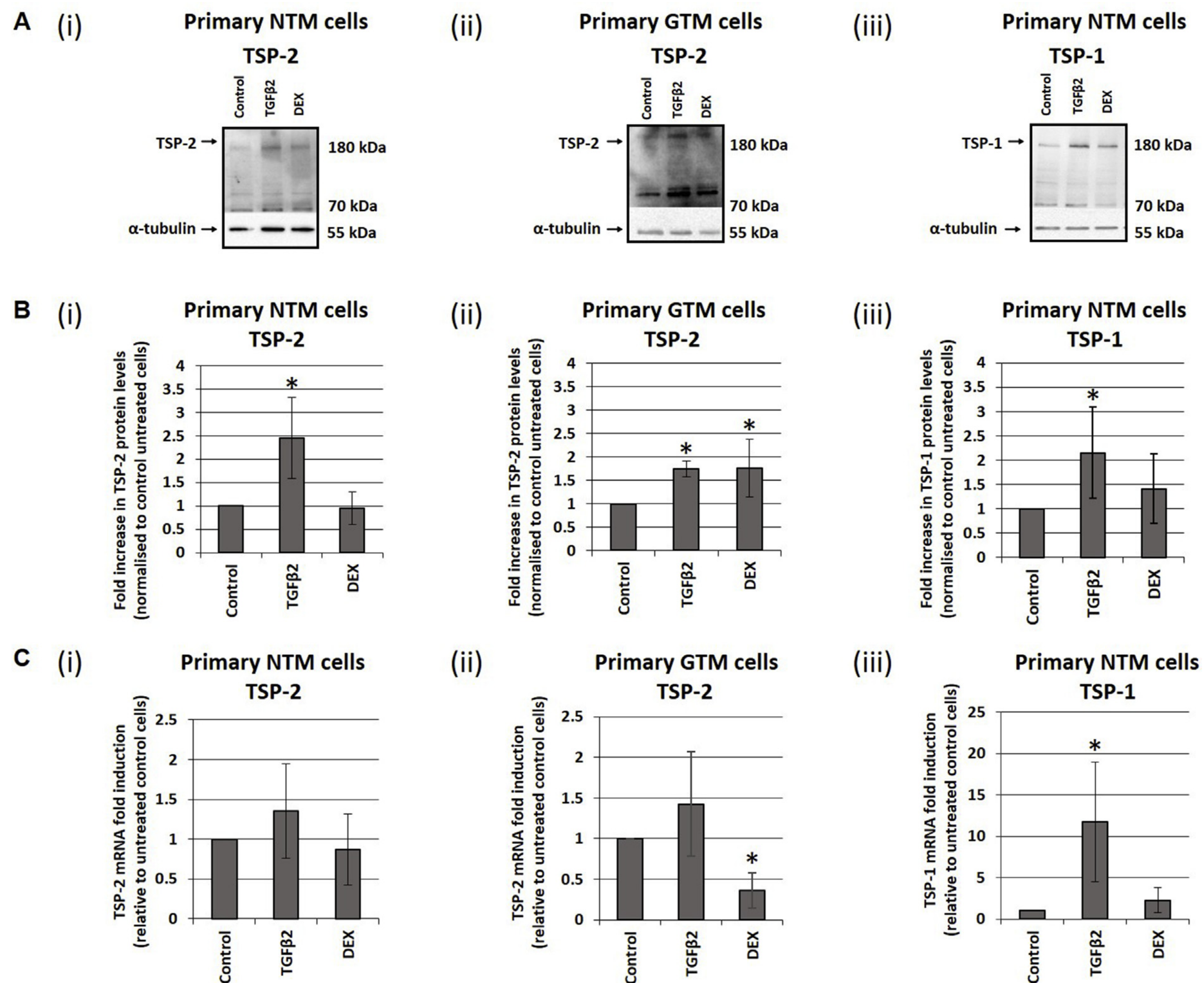


**Fig. 3. TSP-2 and -1 protein and mRNA expression in primary human TM cells in response to TGFβ2 and DEX.** Primary TM cells were exposed to 2 ng/ml TGFβ2 or 100 nM DEX for 3 days. TSP-2 and -1 protein was measured by immuno-blot analysis (A) Representative immuno-blots showing levels of TSP-2 protein expression in (i) primary NTM cells and (ii) primary GTM cells and TSP-1 expression (iii) in primary NTM cells following treatment with TGFβ2 and DEX. (B) Histograms of densitometry analysis showing TSP-2 and TSP-1 protein fold increases in primary NTM and GTM cells, following treatment with TGFβ2 and DEX, relative to untreated control cells. (B) (i) represents TSP-2 expression in primary NTM cells and (ii) in primary GTM cells and TSP-1 expression in (iii) in primary NTM cells. (C) TSP-2 and TSP-1 mRNA levels were measured using qPCR and mRNA levels normalised to housekeeping gene (GAPDH) in primaryTM cells. Results shown as fold change compared to control untreated cells. (C) (i) Represents TSP-2 mRNA expression in primary NTM cells, (ii) represents TSP-1 mRNA expression in primary GTM cells and (iii) represents TSP-1 mRNA expression in primary NTM cells. Error bars represent standard deviation, n = 6 for NTM and n = 3 for GTM. Symbol * represents significantly different ($p < 0.05$) using One way ANOVA and post hoc Tukey's analysis.

## 4. Discussion

Thrombospondins are important regulators of ECM interactions and modulating cellular responses *in vivo*. TSP-1 levels are elevated in POAG patients, however less is known about the expression and function of TSP-2 of which it shares similar structural and sequence homology (Bornstein, 1995; Bornstein and Sage, 2002). On this basis, we have investigated if TSP-2 is expressed within the TM in vitro, whether its levels are increased via TGFβ2 stimulation, and also the downstream effects