Differential Gene Expression in Pseudoexfoliation Syndrome

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Purpose. To identify and characterize genes differentially expressed in anterior segment tissues of eyes with pseudoexfoliation (PEX) syndrome and glaucoma.

Methods. Anterior segment tissues (iris, ciliary processes, lens epithelium) were obtained from eight surgically enucleated eyes with PEX-associated open-angle or closed-angle glaucomas and eight age-matched glaucomatous control eyes without PEX. cDNA libraries were generated from three PEX and three control specimens, and their gene expression patterns were compared by means of cDNA subtraction. Differentially expressed clones from the subtracted cDNA libraries were sequenced, and their differential expression was verified by means of RT-PCR, virtual Northern blot analysis, and in situ hybridization with specific RNA probes.

Results. Subtraction of cDNA libraries identified 27 candidate genes for differential expression in PEX tissues, of which 23 genes were confirmed by virtual Northern blot, RT-PCR, and in situ hybridization. One set of genes consistently upregulated in anterior segment tissues from different patients with PEX comprised latent transforming growth factor binding proteins (*LTBP-1* and *-2*), which are structural components of elastic microfibrils, the cross-linking enzyme transglutaminase-2 (*TGase-2*), tissue inhibitor of matrix metalloproteinase-2 (*TIMP-2*), A-kinase anchor protein-2 (*AKAP-2*), apolipoprotein D, and the adenosine receptor-A3 (*AdoR-A3*). Genes reproducibly downregulated in PEX tissues included *TIMP-1*, clusterin, microsomal glutathione-*S*-transferase-1 (*mGST-1*), and serum amyloid A1. Further transcripts, such as elastase, *GST-T1*, integrin $\beta 4$, and dehydrocholesterol reductase, did not show a consistent differential expression pattern in tissues obtained from different patients. Although fibrillin-1 was not isolated from subtracted cDNA libraries, upregulated expression of this elastic microfibrillar component was also demonstrated by RT-PCR and in situ hybridization.

Conclusions. Differentially expressed genes with a high level of reproducibility in different tissues and different patients with PEX syndrome are mainly related to extracellular matrix metabolism and cellular stress. The underlying pathophysiology of PEX syndrome appears to be associated with an excessive production of elastic microfibril components, enzymatic cross-linking processes, a proteolytic imbalance between matrix metalloproteinases and their inhibitors, and increased cellular and oxidative stress supporting the notion of PEX syndrome as a stress-induced elastic microfibrillopathy. (*Invest Ophthalmol Vis Sci.* 2005;46:3742–3752) DOI:10.1167/iovs.05-0249

Pseudoexfoliation (PEX) syndrome is the most commonly identified specific cause of open-angle glaucoma, which is characterized by rapid progression, high resistance to medical therapy, and an overall poor prognosis.[1] It accounts for most cases of glaucoma in some countries and for approximately 25% of all cases of open-angle glaucoma worldwide. The hallmark of the underlying disease is the pathologic production and accumulation of an abnormal fibrillar extracellular material (PEX material) in many intra- and extraocular tissues.[2,3] Despite its systemic nature, the most prominent accumulations of PEX material are usually found in the anterior segment of the eye, leading to numerous clinical complications apart from secondary glaucoma development.[4] Ultrastructural evidence suggests that, among other intraocular cell types, PEX material is mainly produced by epithelial cells of the iris, lens, and ciliary body.[4,5] However, the exact composition of the pathologic material, a glycosylated glycoprotein–proteoglycan complex bearing epitopes of the basement membrane and the elastic fiber system, as well as the molecular mechanisms responsible for its excessive production and progressive accumulation still remain elusive.[5] This obvious deficiency may be mainly attributable to the lack of any molecular biological approaches to clarification of this matrix process.

To elucidate the molecular mechanisms underlying PEX syndrome, comparative analyses of mRNA expression patterns of anterior segment tissues obtained from PEX and control eyes were performed. We hypothesized that PEX cells, showing ultrastructural signs of active fibrillogenesis and metabolic activation,[4,5] should differ from normal cells also at the transcriptional level. Because of significant involvement of the preequatorial lens epithelium, the nonpigmented ciliary epithelium, and virtually all cell types of the iris in PEX fibril formation,[4] we selected the entire lens epithelium, iris tissue, and ciliary process tissue obtained from PEX eyes for gene expression analyses in comparison with the respective control tissues. Because of low mRNA yields of ocular tissues, cDNA had to be amplified by the use of cDNA libraries (SMART; BD-Clontech, Heidelberg, Germany), which has been shown to maintain the relative representation of each transcript in the original sample.[6,7] To identify differentially expressed genes in PEX tissues, we compared the gene expression patterns in PEX and control tissues by using suppression-subtractive hybridization (SSH) of cDNA and differential screening. Potentially disease-relevant gene products related to matrix turnover, cellular stress, and regulation were verified by further molecular biological methods, including virtual Northern blot analysis, semiquantitative RT-PCR, and in situ hybridization.

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METHODS

Tissues

For RNA extractions, anterior segment tissues (iris, lens capsules with epithelium, ciliary processes) were obtained from two eyes with PEX-associated open-angle glaucoma (77 and 86 years; a man and woman, respectively), one eye with PEX-associated angle-closure glaucoma (85 years; a woman), two eyes with angle-closure glaucoma without evidence of PEX syndrome (77 and 90 years; a man and woman, respectively), and one eye with open-angle glaucoma without PEX (68 years; a man). All PEX (mean age, 82.6 ± 4.9 years) and control (mean age 78.3 ± 11.1 years) eyes had to be surgically enucleated because of painful absolute glaucoma. Immediately after enucleation, tissues were prepared under a dissecting microscope and shock frozen in liquid nitrogen in RNA extraction buffer.

For in situ hybridizations, another four eyes with PEX-associated open-angle glaucoma (77.5 ± 4.9 years; three men, one woman), one eye with PEX-associated angle-closure glaucoma (83 years; a man), four eyes with absolute open-angle glaucoma (78.0 ± 6.7 years; two men and two women) and one eye with angle-closure glaucoma (80 years; a man) without evidence of PEX syndrome were obtained by surgical enucleation. The eyes were fixed in buffered 4% paraformaldehyde immediately after enucleation for optimal preservation of RNA. In addition, we included one donor eye with PEX syndrome without glaucoma (64 years, male) and one normal-appearing donor eye (64 years, male donor) without PEX syndrome or any other known ocular disease, which were obtained at autopsy and fixed within 8 hours of death.

Informed consent to tissue donation was obtained from the patients and the research followed the tenets of the Declaration of Helsinki.

RNA Extraction and Generation of cDNA Libraries

Tissues were homogenized with a rotor-stator homogenizer in 350 μL guanidine thiocyanate buffer. RNA was extracted with a kit (RNeasy kit; Qiagen, Hilden, Germany) that included an on-column DNase I digestion step, according to the manufacturer's instructions. Representative cDNA libraries from PEX and control tissues were generated with a PCR cDNA synthesis kit (SMART; BD-Clontech, Heidelberg, Germany), according to the manufacturer's instructions. Briefly, 1 μg of total RNA from PEX or control tissues was reverse transcribed with the kit oligonucleotides.[6] To determine the linear range of cDNA amplification, first-strand cDNA was subjected to PCR for 15, 18, 21, and 24 cycles. Amplification reactions were analyzed on 1.2% agarose gels, whereas 19 cycles were considered to be within the linear range and to represent the optimal number of cycles for preparation of cDNA libraries. The amplified cDNA was subsequently used for suppression-subtractive hybridization (SSH) of the cDNA, differential screening, and virtual Northern blot analysis.

