Constitutive Expression and Release of TGF-β2 by Human Trabecular Meshwork Cells

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INTRODUCTION

Primary open-angle glaucoma (POAG) is a common cause of blindness worldwide, affecting nearly 2 million individuals 40 years of age and older in the US. Elevated intraocular pressure (IOP) is a poorly-understood risk factor for the initiation and progression of POAG. In healthy eyes, normal IOP is sustained through balanced production and outflow of aqueous humor (AH). In adults, the majority (>50%) of AH exits the eye through a conventional outflow pathway involving the trabecular meshwork (TM). Resistance to AH outflow through the TM is mediated, in part, through enhanced cellular contractility and increased extracellular matrix deposition in the TM.

Whereas the cause of outflow resistance and elevated IOP in POAG patients remains unclear, pathogenesis of POAG has been strongly correlated with aberrantly-elevated levels of a variety of soluble factors in the AH. Of particular significance, levels of biologically-active transforming growth factor (TGF)-β2 are known to be increased by 60-70% in the AH of POAG patients as compared to healthy control subjects. *Despite a clear link between aberrantly-elevated content of TGF-β2 and increased resistance to AH outflow through the TM, there remains a paucity of data on the mechanisms regulating constitutive TGF-β2 content in the eye.*

Within the human anterior segment, TGF-β2 immunoreactivity has been localized to limbal and lens epithelial cells, the ciliary body, and the conjunctival stroma. In contrast, only TGF-β1 mRNA expression and protein secretion has been localized to human TM cells.

The purpose of this study was to investigate whether TM cells serve as a constitutive source of TGF-β2 in the human anterior segment, and determine the mechanisms underlying constitutive TGF-β2 expression and release.

METHODS

Porcine Anterior Segment Perfusion: Anterior segment perfusion experiments are performed using fresh porcine eyes obtained from a local abattoir. Globes are bisected aseptically at the equator, and the iris, lens, and vitreous gently removed. The prepared anterior segment is continuously perfused at a constant flow rate of 4.5 µl/min with pre-warmed DMEM supplemented with antibiotics and antimycotics. Anterior segments are cultured under a humidified atmosphere of 5% CO_2/95% air at 37°C and allowed to stabilize overnight. Following stabilization, media perfusing porcine anterior segments was exchanged with DMEM containing either vehicle (400 nM HCl) or recombinant active human TGF-β2 (10 ng/ml; R&D Systems). In some cases, stabilized porcine anterior segments were perfused with either vehicle (0.01% DMSO) or the TGFβRI/ALK-5 antagonist SB-431542 (1 µM). IOP was monitored and recorded every 3 minutes (PowerLab 8/35 with LabChart Pro).

Human TM Cell Culture: Primary human TM cells were harvested from discarded human corneoscleral rims and cultured to confluence as we have previously described (Von Zee *et al.*, 2012). An SV40-transformed human TM cell line (GTM3) derived from a male glaucomatous patient was a generous gift from Alcon Labs. All cultures were maintained at 37°C under an atmosphere of 5% CO_2/95% air.

Treatment of Human TM Cells: Human TM cells were cultured to confluence and treated x24h with **(i)** vehicle (0.01% ethanol) or chemically activated lovastatin (10 µM), **(ii)** vehicle (0.6% DMSO) or a geranylgeranyl transferase-I inhibitor (GGTI-298; 20 µM), or **(iii)** vehicle (PBS) or C3 exoenzyme (10 µM).

siRNA-Targeted Knockdown: GTM3 cells were transfected x24h with siRNA (100 nM) directed against a scrambled sequence (conrtrol), RhoA, or RhoB using Lipofectamine in a 1:1 mixture of OptiMEM and cell culture medium without serum or antibiotics/antimycotics.

