Characterization of Gene Expression in Human Trabecular Meshwork Using Single-Pass Sequencing of 1060 Clones

Pedro Gonzalez, David L. Epstein, and Teresa Borra’s

PURPOSE. To study the gene expression profile of the human trabecular meshwork (HTM).

METHODS. A polymerase chain reaction (PCR)-amplified cDNA library was constructed using RNA from the TM of a 67-year-old normal, perfused human eye. A total of 1060 clones were randomly selected for sequencing of one end. These sequences were searched against nonredundant GenBank and dbEST databases for similarity comparison by using a FASTA file and the BLASTc3 program. Relative expression patterns of those clones that matched other expressed sequence tags (ESTs) were determined using the National Center for BiotecnoInformatIon (NCBI) Unique Human Gene Sequence Collection (UniGene) database.

RESULTS. Of the 1060 clones analyzed, 519 (48.9%) had sequences identical with known genes, 125 (11.8%) matched ESTs, and 189 (17.8%) did not match any database sequences. Of the remaining clones, 31 (3%) corresponded to mitochondrial transcripts and 196 (18.5%) to repetitive and noninformative sequences. It is notable that some of the genes highly represented in this library are not ubiquitously expressed in other tissues, which suggests a potentially important role in the HTM. As evidence for the presence of true novel genes in the library, one of the clones was fully sequenced. This clone comprised a complete open reading frame of 966 nucleotides, and its deduced amino acid sequence corresponded to a protein 53% similar to the MAS-related G-protein-coupled receptor.

CONCLUSIONS. The identification of the more highly expressed genes in HTM and the discovery of novel genes expressed in this tissue provides basic information for further research on the physiology of the TM and for the identification of glaucoma candidate genes. (Invest Ophthalmol Vis Sci. 2000;41:3678–3693)

The trabecular meshwork (TM) is known to play a critical role in the maintenance of the intraocular pressure (IOP) of the eye. In humans, approximately 85% to 90% of the aqueous humor generated by the ciliary body exits the eye through Schlemm’s canal (SC) and the collector channels after being filtered by the TM. The distal TM generates most of the eye through Schlemm’s canal (SC) and the collector channels of the aqueous humor generated by the ciliary body exits the eye through Schlemm’s canal (SC) and the collector channels after being filtered by the TM. The distal TM generates most of the normal resistance to aqueous humor outflow, and it is also the site of the abnormal increased resistance that leads to the normal resistance to aqueous humor outflow.1–4 In addition to these three main cell types, electron microscopy of HTM sections often reveals the presence of other cells, such as macrophages.8 These cells are found within the intertrabecular spaces, are usually loaded with pigment granules, and probably leave the meshwork through paracellular routes of the inner wall.9

The TM is implicated in functions such as phagocytosis, extracellular matrix dynamics, secretion, cytoskeletal reorganization, and detoxification of aqueous humor.10,11 It is also believed to be involved in the maintenance of the immune

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privilege in the eye. However, most of the specific mechanisms by which the TM accomplishes these functions are not well understood.

The biologic features of TM, like those of any tissue, are determined largely at the level of gene expression. The identification of the genes expressed in TM constitutes a necessary step toward the understanding of its physiology. A powerful approach to the analysis of expression in a particular tissue or cell type involves single-pass partial sequencing of clones from a cDNA library together with the comparative analysis of the resultant expressed sequence tags (ESTs) with entries in public databases. A number of sequencing projects have provided a large collection of human ESTs from a variety of tissues.

However, the gene expression profile in the HTM has not been studied—in part, because of difficulties in obtaining enough RNA from such a small tissue that in turn preclude the construction of cDNA libraries by conventional methods. Polymerase chain reaction (PCR)-based methods for construction of cDNA libraries have been developed to circumvent these problems.

However, libraries generated using standard PCR methods tend to include a number of artifacts because of the overcycling of the PCR reaction. In addition, these libraries result in an enrichment of small cDNA clones that do not possess the entire open reading frame (ORF) of the genes. Instead, a recently developed, exponential PCR-based method generates high-quality cDNA libraries, enriched for full-length clones. The new method has been used to make good cDNA libraries from mouse blastocysts and human CD34 + hematopoietic stem–progenitor cells. It also has been successfully used in our laboratory to generate HTM cDNA probes for the hybridization of gene arrays. Using this new approach, we constructed a cDNA library from the TM of a 67-year-old human donor with no history of glaucoma, and analyzed 1060 clones by single-pass sequencing.