Suppression-Subtractive cDNA Hybridization

SSH was performed with cDNA derived from PEX or control tissues with a kit (PCR-Select cDNA Subtraction Kit; BD-Clontech) according to the manufacturer's recommendations. In brief, 2 μg of amplified tester cDNA was digested with *Rsa*I and divided into two portions, and each was ligated to a different DNA adaptor. Each of the ligation reactions was then hybridized with driver cDNA, leading to the enrichment of differentially expressed genes in the population of single-stranded adaptor ligated tester cDNA (sA-cDNA). The two primary hybridization samples were combined without prior denaturation, allowing sA-cDNA to associate into double-stranded tester cDNA with different adaptors (dA-cDNA). Subsequently, a primary PCR (27 cycles) and a secondary PCR amplification (12 cycles) were performed with adaptor-specific primers, which resulted in exponential amplification of dA-cDNA (i.e., differentially expressed genes). The amplified products were cloned into the T/A cloning vector pCRII-TOPO and chemically transformed into *Escherichia coli* TOP10F′ with a kit (TOPO TA Cloning kit; Invitrogen, Karlsruhe, Germany). To estimate the efficiency of subtraction, indicated by the reduction of equally expressed transcripts in the subtracted sample, the secondary PCR products of subtracted and unsubtracted samples were amplified with primers for glycerinealdehyde-3-phosphate dehydrogenase (GAPDH) for 18, 23, 28, and 33 cycles and analyzed on 1.2% agarose gels.

Differential Screening

To exclude false-positive results of SSH, the subtracted cDNA libraries were subjected to differential screening.[8] Five hundred clones from each cDNA library were grown in 96-well microtiter plates on a gyratory shaker for 4 hours at room temperature. Bacterial cultures (2.5 μL) were subjected to PCR amplifications of cDNA inserts in a 25-μL reaction volume containing 0.6 U *Taq* DNA polymerase (HotStar; Qiagen), 400 μM of each dNTP, and 0.2 μM standard M13 primers, with the following program: initial denaturation step of 95°C for 15 minutes, and 25 cycles of 95°C for 15 seconds, 51°C for 30 seconds, and 72°C for 90 seconds. PCR products (2 μL) were diluted 1:25 in 0.5 M NaOH and 1.5 M NaCl, spotted on duplicate nylon membranes (ZetaProbeGT; Bio-Rad, Munich, Germany), and UV cross-linked. Two identical nylon membranes, including GAPDH cDNA for normalization, were hybridized with buffer solution and protocol (ExpressHyb; BD-Clontech), with equivalent amounts (250 ng) of ^{32}P-labeled (PrimeIt II Random Primer Labeling kit; Stratagene, Heidelberg, Germany) amplified cDNA (SMART; BD-Clontech) from either PEX or control tissues. Membranes were exposed to imaging plates and analyzed with a phosphorescence imager (BAS-2000; Fuji, Düsseldorf, Germany).

Putative differentially expressed clones were subjected to DNA sequencing with dye termination chemistry (BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Inc. [ABI], Foster City, CA) and a DNA sequencer (model 310; ABI). Database searches were performed with the Basic Local Alignment Search Tool (BLAST) algorithm.[9]

Virtual Northern Blot Analysis

Equal quantities (2 μg) of SMART-amplified cDNA from PEX and control tissues were electrophoretically separated on a 1.2% agarose gel, transferred to nylon membranes (ZetaProbeGT; Bio-Rad) in a standard capillary Southern blot protocol, and UV cross-linked. Hybridization was performed overnight with ^{32}P-labeled (PrimeIt II Random Primer Labeling kit; Stratagene) inserts of the SSH-clones with a hybridization buffer solution and protocol (ExpressHyb; BD-Clontech). To assure equal loading of lanes, each blot was hybridized with labeled GAPDH cDNA. Membranes were exposed to imaging plates and analyzed with the phosphorescence imager (BAS-2000; Fuji).

Semiquantitative RT-PCR

Total RNA (1 μg) was reverse transcribed in 20-μL reaction volumes, with 500 ng oligo dT primer and 200 U reverse transcriptase (Superscript II; Invitrogen). Primers (Table 1) were designed to anneal with sequences located in different exons, by using the program Primer 3.[10] Normalization of cDNA samples from normal and PEX tissues was performed in 25-μL PCR reaction volumes with primers for the housekeeping gene GAPDH and 2.5 μL of dilutions (1:5–1:20) of the first-strand products. Dilutions resulting in the same band intensities were used for analytic amplifications. Amplification of each of the candidate genes for differential expression was performed within the range of exponential amplification (individually tested for each gene) in 25-μL reaction volumes with normalized templates, DNA Polymerase (HotStar*Taq*; Quiagen) and a program with an initial denaturation step of 95°C for 15 minutes, and 30 to 40 cycles of 95°C for 15 seconds, 56°C (or 57°C and 58°C, respectively) for 30 seconds, and 72°C for 90 seconds. PCR products (10 μL) were analyzed in 1.2% agarose gels containing 250 ng/mL ethidium bromide. Images were captured and differences of band intensities quantified (Eagle Eye II Computerized Densitometry; Stratagene). The identity of PCR fragments was subsequently confirmed by sequence analysis (model 310 DNA sequencer; ABI).

TABLE 1. Primers Used for Semiquantitative RT-PCR

Gene	Accession No.	Product (bp)	T_{an} (°C)	Sequence (5′–3′)
AdoR-A3	NM_000677	400	56	GGC TGG AAC ATG AAA CTG AC CGA TAG GGT TCA TCA TGG AG
AKAP-2	NM_007203	516	56	CTC TTT GAG GAT GAC GAG CA TGA GTT GAC TGA CCC CAG AG
Clusterin	M74816	445	56	GAA TTG GAG GCA TGA TGA AG TTC AGG AAC TCC TCA AGC TG
Fibrillin-1	X63556	475	58	ACA TCA TTG GGG GCT ACA GG ATA TTG GAG GCA TCA GTT TCG
GAPDH	M33197	452	58	ACC ACA GTC CAT GCC ATC AC TCC ACC ACC CTG TTG CTG TA
LTBP-1	NM_000627	525	58	GAA CTG CTC AGT GGG GTG TGT GCA CTG CAG TTT CAC AGG ATC ATA
LTBP-2	NM_000428	400	58	CCC ATC CTT GAG TCT CCT TT GAG GCC ATT TCC AGG TAG TT
MAGP-1	NM_017459	262	58	ACC AGA TCG ACA ACC CAG AC CGG AGG CTG TAG AAG CAG AC
mGST-1	J03746	311	58	ATT GGC CTC CTG TAT TCC TTG TAA TTC CTC TGC TCC CCT CC
TGase-2	NM_004613	518	56	ATG TCA ACC CCA AGT TCC TG GCA GTA CGT TCC TTC GCT CT
TIMP-1	NM_003254	551	57	TGC ACC TGT GTC CCA CCC GCT ATC TGG GAC CGC AGG
TIMP-2	NM_003255	400	57	GGA AGT GGA CTC TGG AAA CG GTG CCC GTT GAT GTT CTT CT
TIMP-3	NM_003262	270	57	CCA TCA AGC AGA TGA AGA TGT ACC GGT AGT AGC AGG ACT TGA TCT TGC

T_{an}, annealing temperature.

