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Differential Expression of Genes in Cells Cultured from Juxtacanalicular Trabecular Meshwork and Schlemm's Canal

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Abstract

Purpose: The purpose of this study was to distinguish differences in gene expression between cells cultured from the juxtacanalicular trabecular meshwork (JCTM) and those from Schlemm's canal (SC), to gain clues to differences between those cell types, and to add to our baseline knowledge of gene expression differences in these cell types for later comparison between cells from nonprimary open-angle glaucoma (POAG) and POAG outflow tissues.
Methods: A set of JCTM and SC cells was cultured from each of 2 donor eyes by an explant method, grown to passage 3, and frozen in liquid nitrogen. The cells were thawed, total RNA was extracted, and the probes made from total RNAs were hybridized to MICROMAX human cDNA microarray slides in 2 separate trials. Differentially expressed genes were analyzed using PubMed, Prosite, and IPA software, and the expression of several of the genes including intercellular adhesion molecule-1 (ICAM-1), tenascin, and β-spectrin was assessed by immunofluorescence.
Results: Schlemm's canal cells differentially expressed ICAM-1, spectrin, complement, fibulin-1, and several genes consistent with an endothelial origin in both arrays, while the JCTM cells more often overexpressed genes consistent with contractile, matrix function, and neural character. At the same time, many genes highly expressed in the first array were not highly overexpressed in the second. One highly overexpressed gene in the JCTM in both arrays, that for heparan sulfate 3-*O*-sulfotransferase-1 precursor, is thought to be somewhat unique, and could affect the glycosaminoglycan functionality in the extracellular matrix (ECM).
Conclusions: We found generally good agreement between the 2 array trials, but some contradictions as well. Many of the genes overexpressed in each cell type had been described in earlier work, but several were new. Tables of genes, grouped by cellular function, and the complete datasets are provided for the development of new hypotheses.

Introduction

The aqueous outflow pathway is considered to be the likely site of the increased hydrodynamic resistance to outflow associated with increased intraocular pressure in primary open-angle glaucoma (POAG).[1–6] The normal and abnormal functioning of the cells that line Schlemm's canal (SC) and those that generate, maintain, and line the trabecular meshwork (TM) are therefore of continuing interest. We prepared cultures of SC and juxtacanalicular, corneoscleral, and uveal TM cells by fine dissection and explant culture, and were able to obtain cells that were typical for each tissue. Our long-term goal was to compare cells from each subdivision of the outflow pathway with comparable cells from POAG donor eyes. Our early experience had found that cells obtained from POAG tissue grew more slowly, and individual cells had aberrant vesicle movements and actin-based motility. However, this pattern was not apparent when POAG anterior segments were supported with pH-stabilized growth medium within 12 h of death. In that case, cells from each section of POAG eyes grew as well as and sometimes better than comparable non-POAG tissue. The present study was undertaken in 1999–2000 as a baseline comparison to test the reproducibility of the

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differential dissection method in non-POAG tissue, and to determine whether recognizable characteristics of the specific cell types would persist for several generations in culture. We compared the relative gene expression of SC and the juxtacanalicular TM (JCTM) cells derived from nonglaucomatous donors by the explant method. We tested 2 sets of cells from 2 donors, and 2 differential cDNA screens. The screens differed by only 4 genes in total out of 2,400. We particularly looked at those genes that were most differentially expressed in both assays. The patterns obtained generally support the utility of the subdivided tissue process, while the patterns themselves highlight interesting differences between the SC and JCTM cells that may be exploited in future studies.

Methods

Cells were isolated and cultured by our previously published explant method,[7] with several additional steps to isolate the finer JCTM from the corneoscleral and uveal layers. The principal difference was not using blunt dissection to remove all of the TM at once, but rather using fine forceps to separately tease apart short, ~1-cm-long strips of uveal, corneoscleral, and then JCTM. Each was placed into a shallow sterile plastic culture dish, a sterile cover glass was placed firmly over the tissue, and medium was added. The JCTM was usually removed without a portion of the inner wall, but when a small portion of the canal was removed with the JCTM, the orientation of the tissue was recorded, and only the JCTM side was used to grow JCTM cells. To remove SC, deeper radial cuts were made through the canal about 1 cm apart. A deeper circumferential cut was then made between the canal and the iris root, into the limbal stroma. Finally, a fresh scalpel was used to make a ''filet'' cut ''under'' the canal, parallel with the outer wall, to excise the walls of the canal. The canal was subsequently spread open and placed face down on the tissue culture plastic. Our identification of SC cells was at first primarily dependent on the subdivision and orientation of the tissue, and our knowledge of what the TM cells look like. Over many dissections, the SC cells were found to consistently exhibit a canoe-like fusiform shape, most evident in reference 7. They also formed regular side-to-side junctions with little overlap. The JCTM cells were essentially TM-like in form, somewhat ''triangular'' in shape, did not make as consistent and regular intercellular junctions, and appeared smaller on average than uveal TM cells. Cells were frozen back before reaching full confluence after passage 3 and stored on liquid N_2 until shipment on dry ice to the Yue laboratory for extraction. Immunofluorescence microscopy was performed using the cells from the older donor that had been frozen at passage 3. Cells were thawed, passaged one more time and plated onto gelatin-coated glass coverslips. When confluent for at least 1 day, the cells were fixed with 4% formaldehyde and stained using monoclonal antibodies to ICAM-1 (R & D Systems, Minneapolis, MN), and polyclonal antibodies to tenascin (R & D Systems) and spectrin (Sigma Chemical, St. Louis, MO) and the appropriate secondary antibodies.

