Quantitative Proteomics: TGF β ₂ Signaling in Trabecular Meshwork Cells

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PURPOSE. Transforming growth factor beta 2 (TGF β ₂) is often elevated in the aqueous humor (AH) and trabecular meshwork (TM) of patients with primary open-angle glaucoma (POAG) and appears to contribute to POAG pathogenesis. To better understand TGF β ₂ signaling in the eye, TGF β ₂-induced proteomic changes were identified in cells cultured from the TM, a tissue involved in intraocular pressure (IOP) elevation in glaucoma.

METHODS. Primary cultures of human TM cells from four donors were treated with or without TGF β ₂ (5 ng/mL) for 48 hours; then cellular protein was analyzed by liquid chromatography-mass spectrometry iTRAQ (isobaric tags for relative and absolute quantitation) technology.

RESULTS. A total of 853 proteins were quantified. TGF β ₂ treatment significantly altered the abundance of 47 proteins, 40 of which have not previously been associated with TGF β ₂ signaling in the eye. More than half the 30 elevated proteins support growing evidence that TGF β ₂ induces extracellular matrix remodeling and abnormal cytoskeletal interactions in the TM. The levels of 17 proteins were reduced, including four cytoskeletal and six regulatory proteins. Both elevated and decreased regulatory proteins implicate TGF β ₂-altered processes involving transcription, translation, and the glutamate/glutamine cycle. Altered levels of eight mitochondrial proteins support TGF β ₂-induced mitochondrial dysfunction in the TM that in POAG could contribute to oxidative damage in the AH outflow pathway, TM senescence, and elevated IOP.

CONCLUSIONS. The results expand the repertoire of proteins known to participate in TGF β ₂ signaling, provide new molecular insight into POAG, and establish a quantitative proteomics database for the TM that includes candidate glaucoma biomarkers for future validation studies.

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Primary open-angle glaucoma (POAG) is the most common form of the primary glaucomas and affects approximately 3 million Americans and more than 70 million people worldwide.¹ Visual loss in POAG results from damage to retinal ganglion cells and the optic nerve; advanced age and elevated intraocular pressure (IOP) are risk factors.^{2,3} A significant proportion of POAG patients have what is termed normal-tension glaucoma in which IOP remains in the normal range and the cause of the neuropathy is unclear.⁴ Nevertheless, IOP is still a major risk factor in normal-tension glaucoma because further lowering of IOP decreases disease progression. Despite the high prevalence of POAG and the identification of glaucoma susceptibility genes,⁵ the molecular mechanisms of glaucoma are poorly understood. Elevated IOP in POAG appears related to pathologic changes in the aqueous humor (AH) outflow pathway that cause increased outflow resistance, particularly in the trabecular meshwork (TM). Abnormal accumulation of extracellular matrix (ECM),^{6,7} abnormal protein expression,⁸ and changes in cytoskeletal interactions^{9,10} within the TM have been associated with increased AH outflow resistance and elevated IOP. Oxidative damage may also contribute to elevated IOP¹¹ and play a role in POAG.^{12,13}

Transforming growth factor beta 2 (TGF β ₂) is an immunosuppressive factor in normal human AH that helps maintain the immune privilege of the eye.^{14,15} In addition to elevated levels of TGF β ₂ in the AH of POAG patients, as shown in numerous studies,^{16–18} TGF β ₂ is elevated in glaucomatous TM tissues and cultured glaucomatous TM cells.¹⁹ In vitro studies in the TM show that TGF β ₂ can induce ECM remodeling,^{20,21} inhibit cell proliferation,²² induce senescence-like changes,²³ and alter the actin cytoskeleton. Elsewhere in the body, TGF β ₂ mediates increased ECM deposition and has been implicated in fibrosis of the liver,²⁴ kidney,²⁵ and lung.²⁶ Gene expression studies have reported changes in TGF β ₂-treated TM cells,^{21,27,28} including increased levels of transcripts encoding ECM and cytoskeletal components. Organ culture studies in which the anterior segment of human and porcine eyes is perfused with TGF β ₂ have reported increased IOP and ECM deposition in the AH drainage pathway.^{21,29,30} More recently, adenoviral gene transfer of TGF β ₂ in vivo to rodent eyes has led to reduced AH outflow and increased IOP.³¹ This growing body of evidence implicates TGF β ₂ in POAG pathology.