In Situ Hybridization

In vitro transcription of the linearized constructs was performed with SP6 and T7 RNA polymerase in the presence of digoxigenin-11-uridine triphosphate (DIG-UTP) to produce DIG-labeled single-strand antisense or sense RNA probes (DIG RNA Labeling Kit; Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. The quality of the transcripts was controlled with denaturing formaldehyde-agarose gels before and after DNase I digestion. Hybridizations of 4-μm paraffin-embedded sections of six eyes with PEX syndrome and six control eyes were performed according to standard protocols. Hybridization with sense strand riboprobes served as the negative control, and hybridization with 18S rRNA probes served as the positive control.

RESULTS

SSH and Differential Screening

To identify differentially expressed genes in anterior segment tissues of patients with PEX, we used SSH, a technique that enriches the amount of cDNA fragments from transcripts that are upregulated in one population of mRNAs (tester) but that are normally expressed in another mRNA population (driver). Because of the low availability of RNA from iris, lens epithelial, and ciliary process tissues, which is usually not sufficient for complex gene expression analyses, we first generated 18 representative cDNA libraries (three PEX and three control libraries for each tissue type) by exponential cDNA amplification (SMART; BD-Clontech). Subtractions of cDNA libraries were then performed as forward (tester: cDNA from patients with PEX) and reverse (tester: cDNA from control subjects) reactions, to identify genes up- and downregulated in PEX tissues, resulting in three forward and three reverse subtracted libraries for each tissue type. Along with enrichment of upregulated transcripts, SSH excludes sequences equally expressed in tester and driver. To monitor the efficiency of each subtraction, aliquots of subtracted and nonsubtracted cDNA libraries were amplified with primers for the housekeeping gene GAPDH. A successful subtraction was indicated by the appearance at least five cycles later of the product in the subtracted sample. In all subtracted libraries, GAPDH appeared 10 cycles later, which indicates a 2^{10}-fold enrichment of differentially expressed transcripts and high performance of cDNA subtraction (Fig. 1).

To exclude false-positive results, the subtracted cDNA libraries were subjected to differential screening. The inserts of 470 clones from each of the 18 forward and reverse subtracted libraries (2820 clones for each tissue; 8460 clones in total) were PCR amplified and spotted onto duplicate nylon mem-

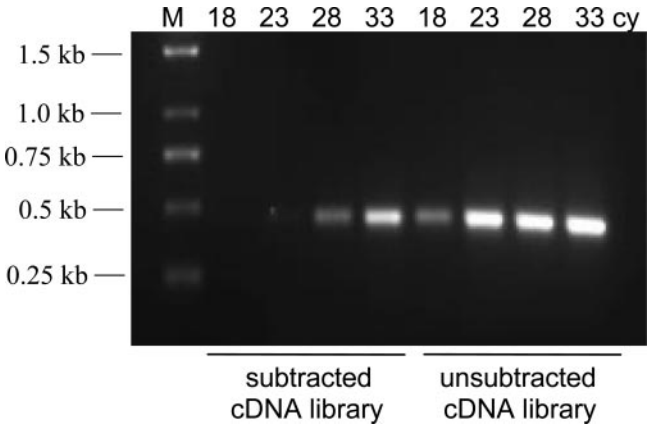


FIGURE 1. Subtraction efficiency test. Subtracted and unsubtracted cDNA libraries were subjected to PCR amplification for 18, 23, 28, and 33 cycles (cy) with primers for GAPDH. As subtractive cDNA hybridization excludes sequences equally expressed in PEX and control tissues, the later appearance (10 cycles) of PCR product in all forward and reverse subtracted libraries indicates a 2^{10}-fold enrichment of differentially expressed transcripts and a high performance of cDNA subtraction. M, molecular weight marker.

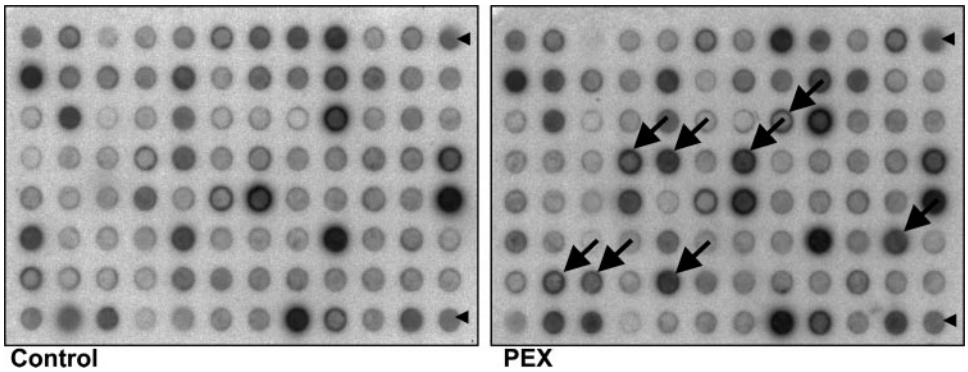


FIGURE 2. Differential screening of subtracted cDNA libraries. Approximately 8500 independent clones from 18 subtracted cDNA libraries were replica spotted to generate identical sets of arrays, which were subsequently hybridized with radioactively labeled cDNA from control and PEX tissues. Hybridization signals were normalized with GAPDH (*arrowheads*) as an internal control. Shown is one representative set of hybridized arrays with the *arrows* designating clones with increased expression in ciliary process tissue of an eye with PEX-associated angle-closure glaucoma compared to angle-closure glaucoma without PEX syndrome.

branes, and subsequently hybridized with radioactively labeled, exponentially amplified cDNA from either PEX or control tissues (Fig. 2). A large percentage of clones (7131 [84%] of 8460;) did not show differential expression by differential screening. The sequencing of the resultant 1329 clones, showing expression differences higher than twofold by differential screening, resulted in 27 different cDNAs, including gene products related to extracellular matrix turnover, stress response, and regulation. Twenty-three of these 27 cDNAs were confirmed by virtual Northern blot or RT-PCR (Table 2). One set of genes consistently upregulated in iris tissue, lens epithelium, and ciliary processes from three different patients with PEX comprised latent transforming growth factor binding proteins (*LTBP-1*) and *-2*, which are structural components of elastic microfibrils; transglutaminase 2 (*TGase-2*), an extracellular matrix cross-linking enzyme; and tissue inhibitor of matrix metalloproteinase 2 (*TIMP-2*). Another set of genes, however, showed a tissue-specific differential expression pattern: A-kinase anchor protein 2 (*AKAP-2*), which localizes protein kinase A to specific subcellular destinations, was consistently upregulated both in the iris and the lens epithelium; adenosine receptor A3 (*AdoR-A3*) and apolipoprotein D (*Apo D*) in the ciliary processes only; and clathrin, lengsin, and crystallin βA3 only in the lens epithelium of all PEX eyes examined.

Genes reproducibly downregulated in all analyzed tissues from different patients with PEX included *TIMP-1*, microsomal glutathione-*S*-transferase (*mGST-1*), serum amyloid A1 (*SAA1*), and the extracellular chaperon clusterin. Further transcripts, such as elastase, glutathione-*S*-transferase T1, integrinβ4, dehydrocholesterolreductase, and others, did not show a consistent differential expression pattern in subtracted cDNA libraries from different patients with PEX (Table 2).