For this first analysis of the HTM gene expression profile, we chose a TM sample that had been perfused under organ culture conditions. Perfusion of the anterior segment of the eye is one of the best and most commonly used models for studying outflow physiology. Although perfused HTMs may not exactly reproduce the pattern of gene expression of the in vivo tissue, they are one of the closest. The model helps recover TM cell activity and maintains the expression of genes responsive to flow and IOP. In comparison, gene expression of a nonperfused HTM sample from a postmortem donor diverges from the in vivo expression pattern because of ongoing cell death and absence of IOP and aqueous humor flow.

Our results provide the first profile of gene expression in a human TM. This information will undoubtedly help in understanding the normal physiology of the TM and will provide new candidate genes potentially involved in the pathophysiology and the susceptibility to glaucoma.

**MATERIALS AND METHODS**

**Perfused Human Anterior Segment Organ Culture**

One pair of normal human eyes was obtained from The National Disease Research Interchange (NDRI), a nonprofit organization engaged in the procurement and distribution of human tissues for biomedical research in the United States. It was obtained with the signed consent of the patient according to the Tenets of the Declaration of Helsinki. The age of the donor was 67 years, the eyes were dissected within 30 to 40 hours of death, and no glaucoma was diagnosed. To revive the tissue and recover cell activity, the anterior segments were perfused as described earlier. Briefly, eyes were bisected at the equator and the lens, iris, and vitreous removed. The anterior segment was then clamped to a modified petri dish and perfused at 3 μl/min of constant flow using a microinfusion pump. The culture medium was Dulbecco’s modified Eagle’s medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 170 μg/ml gentamicin, and 250 μg/ml amphotericin B (DMEM+). Anterior segments were maintained at 37°C in 5% CO₂. IOPs were continuously monitored with a pressure transducer connected to the dish’s second cannula and recorded with an automated computerized system. For this experiment, perfusion was performed for a total of 27 hours. At this time, the outflow facility baseline was stable and had a value of 0.101 μl/min per mmHg. At the end of the experiment, anterior segments were frozen in liquid nitrogen within 2 minutes of turning off the pumps and stored at −70°C for later dissection of the TM and RNA extraction.

**Light and Electron Microscopy**

A second pair of eyes from a 32-year-old normal donor was perfused in similar conditions and used for morphology analysis. After 48 hours in culture, whole anterior segments were fixed by overnight perfusion with 2% glutaraldehyde at a pressure of 15 mm Hg. The anterior segments were then cut meridionally into quadrants and immersed in the same fixative until processing. From each of two quadrants, wedge-shaped specimens containing the angle region with the TM were cut and embedded in glycol methacrylate using an embedding kit (JB-4; Polysciences, Warrenton, PA). Blocks were counterstained with eosin and hematoxylin and sectioned to 2 μm. At least two sections from each of the two quadrants of the processed eyes were analyzed by light microscopy to examine the morphology of the tissue.

For electron microscopy, wedges of meridional sections of the TM were postfixed in 1% osmium tetroxide, block stained in 2% uranyl acetate, dehydrated, and embedded (Spurr embedding medium; Polysciences). Ultrathin sections were stained with potassium manganate and lead and examined with a transmission electron microscope (1200Ex; JEOL, Tokyo, Japan).