Total cellular RNA was extracted from 2 sets of JCTM and SC cells (2–3 vials, each containing approximately 500,000 cells per vial), 1 set from each of 2 donors. For the first test, we compared expression of JCTM and SC cells from an 18-year-old normal human donor using an early version of the MICROMAX Human cDNA Microarray System I, AB581 (NEN, Boston, MA). For the second test, cells were from a normal 68-year-old donor and a second version of the array was used (AG694). Surprisingly, cells from older donors grew successfully at rates similar to those from younger donors, as well as eyes from POAG donors, if the eyes were opened and placed in medium within 12 h of death. Thus, it was of interest to observe whether the similarity in observed growth would be reflected in similarities in the gene expression levels of donors of such disparate ages. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). The integrity of RNA was assessed by visualization of intact 28S and 18S ribosomal RNA on 1% agarose gels. The $OD_{260/280}$ ratio was 1.7–1.9. The MICROMAX System I comprised 2 identical 1'' × 3'' glass microarrays, each pre-spotted with 2,400 or 2,396 known genes. Except for a small number of plant control genes, all genes were from over 50 human cDNA libraries. In this microarray, over 10 tissue sources were represented, over 40% of which were full-length genes. The 2,400 genes in the first array and 2,396 genes in the second were characterized functionally based on Prosite ''motif'' research, and were readily identifiable through a gene database on NEN's website, and linked to the NIH GenBank. MICROMAX can

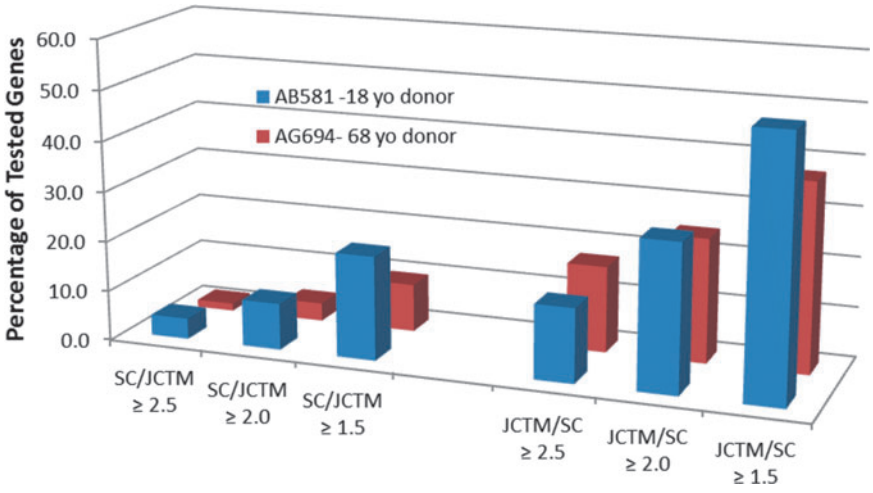

FIG. 1. Bar graphs of the overall fraction of genes that were relatively overexpressed at three cutoff levels: ≥2.5, 2.0, or 1.5-fold in Schlemm's canal (SC) over juxtacanalicular trabecular meshwork (JCTM) or JCTM over SC cell type. yo, year-old.