To better understand the molecular consequences of TGF β ₂ signaling in the anterior segment, we compared human TM cells with and without TGF β ₂ treatment using global quantitative proteomics methods. Liquid chromatography-mass spectrometry (LC MS/MS) isobaric tags for relative and absolute quantitation (iTRAQ; Applied Biosystems, Foster City, CA) technology was used to quantify TGF β ₂-induced protein changes in TM cells. Forty-seven significantly altered proteins were identified, 40 of which have not previously been associ-

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TABLE 1. Trabecular Meshwork Cell Samples

Experiment	Cell Culture	Donor Age and Sex	Cell Passage	Protein Analyzed (μ g)
1	NTM416-07	78/M	4	47
2	NTM496-05	82/F	3	54
3	GTM304-4	75/F	3	38
4	GTM477-02	85/F	4	67

Each of the above primary TM cell cultures was treated with or without TGF β_2 , then analyzed by LC MS/MS iTRAQ technology. NTM, normal trabecular meshwork; GTM, glaucomatous trabecular meshwork; M, male; F, female.

ated with TGF β_2 signaling in the TM. The results provide new molecular insight into the consequences of TGF β_2 signaling in ocular hypertension and POAG.

METHODS

TM Cell Cultures

TM cells were isolated from postmortem human TM tissue explants derived from open-angle glaucoma and nonglaucomatous control donor eyes. Glaucoma status was indicated from donor medical histories. The average death to preservation time was 7.75 ± 3.3 hours. Eyes were stored at 4°C until the TM was dissected, generally within 24 to 36 hours. Primary cultures were established, and TM cell morphology and purity of these cultures were characterized as previously described.²² Human TM cells were grown in Dulbecco's modified Eagle's medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 1% penicillin-streptomycin (HyClone), and 1% L-glutamine (Thermo-Scientific, Waltham, MA) to confluence in T-25 flasks or in six-well plates. Primary cultures of human TM cells from two POAG and two nonglaucomatous donors were treated with or without TGF β_2 (5 ng/mL) for 48 hours, yielding four TGF β_2 -treated and four untreated TM cultures.

Sample Preparation, iTRAQ Labeling, SCX Chromatography, Protein Identification and Quantitation, and Bioinformatic and Western Blot Analysis

Detailed methods are provided in the Supplementary Methods (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental>) available online. Briefly, for proteomic analyses, proteins were extracted from TM cells, quantified by amino acid analysis, reduced, alkylated, and digested with trypsin. Tryptic peptides from TGF β_2 -treated TM cells were labeled with iTRAQ tag 117 and mixed with an equal amount of tryptic peptides from the corresponding untreated cell sample labeled with iTRAQ tag 115.^{32,33} Each peptide mixture was fractionated by strong cation exchange (SCX) chromatography, and fractions were collected for LC MS/MS. LC MS/MS was performed with a QTOF2 mass spectrometer equipped with a capillary column system (Cap LC; Waters Corporation, Milford, MA).^{32,33} Protein identification used MASSLYNX 4.1 software (Waters), the Mascot search engine (Matrix Science), and the Swiss-Protein human sequence database. Protein quantitation from iTRAQ labeling was achieved with code written in the statistical program R.^{32,33} Bioinformatic analyses were performed with The Protein Analysis THrough Evolutionary Relationships (PANTHER) Classification System, pathway analysis (Ingenuity Pathways Analysis 8.5; Ingenuity Systems, Redwood City, CA), and the Swiss Protein database. Western blot analyses were performed on polyvinylidene difluoride membranes using established protocols and commercially available antibodies.

RESULTS

From four primary cultures of human TM cells treated or untreated with TGF β_2 , a total of 853 proteins were identified and quantified with two or more peptides by LC MS/MS and iTRAQ quantification code written in R. The TM cell samples were from normal ($n = 2$) and glaucomatous ($n = 2$) donor eyes (Table 1). Quantitative results from each cell sample are presented in Supplementary Tables S1–S4 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental>), including protein ratios, SDs, P values, number of unique peptides quantified, percentage sequence coverage for each protein, and peptide false discovery rates. The data from all four TM samples were of comparable quality and appropriate for averaging based on consistently low peptide false discovery (average rates, 2.1% identity, 3.0% homology) and similar distributions of protein ratios. The average relative abundance of the 853 quantified proteins is presented in Supplementary Table S5 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental>), and the distribution of mean protein ratios for all proteins and those quantified in three or more TM samples ($n = 450$) are shown in Figure 1. The distributions in Figure 1 are slightly skewed, indicating that more proteins were increased in abundance than were decreased by TGF β_2 treatment. Criteria for determining whether a protein was elevated or decreased included the average adjusted protein ratio and P value, with only proteins quantified in three or more cell