**Construction of an HTM cDNA Library**

RNA extraction and exponential amplification of the reverse transcribed cDNA were performed as previously described. Briefly, the TM from a single eye was dissected and its RNA extracted with an RNeasy kit (Qiagen, Chatsworth, CA). One forth of the total RNA was lyophilized and used as template for the generation of a reversed transcribed first cDNA using a DNA synthesis kit (SMART-PCR; Clontech, Palo Alto, CA) and Superscript II, RNase H− reverse transcriptase (Gibco, Gaithersburg, MD) to a final volume of 50 μl. The number of cycles needed for exponential phase amplification of this cDNA was determined by running a series of 16 μl analytical PCR amplifications at 17, 20, and 23 cycles using the same kit. To prevent overrepresentation of clones with short inserts (which would ligate to the vector more efficiently than larger ones), the resultant cDNAs were size fractionated using...
agarose gel electrophoresis. For that, the PCR-amplified sample was purified and concentrated in an elution column (QIAquick; Qiagen) to 30 μl and loaded onto a single well of a 2% low-melting agarose gel. Four separate slices corresponding approximately to molecular weights of 0.6 to 1 kb (fraction I), 1 to 2 kb (fraction II), 2 to 3 kb (fraction III), and more than 3 kb (fraction IV) were cut from the gel and melted at 65°C for 10 minutes. The cDNA from each slice was purified and concentrated to a total of 30 μl with an elution column (QIAquick; Qiagen). One tenth of each eluted cDNA was used for ligation into a cloning vector (pCR2.1-TOPO; Invitrogen, Carlsbad, CA) followed by transformation of Escherichia coli competent cells (TOP10F‘ One Shot; Invitrogen) and plating onto ampicillin-agar plates with 5-bromo-4-chloro-3-indoyl-β-D-galactosidase (X-gal). A similar number of white cell colonies from each fraction were considered for DNA sequencing. Glycerol stocks from each of these clones have been kept under identification numbers HTM1-0001 to HTM1-1060.

Template Preparation and Sequencing

Plasmid purification and automatic DNA sequencing were performed by Midland Certified Reagent (Midland TX). Briefly, plasmid DNAs were purified by the alkaline lysis method and 0.5 to 1 μg used as template for cycle sequencing reactions (Thermosequenase kit; Amersham, Arlington Heights, IL) with IRD800-labeled M13 as the forward primer. Electrophoresis was then conducted (model LS4200; Li-cor, Lincoln, NE). For clones in which the M13 primer produced a poor-quality sequence, a second reaction was prepared using the M13 reverse primer. Because the SMART PCR library cloned by the TA method is not directional, the ESTs were generated from either the 5’ or the 3’ end of the cDNA clones. In the case of full-length cDNA sequencing, custom-made oligonucleotides were used instead of the clone insert, and the full sequence was obtained by overlapping readings of both strands of the cDNA.

Sequence Analysis

Sequences corresponding to the vector and to the PRC primer used to construct the library were deleted manually from each clone. All sequences were arranged in a unique FASTA file and searched against databases using the network BLAST client program (BLASTcl3) of the National Center for Biotechnology Information (NCBI; URL: http://www.ncbi.nlm.nih.gov/blast/blast.cgi). The searches were performed between September and November 1999.

The first sequence search was conducted using BlastN against all entries in the nonredundant GenBank database. Sequences with an E-value expected (number of matches to be found merely by chance) equaling zero were considered to identify known genes or to have partial similarity to known genes. Sequences with E-values close to zero, were manually analyzed for overall similarity or identical stretches that could indicate a different gene from the same family, small sequencing errors, alternative splicing, chimeric clones or presence of vector sequences. Sequences including fragments with similarity with repetitive sequences (e.g., ALU, LINE) were considered perfect matches only when BLAST searches performed after eliminating the nonrepetitive part of the sequence also provided an E-value equal to zero.

DNA sequences for which no match was found in the nonredundant GenBank database were subjected to a second search with the BlastN against the dbEST database. From those, DNA sequences that matched dbESTs were searched against the Unique Human Gene Sequence Collection (UniGene) database (URL: http://www.ncbi.nlm.nih.gov/UniGene/) to determine whether they were integrated in a gene cluster. Expression patterns and chromosomal localization of these DNA sequences found to belong to a cluster were thus obtained.

Last, DNA sequences found to contain no similarities to any database were translated in all reading frames and further searched against the nonredundant protein database using the BLASTX program. The same DNA sequences were also searched for the presence of open reading frames using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Hybrid Panel Analysis

To determine the chromosomal location of the gene represented by HTM1-0025 cDNA, we used the hybrid panel G3 from Stanford University (Stanford, CA). We designed primers for the specific PCR amplification of a fragment of the gene. Positive amplification was seen only with the human genomic DNA control but not with the hamster DNA. After analyzing PCR reactions from the 83 hybrids in the G3 panel, we submitted the results to the SHGC server (rhserver@pixil.stanford.edu).