Virtual Northern Blot Analysis

For an initial verification of differential expression, clones were hybridized to virtual Northern blot analysis (use of cDNA instead of RNA) containing cDNA from iris, lens epithelium, and ciliary processes of three different pairs of patients with PEX and control subjects (Fig. 3). These analyses confirmed the upregulation of *LTBP-1*, *LTBP-2*, *TGase-2*, and *TIMP-2*, as well as the downregulation of *TIMP-1*, *SAA1*, clusterin, and *mGST-1* in all tissues analyzed. Furthermore, the tissue-specific upregulation of *AdoRA3* and *ApoD* in ciliary processes and that of clathrin, lengsin, and crystallin βA3 in lens epithelium of patients with PEX was confirmed. Further transcripts (Table 2) were verified but yielded no consistent differential signal in virtual Northern blot analyses with cDNA from different patients. The hybridization of probes for *AKAP-2* to virtual Northern blot analysis yielded no signals, but its differential expression was confirmed later with other methods.

From this initial pool of differentially expressed genes, a subset of promising candidate genes related to matrix turnover (*LTBP-1*, *LTBP-2*, *TGase-2*, *TIMP-1*, and *TIMP-2*), cellular stress (*AdoRA3*, Clusterin, *mGST-1*), and regulation (*AKAP-2*), showing consistent differential expression in different patients and tissue types, was selected for further analysis. We also included several related genes (fibrillin-1, *MAGP-1*, *TIMP-3*; discussed later) in the candidate analysis, because it cannot be ruled out that certain differentially expressed gene products were not found by means of SSH.

Semiquantitative RT-PCR

The densitometric quantification of PCR band intensities after amplification from iris, lens epithelium, and ciliary process cDNA confirmed a consistent mRNA upregulation for *LTBP-1* (up to 3.4-fold), *LTBP-2* (up to 4.2-fold), *TGase-2* (up to 5.6-fold), and *TIMP-2* (up to 3.1-fold) in all PEX tissues obtained from three different patients (Fig. 4). The mRNA amount of *AKAP-2* was enhanced threefold in the iris and lens epithelium of PEX eyes, but was equally expressed in the ciliary processes of PEX and control eyes confirming the SSH findings. Expression of *AdoR-A3* was only upregulated 8.3 ±1.3-fold in the ciliary processes of patients with PEX. Semiquantitative RT-PCR further confirmed a consistent downregulation of mRNA coding for *TIMP-1* (up to 2.7-fold), clusterin (up to 3.7-fold), and *mGST-1* (up to 3.5-fold) in all PEX tissues analyzed (Fig. 4).

Although other microfibrillar components (e.g., fibrillin-1 and microfibril associated glycoprotein 1 (*MAGP-1*), are known to be major components of PEX material,[11] only LTBPs were isolated from subtracted cDNA libraries. Therefore, we decided to include fibrillin-1 and *MAGP-1* in the analysis. In fact, RT-PCR showed an up to 5.6-fold upregulation of fibrillin-1 mRNA, particularly in the ciliary processes, of all PEX eyes (Fig. 4), whereas *MAGP-1* was not differentially expressed (data not shown). *TIMP-3*, which has been previously shown to bind strongly to PEX tissue (Schlötzer-Schrehardt U, et al. *IOVS* 2002;43:ARVO E-Abstract 3369), was also included. In contrast to *TIMP-1* and *TIMP-2* (Fig. 4), *TIMP-3* showed no differential expression in PEX tissues (data not shown).

TABLE 2. Differentially Expressed Genes in Ocular Tissues of Eyes with PEX

Gene	Accession No.	Iris Tissue			Lens Epithelium			Ciliary Processes		
		A	B	C	A	B	C	A	B	C
Extracellular matrix metabolism										
Latent TGFβ binding protein 1 (*LTBP-1*)	NM_000627	+3	+2	+3	+2	+3	+2	+3	+3	+4
Latent TGFβ binding protein 2 (*LTBP-2*)	NM_000428	+4	+2	+4	+3	+2	+3	+3	+4	+2
Transglutaminase 2 (*TGase-2*)	NM_004613	+4	+3	+6	+3	+4	+3	+3	+3	+3
Tissue inhibitor of MMP 1 (*TIMP-1*)	NM_003254	−4	−2	−3	−3	−2	−2	−2	−2	−2
Tissue inhibitor of MMP 2 (*TIMP-2*)	NM_003255	+2	+3	+3	+4	+2	+3	+3	+3	+4
Elastase	NM_002777	EE	−2	EE	EE	−2	EE	EE	−2	EE
Cellular stress										
Adenosine receptor A3 (*AdoR-A3*)	X76981	NE	NE	NE	NE	NE	NE	+9	+6	+8
Clusterin	M74816	−4	−2	−4	−2	−2	−2	−4	−3	−4
Glutathione-*S*-transferase (*GST-T1*)	X79389	EE	−2	+2	EE	EE	−2	EE	+3	−3
Microsomal GST-1 (*mGST-1*)	J03746	−2	−2	−3	−2	−2	−3	−4	−3	−4
Serum amyloid A1 (*SAA1*)	M26152	−11	−5	EE	−2	−3	EE	−2	−2	EE
Regulatory gene product										
A-kinase anchor protein 2 (*AKAP-2*)	NM_007203	+3	+3	+3	+4	+2	+3	EE	EE	EE
Integrin β4	X53578	+2	−2	+3	EE	+2	+2	EE	EE	−2
Clathrin	X95487	EE	+2	−3	+3	+2	+2	EE	EE	EE
Other										
Apolipoprotein D (*ApoD*)	J02611	+3	EE	+4	+2	EE	EE	+3	+3	+4
Dehydrocholesterolreduktase (*DHCR*)	AF062481	+15	+7	EE	+4	EE	EE	+5	+3	EE
Lengsin	AF242388	NE	NE	NE	+16	+9	+4	NE	NE	NE
Crystallin βA3 (*CrybA3*)	M14302	NE	NE	NE	+4	+4	+2	NE	NE	NE
Crystallin γD3 (*CryγD3*)	U66583	NE	NE	NE	+5	EE	EE	NE	NE	NE
Crystallin γS (CryγS)	AF161703	NE	NE	NE	+4	EE	−2	NE	NE	NE
Clone L166 (expressed sequence tag)	CD675761	EE	EE	EE	+4	+3	−2	NE	NE	NE
Clone L260 (expressed sequence tag)	BG034301	EE	EE	EE	+25	+7	−3	NE	NE	NE
Clone L543 (expressed sequence tag)	BU686492	NE	NE	NE	+3	EE	EE	NE	NE	NE

Data were collected after cDNA subtraction and differential screening of three individual pairs (A, B, C) of PEX and control tissues and are expressed as the approximate level of differential expression in PEX syndrome (*x*-fold change). +, enhanced; −, reduced expression in PEX tissues; EE, equally expressed; NE, not expressed.

In Situ Hybridization

To verify further and localize the differential expression of potentially disease-related genes in the anterior segment tissues analyzed, we performed in situ hybridizations with six PEX and six control eyes. Signals for fibrillin-1, *LTBP-1*, and *TGase-2* showed a largely equal distribution in anterior segment tissues and these signals were detected in the preequatorial lens epithelium, in vascular endothelial cells, stromal cells, and smooth muscle cells of the iris (Figs. 5A, 5B, 5E, 5F) as well as in the nonpigmented epithelium (Figs. 5C, 5D) and muscle cells of the ciliary body of both normal and PEX eyes. Signals for these genes, however, were generally more pronounced in all PEX eyes, particularly in the nonpigmented epithelium of the ciliary processes and in iridal stromal and vascular endothelial cells (Figs. 5A–F).

