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Novel protein constituents of pathological ocular pseudoexfoliation syndrome deposits identified with mass spectrometry

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Purpose: Pseudoexfoliation (PEX) syndrome is an age-related progressive disease of the extracellular matrix with ocular manifestations. PEX is clinically diagnosed by the presence of extracellular exfoliative deposits on the anterior surface of the ocular lens. PEX syndrome is a major risk factor for developing glaucoma, the leading cause of irreversible blindness in the world, and is often associated with the development of cataract. PEX reportedly coexists with Alzheimer disease and increases the risk of heart disease and stroke. PEX material deposited on the anterior surface of the ocular lens is highly proteinaceous, complex, and insoluble, making deciphering the protein composition of the material challenging. Thus, to date, only a small proportion of the protein composition of PEX material is known. The aim of this study was to decipher the protein composition of pathological PEX material deposited on the ocular lens in patients and advance the understanding of pathophysiology of PEX syndrome.

Methods: Liquid-chromatography and tandem mass spectrometry (LC-MS/MS) was employed to discover novel proteins in extracts of neat PEX material surgically isolated from patients (n = 4) with PEX syndrome undergoing cataract surgery. A sub-set of the identified proteins was validated with immunohistochemistry using lens capsule specimens from independent patients (n=3); lens capsules from patients with cataract but without PEX syndrome were used as controls (n=4). Expression of transcripts of the validated proteins in the human lens epithelium was analyzed with reverse transcription PCR (RT–PCR). Functional relationships among the proteins identified in this study and genes and proteins previously implicated in the disease were bioinformatically determined using InnateDB.

Results: Peptides corresponding to 66 proteins, including ten proteins previously known to be present in PEX material, were identified. Thirteen newly identified proteins were chosen for validation. Of those proteins, 12 were found to be genuine components of the material. The novel protein constituents include apolipoproteins (APOA1 and APOA4), stress response proteins (CRYAA and PRDX2), and blood-related proteins (fibrinogen and hemoglobin subunits), including iron-free hemoglobin. The gene expression data suggest that the identified stress-response proteins and hemoglobin are contributed by the lens epithelium and apolipoproteins and fibrinogen by the aqueous humor to the PEX material. Pathway analysis of the identified novel protein constituents and genes or proteins previously implicated in the disease reiterated the involvement of extracellular matrix organization and degradation, elastic fiber formation, and complement cascade in PEX syndrome. Network analysis suggested a central role of fibronectin in the pathophysiology of the disease. The identified novel protein constituents of PEX material also shed light on the molecular basis of the association of PEX syndrome with heart disease, stroke, and Alzheimer disease.

Conclusions: This study expands the understanding of the protein composition of pathological PEX material deposited on the ocular lens in patients with PEX syndrome and provides useful insights into the pathophysiology of this disease. This study together with the previous study by our group (Sharma et al. Experimental Eye Research 2009;89(4):479–85) demonstrate that using neat PEX material, devoid of the underlying lens capsule, for proteomics analysis is an effective approach for deciphering the protein composition of complex and highly insoluble extracellular pathological ocular deposits present in patients with PEX syndrome.

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Pseudoexfoliation (PEX) syndrome is an age-related systemic disease of the extracellular matrix. Clinically, PEX is characterized by the presence of abnormal fibrillar deposits, referred to as PEX material, on the anterior surface of the ocular lens. Histologically, pathological PEX deposits are present in all the aqueous bathed tissues of the anterior ocular segment [1]. These deposits compromise aqueous humor outflow that often leads to increased intraocular

pressure, which, if left untreated, can lead to vision loss as a result of glaucomatous optic neuropathy. PEX syndrome is a leading risk factor for glaucoma, the most common cause of irreversible blindness worldwide. Cataract, the leading cause of blindness in the world, is also strongly associated with this disease. Pathological deposits have been also reported in systemic organs, such as blood vessels, skin, heart, lung, liver, and cerebral meninges, in patients with PEX syndrome and are likely related to increased risk of cardiovascular and cerebrovascular diseases, hearing loss, and possibly coexistence of Alzheimer disease (AD) [2-7].

PEX deposits are composed of a complex highly proteinaceous material. Some of the component proteins identified to date include extracellular matrix (ECM) proteins (laminin, fibronectin, vitronectin, entactin/nidogen, elastin, fibrillin-1, fibulin-2, and serum amyloid P component, proteoglycan core proteins versican and syndecan-3, and proteoglycans heparan sulfate and chondroitin sulfate) and remodeling enzymes (TIMP3, ADAM-19, ADAM-20, and ADAMTS-8), complement proteins (C1q, C3c, and C4c), cell adhesion molecule desmocollin-2, stress response protein clusterin, transforming growth factor-β1 (TGF-β1), and its binding proteins (LTBP-1 and LTBP-2) [8]. Correspondingly, genes encoding elastic fiber components, latent TGF-β1 binding proteins, ECM remodeling enzymes, ECM cross-linking proteins, and cellular stress proteins are dysregulated in the anterior ocular segment tissues of PEX-affected individuals compared to healthy individuals [9]. Thus, increased ECM production or reduced turnover, inflammation, and oxidative stress are thought to contribute to pathogenesis of the disease.

Clustering of the disease in families indicates the involvement of genetic factors. Consistently, genetic variants in several genes have been found to be associated with the disease risk in various populations in the world. Common single nucleotide polymorphisms (SNPs) in the lysyl oxidase like-1 (*LOXL1*; Gene ID 4016, OMIM 153456) gene are the strongest associated genetic risk factor for PEX syndrome in populations worldwide [10-14]. SNPs in the clusterin (*CLU*; Gene ID 1191, OMIM 185430) gene are nominally associated with the disease in Australian, German, and Indian cases [15-17], and SNPs in the contactin associated protein-like 2 (*CNTNAP2*; Gene ID 26047, OMIM 604569) gene in German cases [18]. Association of an SNP in the calcium channel, voltage-dependent, P/Q type, alpha 1A subunit (*CACNA1A*; Gene ID 773, OMIM 601011) gene has been reported in populations from multiple ethnicities [19] and of SNPs at loci near proteasome maturation protein (*POMP*; Gene ID 51371, OMIM 613386), transemembrane protein 136 (*TMEM136*; Gene ID 219902), 1-acylglycerol-3-phosphate O-acyltransferase 1 (*AGPAT1*; Gene ID 10554, OMIM 603099), RNA binding motif single stranded interacting protein 3 (*RBMS3*; Gene ID 27303, OMIM 605786), and semaphorin 6A (*SEMA6A*; Gene ID 57556, OMIM 605885) genes, in cases from populations across the world [20]. SNPs in olfactory receptor family 11 subfamily L member 1 (*OR11L1*; Gene ID 391189), CD80 molecule (*CD80;* Gene ID 941, OMIM 112203), TRAF2 and NCK interacting kinase (*TNIK*; Gene ID 23043, OMIM 610005), cell adhesion molecule 2 (*CADM2*; Gene ID 253559, OMIM 609938), sorbin and SH3 domain containing 2 (*SORBS2*; Gene ID 8470, OMIM 616349), ring finger protein 180 (*RNF180* Gene ID 285671, OMIM 616015), fibroblast growth factor 14 (*FGF14*; Gene ID 2259, OMIM 601515), formin 1 (*FMN1*; Gene ID 342184, OMIM 136535), and RNA binding fox-1 homolog 1 (*RBFOX1*; Gene ID 54715, OMIM 605104) genes have been reported to be associated with PEX syndrome without glaucoma in Polish cases [21]. Despite the identification of several genetic risk factors, the mechanisms underlying their involvement in the disease remain poorly understood although some studies suggest a regulatory role of associated variants in *LOXL1* in the expression of this gene in affected ocular tissues [22]. Moreover, the identified genetic variants do not explain the entire disease burden or the difference in disease prevalence between populations indicating that the genetics approach alone is not sufficient to understand the pathogenesis of this disease.