RESULTS

Morphology of the Perfused HTM

The perfused human anterior segment organ culture keeps the majority of the cells of the TM tissue alive.26 In our experiments, after a period of 30 to 40 hours after death, the cells of the TM were revived by the flow of serum-free medium pumped through the outflow pathway at the constant rate of 3 μl/min. Figure 1 shows the histologic examination of a representative pair of human anterior segments perfused for 48 hours. The light microscopy photograph (Fig. 1A) reveals the well-preserved architecture of the TM tissue and the presence of a good number of cells in all three layers. Although some loss of the uveal cells was evident, a large percentage of cells from the corneoscleral and juxtacanalicular regions were conserved. The electron microscopic analysis (Figs. 1B, 1C, 1D) confirmed the healthy appearance of the cells. In the corneoscleral layer, cells properly lined the trabecular beams (Fig. 1B) and the juxtacanalicular layer was intact. SC was well formed, and the endothelial cells of the inner wall appeared to form tight intercellular contacts.

Characteristics of the HTM Library and General Data of the ESTs

The electrophoretic analysis of the amplified HTM cDNA yielded the expected smear pattern with visible, discrete bands corresponding to the more abundant transcripts of the tissue (Fig. 2). The smear extended from approximately 0.2 to more than 5 kb and showed clear, visible bands that indicate high abundance of full-length cDNAs. To determine whether significant genomic DNA contamination was present in the library, we amplified library genes using primers spanning short introns as well as other genes not found in HTM. We found that the corresponding PCR products from those amplifications never included intron sequences (even in cases in which in-
trons were only a few hundred base pairs long). In addition, PCR reactions with primers for genes not expressed in the HTM did not generate any product (data not shown).

The analysis of 1060 ESTs revealed the five groups of cDNA sequences: Group I (519 ESTs, 48.9%) showed identity with the sequences of the nonredundant GenBank database (using BlastN and E values of zero). They were labeled known genes. Group II (125 ESTs, 11.8%) matched well to EST sequences from unknown genes reported in other tissues in the public domain database (dbEST). They were labeled known ESTs–unknown genes. Group III (189 ESTs, 17.8%) exhibited no significant similarity to known genes or known ESTs in the public databases and were thus defined as novel ESTs. Group IV (31 ESTs, 2.9%) were of mitochondrial DNA. Group V (196 ESTs, 18.5%) included either repetitive elements (ALU, L1, MER), short tandem repeats, or clones with none or short inserts with no similarity in any database (192 ESTs). Four ESTs were found to be chimeras (β-mannosidase, Bir L37, ferritin L, proline-rich calmodulin) with no introns, possibly the result of rearrangements during the cDNA synthesis or PCR amplification. These were classified as uninformative sequences. Finally, in this library there were no sequences corresponding to rRNA (Table 1).

Some of the genes were represented in the library by more than one clone. The level of redundancy of the different cDNAs is a reflection of the relative abundance of expression of each of the library genes. The total number of unique cDNA species in each of the five groups is represented in the right column of Table 1. All clones from the same gene (identical or nonoverlapping) corresponded to one cDNA species. The 519 ESTs of group I corresponded to 338 unique cDNA sequences and the 125 ESTs of group II to 120; the 189 novel ESTs of group III were all found to be single-copy cDNAs.

Other potentially relevant genes were present in this library but did not show in the 1060 clones analyzed by single-pass sequencing. For instance, the presence of TIGR/MYOC as well as that of other olfactomedins was confirmed by PCR, but none of the 1060 clones corresponded to these genes. Despite the importance of TIGR/

### Table 1. Summary of the ESTs Found in the HTM Library

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<th>cDNA Category</th>
<th>Clones</th>
<th>cDNA Species</th>
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<td></td>
<td>n</td>
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<tr>
<td>I Known genes</td>
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<tr>
<td>II Known ESTs/unknown genes</td>
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<td>IV Mitochondrial transcripts</td>
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<td>2.9</td>
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<td>V Uninformative sequences</td>
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**Metabolism**