TIMP-1 mRNA was found to be expressed in both normal and PEX eyes in the preequatorial lens epithelium, in the nonpigmented ciliary epithelium and ciliary muscle cells, and in iridal stroma and iris dilator muscle cells (Figs. 5G, 5H). Expression of *TIMP-2* mRNA was less frequently observed in the nonpigmented ciliary epithelium (Figs. 5I, 5J), in iridal and ciliary stroma cells, and in ciliary muscle cells. Signals for *TIMP-1* were weaker in all anterior segment tissues of PEX eyes, but signals for *TIMP-2* were generally more pronounced in PEX tissues, particularly in the nonpigmented epithelium of the ciliary processes (Figs. 5G–J).

In situ hybridizations with the antisense probe for clusterin resulted in strong signals in most tissues and cell types of the anterior segment, particularly in the ciliary epithelium, with distinctly weaker expression levels in PEX eyes (Figs. 5K, 5L). *AdoR-A3* mRNA was found to be mainly expressed in cells of the nonpigmented ciliary epithelium and was confirmed to be markedly increased in PEX eyes compared with control eyes (Figs. 5M, 5N). In addition, weaker signals for *AdoR-A3* mRNA were detected in walls of iridal blood vessels, in dilator and sphincter muscles of the iris, and in ciliary muscle cells of PEX and control eyes. Weak expression of *AKAP-2* mRNA was localized to the epithelia of ciliary body and lens, to ciliary and iridal muscle cells, and iridal stromal cells of both normal and PEX eyes. Moderately increased signals were observed in the ciliary epithelium and iridal vessel walls of PEX eyes (Figs. 5O, 5P).

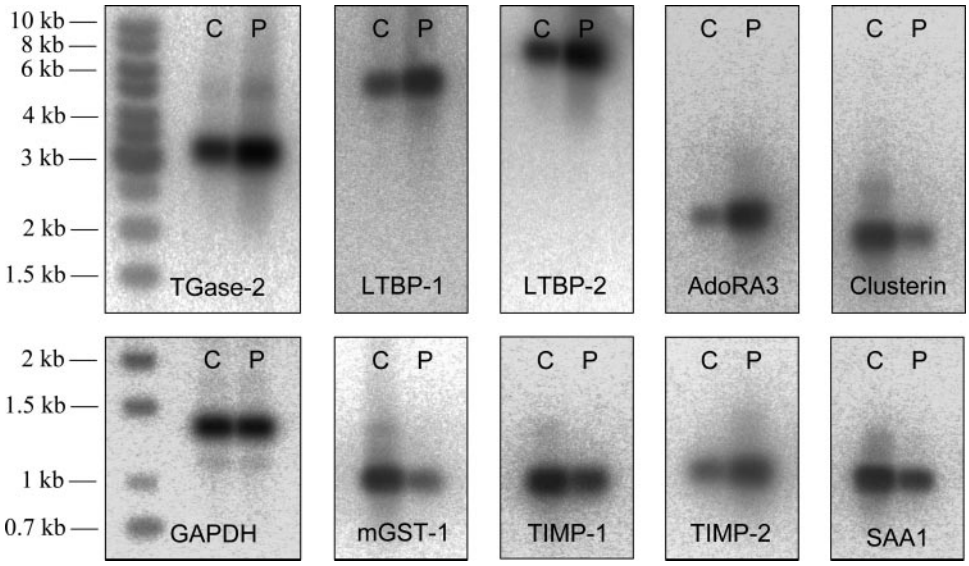


FIGURE 3. Confirmation of differential expression by means of virtual Northern blot analysis. Clones from the subtracted libraries were hybridized to Southern blots containing equal amounts (2 μg) of PCR-amplified cDNA from PEX (P) and control (C) tissues. For example, *TGase-2*, *LTBP-1* and *-2*, *AdoR-A3*, and *TIMP-2* were upregulated, whereas clusterin, *mGST-1*, *TIMP-1*, and *SAA1* were downregulated in the ciliary processes of eyes with PEX-associated open-angle glaucoma compared with specimens with open-angle glaucoma without PEX. A clone for the housekeeping gene *GAPDH*, which exhibits identical expression levels in PEX and control, was used for normalization.

Hybridizations with sense RNA probes served as negative controls and yielded no signals (Figs. 5Q, 5R), while hybridization with 18S rRNA antisense probes served as positive controls and yielded strong, ubiquitous, and equally distributed signals in all tissue sections (data not shown).

Discussion

PEX syndrome is an age-related, generalized disorder of the extracellular matrix characterized by the intra- and extraocular formation and progressive accumulation of abnormal fibrillar material, which may be either the result of an excessive production or an insufficient degradation.[4,5] The characteristic fibrils, which are composed of microfibrillar subunits surrounded by an amorphous matrix, contain predominantly epitopes of elastic fibers, such as elastin, tropoelastin, amyloid P, vitronectin, and components of elastic microfibrils, such as fibrillin-1, MAGP-1, LTBP-1, and LTBP-2 by immunohistochemistry.[5] This fibrillar matrix product appears to be primarily produced by the preequatorial lens epithelium, the nonpigmented ciliary epithelium, and the posterior iris pigment epithelium, whereas the trabecular cells, corneal endothelial cells, and vascular endothelial and smooth muscle cells of the iris have also been implicated as being contributory.[4] Increased growth factor activity, particularly of transforming growth factor (TGF)-β1,[12–14] proteolytic enzymes, and their inhibitors,[14,15] increased oxidative stress,[16,17] and anterior chamber hypoxia[18] have been suggested to be involved in the pathobiology, although the exact pathogenesis and etiology of PEX syndrome have still not been elucidated.

This study represents the first attempt to identify differentially expressed genes in anterior segment tissues from patients with PEX syndrome compared with those without PEX. We identified and extensively verified several differentially expressed genes, with a high level of reproducibility in different tissues and different patients, that are involved in extracellular matrix metabolism and cross-linkage as well as in cellular stress and regulation.