Interestingly, our group and other groups found that LOXL1 and CLU proteins are present in pathological PEX deposits [23-25]. We also found that a disease-associated coding SNP in *LOXL1* affects extracellular cleavage of the encoded protein that may affect protein activity in carrier individuals [26]. Similarly, a rare protective coding SNP in *LOXL1* has been shown to increase extracellular deposition of elastin and fibrillin-1 that may prevent stiffening of ocular tissues seen in patients with PEX syndrome [20]. Therefore, deciphering the composition of PEX material can advance the understanding of pathophysiology of the disease. Thus, the aim of this study was to identify novel protein constituents of pathological PEX material to advance the understanding of its composition, and pathophysiology of the disease.

The current understanding of the protein composition of PEX material has been gained through immunolabeling studies performed on affected anterior lens capsules for proteins of interest and from a mass spectrometry-based proteomic analysis of affected and unaffected anterior lens capsules [1,8]. The former approach was limited to identifying predetermined proteins, and the latter, in addition to identifying PEX material proteins, identified contaminating

lens capsule proteins. Thus to overcome these limitations, we developed a novel analytical approach for identifying as yet undiscovered protein components of PEX material that involves mass spectrometry of protein extracts of neat PEX material isolated from affected eyes; the neat PEX material is surgically isolated from the anterior surface of the ocular lens during cataract surgery before capsulorhexis [23]. This approach avoids contamination of PEX material by lens capsule proteins and facilitates efficient identification of genuine components of the material. Using this approach, we previously reported identification of LOXL1 and for the first time, apolipoprotein E (APOE) in PEX material [23]. Using the same approach, we report identification of several novel protein constituents of PEX material and demonstrate its utility for deciphering the protein composition of this complex and highly insoluble pathological material. The study expands understanding of the composition of PEX material and provides insight into the pathophysiology of PEX syndrome. This study also sheds light on the molecular basis of association of PEX syndrome with heart disease, stroke, and AD.

METHODS

Specimens: Collection of surgical specimens and human tissues used in this study was approved by the Flinders University Human Research Ethics Committee, Adelaide, Australia; surgical specimens were collected without informed consent because they are routinely discarded after surgery; verbal informed consent was obtained from next-of-kin for collection of tissues from deceased donors. The samples were collected following the approved ethical guidelines and the tenets of the revised Declaration of Helsinki. The study adhered to the Association for Research in Vision and Ophthalmology statement on the use of human subjects for research. PEX material deposited on the anterior lens capsule of four patients severely affected with PEX syndrome and undergoing cataract surgery was removed during surgery before capsulorhexis and stored as previously described [23] for later protein analysis. The first patient, a 77-year-old female, presented with bilateral mixed cortical and nuclear cataracts and reduced visual acuity, and was a glaucoma suspect based on asymmetry of the vertical cup-to-disc ratio (VCDR). Intraocular pressure (IOP) was 21 mmHg in the right eye and 14 mmHg in the left eye. VCDR was 0.65 in the right eye and 0.4 in the left eye. Automated perimetry was normal in both eyes. Abundant PEX material was noted on both anterior lens capsules. She had cataract surgery in both eyes with good visual outcome. The second patient, an 84-year-old female, presented with moderately advanced pseudoexfoliative glaucoma on treatment with topical latanoprost, with bilateral superior visual field loss. Visual acuity was markedly reduced in the left eye at count fingers consistent with advanced nuclear and cortical cataract. The right eye was similarly but less severely affected. In both eyes, abundant PEX material was found on the anterior lens capsule. Initially, IOP was 22 mm Hg in the right eye and 17 mmHg in the left eye. Further topical therapy was added (brinzolamide two times a day both eyes), and left cataract surgery was performed with an excellent visual outcome. The third patient, a 76-year-old male, presented with mixed cortical and nuclear cataracts in both eyes, worse in the left eye. PEX syndrome was noted in the right eye only. Cataract surgery was performed initially in the left eye and following a successful outcome, 2 months later, in the right eye with collection of the PEX material. No glaucoma was present in either eye. The fourth patient, an 80-year-old male, presented with bilateral moderate mixed cortical and nuclear cataracts, advanced pseudoexfoliative glaucoma in the right eye, and less severe field loss in the left eye. Combined cataract and glaucoma filtration surgery was performed initially on the right eye, and following a good result, 1 year later, on the left eye. Current treatment is combined bimatoprost and timolol eye drops in the left eye only, with the glaucoma in the right eye controlled following the trabeculectomy. A peripapillary choroidal neovascular membrane was subsequently diagnosed in the left eye and treated with intravitreal bevacizumab. From independent, 70- to 85-year-old, PEX-affected and unaffected patients with cataract, anterior lens capsules were obtained with capsulorhexis during cataract surgery, stained with Vision Blue™ (D.O.R.C. International b.v., Zuidland, The Netherlands), fixed in buffered formalin, and embedded in paraffin for later sectioning.

Mass spectrometry: For protein extraction, PEX material from each patient was separately incubated in 100% formic acid at 30 °C overnight, cleaved with 5 mg/ml cyanogen bromide in 70% formic acid at 30 °C overnight, dried under vacuum, and digested twice each with 400 ng of Trypsin Gold (Promega Corporation, Alexandria, NSW, Australia) in 50 mM NH_4HCO_3 at 37 °C. The initial digestion was performed for 4 h and the subsequent digestion performed overnight. The tryptic peptides of the extracted proteins from the specimens were analyzed with a Thermo LTQ XL linear ion trap (the first three patients) or Thermo Orbitrap XL hybrid (the fourth patient) mass spectrometers, each fitted with a nanospray source as previously described [23]. The Orbitrap identifies precursor ions with higher resolution and mass accuracy although the spectrometer utilizes the same ion trap as for LTQ XL. Each sample was analyzed in duplicate. Digested peptides were applied and separated as

previously described [23]. The mass spectrometers were operated in positive-ion mode with collision-induced dissociation (CID) fragmentation of the six most intense ions with only the multiple-charged ions fragmented for Orbitrap analysis. A dynamic exclusion of 30 s was used with an exclusion list of 500 and collision energy of 35%. The MS spectra were searched against the UniprotKB 2014–09 database with Proteome Discoverer v1.4 (Thermo Electron Corporation, San Jose, CA) using trypsin as the protease, allowing for two missed cleavages with the following variable modifications: oxidation of methionine; phosphorylation of serine, threonine, and tyrosine; deamination of asparagine; and fixed carboxymethylation of cysteine. The mass tolerance for peptide identification using the ion trap data was 1 Da for precursor ions and 0.8 Da for product ions and for Orbitrap data, 10 ppm for precursor ions and 0.8 Da for product ions. A false discovery rate of 1% was employed, and peptide confidence was set to high. Spectra from all matched peptides were manually inspected to confirm spectral quality and sequence match.