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<td>Cytochrome C oxidase subunit IV</td>
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<td>Somatic cytochrome C</td>
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<td>ATP synthase subunit C (ATPSCP2)</td>
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<td>Mitochondrial ATPase-coupling factor 6 (MATPSY)</td>
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<td>5-Adenosylhomocysteine hydrolase</td>
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<td>5-Adenosylmethionine synthase</td>
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<td>Proline synthetase</td>
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<td>Pyrroline-5-carboxylate synthase</td>
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<td>Carbonic anhydrase III (CAIII)</td>
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<td>Site-1 protease subtilisin-1 (SP1)</td>
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<td>Ariadne homologue (ARI)</td>
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<td>Erythrocyte membrane protein 72, stomatin (EPB72)</td>
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<tr>
<td>Integral transmembrane protein 1 (ITM1)</td>
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<td>Novel gene similar to C. elegans hypoth 55.2-kDa protein F16A11.2</td>
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<td>P97 Homologue to bovine BCNT</td>
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<td>Zona pellucida B Protein</td>
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<tr>
<td>Gs1 (protein of unknown function)</td>
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<tr>
<td>Expressed in fibroblasts of periodontal ligament PL108</td>
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<tr>
<td>LR8 Expressed by a subpopulation of human lung fibroblasts</td>
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</table>
MYOC in glaucoma, this result is not surprising. In our laboratory, Northern blot analysis consistently shows that the expression of this gene in the HTM is lower than that of αB-crystallin (represented by only one clone in the library).

### Gene Expression Profile in HTM

#### Group I: Clones Similar to Known Genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession Number</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ring zinc finger (RZF)</td>
<td>AF037204</td>
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</tr>
<tr>
<td>AF1q (expressed in leukemic and immature hematopoietic cells)</td>
<td>U16954</td>
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<tr>
<td>Okadaic acid-inducible phosphoprotein (OAAB18)*</td>
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<tr>
<td>CG1-21</td>
<td>AF132955</td>
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<td>HS1 binding protein HAX-1 (HAX1)</td>
<td>U68566</td>
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</tr>
<tr>
<td>23-kDa highly basic protein (23KDHBP)</td>
<td>X56932</td>
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<tr>
<td>NEL-related protein 2</td>
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<tr>
<td>Nuclear protein (NP220)</td>
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<td>Homo sapiens TDE homologue (DIFF33)</td>
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<td>OTK27 mRNA, highly homologous to yeast nuclear protein NHP2</td>
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<tr>
<td>RNA-binding protein regulatory subunit (DJ1)*</td>
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<td>N33 candidate tumor suppressor gene</td>
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<td>RRM RNA-binding protein GRY-RBP (GRYRBP)</td>
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</tr>
<tr>
<td>Type II membrane protein similar to CD69 (CLECSF2)</td>
<td>AB015628</td>
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<tr>
<td>Divalent cation tolerant protein (CUTA)</td>
<td>AF106943</td>
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<tr>
<td>Homo sapiens homologue of yeast (S. cerevisiae) ufd2 (UFD2)</td>
<td>NM_004788</td>
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<td>4F5rel candidate modifying gene for spinal muscular atrophy</td>
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<td>sp25L2 protein (GP25L2)</td>
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<td>HSPC003</td>
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<td>HSPC013/cDNA DKFZp566G1246</td>
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<td>HeptG2 3' region Mbol cDNA</td>
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<tr>
<td>JWA protein</td>
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<tr>
<td>Josephin MJ1, Machado-Joseph disease (SCA3)*</td>
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<td>Similar to human 7S L/1 EST (7S L pseudogene)</td>
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<td>SH3 domain–binding glutamic acid-rich protein-like (SH3BGRL)</td>
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<td>Human lupus p70 (Ku) autoantigen</td>
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<td>CaX box 1 (CXX1)</td>
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<tr>
<td>mRNA, chromosome 1 specific</td>
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<td>Testis-enhanced gene transcript (TEGT)</td>
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<td>Clone 25071 and 25177 mRNA</td>
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<td>Clone 25022 mRNA</td>
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<td>DJ0614C 10/Hs. 56729 lymphocyte-specific protein 1</td>
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<td>Hypothetical protein</td>
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<td>Major nuclear matrix protein (MNMP)</td>
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<td>cDNA DKFZp566D116</td>
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<td>cDNA DKFZp564L0822</td>
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* EST in HTM library shows a possible alternative splicing with respect to the GenBank entry.
The accession number of each of these genes, along with their distribution into nine different functional categories are summarized in Table 2. The number of clones and cDNA species from each category are represented in Figure 3.