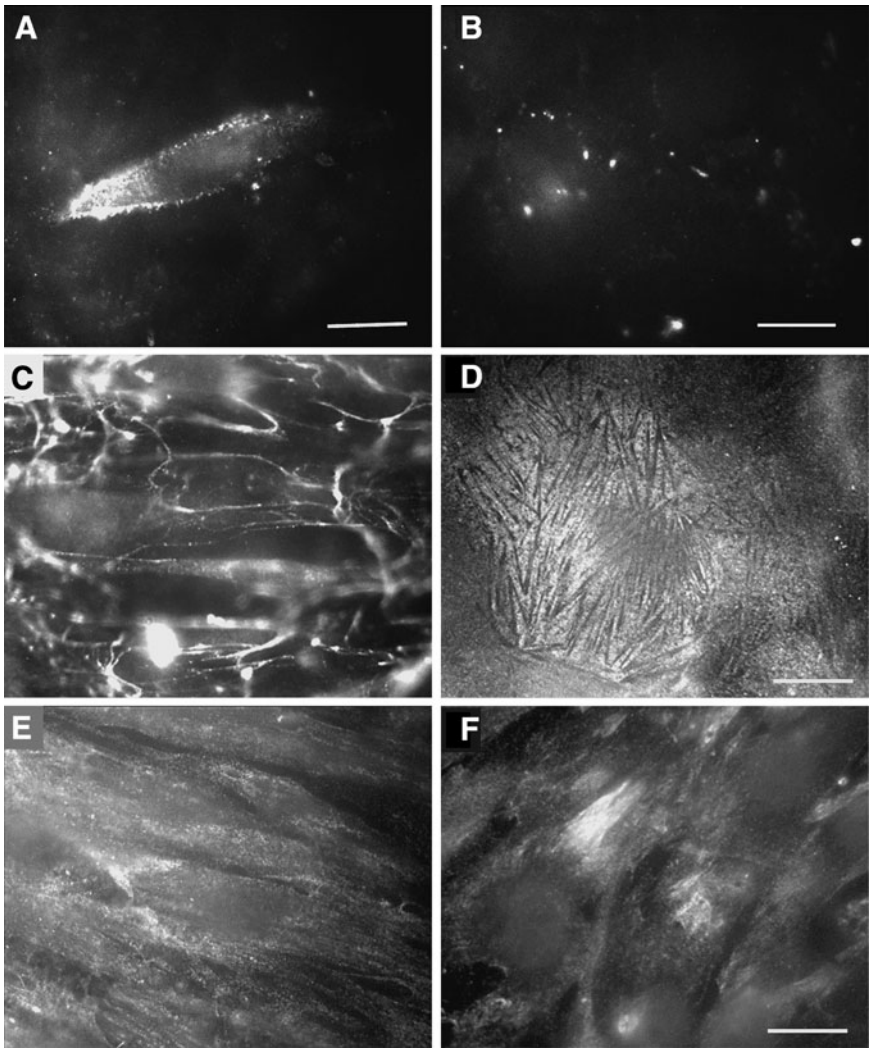

FIG. 2. Staining for human intracellular adhesion molecule 1 (ICAM-1), tenascin, and β-spectrin in confluent SC and JCTM cultures from the same donors. Antibodies to ICAM-1 **(A,B)**, tenascin **(C,D)**, and β-spectrin **(E,F)** were used to stain cultures of SC (A,C,E) and JCTM (B,D,F) cells. As evident in panel A, staining for ICAM-1 in SC cells was sporadic, being seen brightly in only a small proportion of cells, but was not observed in any structures stained in JCTM cells (B). Staining of tenascin protein was observed in outlines of SC cells throughout the culture (C). Tenascin was also present in JCTM cells, but appeared primarily between the ventral surface of each cell and the glass surface. Areas of exclusion of the stain appeared to outline the pattern of actin stress fibers within the cell (D). Staining of β-spectrin was seen in both cell populations to be primarily in the lamellapodial regions, but was more widely and uniformly observed in SC cell cultures (E) than JCTM (F). Three pairs of cultures showed similar results. Bar = 10 μm.

detect as few as 1.2 copies per cell of a gene starting with RNA from 2.0×10^5 cells.