FIGURE 1. Distribution of protein ratios. The log₂ mean distribution of protein ratios (TGF β_2 -treated TM/untreated TM) are shown for (A) all 853 quantified proteins and (B) 450 proteins quantified in three or more cell samples. Median, mean, and SD values are indicated; protein ratios between 1 and 2 SD from the mean are shaded. The distribution of protein ratios is near normal for all proteins but with some spread above 1.0 (log₂ mean ratio 0), suggesting the increased abundance of several proteins after TGF β_2 treatment.

samples used for comparative purposes. Proteins exhibiting average protein ratios (adjusted by SEM) above or below the mean by at least 1 SD (Fig. 1) and $P < 0.06$ were considered of higher or lower abundance.

Majority of the Determined Proteome Reflects That of Untreated TM Cells

Approximately 95% of the 853 quantified proteins and ~90% of the 450 proteins quantified in three or more samples (Supplementary Table S5, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental>) were present in similar amounts in TGF β_2 -treated and untreated TM cells. Accordingly, the overall determined proteome largely reflects that of untreated TM cells. The quantified proteins include ~5% secreted, ~46% cytoplasmic, ~22% membrane and membrane-associated, ~15% nuclear, and ~12% mitochondrial proteins. Among the significant proteomic changes after TGF β_2 treatment, more secreted proteins were elevated (~20%) than decreased (0%) and twice as many mitochondrial proteins were decreased (~41%) than elevated (~20%).

TM Proteins Increased by TGF β_2

Thirty proteins were elevated ≥ 1 SD ($P < 0.06$) above the mean ratio after TGF β_2 treatment, including 11 proteins elevated ≥ 2 SD (Table 2). The majority of these proteins have not previously been associated with TGF β_2 signaling in the TM; however, more than 50% can be grouped by function into processes previously associated with TGF β_2 treatment, such as ECM remodeling and cytoskeletal interactions. For example, among the most elevated proteins were ECM-associated SPARC (secreted protein acidic and rich in cysteine), CTGF (connective tissue growth factor), cytoskeletal-associated myosin regulatory light chain 9, and caldesmon. Approximately 20% of the elevated proteins can be grouped into protein binding, folding, and chaperone processes, and another ~26% can be grouped into regulatory processes involving transcription, translation, steroid binding, and mitochondrial metabolism. Proteins significantly increased in abundance by TGF β_2 treatment account for ~7% of those quantified in three or more samples.