Immunohistochemistry: Immunohistochemistry on sections of three PEX-affected and four unaffected lens capsules was performed as previously described [23,27] using the rabbit anti-APOA1 (1:500; Abcam, Cambridge, MA), or anti-APOA4 (1:100; Sigma-Aldrich, St. Louis, MO), or anti-FGB (1:500; Sigma-Aldrich) or anti-HB (1:4,000; MP Biomedicals Australia, Seven Hills, NSW, Australia), or anti-HSPB1 (1:50; Sigma-Aldrich), or anti-PRDX2 (1:1,000; Antibody Technology Australia Pty Ltd.), or sheep anti-CRYAA (1:1,000; Flinders University Antibody Production Facility, Bedford Park, SA, Australia) primary antibody. As commercially available anti-CRYAA antibodies were found to be either non-specific or not suitable for immunodetection applications, a sheep polyclonal anti-CRYAA antibody against a human CRYAA peptide in the protein region highly similar among humans, mice, and rats, raised in-house, was used [28]. Anti-APOA1, anti-APOA4, and anti-HSPB1 antibodies were detected with the Novolink polymer detection system (Leica Microsystems, Pty Ltd., North Ryde, NWS, Australia), and anti-FGB, anti-HB, and anti-PRDX2 antibodies with EnVision+ Dual Link System-HRP (Dako, Australia Pty, Ltd., North Sydney, NSW, Australia) and DAB. The anti-CRYAA antibody was detected with biotinylated donkey anti-sheep immunoglobulin G (IgG) secondary antibody followed by the Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) and FAST DAB tablets (Sigma-Aldrich). Sections were counterstained and mounted, and microscopy performed as previously described [23,27].

Western blotting: For determining the specificity of the antibodies used in the study, western blotting was performed on lysates of appropriate control tissues, SRA 01/04 human lens epithelial cells and human lens. Human serum was used as positive control for determining the specificities of the anti-APOA1, anti-APOA4, and anti-FGB antibodies, human red blood cells for the anti-HB and human embryonic kidney (HEK293A) fibroblast cells for the anti-PRDX2 antibodies. Human lens served as positive control for the anti-CRYAA and SRA 01/04 cells for the anti-HSPB1 antibodies. Proteins from red blood cells, and HEK293A and SRA 01/04 cells were extracted in radioimmunoprecipitation assay (RIPA) buffer as previously described [29]. Lens proteins were extracted in TUC buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.6% DTT). Protein lysates were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting prepared as previously described [15]. Western blots were hybridized with the rabbit anti-APOA1 (1:20,000), anti-APOA4 (1:250), anti-FGB (1:200), anti-HB (1:500 or 1:1,000), anti-HSPB1 (1:250), and anti-PRDX2 (1:5,000) and sheep anti-CRYAA (1:1,000) primary antibodies. Binding of the rabbit anti-APOA1, anti-APOA4, anti-FGB, anti-HB, anti-HSPB1, and anti-PRDX2 primary antibodies were detected with the donkey anti-rabbit IgG HRP-conjugated secondary antibody (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Anti-CRYAA antibody was detected with the biotin-conjugated donkey anti-sheep IgG secondary antibody (Jackson ImmunoResearch Laboratories) and streptavidin-horseradish peroxidase conjugate. Blots were developed with chemiluminescence reagent (Pierce Biotechnology, Inc., Rockford, IL) or the ECL Standard/ECL Advance Western Blotting System (GE Healthcare Australia and New Zealand) and imaged on the LAS 4000 imager using Image Reader LAS 4000 software (FujiFilm).

Histology: Perls' Prussian blue staining was performed as described elsewhere [30]. Briefly, sections were stained with a 1:1 mixture of potassium ferrocyanide and hydrochloric acid, and counterstained with acidified neutral red stain. Sections of human liver tissue of a patient with hemochromatosis were used as positive control.

Reverse transcription–PCR: RNA was extracted from lens epithelial cells adherent to the lens capsule using the RNeasy Micro Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA from blood collected in Tempus Blood Collection Tubes (Applied Biosystems, Foster City, CA) was extracted using the Tempus Blood RNA Extraction Kit (Applied Biosystems) according to the manufacturer's protocol. From RNA, cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamers. Reverse transcription PCR (RT–PCR) was performed on cDNA templates using gene-specific primers

and HotStarTaq Plus (Qiagen) under the following conditions: enzyme activation at 95 °C 5 min; 30–45 cycles of denaturation at 95 °C 30 s, annealing at 56 °C 30 s, and elongation at 72 °C 30 s, followed by extension at 72 °C 5 min. The primer sequences used for amplification of each gene are listed in Appendix 1. The resulting PCR products were analyzed with agarose gel electrophoresis. Specificity of the amplified products was confirmed with sequencing.

Bioinformatics analysis: Functional relationships of the PEX material proteins validated in this study, those reported in previous studies, and the genes implicated in PEX syndrome were explored with each other and with experimentally validated interactors in InnateDB [31]. Gene names (see Appendix 2) were uploaded and analyses performed using the pathway analysis tool. A network of molecular interactions was constructed using all PEX-associated genes and their interactors using the InnateDB web tool. Henceforth, all PEX-associated genes and their interactors are referred to as nodes and the interactions as edges. Any redundant edges between two nodes, self-interactions, and edges involving ubiquitin C (UBC) were removed. Cytoscape v3.4.0 was used to visualize the generated network and identify the top hub nodes. Hub nodes are genes with a large number of interactions with other genes within the network and potentially of great functional significance. The Cytoscape plugin jActiveModules was used to highlight sub-networks with more than 20 nodes that were highly enriched for nodes associated with PEX syndrome using the following parameters: number of modules = 5, overlap threshold = 0.3, and search depth = 2. Enriched sub-networks were analyzed with InnateDB pathway analysis tools to recognize the sub-networks' biologic functions.

RESULTS

Novel proteins identified in pathological PEX material: In our previous work, liquid-chromatography and tandem mass spectrometry (LC-MS/MS) of proteins extracted from neat PEX material with chemical cleavage, apart from identifying the reported proteins [23], identified peptides corresponding to several additional proteins in one of the analyzed samples. Thus, to further decipher the protein composition of PEX material, in the present study, we performed LC-MS/MS on extracts of neat PEX material isolated from four independent patients with PEX syndrome. Small amounts of surgically isolated neat PEX material obtained from each patient were subjected to chemical cleavage with formic acid and cyanogen bromide for cleavage of Asp-Pro and Met-X peptide bonds, respectively [32,33]. As previously reported by us [23] and Ovodenko et al. [8], protein extraction by this method did not alter the physical appearance of the material suggesting that only a very small proportion of pathological proteins are extracted with chemical cleavage. Additionally, PEX material is a pathological extracellular deposit; thus, an equivalent control tissue does not exist and was not analyzed.