The genes more highly represented in the HTM library (by four or more clones) are summarized in Table 3. The right column of this table shows the number of ESTs reported in the UniGene database for each of these genes. This information helps to differentiate between genes with ubiquitous high expression in most tissues and those in which high expression in TM may be associated with the specific functions of the tissue.

Group II: Clones Similar to Reported ESTs from Unknown Genes. Of the 1060 clones sequenced, 125 (11.8%) produced sequences matching ESTs from unknown genes previously reported in other tissues. Ninety-three clones can be classified as part of clusters in the UniGene database, whereas 32 where identical with ESTs from the GenBank with no UniGene cluster. Table 4 shows the complete number of HTM ESTs identical with UniGene clusters from unknown genes. The table also includes their chromosomal localization (when available), number of ESTs in the cluster, and information regarding their similarity to other known genes. In addition, those ESTs in the cluster showing a pattern of expression restricted to few tissues are also indicated.

Group III: Novel ESTs. Clones with No Similarity to Sequences in the GenBank. A relatively high number of clones, 189 (17.8%), showed no similarity with any entry of the public domain databases and were classified as previously unreported or novel HTM ESTs. Recently, a human hematopoietic cell line exhibited a 14.3% of novel ESTs, whereas a mouse blastocyst had only 2%.22 However, a similar percentage of novel sequences have recently been reported in another eye tissue, a rabbit corneal endothelial library.17 To demonstrate that some of the unreported ESTs truly corresponded to novel genes previously unreported in other tissues, we selected one
### Table 4. UniGene Clusters for ESTs from Unknown Genes Represented in the HTM Library

<table>
<thead>
<tr>
<th>UniGene ID</th>
<th>Accession Number</th>
<th>Frequency in the HTM Library</th>
<th>Chromosome Localization</th>
<th>ESTs in the UniGene Cluster (n)</th>
<th>Comments</th>
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<td>Chr.20</td>
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<td>235</td>
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<td>Chr.14</td>
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<td>Hs.3390</td>
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<td>Chr.17</td>
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<td>Hs.3420</td>
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<td>Chr.X</td>
<td>DXS990-DXS1059</td>
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</table>

... (Continues with similar entries)
of them, clone HTM1-0025, for full characterization. The complete insert of this clone was sequenced in both orientations using the forward and reverse M13 flanking primers. The sequence revealed an ORF of 966 nucleotides (nt) flanked by a 5' untranslated region (UTR) leader of 58 nt and a 3' UTR of 343 nt (Fig. 4A). The deduced amino acid sequence of the ORF corresponded to a protein of approximately 33 kDa (322 amino acids). The 5' leader cDNA contains a stop codon 31 nt upstream of the first methionine of the ORF which identifies this ATG as the first translated codon. Moreover, the nucleotides surrounding this ATG match the consensus sequence for the translation initiation region of vertebrate mRNAs.31 On the 3' end, the cDNA contains a putative polyadenylation signal located 22 nt upstream of a polyA tail.

The protein encoded by this cDNA had no match in any database and was therefore classified as a novel gene expressed in the human TM. The closest similarity found for the new protein was that of a 33% with the MAS-related G-protein-coupled receptor (343 amino acids, GenBank accession number P35410; Fig. 4B).32 The similarity extended along most of the ORF and had a 14–amino acid stretch of identical residues close to the C-terminal end. The hybrid panel analysis of this clone provided a lod score of 7.28 with the marker SHGC-6030, and located the gene to chromosome 11.