TABLE 2. Elevated Proteins in TGF β_2 -Treated Human TM Cells

SwissProt Accession	Protein	Subcellular Source	Sample Frequency (Total = 4)	Mean Protein Ratio	SEM	P
ECM Remodeling						
P09486	SPARC†	AC	3	6.70*	0.14	0.006
P29279	Connective tissue growth factor	A	4	2.94*	0.18	0.009
P02461	Collagen $\alpha 1$ (III)	A	4	2.89*	0.13	0.004
O00469	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2†	C	4	2.58*	0.09	0.002
P07996	Thrombospondin-1†	A	3	2.41*	0.15	0.030
Q16270	Insulin-like growth factor-binding protein 7	A	3	2.40*	0.17	0.036
O15460	Prolyl 4-hydroxylase subunit $\alpha 2$ †	C	4	2.11*	0.22	0.042
Q96CG8	Collagen triple helix repeat-containing protein 1	A	3	2.04*	0.14	0.036
P13674	Prolyl 4-hydroxylase subunit $\alpha 1$ †	C	4	1.53	0.14	0.057
P55145	Mesencephalic astrocyte-derived neurotrophic factor	A	4	1.45	0.08	0.020
Cytoskeletal Interactions						
P24844	Myosin regulatory light chain 9†	B	4	2.12*	0.11	0.006
Q05682	Caldesmon	B	4	2.06*	0.21	0.042
Q8WX93	Palladin	B	4	1.70	0.06	0.003
Q9HBL0	Tensin-1	B	3	1.67	0.07	0.017
P37802	Transgelin-2	B	4	1.56	0.08	0.012
Q9Y2B0	Protein canopy homolog 2	C	3	1.45	0.03	0.008
Protein Folding and Chaperone Processes						
P02511	α -Crystallin B	B	4	3.37*	0.16	0.004
Q8NBS9	Thioredoxin domain-containing protein 5	C	4	1.66	0.06	0.003
Q9Y4L1	Hypoxia upregulated protein 1	C	4	1.49	0.09	0.020
Q96AY3	FK506-binding protein 10†	C	4	1.48	0.05	0.005
P11021	78 kDa glucose-regulated protein	C	4	1.43	0.06	0.008
Q15084	Protein disulfide-isomerase A6	C	4	1.41	0.10	0.041
Regulatory Processes						
P50479	PDZ and LIM domain protein 4†	B	4	1.68	0.14	0.036
Q14192	Four and a half LIM domain protein 2†	BD	4	1.67	0.09	0.012
Q9P2E9	Ribosome-binding protein 1	C	4	1.65	0.07	0.006
O00264	Membrane-associated progesterone receptor component 1	C	4	1.53	0.14	0.059
P62851	40S ribosomal protein S25	B	4	1.50	0.12	0.045
O94925	Glutaminase, kidney isoform	E	3	1.43	0.07	0.037
P24534	Elongation factor 1- β	B	4	1.42	0.06	0.009
O15173	Membrane-associated progesterone receptor component 2	C	4	1.39	0.10	0.052

Adjusted mean protein ratios (TGF β_2 -treated/control), standard error of the mean (SEM). P values, and subcellular source for abundant proteins (from Supplementary Table S5 [<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental>]). Abundant proteins exhibited mean ratios at least 1 SD above the mean and $P < 0.06$ (Fig. 2). All proteins were quantified in ≥ 3 TM cell samples (median ratio = 1.00; mean = 1.01; SD = 0.31; $n = 450$).

* Ratios at least 2 SD above the mean. Protein subcellular source from the Swiss Protein database: A, secreted; B, cytoplasmic; C, membrane; D, nuclear; E, mitochondrial.

† Calcium or metal ion-binding proteins.

TABLE 3. Decreased Proteins in TGF β_2 -Treated Human TM Cells

SwissProt Accession	Protein	Subcellular Source	Sample Frequency (Total = 4)	Mean Protein Ratio	SEM	P
Signaling/Regulation						
P39023	60S ribosomal protein L3	B	4	0.69	0.11	0.044
Q71DI3	Histone H3.2	D	4	0.68	0.08	0.014
Q99536	Synaptic vesicle membrane protein VAT-1 homolog	B	4	0.66	0.05	0.004
P01891	HLA class I histocompatibility antigen, A-68 α	C	3	0.65	0.08	0.032
P04899	Guanine nucleotide-binding protein G(i), $\alpha 2$	B	4	0.59	0.10	0.012
P21926	CD9 antigen	C	3	0.36*	0.24	0.051
Cytoskeletal Interactions						
P07737	Profilin-1	B	4	0.70	0.12	0.059
Q9ULV4	Coronin-1C	B	3	0.60	0.05	0.011
O00159	Myosin-1c	BC	3	0.58	0.12	0.047
P68371	Tubulin β -2C	B	3	0.57	0.05	0.007
Mitochondrial Metabolism						
P45880	Voltage-dependent anion-selective channel protein 2	CE	4	0.73	0.05	0.011
P25705	ATP synthase subunit α	CE	4	0.72	0.05	0.006
P55084	Trifunctional enzyme subunit β	E	4	0.70	0.06	0.008
P24539	ATP synthase subunit β	CE	4	0.68	0.11	0.037
P21796	Voltage-dependent anion-selective channel protein 1	CE	4	0.66	0.13	0.044
P00367	Glutamate dehydrogenase 1	E	4	0.60	0.10	0.012
P04179	Superoxide dismutase 2 [Mn]	E	4	0.054*	0.06	0.002

Adjusted mean protein ratios (TGF β_2 -treated/control), SEM, *P* values, and subcellular source for less abundant proteins (Supplementary Table S5 [http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental]). Less abundant proteins exhibited mean ratios at least 1 SD below the mean and *P* < 0.06 (Fig. 2). All proteins were quantified in ≥ 3 TM cell samples (median ratio = 1.00; mean = 1.01; SD = 0.31; *n* = 450).