Total RNA (4 μg) from JCTM cells was used to make dinitrophenyl (DNP)-labeled cDNA employing reverse transcriptase and nucleotide analogs. RNA (4 μg) from SC cells was used to synthesize biotin-labeled cDNA. The quality and efficiency of labeled cDNAs was checked using membrane-based chemiluminescence methods. Equal volumes of DNP and biotin cDNA samples were mixed and applied onto 1 microarray slide. Hybridization was done overnight at 65°C. After hybridization, the slide was washed with 0.5x SSC (sodium chloride–sodium citrate buffer), 0.01% sodium dodecyl sulfate (SDS) (wt/vol) for 5 min, 0.06x SSC, 0.01% SDS for 5 min, and 0.06x SSC for 2 min. The hybridized DNP and biotin-labeled cDNAs were sequentially detected with a series of conjugate reporter molecules using the NEN's Tyramide Signal Amplification system. Each of the 2 fluorescent reporter molecules (cyanine 3 or Cy3 and cyanine 5 or Cy5) was associated with the hybridized material from each of the starting samples, Cy3 with JCTM and Cy5 with SC. Microarray slides were sent back to NEN for scanning and data processing. A ScanArray 3000 laser detection system (GSI Lumonics Inc., Watertown, MA) was used to scan and report the relative quantity of the 2 dyes at any given gene spot. The ratio of each spot was calculated by dividing the volume of Cy5 channel with the volume of Cy3 channel. Normalization factor was calculated based in the ratios obtained by 50 control gene spots or by densities of all gene spots. The factor was nearly identical. A Cy3/Cy5 ratio larger than 1.0 indicates that the JCTM samples were greater in expression than the SC samples and a Cy5/Cy3 ratio >1.0 was indicative of SC cells having a greater expression. We looked at the number of genes relatively overexpressed in 1 cell type at ratios of 1.5 and greater, 2 or greater, and 2.5 or greater over the other cell type (''cutoff ratios'') using Excel (Microsoft, Inc.), and compared these values in the 2 donor runs. We then compared the actual genes that were overexpressed in each at the higher cutoffs. We grouped genes overexpressed in each cell type by putative function using the gene name and PubMed and the Prosite software, to group genes by general relationships. Finally, we used a commercial software package, Interactive Pathway Analysis (IPA), (Ingenuity Systems, Inc., Redwood City, CA) to obtain a third perspective on the relationships of relatively overexpressed genes in both arrays (at the 2 or greater cutoff ratio) in terms of signaling pathways, metabolic pathways, molecular networks, and biological processes.

Results

We were first interested to know whether the 2 independent array experiments, using cells from a very young and an older donor, would yield consistent results. As an overview, we looked at the overall proportion of genes that were expressed more highly in 1 cell type relative to the other. Three ''cutoff'' ratios were used. A 2.5-fold ratio or greater was the most stringent, and 2- and 1.5-fold ratios were also compared. Figure 1 shows the comparisons at each ratio. Although we used SC and JCTM cells from donors of widely different ages, the results at this level were remarkably consistent. The percent of genes overexpressed in SC relative to JCTM cells at each fold difference was about 3-fold smaller in both sets than the relative overexpression seen in JCTM. Moreover, the proportions were similar in both sets. These general trends lend support to the reproducibility of the assay, as well as the differential dissection protocol. On the other hand, it is interesting that what could be interpreted as the ''tails'' of what one might expect to be a normal distribution of ratios is so asymmetric. This most probably reflects a nonrandomness in the pattern of genes chosen in the Micromax array. Also interesting was that, while the proportion of genes overexpressed in JCTM relative to SC was greater in both arrays, the genes that were markedly overexpressed (>2-fold) in both arrays were much larger for the SC cells (Tables 1 and 2). This may reflect more heterogeneity in the JCTMs, or perhaps a difference in donor ages makes more of a difference in the JCTMs (see Discussion).

Table 1 shows the gene transcripts overexpressed above 2-fold in JCTM cells in both arrays. They are presented in the approximate order of overexpression in both arrays. Alkali myosin light-chain 1 is associated with skeletal muscle and other muscle cell types.[8] Heparan sulfate 3-*O*-sulfotransferase-1 precursor is an interesting protein related to glycosaminoglycan function and thus might affect hydrodynamics near the surface of the TM[9] (see Discussion). Several of the transcripts are associated with neuronal origins. Microtubule-associated τ protein is enriched in brain neurons, and is a core component of neurofibrillary tangles related to Alzheimer disease.[10,11] MEGAP/srGAP3b (KIAA0411) is important in brain development,[12] and NeuroD has been associated with inhibition of cell motility in glioma.[13,14] Thus, there is some indication of neuronal character in the genes overexpressed in the JCTM. This is discussed further below.