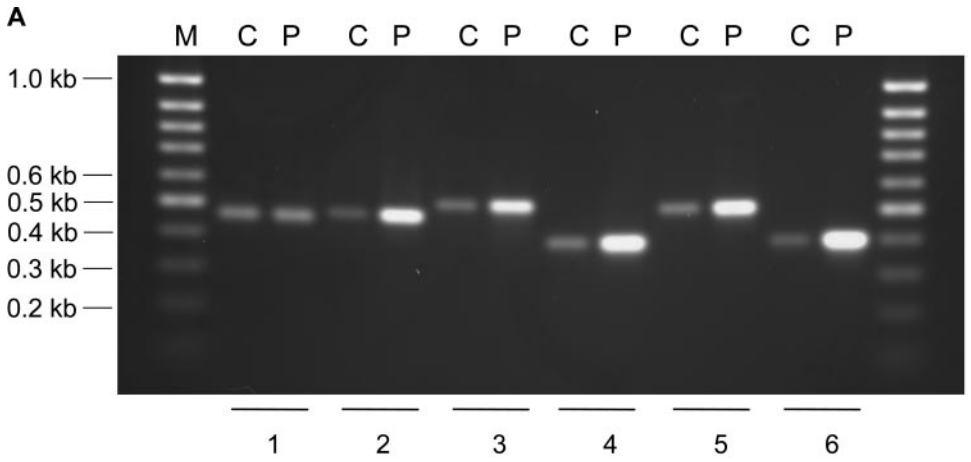
Genes Related to Extracellular Matrix Metabolism

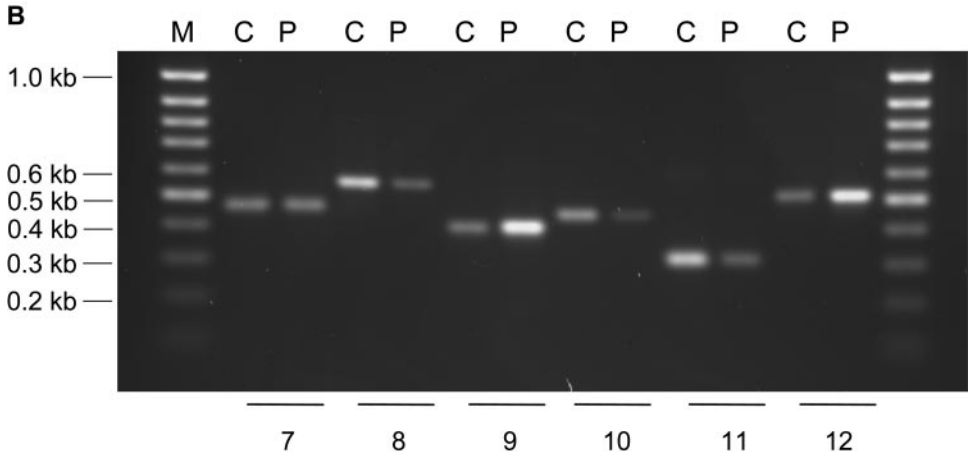
Our study provided evidence of overexpression of the elastic microfibrillar components fibrillin-1, *LTBP-1* and *LTBP-2* in ciliary processes, iris tissue, and lens epithelium of PEX eyes. Elastic microfibrils, 10 to 12 nm in diameter, are distinct structural elements of connective tissues throughout the body, including all ocular tissues,[11] where they form a scaffold for tropoelastin deposition and elastic fiber formation, contribute to the elastic and biomechanical properties of tissues, and provide anchorage between cells and matrix structures.[19] Elastic microfibrils represent complex assemblies of several glycoproteins with fibrillins constituting their structural backbone.[20] Fibrillin-1, a large (350-kDa) glycoprotein, has been also shown to represent an intrinsic component of PEX fibers by immunohistochemistry.[11,21]

The LTBPs are a family of glycoproteins that show pronounced structural similarities with fibrillins.[22] LTBPs have been found to serve as a vehicle for secretion of latent TGF-β and to target latent TGF-β to the extracellular matrix.[23] In addition, LTBPs have been shown to be structural components of fibrillin-containing microfibrils.[24] LTBP-1 and -2 have also been demonstrated to constitute integral components of PEX fibers, binding latent TGF-β1 to the abnormal matrix product.[12] Thus, LTBP-containing PEX fibers potentially play a role in storage, presentation, and activation of TGF-β1 in the extracellular matrix of PEX eyes.

The present findings provide evidence that overexpression, excessive production, and abnormal aggregation of fibrillin-containing elastic microfibrils represent pivotal processes in the pathogenesis of PEX syndrome, as has been shown to occur in other fibrotic diseases.[25] Thus, the findings give strong support to the elastic microfibril theory of pathogenesis of PEX syndrome, which was first proposed by Streeten et al.[21] on the basis of histochemical similarities between PEX and zonular fibers. Aggregated elastic microfibrils appear to form the protein core of PEX fibrils,[5] which may serve as a scaffold for secondary interaction with other matrix components, such as elastic fiber and basement membrane components, which may become incorporated into the mature PEX fiber aggregates.

Covalent cross-linking of proteins is an important mechanism for stabilization of extracellular matrix molecules and contributes to their mechanical strength and resistance to enzymatic degradation. The TGase gene family catalyzes the posttranslational modification of proteins through formation of covalent intra- and intermolecular ε-(γ-glutaminyl)-lysine bonds.[26] TGase-2, also termed tissue-type TGase, is the most widespread member of this family and has been found to be the major cross-linker and stabilizer of ocular connective tissues.[27] An increase in TGase activity has been shown in several ocular fibrotic conditions that are accompanied by an increase





C

Gene Product	Iris Tissue	Lens Epithelium	Ciliary Processes
Fibrillin-1 (2)	2.6 ± 0.5	2.3 ± 0.7	5.6 ± 1.4
LTBP-1 (3)	2.7 ± 0.5	2.6 ± 0.4	3.4 ± 0.6
LTBP-2 (4)	3.6 ± 0.9	3.2 ± 0.5	4.2 ± 1.0
TGase-2 (5)	5.6 ± 1.1	4.1 ± 1.5	3.3 ± 0.6
AdoRA3 (6)	ne	ne	8.3 ± 1.3
TIMP-1 (8)	-2.7 ± 0.2	-2.1 ± 0.2	-1.9 ± 0.2
TIMP-2 (9)	2.6 ± 0.7	3.1 ± 0.6	3.1 ± 0.5
Clusterin (10)	-3.1 ± 0.7	-1.5 ± 0.4	-3.7 ± 0.3
mGST-1 (11)	-2.3 ± 0.3	-2.2 ± 0.4	-3.5 ± 0.6
AKAP-2 (12)	3.0 ± 0.3	2.9 ± 0.7	1.1 ± 0.2

FIGURE 4. Confirmation of differential expression by means of semiquantitative RT-PCR. (**A**, **B**) RT-PCR products of total RNA from ciliary process (**A**) and iris tissue (**B**) of an eye with PEX-associated open-angle glaucoma compared with open-angle glaucoma without PEX syndrome. Lane numbers cover two lanes, each indicating the amplification from control (C) and PEX (P) cDNAs: *lane 1*: *GAPDH*; *lane 2*: fibrillin-1; *lane 3*: *LTBP-1*; *lane 4*: *LTBP-2*; *lane 5*: *TGase-2*; *lane 6*: *AdoRA3*; *lane 7*: *GAPDH*; *lane 8*: *TIMP-1*; *lane 9*: *TIMP-2*; *lane 10*: clusterin; *lane 11*: *mGST-1*; *lane 12*: *AKAP-2*. *Lane M*: 600 ng of low-range DNA-ladder. (**C**) Intensities of the PCR bands were quantified by computerized densitometry. The densitometric data are the mean ± SD of separate experiments ($n = 3$) and indicate the relative expression difference of genes in PEX samples compared with control samples, normalized to *GAPDH*. Positive values, enhanced expression; negative values, reduced expression in PEX; The numbers next to each gene product correspond to the lane numbers in (**A**) and (**B**). ne, not expressed.

in cross-linked matrix proteins, such as proliferative vitreoretinopathy.[28]