* Ratios at least 2 SD below the mean. Protein subcellular source from the Swiss Protein database and Ingenuity Pathway Analysis 8.5: A, secreted; B, cytoplasmic; C, membrane; D, nuclear; E, mitochondrial.

TM Proteins Decreased by TGF β_2

In response to TGF β_2 treatment, 17 proteins were decreased in abundance ≥ 1 SD (*P* < 0.06) below the mean ratio (Table 3); none of these proteins have previously been associated with TGF β_2 signaling in TM cells. CD9 antigen and mitochondrial superoxide dismutase 2 (SOD2) were the most significantly reduced proteins (decreased $\sim 64\%$ and $\sim 46\%$, respectively). Four of the reduced proteins are associated with cytoskeletal interactions and six with a variety of regulatory processes, including transcription, translation, and the immune response. Notably, more than 40% of the decreased proteins were from the mitochondria and are involved in critical metabolic functions. Proteins significantly reduced in abundance accounted for $\sim 4\%$ of those quantified in three or more samples.

Independent Evidence Supporting the ITRAQ Protein Quantitation

Western blot analysis was used to independently evaluate proteomic changes in the TM cell samples with and without

TGF β_2 treatment. Densitometric results (Fig. 2) from immunoblots corroborated increased amounts of caldesmon and decreased amounts of SOD2 after TGF β_2 treatment and were in reasonable agreement with the iTRAQ data in both ratios and *P* values. Reports in the literature support TGF β_2 -induced changes observed for at least six other proteins. Specifically, Western blot analyses published by others show that TGF β_2 treatment of human TM cells results in increased amounts of SPARC,³⁴ CTGF,³⁵ thrombospondin-1,³⁵ and α -crystallin B³⁶ and that CTGF treatment of human TM cells results in elevated levels of collagen $\alpha 1$ type III.³⁷ In addition, a previous proteomic study reported TGF β_2 induced increased amounts of transgelin-2, α -crystallin B, and possibly 78 kDa glucose-regulated protein in TM cells based on 2D gel spot staining intensity.²⁷

DISCUSSION

To better understand TGF β_2 signaling in the anterior segment, we quantified proteomic changes in four primary cultures of

FIGURE 2. Western blot analysis. Western blot analysis was used to evaluate the relative amounts of proteins in TGF β_2 -treated and untreated TM cells (*n* = 4 samples each). Average densitometry ratios (TGF β_2 -treated/untreated) and *P* values (two-sided *t*-test) for caldesmon and SOD2 support the quantitative data shown in Tables 2 and 3. Previous protein analyses corroborate at least six additional TGF β_2 -induced proteomic changes detected in this study (see Results).

human TM cells after TGFβ₂ treatment using LC MS/MS iTRAQ technology. The study was not designed to reveal differences between glaucomatous and nonglaucomatous cell cultures but rather to probe the impact of TGFβ₂ signaling on TM cells. Although glaucomatous and normal TM cells may exhibit differences in TGFβ₂ signaling yet to be identified, all TGFβ₂-altered proteins in this study were derived from both TM donor populations and definitively demonstrate common consequences of TGFβ₂ signaling in normal and glaucomatous TM cells. Most of the 853 quantified proteins (~90%) were present in similar amounts in TGFβ₂-treated and untreated cells, indicating that the determined proteome largely reflects that of untreated TM cells. TGFβ₂ treatment significantly altered the abundance of 47 proteins; independent data from Western blot analysis and 2D-PAGE corroborated eight or more (~17%) of these TGFβ₂-induced proteomic changes. Bioinformatic analysis suggested that major biological processes associated with the quantified proteins (Fig. 3) include cellular metabolism (~28% of the 853 proteins), signal transduction (~8%), cell structure/motility (~8%), immunity and defense (~5%), and cell adhesion (2%). Biological analysis and interpretation (Ingenuity Pathway Analysis [IPA]; Ingenuity) of these proteins implicated gene expression and cellular assembly and organization as the highest scoring networks.