Even though there were fewer genes overexpressed in SC cells relative to JCTM, many more genes were found strongly overexpressed in both arrays (Table 2). SC cells expressed genes such as ICAM-1, hexabrachion (tenascin), β-spectrin, high-density lipoprotein binding protein (HDLBP), and complement proteins that are relevant to functions of the vascular endothelium. ICAM-1 is expressed on the surface of endothelial cells and macrophages and plays an important role during inflammation by facilitating the tight adhesion of leukocytes to the vascular wall.[15] Hexabrachion is an anti-adhesive ECM protein with a fascinating 6-armed structure.[16,17] It is strongly associated with the basement membrane of the vascular endothelium, and may play a role in epithelial-to-mesenchymal transition.[18,19] Tenascin is not in Table 2 as a separate entry, but it is the same molecule as hexabrachion, which was highly overexpressed in both arrays.[17] Tenascin (presumably hybridizing with a different section of the gene sequence) was separately found very highly overexpressed in the AG694 array but not AB581. This may reflect the age of the donors, or an inherent variability in the expression. HDLBP assists uptake of high-density lipids and lipoprotein lipase in the vascular system.[20] Complement proteins provide the link for immune cells to bind to the endothelium and can activate endothelial cells to induce production of cytokines and pro-inflammatory responses, and may also connect to the thrombotic cascade.[21] Spectrin is a prominent component of the plasma membrane cytoskeleton in endothelial cells, where it has been suggested to have a role in regulation of store-operated calcium entry.[22] Of note is that, among the cadherin family junctional genes being expressed, VE-cadherin was not tested in these arrays, nor was PECAM, both reported to be expressed in SC cells in culture.[23,24] Fibulin 2, also reported in SC cells,[24,25] was also not tested in this array, but fibulin 1 was much higher in SC cells. The fibulins are interesting as an elastic component of the ECM and basement membrane.[26,27] As such, differences in expression of this glycoprotein could affect the elastic ''tone'' of the cribriform ECM. Thus, our results are consistent with

Table 1. Genes Highly Expressed (>2.0 Fold) in Juxtacanalicular Trabecular Meshwork Cells Relative to Schlemm's Canal Cells in Both Array Experiments

Accession #	*Gene product*
M20642	Alkali myosin light-chain 1—skeletal muscle related
AF019386	Heparan sulfate 3-*O*-sulfotransferase-1 precursor
Y15409	Putative glucose 6-phosphate translocase
X14474	Microtubule-associated τ protein—brain—found in neurofibrillary tangles
D50419	OTK18—possible antiretroviral transcriptional factor
U40490	Nicotinamide nucleotide transhydrogenase, nuclear gene encoding mitochondrial protein
D11094	MSS1—mammalian suppressor of sgv1
M12783	C-sis/platelet-derived growth factor 2
M83670	Carbonic anhydrase IV
Y07512	Type I beta cGMP-dependent protein kinase (EC 2.7.1.37)
AB007871	KIAA0411- MEGAP/srGAP3b—brain development—role in mental retardation
M29064	hnRNP B1 protein
U07361	Sorbitol dehydrogenase gene—loss related to cataract
U59309	Fumarase precursor (FH) mRNA, nuclear gene encoding mitochondrial protein
D82347	NeuroD—cerebellum—inhibits motility in glioma

Table 2. Genes Overexpressed at >2.5 Fold in Schlemm's Canal Cells in Both Arrays

Accession #	*Gene product*
M80927	Glycoprotein—member of chitinase protein family
U03877	Extracellular protein (S1-5) fibrillin-like protein
L13698	*gas1* gene—growth suppressor
X73478	hPTPA—activates the tyrosyl phosphatase activity of protein phosphatase 2A
U05572	Lysosomal α-mannosidase
X53743	Fibulin-1 C
M55618	Hexabrachion
X06990	Intercellular adhesion molecule-1
X04701	mRNA for complement component C1r
X03445	mRNA for nuclear envelope protein lamin C precursor
M64098	High-density lipoprotein binding protein
X53416	Actin-binding protein (filamin)
M22960	Protective protein
M63959	α-2-Macroglobulin receptor–associated protein
AF000381	Nonfunctional folate-binding protein
L37033	FK-506 binding protein homologue (FKBP38)
AF014807	Phosphatidylinositol synthase (PIS)
U22526	2,3-oxidosqualene-lanosterol cyclase
M24097	MHC class I HLA-C-α-2 chain and alternative, clones 4 and 10
D67025	Proteasome subunit p58
X72018	hTGR 1 mRNA (NAGR1)
M73778	PML-1 protein
L14754	DNA-binding protein (SMBP2)
D26181	Novel protein kinase
M33294	Tumor necrosis factor receptor
Z74615	Prepro-α1(I) collagen
M64571	Microtubule-associated protein 4
U58856	Chromosome 17 unknown product
Z29481	3-Hydroxyanthranilic acid dioxygenase
D21089	XP-C repair complementing protein (p125)
U29089	Proline arginine-rich end leucine-rich repeat protein
X64364	M6 antigen
J02939	Membrane glycoprotein 4F2 antigen heavy-chain mRNA
M27110	Proteolipid protein mRNA
L42024	MHC HLA-B39
X64330	ATP-citrate lyase
M23725	M2-type pyruvate kinase
L19871	Activating transcription factor 3
M62994	Thyroid autoantigen (truncated actin-binding protein)
X58141	Erythrocyte adducin α subunit
M59807	NK4
X87949	BiP protein
M88338	Serum constituent protein (MSE55)
U4106	Breast cancer, estrogen-regulated LIV-1 protein
D67029	SEC14L
V00572	Phosphoglycerate kinase
X62137	Anion exchange protein 2
X98893	TBP-associated factor, TAFII68
M14221	Cathepsin B proteinase
L77964	ERK3 protein kinase

hPTPA, human phosphotyrosyl phosphatase activator; MHC, major histocompatibility complex; hTGR, human thyroid glucosamine receptor; PML, promyelocytic leukemia; TBP, TATA binding protein.