Multiple peptides with statistically significant corrected p values corresponding to 66 proteins were detected in the analyzed samples (Appendix 3). These peptides included peptides corresponding to ten proteins known to be present in PEX material, proving the efficacy of the approach: LOXL1, APOE, CLU, complement 3, emilin-1, fibrillin-1, fibronectin, LTBP-2, TIMP3, and vitronectin. Peptides corresponding to LOXL1 were detected in all the analyzed samples and those corresponding to the other PEX material proteins in one or more samples (Appendix 3); variability in detection of peptides corresponding to a particular protein between samples was most likely due to the variable amount of neat PEX material available from each patient and thus, the variable amount of proteins extracted from each sample. Additionally, peptides corresponding to hemoglobin (HB) subunits were detected; HB alpha, beta, and delta chain peptides were detected in two or more samples and epsilon and gamma-1 chains in one sample each. We previously also detected peptides corresponding to HB alpha, beta, delta, and epsilon chains in PEX material [23]. However, whether the peptides indicated potential erythrocyte contamination or were genuine components of the material was not investigated. Lee also reported identification of HB alpha and delta chains in PEX-affected lens capsule [34]. To date, none of the peptides from the other 51 proteins have been reported in pathological PEX deposits; thus, these proteins are likely novel constituents of the material. Multiple peptides were detected for some of these proteins, and a single peptide in one of the samples for the majority of proteins likely due to the availability of an extremely low amount of starting material. As some of the known PEX material proteins were also represented by a single peptide, the novel proteins with a single detected peptide were considered potentially actual constituents of the material. To determine whether the newly identified proteins were genuine components of the material, 13 proteins were chosen for validation. They were either represented by multiple peptides in multiple samples, or multiple peptides in a single sample, or a single peptide in one or duplicate runs of a sample. The chosen proteins were apolipoprotein A-I (APOA1), apolipoprotein A-IV (APOA4), crystallin alpha A (CRYAA), fibrinogen alpha (FGA), beta (FGB) and gamma (FGG) chains, HB alpha, beta, gamma-1, delta, and epsilon chains, heat shock protein beat-1 (HSPB1), and peroxiredoxin-2 (PRDX2; Appendix 3). A single peptide each was detected for APOA4, CRYAA, HBG1, HSPB1, and PRDX2 and multiple peptides for other chosen proteins

(Appendix 4). The MS/MS spectra of representative or the detected peptides of these proteins are shown in Appendix 5.

Confirmed novel protein constituents of PEX material: For validation of the chosen identified proteins, using protein-specific antibodies, immunohistochemistry was performed on sections of anterior lens capsules from patients with PEX syndrome independent from those from whom PEX material was analyzed. Lens capsules from patients with cataract without PEX syndrome served as controls. For proteins with multiple subunits, instead of validating each identified subunit either a representative subunit or the protein complex was validated. The specificity of each antibody was demonstrated with western blotting (Appendix 6).

Immunolabeling with the anti-APOA1, anti-APOA4, anti-CRYAA, anti-FGB, anti-HB, and anti-PRDX2 antibodies revealed positive labeling of the pathological deposits accumulated on PEX-affected capsules (Figure 1, left panels) confirming that the respective proteins are present in PEX material. However, labeling with the anti-HSPB1 antibody was very faint; thus, the presence of HSPB1 in PEX material could not be unequivocally confirmed (Appendix 7, left panel). With each of these antibodies, similar labeling was absent on the control capsules that were devoid of PEX material deposits (Figure 1, right panels). Instead, in the control capsules, with each antibody, positive immunolabeling was observed in adherent lens epithelial cells (Figure 1, right panels; Appendix 7, right panel) suggesting that the respective proteins are either expressed or present in the human lens epithelium. The absence of lens epithelial cells in PEX capsule sections is possibly related to the pathology because capsules from patients with PEX syndrome were often found to either be devoid of or have remnants of the lens epithelium (Figure 1, left panels). The expression or presence of APOA1, APOA4, FGB, and PRDX2 proteins in the lens epithelium has not been reported before and thus, is a novel finding.

To confirm the specificity of positive immunolabeling of pathological material deposited on PEX capsules with each antibody, immunolabeling of ZO-1, a cell membrane–associated protein, was performed. ZO-1 is expressed in the lens epithelium in vivo [35] but was not detected in PEX material with LC-MS/MS (Appendix 3). Consistently, immunolabeling with an anti-ZO-1 antibody did not show any labeling of PEX deposits on lens capsules from affected eyes (Figure 1, ZO-1, left panel), and as expected, the lens epithelial cells in the control lens capsules showed ZO-1-positive labeling (Figure 1, ZO-1, right panel). Thus, positive labeling of PEX material for each identified protein is specific and not due to non-specific binding of antibodies to the highly proteinaceous material. Taken together, the immunohistochemistry data confirmed that APOA1, APOA4, CRYAA, FGB, and PRDX2 are novel components of PEX material. Furthermore, these data confirmed that HB is an integral component of the material.

To determine whether the HB present in PEX deposits is iron-bound, Perls' Prussian blue staining was performed on PEX capsule sections. Negative staining of PEX deposits was observed indicating that the HB present in the deposits is iron-free (Figure 2A). Similarly, negative staining of sections of the control capsules indicated that the HB expressed in lens epithelial cells is also free of iron (Figure 2B). Sections of human liver tissue of a patient with hemochromatosis, used as positive control, showed blue staining of hepatocytes and Kupffer cells indicating iron overload (data not shown).

Expression of novel PEX material proteins in lens epithelium: To determine whether the genes encoding the newly identified PEX material proteins are expressed in the lens epithelium, we performed RT–PCR on mRNA of lens epithelial cells adherent to the lens capsule; expression of *HSPB1* was also determined. PCR products of the expected sizes corresponding to the *HB*, *HSPB1*, and *PRDX2* genes were readily obtained (Figure 3). However, expression of *APOA1*, *APOA4*, and genes encoding *FG* subunits (*FGA*, *FGB*, and *FGG*) was undetectable. From transcripts of the latter genes, amplicons of the expected sizes were detected in positive control tissues (blood or placenta) demonstrating efficacy of the RT–PCR assays. Expression of *CRYAA* in the lens epithelium is well-known. Expression of *HB* and *HSPB1* genes in the lens epithelium is consistent with their reported expression in lens epithelial cells [36,37]. Expression of *HSPB1* and *PRDX2* mRNA in the lens epithelium correlates with expression of the corresponding proteins in cultured human lens epithelial cells, SRA 01/04 (Appendix 6). Similarly, absence of detectable *APOA1*, *APOA4*, and *FG* subunit transcripts in the lens epithelium correlates with undetectable expression of the respective proteins in SRA 01/04 cells (Appendix 6). None of the proteins identified in PEX material except CRYAA were detectable in human whole lens lysates (Appendix 6). As 90% of lens proteins are composed of crystallins and only 10% of other proteins, the identified proteins would likely be part of the latter 10% fraction and thus, expressed at lower levels than that detectable with western blotting. Nonetheless, these data confirmed in situ expression of *HB*, *HSPB1*, and *PRDX2* genes in the lens epithelium. In addition, these data suggest that *APOA1*, *APOA4*, and *FG* subunits are either not expressed or are expressed at extremely low levels in the lens epithelium.

Functional relationships among genes and proteins implicated in PEX syndrome: To elucidate functional relationships