of TSP-2 expression upon the ECM. In this study we found that TSP-2 is expressed by both primary and immortalised TM cells and protein levels are up-regulated via TGFβ2 treatment. We further demonstrated that glaucomatous TM cells (GTM3), primary NTM and GTM cells can respond to exogenous TSP-2 treatment and are capable of increasing fibronectin levels at the time point analysed. In contrast, TSP-1 had no significant effect upon the fibronectin levels in GTM3 cells, but in primary NTM and GTM cells fibronectin was increased following both TSP-1 or TSP-2 treatments. To the best of our knowledge, this is the first report which demonstrates that TSP-2 is up-regulated via TGFβ2 treatment in TM cells and can modulate the fibronectin levels within the ECM environment.

Our data corroborates findings observed by others of TSP-2 expression in TM cells and also within TM tissue (Haddadin et al., 2012; Hiscott et al., 2006). Previous studies have shown that TSP-2 is expressed predominantly within the uveal meshwork in healthy TM cells, suggesting that TSP-2 has a role in healthy TM tissue specific to the uveal meshwork (Hiscott et al., 2006). In contrast, TSP-1 is expressed predominantly in the JCT in healthy TM, whereas its distribution is found throughout the TM in glaucomatous patients (Flugel-Koch et al., 2004). This differential localisation between TSP-2 and TSP-1 within the TM may differentially regulate the expression of ECM proteins throughout the different regions of the TM in response to fluctuations in IOP. However, the distribution of TSP-2 in glaucomatous tissue requires further investigation.

Regulation of the TM ECM is essential for the regulation of normal IOP. TGFβ2 is present in the aqueous humour under normal conditions (Granstein et al., 1990) and basal levels of TGFβ2 activation may be required for the continuous secretion of ECM molecules to regulate the