By differential expression analyses, we identified a strong upregulation of *TGase-2* in all PEX tissues examined. Known substrates for TGase-2 include the elastic microfibrillar components fibrillin-1, LTBP-1, LTBP-2, and MAGP-1; the basement membrane components laminin, nidogen, fibronectin, and vitronectin; and apolipoproteins A and B,[26,29,30] all of which have been shown to be present in the extracellular PEX material.[5] One of the most characteristic biochemical features of PEX material is its resistance to degradation by most enzymes including collagenase, trypsin, pepsin, and papain.[31] Therefore, TGase-2 may essentially contribute to the stabilization and enzymatic resistance of the newly produced PEX fibrils and their stable accumulation in tissues over time. TGase-2's effects may be further enhanced by the oxidative microenvironment in the anterior chamber of PEX eyes,[17] since oxidized proteins are known to be better substrates for TGase 2-mediated cross-linking.[32]

Extracellular matrix metabolism and turnover is also greatly influenced by the large family of MMPs and their endogenous inhibitors TIMPs.[33] TIMP-1 controls the activity of most MMPs,

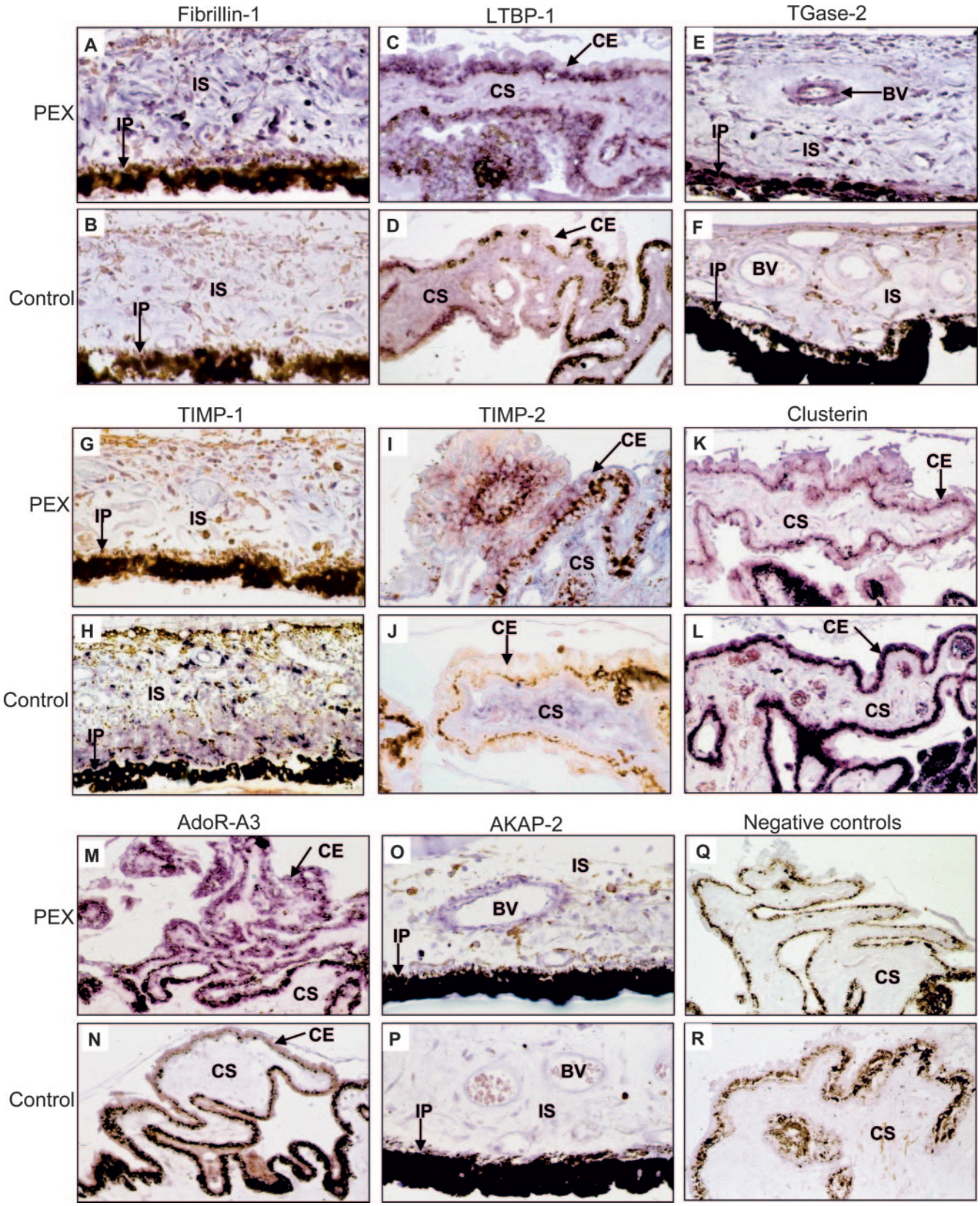


FIGURE 5. Differentially expressed genes in tissues from PEX and control eyes with open-angle glaucoma, as detected by in situ hybridization. Positive signals are indicated by *purple–blue* staining in contrast with the *brownish* lipofuscin and *black* melanin pigment of the cells. (**A**, **B**) Differential localization of fibrillin-1 mRNA to iridal stromal cells. (**C**, **D**) Differential expression of *LTBP-1* mRNA in the ciliary epithelium. (**E**, **F**) Detection of *TGase-2* mRNA in iridal vessels of PEX eyes. (**G**, **H**) Decreased signals for *TIMP-1* mRNA in iridal stromal cells of PEX eyes. (**I**, **J**) Increased signals for *TIMP-2* mRNA in the ciliary epithelium of PEX eyes. (**K**, **L**) Differential expression of clusterin mRNA in the ciliary epithelium. (**M**, **N**) Pronounced expression of *AdoR-A3* mRNA in the ciliary epithelium of PEX eyes. (**O**, **P**) Increased signals for *AKAP-2* mRNA in iridal vessels of PEX eyes. (**Q**, **R**) Negative controls with the *AKAP-2* (**Q**) and clusterin (**R**) sense probes. BV, blood vessel; CE, ciliary epithelium; CS, ciliary stroma; IP, iris pigment epithelium; IS, iris stroma. Original magnification, ×100.

in particular MMP-1, whereas TIMP-2 is the major inhibitor of MMP-2. Dysregulated expression of MMPs and TIMPs has been implicated in many disease processes accompanied by abnormal matrix production.[34] Aqueous humor from patients with PEX had higher levels of MMP-2 and -3 as well as TIMP-1 and -2, compared with control patients.[14,15] However, levels of endogenously active MMP-2, which is the major MMP in human aqueous humor, were significantly decreased, as was the ratio of MMP-2 to TIMP-2.[15] These findings suggest that an excess of TIMP-2 over MMP-2 and a reduced MMP-2 activity in the aqueous humor may promote abnormal matrix accumulation in anterior segment tissues due to impaired matrix turnover. In agreement with this hypothesis, we observed a dysregulation of *TIMP-1* and *TIMP-2* mRNA, but not of *TIMP-3* mRNA, in all PEX tissues examined in the present study. These additional data suggest that the enhanced aqueous protein concentration of TIMP-2 results from an upregulation and increased local production of TIMP-2 by anterior segment tissues. In contrast, a positive correlation of TIMP-1 with total aqueous protein concentration[15] is indicative of a passive influx of TIMP-1 through an impaired blood–aqueous barrier, which may account for a compensatory downregulation of TIMP-1 mRNA expression in PEX tissues.