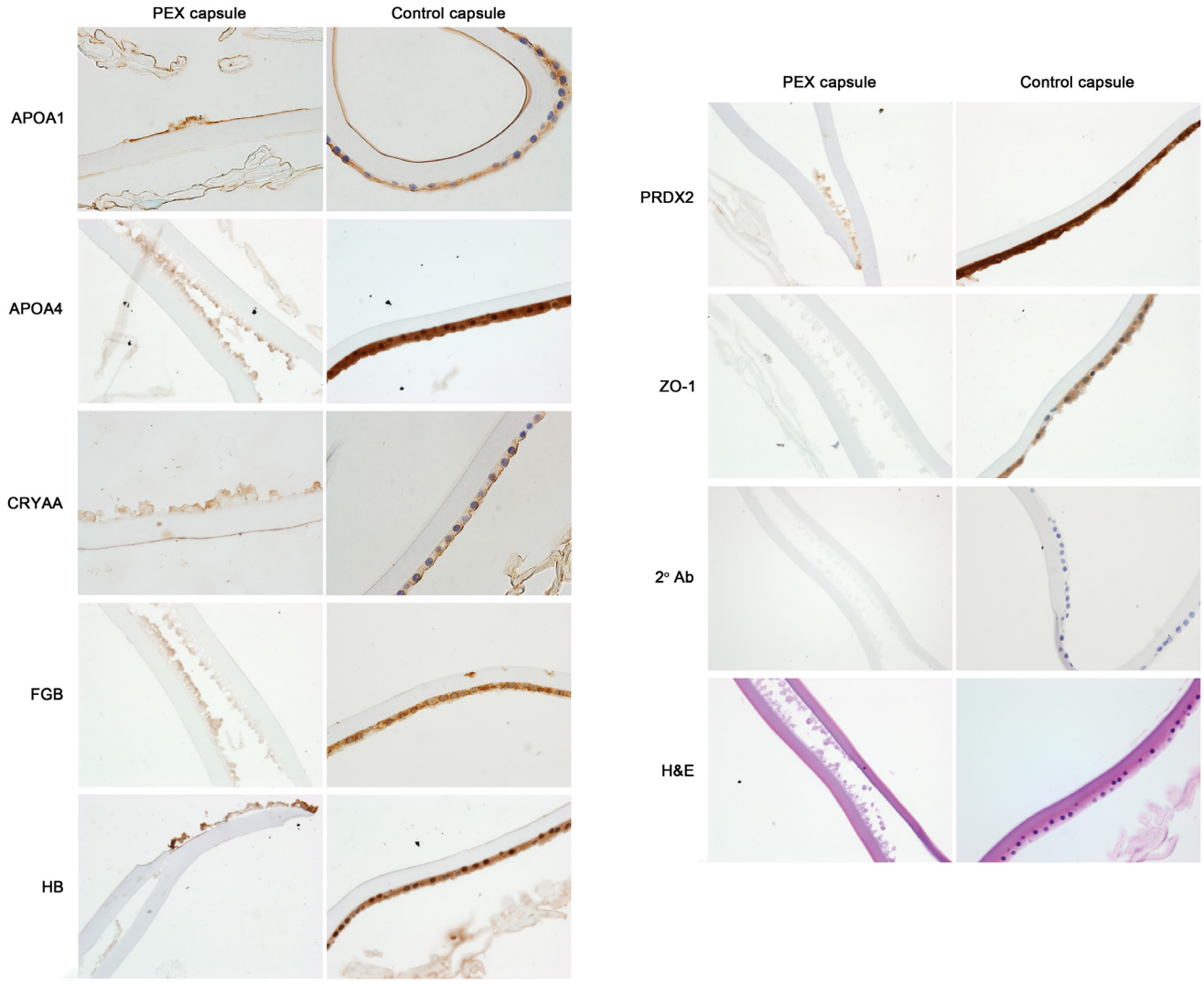

Figure 1. Immunohistochemical labeling of novel proteins identified in PEX material with mass spectrometry in pathological deposits on PEX-affected lens capsules. Sections of pseudoexfoliation (PEX)-affected (left panels) and unaffected (right panels) lens capsules from patients with cataract were immunolabeled with the anti-APOA1, anti-APOA4, anti-CRYAA, anti-FGB, anti-HB, anti-PRDX2, or anti-ZO-1 antibody as indicated. Positive labeling in a section (brown) shows the presence of the indicated protein. Each labeled protein was detected in pathological material deposited on PEX-affected lens capsules (left panels) and in the lens epithelium in unaffected lens capsules (right panels). ZO-1, an irrelevant protein, was absent in PEX material deposited on affected lens capsules proving the specificity of the labeling of the other proteins. ZO-1 protein was detected in the lens epithelium in unaffected lens capsules (right panel), as expected. 2° Ab, negative control sections hybridized with secondary antibody without primary antibody hybridization. H&E, sections stained with hematoxylin and eosin. Two parts of the lens capsule seen in some panels are due to folding of the capsule. Apparent positive immunolabeling of the anterior edge of the control capsule with the anti-APOA1 antibody is a sectioning artifact (APOA1, right panel). Representative images from independent experiments on three PEX-affected and four unaffected lens capsules are shown. Images are at 60X original magnification.

among the PEX material proteins identified in this study and the genes and proteins previously implicated in PEX syndrome (Appendix 2), and to determine biologic pathways involved in the disease, pathway and network analyses were performed in InnateDB. Pathway analysis showed overrepresentation of PEX-associated genes and proteins in pathways involved in extracellular matrix organization (27/266, corrected $p = 4.51 \times 10^{-27}$) and degradation (11/116, corrected $p = 3.52 \times 10^{-10}$), elastin fiber formation (11/45, corrected $p = 2.09 \times 10^{-14}$), and complement cascade (7/47, corrected $p = 5.51 \times 10^{-8}$; Appendix 8), consistent with current understanding of the disease. Network analysis revealed the PEX-associated genes network composed of 1,560 nodes and 2,131 edges (Appendix 9). The top hub nodes included FN1 (755 interactions), CLU

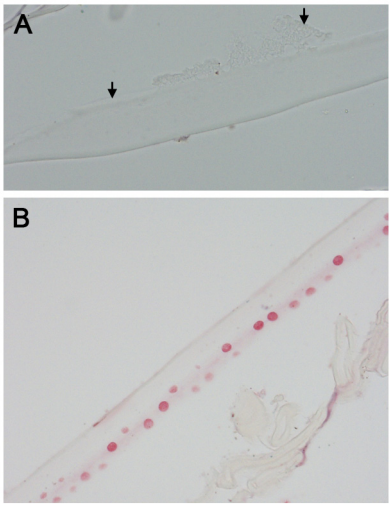

Figure 2. Presence of iron-free hemoglobin in PEX material and lens epithelium. Sections of (**A**) pseudoexfoliation (PEX)-affected and (**B**) unaffected lens capsules were stained with Perls' Prussian blue and imaged in bright-field at 60X original magnification. Positive staining would appear blue. Neither the PEX material deposited on the affected capsule (arrows in **A**) nor lens epithelial cells in the unaffected capsule (**B**) showed positive staining indicating that the hemoglobin present at these sites is free of iron. Lens epithelial cell nuclei are counterstained red in panel **B**.

(90 interactions), and APOA1 (88 interactions; Table 1). Sub-network analysis identified one module highly enriched for PEX-associated genes (Figure 4). The sub-network consisted of 46 nodes and 87 edges. The majority of the top-ranked pathways in this sub-network were similar to those identified in the initial analysis of only PEX-associated genes (Appendix 10). In addition, new significant pathways were identified in the sub-network analysis, including integrin and syndecan-mediated signaling, focal adhesion, integrin signaling, high-density lipoprotein (HDL)-mediated lipid transport, lipid digestion and mobilization, and clotting cascade pathways. Furthermore, sub-network analysis showed the occurrence of direct and indirect functional interactions among many of the PEX-associated genes, explaining their collective involvement in the disease. It showed that fibronectin is the central molecule in these interactions including in functional interactions with PEX material proteins identified in this study (Figure 4). This finding is consistent with fibronectin being the master organizer of extracellular matrix assembly and suggests fibronectin has a central role in the pathogenesis of PEX syndrome.