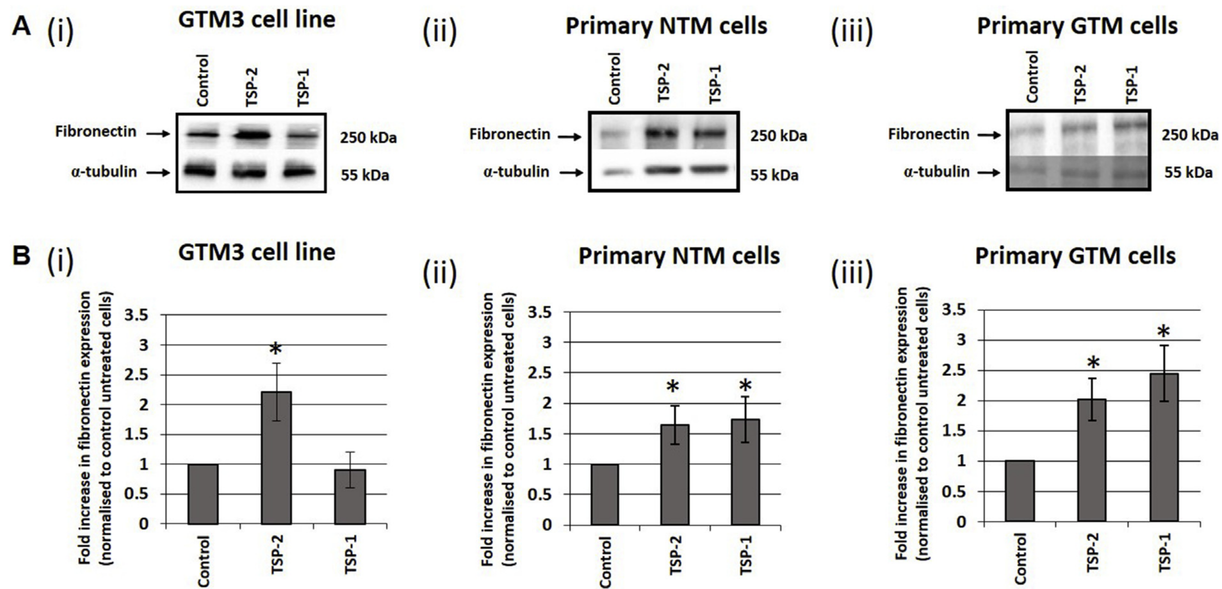


**Fig. 4. Effect of TSP-2 upon the expression of ECM protein fibronectin in TM cells.** Immuno-blot and densitometry analysis of GTM3 and primary non glaucomatous TM (NTM) and glaucomatous TM (GTM) cells treated with either 2 μg/ml TSP-2 or TSP-1 recombinant human protein for 3 days. (A) Shows the immunoblot of (i) GTM3 cell line, (ii) primary NTM cells and (iii) primary GTM cells, samples treated with TSP-2 or -1 for 3 days, probed for fibronectin, shown as a 250 kDa band. (B) Densitometry analysis shows fold change in fibronectin protein expression (i) GTM3 cell line, (ii) primary NTM cells and (iii) primary GTM cells. Bands were normalised to α-tubulin (55 kDa band) and results are shown as fold change compared to untreated control for each cell line. Error bars represent standard deviation (n = 3 for GTM3 and n = 6 for primary NTM cells and n = 3 for primary GTM cells). Symbol * represents significantly different ($p < 0.05$) using One way ANOVA and post hoc Tukey's analysis.

normal turnover by the ECM matrix. It is plausible that TSP-2 may form part of the normal regulation of the TM ECM environment to control the AH outflow in response to TGFβ2 signalling. Studies have shown that TGFβ2 increases ECM protein synthesis in cultured TM cells and is an important mediator of ECM synthesis, secretion and degradation in the human TM (Fleenor et al., 2005, 2006; Gottanka et al., 2004; Shepard et al., 2010, Welge-Lussen and Lutjen-Drecoll, 2000). TGFβ2 is involved in the pathogenesis of glaucoma (Lutjendrecoll et al., 1989; Lutjen-Drecoll, 2005) and is elevated in the AH of POAG patients (Tripathi et al., 1994). In our in vitro model, a dose of 2 ng/ml TGFβ2, which induces glaucomatous effects in TM cells (O'reilly et al., 2011), results in increases in TSP-2, as well as TSP-1. It is well-documented that TSP-1 is up-regulated via TGFβ1 and -2 in the TM (Flugel-Koch et al., 2004), however we have shown that TSP-2 is also up-regulated via TGFβ2 in TM cells.

In contrast with other reports, TSP-2 has not been detected in TM cell microarray or proteomic analysis performed 24 h post treatment with TGFβ2, nor did it show an increase at the protein level (Haddadin et al., 2012; Clark et al., 2013). However, this difference could potentially be due to the differences in the duration of TGFβ2 treatments. Our data suggests that TSP-2 may be secreted at later time points following TGFβ2 treatment, rather than at 24 h as in previous studies (Haddadin et al., 2012). It has previously been reported that TSP-1 is only increased in one third of POAG patients (Flugel-Koch et al., 2004). It is plausible that this might be the case for TSP-2 as well. We observed a significant increase in both TSP-1 and -2 mRNA expression in response to TGFβ2 in GTM3 cell line. We did not observe significant increases in TSP-2 mRNA primary NTM or GTM cells, despite observing significant increases at the protein level. Interestingly, we did observe increases in TSP-2 mRNA expression in half of the donors samples tested, whilst TSP-1 mRNA levels was consistently upregulated in all donors. Whether this difference was due to donor variability is unclear and would warrant further studies.

We identified a consistent lower band in immunoblots for both TSP-2 and TSP-1 in primary NTM and GTM cells around the 70 kDa band. Abu El-Asrar et al. have also reported TSP-2 as a marker of proliferative diabetic retinopathy (PDR) (Abu El-Asrar et al., 2013). TSP-2 ran as two bands with the upper band reportedly correlated with the intact protein and the lower band as cleaved TSP-2, which was increased in vitreous samples from PDR patients. They hypothesised that the increased expression of TSP-2 cleaved product may be due to elevated levels of MMP-9 in PDR inducing proteolytic clearance of TSP-2. Thus, suggesting that a negative feedback system between MMP-9 and TSP-2 requiring further investigation.