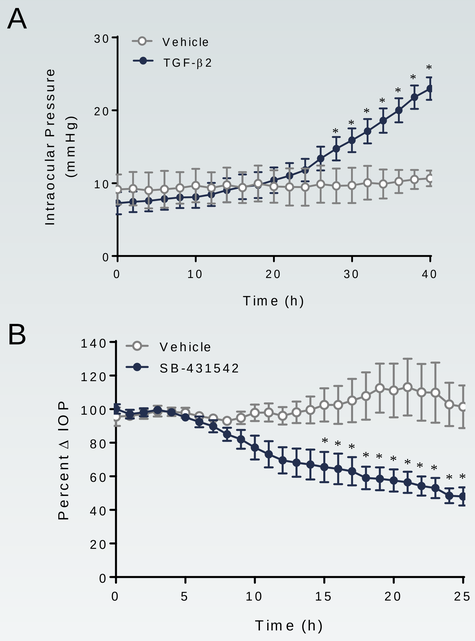
Real-Time RT-PCR: Total RNA was extracted from human TM cells using TRIzol reagent, and 5 µg was reverse-transcribed using Super Script III First Strand Synthesis system. Human-specific TGF-β2 or GAPDH cDNA was amplified by qPCR on a Mini-Opticon PCR detection system. For each sample, the specificity of the reaction product was determined by melt curve analysis. The expression of GAPDH was unaltered by drug treatments; therefore, relative fold-changes in gene expression were normalized to GAPDH.

TGF-β2 ELISA: Levels of biologically-active TGF-β2 in cell culture media harvested from human TM cells were quantified using a commercially-available ELISA kit according to manufacturer's instructions.

Statistical Analysis: Results are expressed as the means ± SEM of triplicate cultures. Parametric data were analyzed by Student's *t-test* or two-way ANOVA with a Bonferroni's multiple comparison post-hoc analysis, as indicated. In all cases, statistical significance was defined as $p < 0.05$.

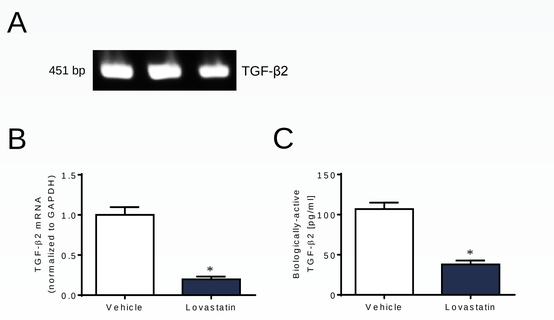
RESULTS

TGF-β2 signaling facilitates elevated IOP in cultured porcine anterior segments



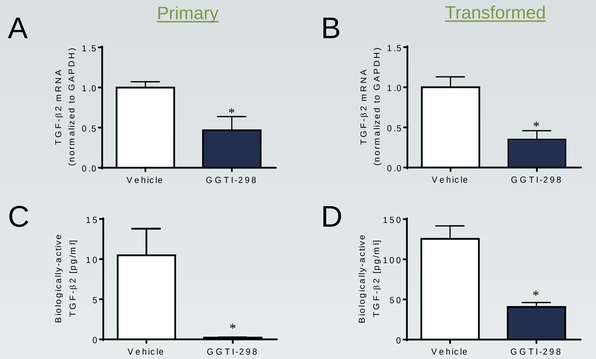
(A) Stabilized porcine anterior segments were perfused with either vehicle (400 nM HCl) or TGF-β2 (10 ng/ml), as indicated. Data shown are the means ± SEM from a single experiment performed in triplicate, representative of two separate experiments. **(B)** Stabilized porcine anterior segments were perfused with either vehicle (0.01% DMSO) or SB-431542 (1 µM), as indicated. Data shown are the means ± SEM of two separate experiments (n = 5-6 per group) and expressed as percent of stabilized baseline pressure. In each case, * $p < 0.05$; two-way ANOVA with Bonferroni's post-hoc analysis.