DISCUSSION

Mass spectrometry of chemical cleavage extracts of neat pathological deposits led to identification of several known and novel protein constituents of PEX material. PEX syndrome is less prevalent in Australia than elsewhere [38,39], and only a few patients present with deposition of sufficient pathological material on the lens capsule to allow isolation of neat material during cataract surgery. Therefore, in this study, neat material from a small number (n=4) of unrelated patients was analyzed. A microscopically small amount of neat PEX material was isolated from each patient, and the chemical cleavage method employed for protein extraction extracted only a very small proportion of proteins without changing the physical appearance of the material. Thus, the concentration of extracted proteins from each sample was extremely low and below the limit of estimation by standard methods. Furthermore, due to the variable amount of PEX material available from each patient, the amount of extracted proteins varied between samples. Therefore, every identified protein was not detected in each analyzed sample or in duplicate analysis of the same sample. To determine whether the identified proteins were genuine components of the material, a subset of proteins was chosen for validation by an independent method using lens capsule specimens obtained from independent and unrelated patients. Validation confirmed 12 of the 13 chosen proteins were genuine and novel constituents of the material. In addition, validation strongly supported that these proteins are general, rather than patient-specific, constituents of the material, and they are not contaminating proteins. Taken together, this study showed that mass spectrometry analysis

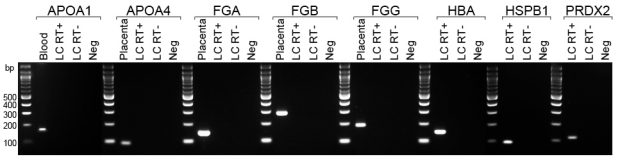

Figure 3. Gene expression analysis of novel identified PEX material proteins in the lens epithelium. Reverse transcription-PCR (RT–PCR) was performed on normal lens epithelium mRNA using gene-specific primers (Appendix 1). The resulting PCR products were analyzed with agarose gel electrophoresis. The products of the expected sizes (see Appendix 1) were observed for the *HB* (subunit A), *HSPB1*, and *PRDX2* genes but were undetectable for the *APOA1*, *APOA4*, and *FG* (subunits A, B, and C) genes. However, the expected sized products for *APOA1*, *APOA4*, and *FG* subunits were observed in the positive control tissues (blood or placenta). The sizes of the molecular weight standards in base pairs (bp) are indicated. LC, lens capsule; RT^+, with reverse transcriptase; RT^-, without reverse transcriptase; neg, negative control.

of extracts of neat pathological PEX deposits is a powerful discovery approach for deciphering the protein composition of PEX material. However, validation with an independent method is required to confirm that the discovered proteins are genuine constituents of the material. Some of the proteins identified by the present approach were also identified with mass spectrometry of chemical cleavage extracts of PEX-affected and unaffected lens capsules, the approach adopted by Ovodenko et al.; both approaches identified CLU, complement 3, fibrillin-1, fibronectin, TIMP3, and vitronectin, the known components, in PEX material (Appendix 3) [8]. However, the other proteins identified by the two approaches are different. The approach used by Ovodenko et al. did not identify LOXL1, an abundant component of PEX material, or any of the novel components we report. This difference in findings between approaches emphasizes the complexity of the material and advocates for the importance of employing diverse approaches for deciphering the material's composition. The novel protein constituents identified in this study include apolipoproteins, blood-related proteins, and stress response proteins.

APOA1 is the major component of HDL. APOA4 is associated with intestinal lipoproteins, particularly chylomicrons. Fibrinogen, formed of alpha, beta, and gamma chains, is a coagulation factor primarily recognized for its function in blood clotting. HB is mainly known for its oxygen transport function in the hematopoietic system. CRYAA is a small heat-shock protein that functions as a molecular chaperone and is a family member of alpha-crystallins, the most abundant proteins in the ocular lens. PRDX2 is a thioredoxin-dependent antioxidant peroxidase [40] that also functions as a molecular chaperone [41]. All these proteins have been implicated in inflammatory diseases, protein aggregation diseases, and/or oxidative stress conditions. Their presence in PEX material is consistent with the involvement of these cellular processes in PEX syndrome.

Interestingly, the majority of these proteins are secreted and present in serum or blood. As PEX syndrome is associated

Table 1. Top hub nodes with more than 20 known interactions with other genes in the constructed network of PEX syndrome associated genes.

Gene symbol	Gene name	Number of interactions
FN1	fibronectin 1	755
CLU	clusterin	90
APOA1	apolipoprotein A-I	88
SEBOX	SEBOX homeobox	83
TGM2	transglutaminase 2	82
APOE	apolipoprotein E	67
TGFB1	transforming growth factor, beta 1	58
PRDX2	peroxiredoxin 2	57
A2M	alpha-2-macroglobulin	42
C3	complement component 3	35
FGB	fibrinogen beta chain	33
VCAN	versican	33
SDC2	syndecan 2	32
FGA	fibrinogen alpha chain	31
FGF2	fibroblast growth factor 2 (basic)	30
MMP2	matrix metallopeptidase 2	29
LAMC1	laminin, gamma 1 (formerly LAMB2)	28
LAMB1	laminin, beta 1	27
C1QA	complement component 1, q subcomponent, A chain	24
CRYAA	crystallin, alpha A	24
APCS	amyloid P component, serum	22
FGG	fibrinogen gamma chain	22
ELN	elastin	21

with breakdown of the blood–aqueous barrier, serum or blood may be the source of some or all of these proteins in PEX material. The present immunohistochemistry data demonstrate that these proteins are present in the lens epithelium suggesting that they are either expressed in situ or translocated from the aqueous humor. The present gene expression data (Figure 3) support in situ expression of HB and PRDX2 in the lens epithelium; expression of CRYAA in the lens epithelium is well-known. Thus, CRYAA, HB, and PRDX2 proteins are likely produced locally in the ocular anterior segment, particularly the lens epithelium, and contribute to the PEX material. Ultramicroscopy findings in the ocular anterior segment tissues in PEX syndrome–affected eyes support this idea [42,43]. Furthermore, although crystallins are primarily intracellular proteins, very low concentrations of alpha and gamma crystallins have been reported in the aqueous humor [44]. Alpha-crystallins are also present in the extracellular pathological deposits associated with age-related macular degeneration, drusen, in the retina [45]. These reports are consistent with the presence of CRYAA in the extracellular PEX material revealed in this study. PRDX1, PRDX4, and PRDX6 are members of the same family of peroxidases as PRDX2 and have been reported to exhibit extracellular localization or secretion [46,47]. This study suggests that PRDX2, normally an intracellular peroxidase or a molecular chaperone, similarly, has an extracellular role. According to the present data, PRDX2 is likely expressed by the lens epithelium and secreted into PEX material; further research is warranted to investigate this possibility.

It is interesting to note the presence of HB in PEX material. Apart from the function in the hematopoietic system, HB is expressed in neuronal, lung epithelial, retinal, and RPE cells [36,48,49]. HB is also expressed in avascular embryonic tissues in the mouse, including the developing eye and lens, and the avascular adult lens [36]. In non-hematopoietic tissues, HB is thought to be involved in tissue