Molecular Clues to POAG from TM Proteins Elevated by TGFβ₂ Treatment

Thirty TM proteins were significantly increased in abundance after TGFβ₂ treatment (Table 2), supporting the upregulated expression of these polypeptides. Analysis (IPA; Ingenuity) of the 30 elevated proteins suggested that TGFβ₂ impacted two major networks, one involving amino acid metabolism, post-translational modification, and small molecule biochemistry

and the other involving gene expression, cellular compromise, and development. Seven of the elevated proteins (or their transcripts) were previously reported to be upregulated by TGFβ₂; the other 23 elevated proteins provide an expanded profile of TGFβ₂ signaling in TM cells. Though yet unconfirmed, calcification and mineralization of the TM have been hypothesized to contribute to elevated IOP in glaucoma,³⁸ and 30% of the elevated proteins bind either calcium or zinc, as noted in Table 2. Consistent with previous reports,^{6,7,20,21} 10 elevated proteins are involved in the structure and regulation of the ECM, and eight of these were increased more than twofold. SPARC, elevated more than sixfold, is a matricellular protein that binds multiple proteins and cell membranes and that has been hypothesized to contribute to elevated IOP in POAG in part because SPARC-null mice exhibit lower IOPs than wild-type controls.³⁹ The SPARC gene, highly expressed in nonglaucomatous TM,⁴⁰ has also been reported to be upregulated by mechanical stretching.⁴¹ CTGF, a mediator of the effects of TGFβs on ECM remodeling, and thrombospondin-1, a major activator of latent TGFβs and elevated in the TM of some POAG patients,⁴² were both found significantly elevated. Also elevated in this study were insulin-like growth factor-binding protein 7, which stimulates cell adhesion and mesencephalic astrocyte-derived neurotrophic factor (also known as Protein ARMET), which binds unfolded proteins and inhibits stress-induced cell death. In addition, levels of collagen α1 type III and several other proteins that modify collagen or collagen deposition in the ECM—namely procollagen-lysine 2-oxoglutarate 5-dioxygenase 2, prolyl 4-hydroxylase α2 and α1, and collagen triple helix repeat-containing protein 1—were elevated.

Previous studies suggest that the TM cytoskeleton is involved in regulating AH outflow and IOP, perhaps in part

FIGURE 3. Biological functions of TM proteins. Functional classification of the 853 proteins quantified in human TM cells was performed with the PANTHER Classification System.

through the formation of cross-linked actin networks (CLANs).^{9,10,43} CLANs are morphologically diverse structures in TM cells, appearing as tangles to geodesic dome-like, and observed in both normal and glaucomatous TM *in vivo*.⁴³ In this study, TGF β_2 induced apparent increased expression of the actin-binding proteins caldesmon, palladin, tensin-1, and transgelin-2, and cytoskeletal regulators like myosin regulatory light chain 9 and its binding partner protein canopy homolog 2. Altered expression of these proteins could promote CLAN formation and impact TM cell shape, motility, proliferation, contractile and tone properties, and cell-cell and cell-ECM interactions.

Six proteins increased in abundance by TGF β_2 were associated with protein folding and chaperone activities, with α -crystallin B the most significantly elevated (>3-fold). Others in this group included thioredoxin domain-containing protein 5, hypoxia upregulated protein 1, FK506-binding protein 10 (also known as peptidyl-prolyl *cis-trans* isomerase FKBP10), 78 kDa glucose-regulated protein, and protein disulfide-isomerase A6. Overall, about twice as many proteins were elevated than decreased by TGF β_2 treatment, and the increased abundance of chaperones and folding proteins appear to be a stress response to metabolic changes, such as in protein synthesis and oxidation reduction.

A variety of regulatory proteins were elevated ~40% to 70% after TGF β_2 treatment. The possibility that TGF β_2 alters translational elongation is suggested by elevated levels of ribosome-binding protein 1, 40S ribosomal protein S25, and elongation factor 1 β . TGF β_2 -altered transcriptional regulation is also suggested by elevated levels of PDZ and LIM domain protein 4 and four and a half LIM domain protein 2, which bind zinc and a zinc finger binding protein. Also elevated were two steroid-binding proteins, membrane-associated progesterone receptor components 1 and 2, suggesting the possibility that TGF β_2 may play a role in steroid responsiveness in glucocorticoid-stimulated secondary glaucoma.⁴⁴ Mitochondrial glutaminase, an enzyme catalyzing the conversion of glutamine to glutamate, was elevated more than 40% after TGF β_2 treatment; notably, increased glutaminase activity in the rat retina has been associated with ocular hypertension.⁴⁵ Seven other mitochondrial proteins were altered by TGF β_2 treatment, as discussed.