Lovastatin attenuates constitutive TGF-β2 mRNA expression and protein secretion



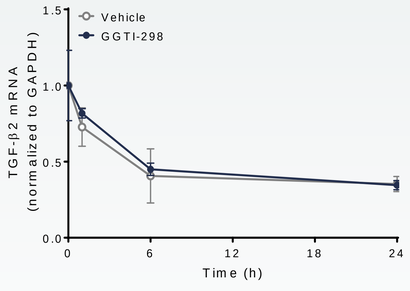
(A) TGF-β2 mRNA in quiescent GTM3 cells (n=3). **(B)** Relative GAPDH-normalized content of TGF-β2 mRNA (n=9) or **(C)** absolute content of secreted biologically-active TGF-β2 protein (n=6) in GTM3 cells incubated x24h with vehicle (0.01% ethanol) or chemically-activated lovastatin (10 µM). *, $p < 0.001$, Student's *t-test.*

Inhibition of geranylgeranylation attenuates constitutive TGF-β2 expression and secretion



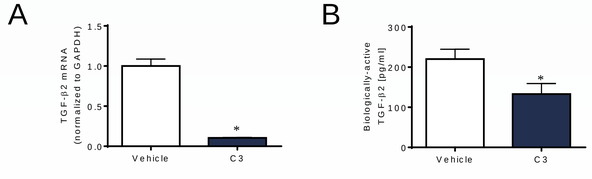
(A, B) Relative GAPDH-normalized content of TGF-β2 mRNA (n=6-9) or **(C, D)** absolute content of secreted biologically-active TGF-β2 protein (n=3-6) in primary or transformed human cells incubated x24h with vehicle (0.06% DMSO) or GGTI-298 (20 µM). *, $p < 0.01$, Student's *t-test.*

TGF-β2 mRNA stability is unaltered by disrupting geranylgeranylation



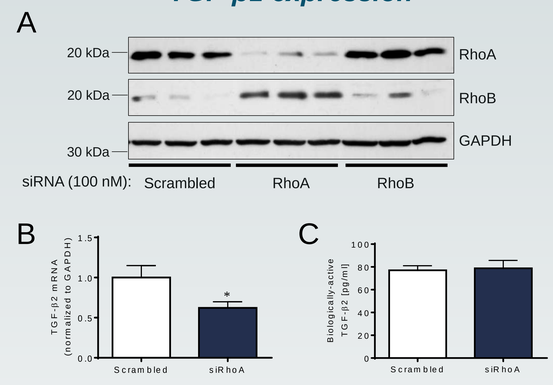
Confluent GTM3 cells were pre-treated x1h with actinomycin D (1 µg/ml) and subsequently incubated in the absence (Vehicle; 0.6% DMSO) or presence of GGTI-298 (20 µM). Relative GAPDH-normalized content of TGF-β2 mRNA (n=3), representative of 2-3 separate experiments, is shown.

TGF-β2 expression and secretion is attenuated by disrupting Rho GTPase signaling



(A) Relative GAPDH-normalized content of TGF-β2 mRNA (n=6) or **(B)** absolute content of secreted biologically-active TGF-β2 protein (n=6) in GTM3 cells incubated x24h with vehicle (PBS) or C3 exoenzyme (10 µg/ml). *, $p < 0.05$, Student's *t-test.*

siRNA-targeted knockdown of RhoA selectively attenuates constitutive TGF-β2 expression



Confluent GTM3 cells were transfected with scrambled, RhoA, or RhoB siRNA (24h, 100 nM each) as indicated. **(A)** Immunoblot of RhoA, RhoB, and GAPDH proteins. Results are representative of 2 separate experiments. **(B)** Relative GAPDH-normalized content of TGF-β2 mRNA (n=12) or **(C)** absolute content of secreted biologically-active TGF-β2 protein (n=12) in GTM3 cells. *, $p < 0.001$, Student's *t-test.*

SUMMARY

- Inhibition of *endogenous* TGF-β/ALK-5 signaling lowers IOP
- Human TM cells express and secrete biologically-active TGF-β2
- Rho GTPases facilitate constitutive TGF-β2 expression and secretion
- siRNA-targeted knockdown of RhoA selectively attenuates constitutive TGF-β2 mRNA expression

CONCLUSION

- Human TM cells represent a source of elevated *biologically-active* TGF-β2 in the aqueous humor of patients with POAG
- Aberrant Rho GTPase signaling mediates TGF-β2 expression and secretion in human TM cells

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