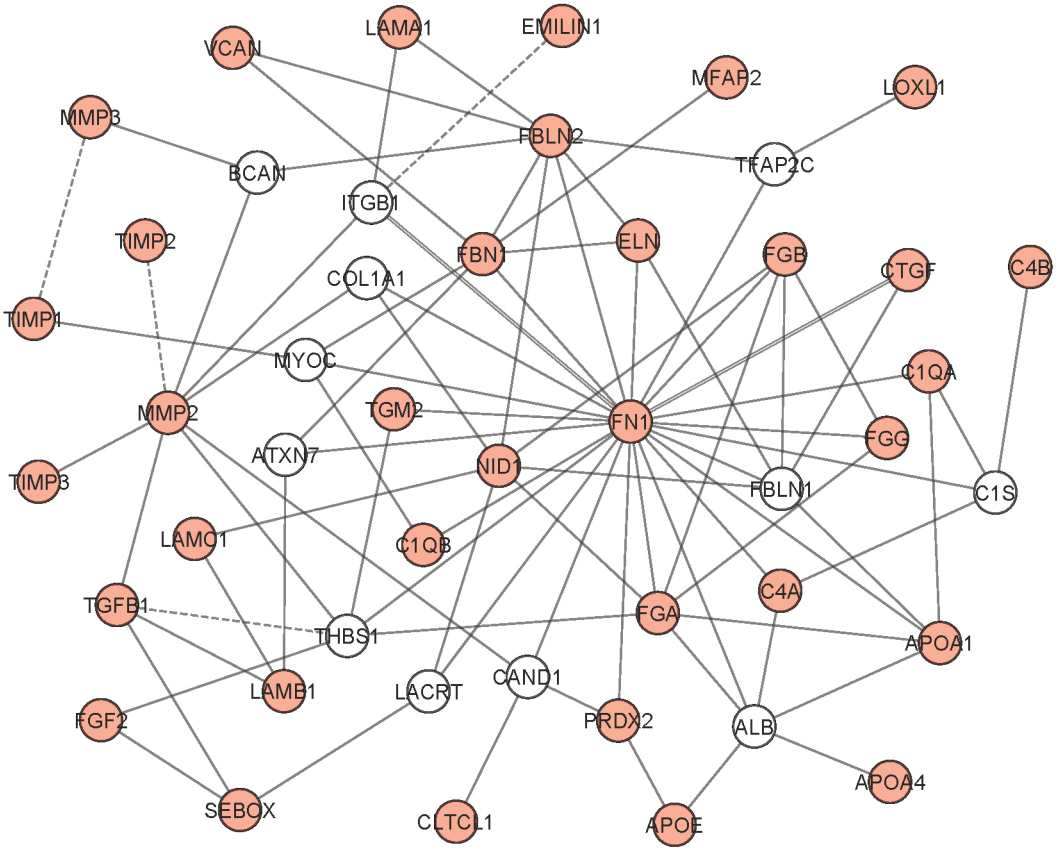

Figure 4. Molecular relationships of proteins identified in PEX material in this study and genes and proteins previously implicated in PEX syndrome. The top sub-network or module consisting of 46 nodes and 87 edges revealed with network analysis in InnateDB is shown. Genes and proteins implicated in pseudoexfoliation (PEX) syndrome are in red; solid line = protein–protein interaction; dashed line = unspecified association; double lines = colocalization.

oxygen transport, nitric oxide detoxification, or free radical scavenging. Notably, unlike the hematopoietic system, HB present in the avascular embryo and embryonic and adult lens is not associated with heme [36]. Heme-free globin reportedly causes apoptosis in cultured cells [50,51]. Thus, it may have a similar function in the former avascular tissues. We argue that HB found in PEX material is not a blood contaminant because first, HB does not carry iron (Figure 2A) and thus, is heme-free whereas in the blood HB is heme-bound. Second, consistent with the report by Mansergh et al. [36], we found that HB protein is expressed in normal lens epithelium (Figure 1, right panel) and is also not bound to iron (Figure 2B). Last, we found expression of the *HB* transcript in the lens epithelium indicating that the encoded protein is produced in situ. Thus, we propose that heme-free HB is expressed in the lens epithelium and secreted in PEX material. This idea is consistent with the reported secretion of HB by the RPE into Bruch's membrane [36]. The significance of the presence of HB in pathological PEX deposits remains unclear.

The secreted proteins DEK (DEK oncogene), human programmed cell death 5 (PDCD5), and Antennapedia and HIV transactivator of transcription (TAT) have been reported to exhibit intercellular translocation [52-54]. Similarly, the presence of APOA1, APOA4, and FG proteins in the lens epithelium without detectable expression of the respective encoding transcripts suggests that these proteins are translocated from the aqueous humor to the lens epithelium. Consistently, these proteins are present in the aqueous humor in the human eye [55]. However, the possibility that serum or blood, due to the breakdown of the blood–aqueous barrier, contributes these proteins to PEX material cannot be excluded.

APOA1 and APOA4 identified in PEX material in this study and APOE in our previous study are components of HDL. Clusterin, another component of PEX material, is also associated with HDL [56]. HDL, depending upon its protein composition, is involved in atheroprotective functions by inhibiting oxidation and inflammatory properties of low-density lipoprotein. However, pathological conditions can alter the protein composition of HDL and in turn, its functional properties and render it inflammatory [56]. Interestingly, HDL is the main lipoprotein in the aqueous humor [57] and may have a similar anti-inflammatory function in the ocular anterior chamber. HDL is likely the source of apolipoproteins identified in PEX material. This idea is consistent with PEX material acting as a sink for aggregation of aqueous humor proteins. Whether this potential phenomenon changes HDL protein composition, and thus, HDL's functional properties, particularly anti-inflammatory properties in the aqueous humor in patients with PEX syndrome, warrants further research.

Fibrinogen, commonly known for its role in blood clotting, also plays a proinflammatory role in extravascular tissues in several diseases and has a role in extracellular matrix physiology [58,59]. Fibrinogen is a component of atherosclerotic plaques [60] and amyloid deposits in AD [61], and fibrin, fibrinogen's insoluble form, is present in pathological lesions in stroke [62]. This study showed the presence of fibrinogen in PEX material and interaction with other PEX material proteins, particularly fibronectin (Figure 4), indicating the important role of fibrinogen in deposition of pathological material in the syndrome. Fibrinogen exerts its inflammatory function either by activation of different immune cell types by binding to their cell surface receptors, such as in diseases with an inflammatory component, or by carrying the inflammatory regulator TGF-β in its latent form to tissues, such as in diseases involving the breakdown of the blood–tissue barrier [58]. Breakdown of the blood–aqueous barrier and upregulation of TGF-β and proinflammatory cytokines in the aqueous humor and outflow pathways reported in patients with PEX syndrome correlate well with a proinflammatory role of fibrinogen in this disease [63]. Furthermore, fibrinogen is known to have an inflammatory role in cardiovascular disease, cerebrovascular disease, and AD, diseases also associated with PEX syndrome. HDL-mediated inflammation, too, has a role in cardiovascular disease. Thus, inflammation is likely the underlying cause of association of these diseases with PEX syndrome.

Reduced HDL, APOA1, and APOA4 levels have been associated with increased risk of cardiovascular disease [64,65]; reduced plasma levels of APOA1 have also been associated with increased risk of AD [66,67]. Consistently, overexpression of APOA1 in a mouse model of AD has been reported to reduce neuroinflammation and alleviate learning and memory defects [68]. In contrast, elevated fibrinogen levels have been associated with increased risk of cardiovascular and cerebrovascular diseases and AD, and upregulation of PRDX2 reported in the brain in patients with AD [58,62,69,70]. Whether the levels of one or more of these proteins are altered in patients with PEX syndrome warrants investigation in future research. Interestingly, APOA4 levels are elevated in the aqueous humor in primary congenital glaucoma [71]. Similarly, PRDX2 is expressed at higher levels in cultured trabecular meshwork cells from patients with glaucoma compared to those from normal individuals, and in cells treated with anti-glaucoma drugs [72]. As PEX syndrome increases the risk of glaucoma, it will be interesting to know whether the levels of these proteins are altered in

the aqueous humor or ocular anterior segment tissues in this disease. Additionally, genetic polymorphisms in the *APOA1*, *APOA4*, and *APOE* genes are associated with the risk of cardiovascular disease and polymorphism in *APOA1* is also associated with the risk of AD [73-76]. The association of polymorphism in *APOE* with PEX syndrome is controversial [77,78]. It will be useful to study whether polymorphisms in these genes collectively confer the risk of PEX syndrome.