Molecular Clues to POAG from TM Proteins Decreased by TGF β_2 Treatment

The levels of 17 TM proteins were significantly reduced after TGF β_2 treatment (Table 3), implying downregulated expression, possible degradation, or both. Little is known about TGF β_2 -downregulated processes in the TM. The highest scoring network from pathway analysis (IPA; Ingenuity) of the 17 decreased proteins concerns developmental disorders, renal and urological diseases, and gene expression. TGF β_2 lowered the abundance of four cytoskeletal components by ~30% to 40%, including the actin-binding proteins profilin-1, coronin-1C, and myosin-1c and microtubule component tubulin β -2C. As noted, altered ratios of cytoskeletal proteins could promote the formation of CLANs and adversely impact TM cell properties and processes.⁴³ In addition to cytoskeletal proteins, a variety of regulatory proteins were decreased by ~30% to 60%. CD9 antigen, a membrane protein involved in cell adhesion and motility, was the most significantly reduced (>60%). Decreased amounts of 60S ribosomal protein L3 and histone H3.2 implicate TGF β_2 -altered translational elongation and transcription, respectively; all these processes are also potentially altered by other proteins elevated by TGF β_2 treatment, as discussed. Altered GTPase and ATPase activities are implied by TGF β_2 -decreased levels of guanine nucleotide-binding protein Gi α 2 and synaptic vesicle membrane protein VAT-1 homolog,

respectively. Reduction of HLA class 1 histocompatibility antigen A68 α and CD9 antigen suggests TGF β_2 -altered immune responses in TM cells, as has been proposed in POAG for retinal glial cells and the optic nerve head.⁴⁶

Mitochondrial Dysfunction in the TM

In addition to elevated mitochondrial glutaminase, seven other mitochondrial proteins were significantly reduced by TGF β_2 treatment (Table 3). These decreased proteins serve important roles in energy production (e.g., ATP synthases), transmembrane transport (e.g., voltage-dependent anion channel proteins), and oxidation reduction (e.g., glutamate dehydrogenase 1 [GLUD1] and SOD2), and their decreased levels could severely impair cellular functions. Mitochondrial dysfunction in retinal ganglion cells has long been associated with glaucoma.^{47,48} However, it is only recently that evidence suggests that elevated reactive oxygen species and impaired calcium regulation in TM cell mitochondria and TM mitochondrial DNA deletions may play a role in POAG.^{49–51} The identification of eight mitochondrial proteins altered in abundance by TGF β_2 adds strong support to the hypothesis that mitochondrial dysfunction in the TM contributes to POAG. For example, TGF β_2 has been reported to induce senescence in the TM,²³ and elevated IOP has been reported to contribute to glutamate toxicity in the rat retina (by increasing glutaminase activity and reducing glutamate uptake).⁴⁵ We found mitochondrial glutaminase elevated by ~43% and mitochondrial GLUD1 decreased by ~40% in TM cells after TGF β_2 treatment. GLUD1 reduces the amount of glutamate by catalyzing its oxidation to 2-oxoglutarate. TGF β_2 may induce TM senescence and cell death through mitochondrial dysfunction that in part promotes glutamate imbalance. Elevated IOP has also been reported to decrease total SOD activity in the rat retina,⁵² and we observed ~46% reduction in mitochondrial SOD2 in TM cells after TGF β_2 treatment. Previous work has shown that ~50% knock-down of mitochondrial SOD2 in mouse retinal pigment epithelium correlates with increased levels of oxidative protein modifications⁵³ and that TGF β_2 increases lipid peroxidation in human TM cells.²³ We and others (Bhattacharya SK, et al. *IOVS*. 2004;45:ARVO E-Abstract 4560)⁵⁴ have also observed increased levels of oxidative protein modifications in trabeculectomy tissues from POAG patients. Accordingly, the present data support the hypothesis that elevated IOP in POAG is due in part to TGF β_2 -induced mitochondrial dysfunction in the TM, with concomitant oxidative damage in the AH outflow pathway.