an endothelial identity of the SC cells.[5,23,24,28] One interesting question is what role chitinase family proteins play in the outflow pathway. Liton et al. showed that the chitinase 3-like 1 gene was strongly overexpressed in TM cells relative to SC.[25] We find that the gene M80927 was highly overexpressed in SC cells, and it is also a member of the chitinase family of glycoproteins.

We subsequently categorized the genes that were expressed differentially in either JCTM (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/jop) or presumptive SC (Supplemental Table S2) cells by their known or postulated general cellular functions using Prosite software (http://www.uniprot.org/) and reading about each gene using Pubmed. Within each category, the genes were listed in descending orders of differential expression using only the results from the AG694 array from the older donor, as more representative of an aging outflow pathway. Comparing the results from Supplementary Tables S1 and S2, one can discern interesting contrasts and similarities between the 2 cell types. For instance, SC cells differentially overexpressed both α and β actin–myosin components, and cell junction and tight junction proteins including cadherin-4, and ZO-1, consistent with a barrier function to paracellular flow and a role for ZO-1.[29,30] Differential expression of these junctional proteins should be taken as a minimum, in that the cells were harvested within a day of reaching confluence. On the other hand, the relative abundance of N-cadherin in SC cells seems unexpected. As discussed above, SC cells appeared to be enriched with ECM genes, especially tenascin and collagen types IV and VI. Although different in specifics, both cell types also express proteases and inhibitors that are responsible for modulation of the ECM and basement membranes. Understanding the pattern of relative overexpression of these proteases and their targets may prove relevant to existing models and ideas of how the ECM and its regulation might affect outflow.[31–34] Also, each cell population expressed in greater amounts a different set of genes for water and ion channels. These channels are of importance for fluid transport and regulation of cell size and thus could directly impact outflow functions.[35–39] At the bottom of both Supplementary Tables S1 and S2, we grouped the genes that are associated with brain or neuronal development or function. The results show that the JCTM cells overexpressed 21 neuronal-related genes down to 1.9x over SC, while the SC cells overexpressed 6 genes at that same level. This is again consistent with the neural crest origin for the TM and possibly the JCTM (see Discussion). We also found many more genes with functions that are related to the vascular endothelium in the SC cells (Supplementary Table S2), but few specifically endothelial in the JCTM.

In addition, we used IPA, a commercial software package (see Methods), to obtain an additional perspective on the relationships of relatively overexpressed genes, using the 2 or greater cutoff ratio for both arrays, in terms of signaling pathways, metabolic pathways, molecular networks, and biological processes. These are presented in Supplementary Table S3 for relatively overexpressed in JCTM, and Supplementary Table S4 for those overexpressed in SC. The molecular and cellular functions of the abundant genes in JCTM cells included carbohydrate metabolism, cellular assembly and organization, cell morphology, and molecular transport. The networks of genes highly expressed in SC

cells included carbohydrate metabolism, cell death and survival, cellular development, and cardiovascular system development and function.