Genes Related to Cellular Stress and Regulation

Adenosine and its receptors (A1, A2a, A2b, and A3) have been shown to be expressed in ocular tissues and to be involved, among other functions, in the regulation of aqueous humor secretion and intraocular pressure.[35] In particular, the A3 receptor subtype has been shown to activate chloride channels in nonpigmented ciliary epithelial cells in vitro and to increase aqueous humor secretion and intraocular pressure on activation in vivo.[36] Moreover, in A3 receptor knockout mice, baseline intraocular pressure was significantly lower than in wild-type animals.[37] Further along this line, topical administration of selective A3 receptor antagonists was recently reported to reduce intraocular pressure in monkey eyes.[38] These observations support the conclusion that adenosine acts mainly through A3 adenosine receptors to regulate intraocular pressure.

Apart from an involvement in intraocular pressure modulation, adenosine receptors have been implicated in systemic and ocular ischemic diseases and in conditions associated with oxidative stress.[39] In metabolic stress conditions, like hypoxia or ischemia, adenosine is increasingly released by cells to mediate cytoprotection against ischemia-induced cell damage. Increasing evidence suggests that adenosine mediates its cytoprotective actions mainly by interacting with A3 receptors. Activation of the A3 receptor is known to activate the cellular antioxidant defense system by increasing the activities of many antioxidant enzymes.[39]

The present findings provide evidence of a significant upregulation of the A3 adenosine receptor mRNA in the nonpigmented ciliary epithelium of eyes with PEX glaucoma. In another study, we were further able to demonstrate, that this upregulation was selective for the A3 receptor subtype and independent of the presence of glaucoma, and that it could be induced in vitro by both hypoxia and oxidative stress.[40] Together, these findings suggest that hypoxia and/or oxidative stress, typical of PEX eyes,[17,18] promote a selective upregulation of A3 adenosine receptors in the ciliary epithelium, which might confer protection against ischemic or oxidative damage. Considering, however, the known role of the A3 receptor in modulating aqueous humor secretion,[35–38] its upregulation may also be a means to counteract an assumed functional deficit of the ciliary epithelium, which is heavily involved in PEX material production,[4,5] to maintain a rather normal rate of aqueous humor formation.

Glutathione-*S*-transferases (GSTs) are a family of detoxification enzymes that catalyze the conjugation of reduced glutathione with a wide variety of reactive compounds and products of oxidative stress.[41] In this regard, the GST isoenzymes play a pivotal role in protection from oxidative damage. A protective role of GST isoenzymes against lipid peroxidation in ocular tissues has been implied—for instance, in cataractogenesis and retinal degeneration.[42] Whereas most GST isoenzymes are located in the cytosol, microsomal GSTs (mGST) are bound to the cell membrane and protect against oxidative insult to the cell membrane.[43] Microsomal GST-1 has been found to be highly expressed in murine retinal pigment epithelium exerting antioxidant functions in vitro and in vivo.[44] Its gradual decline with age was suggested to play a role in the development of age-related retinal diseases associated with oxidative injury, such as age-related macular degeneration.

Microsomal GST-1 was found to be consistently downregulated in iris, lens epithelium, and ciliary processes of patients with PEX. The reduced expression in PEX tissues seems to confirm earlier reports suggesting that protective mechanisms against oxidative stress may be impaired in PEX eyes and may account for the significantly enhanced concentration of 8-iso-prostaglandin $F_{2\alpha}$, a marker of oxidative stress, in the aqueous humor of PEX eyes.[17] Because mGST-1 activity is known to reduce phospholipid hydroperoxides (such as linoleic or arachidonic acid hydroperoxides,[43] the precursor stage of 8-iso-prostaglandin $F_{2\alpha}$), its decreased expression in anterior segment tissues may account for the increased aqueous concentrations of this isoprostane and contribute to increased oxidative stress in PEX eyes.

Clusterin, also termed apolipoprotein J, is a multifunctional, secreted, 80-kDa glycoprotein that is constitutively produced by almost all cell types, mainly at fluid–tissue boundaries, and is found in all body fluids.[45] In the human eye, clusterin has been shown to be present in the retina, cornea, and ciliary body as well as in the aqueous and vitreous humors.[46] Under cellular stress conditions, such as heat shock, hypoxia, and oxidative stress, its expression is strongly induced in vivo and in vitro.[45] Mechanisms, by which clusterin exerts its cytoprotective effects include the inhibition of the complement system, protection of cell membranes, stabilization of cell–cell and cell–matrix contacts, and inhibition of stress-induced precipitation and aggregation of misfolded proteins by acting as an extracellular chaperone.[47] In amyloid disorders, such as Alzheimer's disease, clusterin has been shown to prevent the conversion of the amyloid-β peptide to insoluble forms and their aggregation into fibrillar deposits.[48]

Even in the presence of existing stress conditions in the anterior segment,[17,18] clusterin has been found to be consistently downregulated in anterior segment tissues of PEX eyes. This reduced expression further supports the notion that cellular protection systems may be impaired in PEX eyes. The dysregulation in clusterin expression may contribute to the degeneration of membranes of cells involved in PEX fiber production[5] and may promote the abnormal aggregation of PEX fibers in the extracellular space. In view of the reduced expression of the molecular chaperone clusterin, accumulation of the pathologic matrix product in PEX eyes may partly arise from protein misfolding and deposition of insoluble protein aggregates.

AKAPs target protein kinase A to different subcellular locations, to ensure specificity of signal transduction, and are thought to play a major role in the cAMP signaling pathway.[49] To our knowledge, this is the first report demonstrating the expression and localization of *AKAP-2* in the human eye. Moreover, we identified enhanced expression of *AKAP-2* mRNA in iris and lens epithelium of patients with PEX. Because detailed information on the subcellular localization, interacting pro-

teins, and effects of AKAP-2 is still scarce, speculation about its functional significance in ocular tissues and its upregulation in PEX tissues have to await further investigations.

Pathogenetic Concept

Although not detected by the present differential approach, the growth factor TGF-β1 has been shown to be significantly elevated in the aqueous humor and to be upregulated in anterior segment tissues of patients with PEX.[12,13] TGF-β1 is known to regulate most of the genes found to be differentially expressed in PEX eyes and is therefore considered a key mediator in the fibrotic PEX process. For instance, TGF-β1 has been demonstrated to enhance expression of fibrillin-1,[12] LTBP-1 and -2,[50] and TGase-2,[51] and to suppress clusterin mRNA and protein expression in vitro.[52]

Based on the present and previous findings, PEX syndrome may be described as a specific type of stress-induced elastosis, an elastic microfibrillopathy, associated with the excessive production of elastic microfibrils and their aggregation into typical mature PEX fibers by a variety of cell types. Abnormal glycosylation processes may take place, and other extracellular matrix components, such as basement membrane components derived from ruptured basement membranes, may interact and become secondarily incorporated into the composite PEX fibers. Growth factors, particularly TGF-β1, increased cellular and oxidative stress, an impaired cellular stress response, and possibly a stable aggregation of misfolded stressed proteins appear to be involved in this fibrotic matrix process. Because of an imbalance of MMPs and TIMPs and extensive cross-linking processes involved in PEX fiber formation and stabilization, the newly formed pathologic material is not properly degraded but progressively accumulates within tissues over time, with potentially deleterious effects, such as the development of glaucoma.

Work in progress focuses on differential gene expression analyses of tissues obtained from early disease stages in the absence of glaucoma, to provide clues to the initial molecular pathogenetic processes in PEX syndrome.

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