Comparison with Other Studies of TGF β_2 -Induced Changes in TM Protein and Gene Expression

This is the most extensive quantitative proteomic analysis to date of changes in human TM cells in response to TGF β_2 . The only other global proteomic study of TGF β_2 -treated TM cells used peptide mass fingerprinting for protein identification and 2D gel staining intensity for protein quantification.²⁷ Of the 56 proteins reported in the previous study, we detected 42 and obtained reliable quantitation (i.e., proteins found in three or more TM samples) for 12 of the 24 proteins reported to exhibit a significant change in 2D gel spot intensity.²⁷ For these 12 proteins, two to three exhibited changes in agreement with the present results, namely increased amounts of transgelin-2, α -crystallin B, and possibly 78 kDa glucose-regulated protein (which was reported as 1 of 2 proteins in a gel spot).²⁷ Comparison of these proteomic studies is complicated by the different methods used, including the analysis of pooled rather than individual donor TM samples and a lower concentration of TGF β_2 for treating TM cells in the earlier study. Comparison of the present results with gene expression studies of TGF β_2 -

treated TM cells^{21,27,28} reveals limited correlation, however; only ~20% correlation generally exists between transcript and cognate protein levels in mammals.⁵⁵ From microarray studies, more than 20 transcripts have been reported to be differentially expressed by TGF β_2 in human TM cells.^{21,27,28} Significant proteomic changes in this study are consistent with reported TGF β_2 upregulation of transcripts for thrombospondin 1,^{21,27} SPARC,²³ and prolyl 4 hydroxylase subunit alpha 2.²⁸ Proteases and protease inhibitors function in ECM remodeling in normal TM,⁵⁶ and TGF β_2 has been reported to upregulate the expression of the inactive proform of matrix metalloproteinase-2⁵⁷ and plasminogen activator inhibitor-1 in TM cells.^{21,35,57} Surprisingly, few proteases or protease inhibitors were among the 853 proteins quantified in this study, perhaps because they were below detection limits. Nevertheless, we found almost twice as many proteins increased rather than decreased in abundance, suggesting that TGF β_2 signaling may inhibit proteolytic processes in the TM.⁵⁷ Several proteins previously reported to modify or to be associated with TGF β_2 signaling in the TM or to be overexpressed in POAG TM, including bone morphogenetic protein family members gremlin, Smad 7, and tissue transglutaminase and the lysyl oxidase family of enzymes, were not detected in this study.^{28,35,58–60} Again, these proteins may be below the detection limits of this study. In addition, no known glaucoma susceptibility gene products were detected. We did detect two proteins reportedly upregulated in TM by TGF β_2 , namely senescence-associated- β -galactosidase²³ and fibronectin,^{21,35} but the abundance of these proteins was not significantly different between TGF β_2 -treated and untreated cells. Other TGF β_2 -regulated proteins certainly exist in the TM, and some may be among those (Supplementary Table S5, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8218/-DCSupplemental>) that narrowly miss the criteria used for a significant change in this study.

The mechanism or mechanisms responsible for elevated expression of TGF β_2 in glaucoma patients are unknown. TGF β_2 in the aqueous humor could be made and secreted from one or more tissues surrounding the anterior segment, including the ciliary epithelium, iris, lens epithelium, corneal endothelium, and TM. Elevated levels of TGF β_2 in glaucomatous TM tissues could be derived in part from the aqueous humor and bound to the TM extracellular matrix.¹⁹ However, glaucomatous TM cells express a latent isoform of TGF β_2 of higher molecular weight that must be activated to become biologically active.¹⁹ How latent TGF β_2 is activated in TM cells remains unclear but may involve thrombospondin-1,⁴² which was found elevated in this study.

In summary, the present results add strong support to evidence that TGF β_2 can induce ECM remodeling and alter cytoskeletal interactions in the TM. In addition, the results implicate TGF β_2 -altered regulation in processes such as transcription, translation, steroid metabolism, and the glutamate/glutamine cycle. Finally, the data provide new evidence that TGF β_2 -induces mitochondrial dysfunction in the TM that could contribute to oxidative damage in the AH outflow pathway, TM senescence, and ocular hypertension in POAG. Overall, the results expand the repertoire of proteins known to participate in TGF β_2 signaling and establish a quantitative proteomic database for the TM that includes candidate glaucoma biomarkers for future validation studies.

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