**Table 4 (continued).**

<table>
<thead>
<tr>
<th>UniGene ID</th>
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<th>Frequency in the HTM Library</th>
<th>Chromosome Localization</th>
<th>ESTs in the UniGene Cluster (n)</th>
<th>Comments</th>
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</thead>
<tbody>
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<td>Chr.20</td>
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**DISCUSSION**

With the Human Genome Project nearing completion, a major goal in biology is to provide a link between the genes identified in structural genomic studies and the physiology and pathophysiology of all tissues and cell types. A major step in providing such a link is the identification of ESTs from each particular tissue. Although a number of sequencing projects have already provided a large collection of human ESTs, none of them has focused on the tissues of the outflow pathway. In this study, we present a profile of the gene expression in the HTM based on the analysis of 1060 clones.

The percentage of ESTs obtained in group I (known genes), group II (dbESTs), and group III (novel sequences) in the HTM library was not substantially different from those in other tissues.16–18,22,23 The classification of the known genes by function (Table 2, Fig. 3) also revealed a general pattern similar to that previously described for an average of 37 human tissues.33 The functional group showing a relatively higher representation in the TM library was the one for cell signaling and cell communication. This finding may be an indication of the importance of sensing changes in IOP and sending signals to other cells to respond to these changes. The group with a higher level of gene redundancy was the one involved in...
extracellular matrix synthesis and degradation, which could be a reflection of the importance of extracellular matrix dynamics in the TM metabolism. Although some genes coding for proteins involved in vesicle dynamics were present in the library, genes involved directly in phagocytosis were not highly represented. This could be the result of the perfusion model used. Because the perfusion medium did not contain aqueous humor proteins, DNA, and/or debris, the mechanisms to eliminate them may have been underregulated.

In group I (known genes), some of the observed most abundant transcripts are usually not present at such high frequency in other tissues and may therefore provide useful clues about the biologic features of TM. The high expression of other genes, such as the glycolytic enzymes LDHA, GAPDH, and TPI is expected, given that TM obtains most of its energy though anaerobic metabolism. More surprising is the high abundance of transcripts coding for proteins such as matrix GLA (MGP), chitinase 3-like 1 (CHI3L1), apolipoprotein D, small inducible cytokine (SCYA20), regulator of G protein signal, stromelysin 1, and from the uncharacterized genes KIAA0258 and DKFZp586O0118.

Matrix GLA is the first protein clearly associated with protection against calcification of soft tissues. High levels of matrix GLA expression are observed in response calcification of human vascular cells in vitro and in atherosclerotic lesions. The presence of matrix GLA as one of the more abundant transcripts in our library may indicate that protection against calcification is important in TM. Calcification is associated with the age-related loss of elasticity and contractility in some tissues and could have a similar effect on TM, impairing the ability of TM to regulate outflow. Of note, dexamethasone treatment that can result in extracellular matrix synthesis and degradation, which could be a reflection of the importance of extracellular matrix dynamics in the TM metabolism. Although some genes coding for proteins involved in vesicle dynamics were present in the library, genes involved directly in phagocytosis were not highly represented. This could be the result of the perfusion model used. Because the perfusion medium did not contain aqueous humor proteins, DNA, and/or debris, the mechanisms to eliminate them may have been underregulated.

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in reversible glaucoma, is known to induce calcification in blood vessels.\textsuperscript{42} Chitinase 3-like or human cartilage protein gp-39 is another extracellular protein expressed in cartilage and strongly induced in macrophages from atherosclerotic lesions.\textsuperscript{43} This protein is also one of the antigens implicated in the autoimmune disease, rheumatoid arthritis.\textsuperscript{44, 45} Although its function is not yet clear, its involvement in tissue remodeling and in the pathologic course of blood vessel disease makes this gene a good candidate to play a potential role in TM conductivity and the pathology of glaucoma.

It is more difficult to speculate about the possible role of such proteins as ApoD, SCYA20, and RGS5, because of our incomplete knowledge about their function in other tissues. However, data suggesting the involvement of ApoD in neuronal degeneration and regeneration,\textsuperscript{46} SCYA20 in inflammation and vascular permeability,\textsuperscript{47–49} and RGS5 in the modulation of rectifying potassium (GIRK) channels\textsuperscript{50, 51} suggest possible links to TM physiology.