Finally, we tested the binding of antibodies to several extracellular structural proteins identified in the arrays as being differentially expressed in SC cells (Table 2) using immunofluorescence microscopy. Our purpose was to determine the extent of correlation of the differentially expressed genes with the expression of actual proteins in cultured cells, while recognizing that there are many points of control between mRNA levels and final protein levels in cells.[40,41] Antibodies to ICAM-1 (Fig. 2A, B), tenascin (Fig. 2C, D), and β-spectrin (Fig. 2E, F) were used to stain confluent cultures of JCTM (Fig. 2B, D, F) and SC cells (Figure 2A, C, E). As evident in Fig. 2, staining for ICAM-1 in SC cells was sporadic, being seen brightly in only a small proportion of cells (1 highly expressing cell is shown in Fig. 2A). On the other hand, it was nonexistent in JCTM cells. ICAM staining in SC cells was found around the edges and top (dorsal) surface, and in several instances, staining was found on filopodial-like projections between neighboring cells. Interesting differences between the 2 cell types were also seen with the tenascin staining. The staining in SC cells was more intense than that in JCTM cells. The staining pattern was also different. In SC culture, tenascin staining was observed around the edges of each cell, in dramatic ribbons consistent with extensive extracellular buildup (Fig. 2C). JCTM cells showed almost none of the ribbon-like staining between cells (Fig. 2D). Rather, in places, a very fine, powdery staining between the ventral surface of the cells and the coverglass surface was seen. The bare areas in the powder were very reminiscent of the patterns of F-actin filaments seen in TM cells such as the cross-linked F-actin networks shown by Clark et al.[42] We surmise that tenascin is secreted ''ventrally'' in JCTM cells, but is excluded from the closer contacts between the ventral membrane and glass surface underneath the stress fibers. Why and how this might take place is an interesting question. Fig. 2E and F indicate that both cell types expressed β-spectrin. SC and JCTM cultures both showed areas of spectrin staining, with the areas of most stain found in the lamellipodial areas. It appeared that the level of stain and number of cells with stain was less in JCTM cells. The immunofluorescence results thus are in reasonable agreement with the array data.

Discussion

The experiments described above were carried out using early forms of the Micromax arrays, to ascertain the reproducibility of the method and to gain clues to the cellular basis of outflow pathway physiology. Since the time they were carried out (1999–2000), many studies have clearly shown the utility of the differential expression method, as well as approaches that quantify the relative level of transcripts from a particular tissue or culture individually.[25,43–48] Our results are consistent with many of these studies, and also show some contrasts (discussed below). We also note that the level of agreement between arrays in our study was good, but not perfect. Some genes that were overexpressed in JCTM in the second array, AG694, using cells from the older donor, were often less overexpressed in the first array, or even were higher in SC cells. This can be appreciated most easily by searching for a gene of interest in the final supplemental spreadsheet (Supplementary Table S5), which is the entire raw dataset from both arrays. The spreadsheet is shown with 3 different sortings. Sheet 1 is sorted alphabetically by gene name, so each row shows the results from the same gene in both arrays. Sheet 2 is sorted by expression ratio, low to high based on the JCTM/SC value, for each array independently. Sheet 3 shows both arrays sorted by the expression ratio found in AG694, and thus the genes are still aligned in each row. Using these, one can quickly see whether the arrays showed consistent results for a particular gene, and also assess the values for each. We are not sure at present whether the differences between array results reflect differences in the ages of the donors, or inherent variability in the methods used, yet the many interesting correlations found are worth noting.

Studies that quantify the relative amounts of specific transcripts in the TM from whole eyes,[43–45] cultured TM cells,[25,46] or excised tissue[47] have identified many candidate genes of interest, while differential array experiments have been used to identify differences caused by dexamethasone treatment in TM cells,[48] cultured TM cells versus excised TM,[46] and SC versus TM cell cultures.[25] Our results support many of the previous findings, as described in Results, but conflict with others. For example, we found an increase in mRNAs for GAPDH (M33197) and ferritin light and heavy chain in SC cells in both arrays, while Gonzalez et al. found that whole perfused eyes showed high levels of expression of these genes in the TM.[43] GAPDH was just over 2-fold higher in SC/JCTM in both arrays (Supplementary Tables S5), while ferritin heavy chains (M11146; L20941) and light chain (M11147) were an average of 2.5-fold higher in SC cells (Supplementary Table S5), but 1 value was just below the 2x cutoff for Table 2 in one or the other array for each gene, so they were not included in that Table. This reflects the drawback of the differential array method, in that both cell types showed the transcripts, but in our hands the SC cells had relatively higher levels. We did not quantify the absolute number of transcripts. On the other hand, the TM cells might have lost expression of those genes more quickly in culture, as pointed out by Liton et al.[46] The most directly comparable study to the present work is that of Liton et al.,[25] but our earlier-generation array did not include many of the genes tested in that study. Thus, our data may provide additional and not overlapping information.

An interesting connection to the ECM we see in our data is the overexpression of heparan sulfate 3-*O*-sulfotransferase precursor in JCTM in both arrays (Table 1). The 3-*O* transferases promote sulfation of glucosamine at the 3-*O* position of heparan sulfate, and there is a recent recognition that that reaction may serve to modulate antithrombin activity, lymphocyte binding, growth-factor binding, and possibly other important reactions at the cell membrane or extracellular space.[9] It would be of interest if the modulation of heparan sulfate by this transferase affected the known binding of heparan sulfate to the Hep II domain of fibronectin.[49] As shown by Santas et al., perfusion of excess amounts of isolated Hep II domain can modulate outflow.[50] Perhaps the degree and sites of heparan sulfate ion can affect the binding of syndecans to fibronectin at the Hep II site, possibly affecting outflow.