This study had several limitations. Due to the variable amounts of PEX material available from each patient, variable amounts of proteins would have been extracted from each sample. As chemical cleavage extracted only a small amount of proteins, most likely surface proteins, from the material, the amount of extracted protein was below the estimation by the most sensitive methods. Thus, from each sample equivalent amounts of protein could not be analyzed. Further, one of the samples was analyzed on an instrument coupled with an Orbitrap. However, this did not affect the overall results because despite the higher sensitivity of the Orbitrap for determining precursor ion mass accuracy, the largest number of proteins were detected in the sample analyzed on the Ion trap mass spectrometer (08A2, Appendix 3). As PEX syndrome is less prevalent in Australia than elsewhere [38,39] and only some patients present with deposition of sufficient PEX material on the anterior lens surface for isolation, a small number of neat samples were available for analysis. Despite these limitations, the approach employed in this study led to the identification of genuine novel protein components of PEX material. Chemical cleavage does not alter the physical appearance of PEX material. Thus, alternative methods of protein extraction, with or without chemical cleavage, could be employed to further decipher the composition of this complex material.

In conclusion, mass spectrometry of proteins extracted from neat PEX material is a powerful approach for discovering novel protein components of the material. However, validation with an independent method is necessary for identifying genuine components of the material. This study revealed 12 novel protein constituents of the material. The newly identified proteins provide further insight into the pathophysiology of PEX syndrome and the molecular basis of association of the disease with cardiovascular disease, cerebrovascular disease, and Alzheimer disease. Last, this study raises interesting research questions for further investigation to understand the mechanism of ocular manifestations in PEX syndrome.

APPENDIX 1. SEQUENCES OF PRIMERS USED FOR RT–PCR OF GENES ENCODING THE PROTEINS IDENTIFIED IN PEX MATERIAL.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. LIST OF GENES IMPLICATED IN PSEUDOEXFOLIATION SYNDROME.

To access the data, click or select the words "Appendix 2."

APPENDIX 3. PROTEINS IDENTIFIED WITH LC-MS/MS IN NEAT PATHOLOGICAL PEX MATERIAL.

To access the data, click or select the words "Appendix 3."

APPENDIX 4. NOVEL PROTEINS IDENTIFIED IN PEX MATERIAL WITH LC-MS/MS CHOSEN FOR VALIDATION.

To access the data, click or select the words "Appendix 4."

APPENDIX 5. MS/MS SPECTRUM OF THE MOST FREQUENTLY IDENTIFIED PEPTIDE OF THE PROTEINS DETECTED WITH LC-MS/MS IN PEX MATERIAL CHOSEN FOR VALIDATION.

The protein name, amino acid sequence of the identified peptide and its MS/MS spectrum are shown. The b (red) and y (blue) ion series for each peptide are indicated. The m/z value for each precursor ion and its charge state are also indicated. x-axis=m/z ratio, y-axis=intensity counts. To access the data, click or select the words "Appendix 5."

APPENDIX 6. SPECIFICITIES OF ANTIBODIES USED IN THE STUDY AND EXPRESSION OF NOVEL IDENTIFIED PEX MATERIAL PROTEINS IN THE LENS AND LENS EPITHELIAL CELLS ANALYZED WITH WESTERN BLOTTING.

Human serum proteins (15 µg each), human red blood cell (RBC) lysate or HEK293A cell lysate (50 µg), and SRA 01/04 cell (50 µg each) and human whole lens (50 µg each) lysates were analyzed by SDS–PAGE and western blotting performed with the antibody specified at the top of each panel. The ~26 kDa band detected in serum with the anti-APOA1 antibody corresponds to the expected size of secreted APOA1 and the faint ~50 kDa band is likely a dimer. The faint bands seen in SRA 01/04 cells are most likely non-specific. The ~41 kDa band detected with the anti-APOA4 antibody corresponds to the expected size of secreted APOA4, the bands between 60 and 75 kDa in SRA 01/04 cells are likely non-specific. The ~20 kDa band detected with

the anti-CRYAA antibody in human lens corresponds to the expected size of CRYAA monomer; the higher molecular mass bands likely represent the expected oligomeric forms of the protein. The ~55 kDa protein band in human serum detected with the anti-FGB antibody corresponds to the expected size of FGB. The <15 kDa protein band detected with the anti-HB antibody in RBCs likely represents the monomeric HB subunits. The monomeric subunits migrated slightly faster than their expected sizes of 15 to 17 kDa. The higher ~30 and ~50 kDa protein bands detected in the RBC lysate likely respectively represent di- and tetra- meric forms of HB. The faint 75–100 kDa bands in SRA 01/04 cells are likely non-specific. The ~25 kDa band in SRA 01/04 cells detected with the anti-HSPB1 antibody likely represents HSPB1 but the protein migrated slightly faster than its expected size of 27 kDa. The <25 kDa band in HEK293A and SRA 01/04 cells detected with the anti-PRDX2 antibody likely represents PRDX2 monomer, however, migration of the protein was slightly retarded than its expected size of 22 kDa. The <50 kDa bands in HEK293A and SRA 01/04 cells likely represent PRDX2 dimers. The faint ≥75 kDa bands in the two cell types are most likely none specific. None of the proteins except CRYAA was detected in human lens. Numbers along the panels indicate molecular masses of protein standards in kilo Daltons. Serum, human serum; SRA, SRA 01/04 cells; blood, red blood cells; 293A, HEK293A cells. To access the data, click or select the words "Appendix 6."

APPENDIX 7. IMMUNOHISTOCHEMICAL LABELING OF HSPB1 IN PEX AFFECTED AND UNAFFECTED LENS CAPSULES.

Sections of PEX affected (left panel) and unaffected (right panel) lens capsules from cataract patients were immunolabeled with the anti-HSPB1 antibody. HSPB1-positive labeling (brown) can be seen in the lens epithelium in unaffected lens capsule (right panel) indicating presence of the protein but very weak labeling of PEX material accumulated on affected lens capsule was observed (left panel). Lens capsule in the right hand image is folded over. Representative images from independent experiments on three PEX affected and four unaffected lens capsules are shown. Images are at 60× original magnification. To access the data, click or select the words "Appendix 7."

APPENDIX 8. PATHWAYS OVER REPRESENTED AMONG PEX SYNDROME ASSOCIATED GENES/ PROTEINS.

To access the data, click or select the words "Appendix 8."

APPENDIX 9. MOLECULAR RELATIONSHIPS AMONG PROTEINS IDENTIFIED IN PEX MATERIAL IN THIS STUDY, GENES/ PROTEINS PREVIOUSLY IMPLICATED IN PEX SYNDROME, AND THEIR INTERACTORS.

The network consisting of 1560 nodes and 2131 edges revealed by network analysis in InnateDB is shown. Genes/ proteins implicated in PEX syndrome are in red; solid line=protein–protein interaction; dashed line=unspecified association; double line=co-localization. To access the data, click or select the words "Appendix 9."

APPENDIX 10. PATHWAYS OVER REPRESENTED AMONG PEX SYNDROME ASSOCIATED GENES AND THEIR INTERACTORS.

To access the data, click or select the words "Appendix 10."

ACKNOWLEDGMENTS

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