Our findings showed that the DEX response in the TM cells was more variable and did not result in significant increases in the TSP-1 or TSP-2 expression in TM cells. Other studies have shown that the TSP-1 response was lower with TGFβ1 (2 fold) at 3 days compared to DEX (Flugel-Koch et al., 2004). Glucocorticoids are reported to increase the deposition of ECM material (Acott and Kelley, 2008), in particular the fibronectin levels (Steely et al., 1992; Filla et al., 2017). Unpublished data from our laboratory has also shown an increase in fibronectin in response to DEX, but the extent of the increase was significantly lower to that of TGFβ2. It is therefore possible that DEX treatment on TM cells increases fibronectin levels via a TSP-1 and TSP-2 independent mechanism. Although the effects of DEX upon TM cells can be observed at 3 days (Raghunathan et al., 2015; Jain et al., 2013; Kasetti et al., 2017) investigating the effects of DEX at longer time points would require further investigation.

TSP-2 increased the expression of fibronectin in TM cells, suggesting a possible role for TSP-2 in ECM regulation and hence the pathogenesis of glaucoma. TGFβ2 increases fibronectin levels in TM cells and aqueous humour (Acott and Kelley, 2008; Faralli et al., 2009; Wordinger et al., 2007). Fibronectin is a multifactorial ECM glycoprotein and is not only a major structural component of the ECM, but it also functions as a signalling molecule itself and regulates AH outflow (Acott and Kelley, 2008; Faralli et al., 2009; Keller et al., 2009). Recent data suggests that disruption of fibronectin fibrillogenesis can prevent the incorporation of other ECM proteins into the ECM (Filla et al., 2017). It has been shown that cellular fibronectin, a stretched and insoluble isoform of fibronectin that is secreted and assembled into dense complex fibril networks, affecting overall ECM-cell interactions as well as ECM

homeostasis, is specifically induced by TGFβ2 (Medina-Ortiz et al., 2013). It has been proposed that this might be a novel pathologic mechanism in the TM changes associated with glaucoma (Medina-Ortiz et al., 2013). The present study did not distinguish between the cellular or secreted forms of fibronectin, but the increase in fibronectin levels in response to TGFβ2 treatment support our hypothesis that TSP-2 may be involved within this process. In addition, TSP-2 knockout mice demonstrated lower IOP levels (Haddadin et al., 2012), which suggests TSP-2 may regulate the IOP levels, via regulation of the fibronectin levels, hence suggesting a potential role in the pathogenesis of POAG.

TSP-2 can regulate the levels of MMP-2 by being able to bind and inactivate MMPs (notably MMP-2) (Bornstein and Sage, 2002; Bornstein et al., 2000), which are responsible for the degradation of the ECM environment via its internalisation (Yang et al., 2001). Levels of MMP-2 are also increased in TSP-2 knockout out mice (Kyriakides et al., 2001). There are decreased levels of MMP-2 activity in glaucomatous cells (Schlotzer-Schrehardt et al., 2003) and the levels are further decreased in TM cells treated with TGFβ2 and DEX (Fuchshofer et al., 2003; Snyder et al., 1993), which may be responsible for increasing the deposition of ECM components, giving rise to impaired outflow resistance. MMP-2 levels are also decreased with stretch experiments on the TM (Vittal et al., 2005) increasing the flow rate, whereas TSP-2 levels were increased with the stiffness of the TM. TSP-2 may therefore also contribute to the mechanical properties of the TM through modulation of fibronectin levels.

## 5. Conclusions

In summary, TSP-2 increased the expression of fibronectin in glaucomatous TM cell line, as well as primary NTM and GTM cells and its levels were influenced by TGFβ2. TSP-2 expression within the TM may therefore be involved in the pathogenesis of POAG and contribute to increased IOP levels by increasing the deposition of fibronectin within the ECM. Although the levels of TSP-2 are not likely to be major drivers of POAG, it does however provide further insight into the potential mechanisms of fibronectin deposition and up-regulation within the TM during the pathogenesis of POAG via TGFβ2 signalling. Elucidating the role of TSP-2 in ECM regulation and of TSP-1 and-2 in the regulation of cell matrix interactions may provide future targets for regulation of the ECM in the TM.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2019.107820.

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