We saw no difference in the baseline expression of myocilin in our cell populations. This is not consistent with a previous finding of a higher baseline level of myocilin

protein in more uveal TM cultures compared with SC cultures,[7] but may imply that regulation of baseline myocilin protein may take place at levels other than that of steady-state RNA expression.

Provocative associations have been noted between Alzheimer dementia and glaucoma.[51–57] Although these are generally assumed to be connected via a common pathway of neuronal degeneration, it is intriguing to postulate that the underlying causes of some forms of these insidious diseases of aging might be via common or related effects within the outflow pathway. Consistent with this possibility, the JCTM cells showed relative overexpression of microtubule-associated τ protein, a constituent of neurofibrillary tangles.[9,10,58–60] JCTM cells also expressed a gene bearing missense mutation in Alzheimer and Adaptor protein X11β that binds precursor of amyloid, a primary component of senile plaques (Supplementary Table S1; array 2).[60,61] In SC cells, 4 other genes related to amyloid were found to be enriched (Supplementary Table S2). Thus, the ''raw material'' for a pathology that is mechanistically related to Alzheimer dementia is present in the cells of the outflow pathway, but whether these proteins play a role, either in cell function or via the secretion of misfolded proteins, is not known. Understanding possible connections between the etiology of Alzheimer and POAG will be especially interesting to investigate in the future.

Our results are largely consistent with the TM cells we cultured being derived from the neural crest.[62,63] Three out of the 15 genes in Table 1, for example, appear to be neuronally related, and 21 genes are neuronally related in Supplementary Table S1 compared with 6 in the SC cells in array 2. On the other hand, many of the genes overexpressed in SC relative to JCTM in both arrays (Table 2) are consistent with an endothelial cell type, and several additional endothelial-related genes were identified in Supplementary Table S2. This is not proof of identity, but is consistent with extensive work showing the endothelial nature of SC.[64,65] The origin of JCTM cells from neural crest would be consistent with our having cultured the ''deepest'' (closest to SC) layers of the meshwork, but not the cells embedded within the ECM. By removing the uveal and corneoscleral portions of the meshwork, we endeavored to culture those cells closest to the inner wall. On the other hand, we did not digest the ECM, or finely dice the ''cribriform'' layer, and so we endeavored to select for cells that were accessible to the surface of the trabeculae and cul-de-sacs. However, given the apparent greater variability in the JCTM cultures, it may be that we were only partially successful in isolating a single population. Further work will be necessary to determine whether there are still more subdivisions to be distinguished within the juxtacanalicular region, between cells that are fully embedded within the cribriform ECM matrix, termed ''fibroblasts'' by Grierson et al.[66] and JCTM cells in recent reviews,[67,68] and those cells with areas of their membrane that are exposed to the outflow channels.

As the site of aqueous humor drainage, and of the increased outflow resistance in POAG, the TM, SC, and the ECM, what they construct and maintain are of essential interest. Is proper outflow resistance maintained primarily through adjusting the composition or structure of the ECM, through adhesion to or production of specific glycosaminoglycans, through secreted proteins, or through adjustment of cellular permeability, stiffness, or via the application of contractile forces to the ECM or to other cells? If, as is likely, it is through combinations of these and other factors in a complex network, it is still useful to discern the main components that influence outflow and whether there is a common final pathway to POAG. Differential expression assays have the characteristic of giving an impression of what the cells being compared are like, while in fact they are limited to assessing only the contrasts between cell types. The present study shows fairly large differences between 2 very different cell types within the outflow pathway. A next step would be to combine the subdivided dissection method with approaches such as the differential approach of Lo et al.,[48] to assess more subtle changes in 1 cell type induced by dexamethasone, and as approached by Liton et al.[46] and Liu et al.[47] to assess differences in POAG versus non-POAG tissue. Since the cells from each subdivision of the outflow pathway appear to retain many of their characteristics for several generations in culture, and cells grow well from POAG eyes that have been opened and supported in medium within 12 h of death, a series of comparisons of expression levels between the same cell types from control and POAG eyes would be an attractive approach by which to look for differences that would offer new clues to the etiology of POAG, or to test existing hypotheses. The shortcoming of looking only at the transcription level to infer cell function can now be partially overcome by coupling such experiments with recently greatly improved techniques for identifying the portfolio of expressed proteins and glycoproteins from tissues and cells using mass spectrometry.[69,70] It is hoped that the present results will stimulate new hypotheses toward understanding normal outflow physiology, and the pathophysiology of POAG.

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Disclosure Statement

No competing financial interests exist.

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