Stromelysin 1 already has been identified as an important protein in the regulation of outflow resistance.\textsuperscript{52–54} Stromelysin 1 can be induced by a number of factors.\textsuperscript{55–60} However, it is not usually found as such an abundant transcript in noninduced tissues, with only 21 ESTs reported in the UniGene cluster. Its relative abundance in the TM library (approximately 0.4\%) is consistent with the concept that the TM's ECM is under constant remodeling.

The presence of four clones representing each of the uncharacterized genes KIAA0258 and cDNA DKFZp586O0118 is particularly interesting, because none of them appears to be highly expressed in other tissues, and therefore they may be important for some specific functions of TM. This finding further emphasizes the need for high-throughput sequencing of HTM cDNAs. Some of the key genes essential for the physiology of this tissue may not yet have been fully characterized.

The identification of ESTs from group II (previously reported ESTs from uncharacterized genes), and group III (novel ESTs not previously reported in other tissues) is particularly important for two reasons: First, given the incomplete penetrance, the influence of nongenetic factors, and the late onset of the disease, an efficient search for glaucoma susceptibility genes necessitates not only positional cloning but also a candidate gene approach. The identification of novel genes expressed in HTM together with their chromosome location may greatly contribute to the identification of glaucoma genes. Second, because glaucoma does not appear to be associated with problems in other tissues, proteins involved in this disease may have a TM-preferred tissue expression pattern.

ESTs from uncharacterized genes (group II) can be analyzed for chromosome location and pattern of tissue expression using the information available in databases such as UniGene (Table 4). Some of the ESTs found in our TM library are located in chromosomes with regions linked to glaucoma.\textsuperscript{61–65} As new linkage analysis provides more precise information about all glaucoma loci and more extensive analysis of TM libraries provides more information about ESTs from this tissue, it will be possible to select candidate genes from this group of clones. Furthermore, other ESTs from this group belong to UniGene clusters that appear to have a pattern of expression restricted to very specific tissues. This is the case of clusters such as Hs.97431 which includes only four ESTs, all from a testis library; Hs.132591, which includes five ESTs, all from brain; and Hs.199612, which has only one EST from kidney. This observation suggests that these genes may have some specific functions associated with the particular physiology of these tissues.

The identification of novel ESTs (group III) is particularly important in highly specialized and poorly studied tissues such as TM. However, analysis of novel, previously unreported ESTs has to be undertaken with some caution. cDNA libraries are never totally free of artifacts, which can include chimeric clones and/or fragments of genomic DNA. The presence of four chimeric clones in our library indicates that some of the novel clones could also be artifacts. However, four chimeras in 519 clones in the known gene pool represents a small percentage, probably not higher than that normally found in conventional non-PCR-amplified libraries. More important, the characterization of clone HTM1-0025 shows that true novel genes can be discovered within the subset of novel ESTs. The features of this clone (complete ORF, similarity with a known family, and presence of the polyadenylation signal) confirm its identity as a novel gene and not as an artifact.

Finally, when evaluating single-pass sequencing analysis of cDNA libraries such as the one presented in this study, it is important to remember that any single library has some bias associated with the specific source of RNA. For instance, HTM samples acquired from postmortem donors may not accurately reflect the in vivo expression profile because of selective cell death (uveal layer), absence of IOP, and no aqueous flow. Using perfused TM solves some of these problems by recovering cell activity and maintaining IOP and aqueous flow. However, a limitation of this system is that the TM cells are exposed to tissue culture medium, not to physiological aqueous humor. They therefore do not have the exposure to some components, such as growth factors and reactive oxygen species, that is likely to affect their gene expression. In addition, it is important to consider the high degree of individual variability associated with the analysis of human samples. The particular history of the donor, medication, age, sex, and race also affect gene expression. Further analysis of more individuals is needed for a definitive characterization of the HTM gene expression profile.

In summary, our study describes the first expression profile of known and novel genes in a human TM. We identified 338 known genes, 120 ESTs previously reported in other tissues, and 189 new ESTs. The relative higher abundance of some of the gene species suggests new potential functions of the TM tissue, such as the prevention of cell calcification. The identification of novel ESTs should provide new tools for understanding TM physiology and for identifying genes involved in the genetic susceptibility to glaucoma.

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References


