Proteomics of Human Aqueous Humor

Article *in* Omics: a journal of integrative biology · May 2015

DOI: 10.1089/omi.2015.0029 · Source: PubMed

24 authors, including:

Jayshree Advani
Institute of Bioinformatics
27 PUBLICATIONS **725** CITATIONS
SEE PROFILE

Yashwanth Subbannayya
YU-IOB Center for Systems Biology and Molecul…
26 PUBLICATIONS **1,128** CITATIONS
SEE PROFILE

Gourav Dey
Institute of Bioinformatics
22 PUBLICATIONS **658** CITATIONS
SEE PROFILE

Aditi Chatterjee
Johns Hopkins Medicine
86 PUBLICATIONS **2,546** CITATIONS
SEE PROFILE

OMICS A Journal of Integrative Biology
Volume 19, Number 5, 2015

DOI: 10.1089/omi.2015.0029

Original Articles

Proteomics of Human Aqueous Humor

Krishna R. Murthy,[1–3] Pavithra Rajagopalan,[1,4] Sneha M. Pinto,[1,5] Jayshree Advani,[1,5] Praveen R. Murthy,[3] Renu Goel,[1,#] Yashwanth Subbannayya,[1,6] Lavanya Balakrishnan,[1,7] Mahashweta Dash,[8] Abhijith K. Anil,[8] Srikanth S. Manda,[1,9] Raja Sekhar Nirujogi,[1,9] Dhanashree S. Kelkar,[1] Gajanan J. Sathe,[1,5] Gourav Dey,[1,5] Aditi Chatterjee,[1,4] Harsha Gowda,[1] Shukti Chakravarti,[10] Subramanian Shankar,[11] Nandini A. Sahasrabuddhe,[1] Bipin Nair,[2] Babu Lal Somani,[1] T. S. Keshava Prasad,[1,2,5,9] and Akhilesh Pandey[12–15]

Abstract

The aqueous humor is a colorless, transparent fluid that fills the anterior chamber of the eye. It plays an important role in maintaining the intraocular pressure and providing nourishment to the lens and cornea. The constitution of the aqueous humor is controlled by the blood–aqueous barrier. Though this ocular fluid has been extensively studied, its role in ocular physiology is still not completely understood. In this study, aqueous humor samples were collected from 250 patients undergoing cataract surgery, subjected to multiple fractionation strategies and analyzed on a Fourier transform LTQ-Orbitrap Velos mass spectrometer. In all, we identified 763 proteins, of which 386 have been identified for the first time in this study. Sorbitol dehydrogenase (SORD), filensin (BFSP1), and phakinin (BFSP2) are some of the proteins that have not been previously reported in the aqueous humor. Gene Ontology analysis revealed 35% of the identified proteins to be extracellular, with a majority of them involved in cell communication and signal transduction. This study comprehensively reports 386 novel proteins that have important potential as biomarker candidates for future research into personalized medicine and diagnostics aimed towards improving visual health.

Introduction

The aqueous humor is an optically clear fluid that fills the space between the lens and the cornea. It is a mixture of organic solutes, electrolytes, cytokines, and proteins (Barsotti et al., 1992; Freddo et al., 1990; McLaren et al., 1993; To et al., 2002). Approximately 0.25 mL of aqueous humor is present in each eye in humans and its average rate of formation is 2.75 μL/min (Brubaker, 1991). The aqueous humor is predominantly secreted by the nonpigmented epithelial cells of the ciliary processes by active transport, though diffusion and ultrafiltration also play a role (Green et al., 1972; To et al., 2002).

The aqueous humor is in contact with the avascular structures of the eye such as the crystalline lens, posterior surface of the cornea, the anterior vitreous, and the trabecular meshwork. It exits the eye through the canal of Schlemm into the episcleral veins (Fig. 1A). An alternate exit route for the aqueous exists between the muscle bundles of the ciliary body, eventually draining to the supraciliary and suprachoroidal spaces, commonly called the uveoscleral pathway (To et al., 2002). This constant flow of aqueous humor replenishes the nutrients required for these avascular tissues and carries away their metabolic wastes. The aqueous humor dynamics also help to maintain the intraocular pressure of the eye that is essential for maintaining its optical and refractive

[1]Institute of Bioinformatics, International Tech Park, Bangalore, India.
[2]Department of Biotechnology, Amrita Vishwa Vidyapeetham, Kollam, India.
[3]Vittala International Institute of Ophthalmology, Bangalore, India.
[4]School of Biotechnology, KIIT University, Bhubaneswar, India.
[5]Manipal University, Madhav Nagar, Manipal, Karnataka, India.
[6]Rajiv Gandhi University of Health Sciences, Bangalore, India.
[7]Department of Biotechnology, Kuvempu University, Shankaraghatta, India.
[8]Department of Internal Medicine, Armed Forces Medical College, Pune, India.
[9]Centre of Excellence in Bioinformatics, School of Life Sciences, Pondicherry University, Puducherry, India.
[11]Department of Rheumatology, Medical Division, Command Hospital (Air Force), Bangalore, India.
[12]McKusick-Nathans Institute of Genetic Medicine, and Departments of [10]Medicine, [13]Biological Chemistry, [14]Oncology, and [15]Pathology, Johns Hopkins University School of Medicine, Baltimore, Maryland.
[#]Current Address: Renu Goel, PhD, Central Instrumentation Facility, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110021, India.

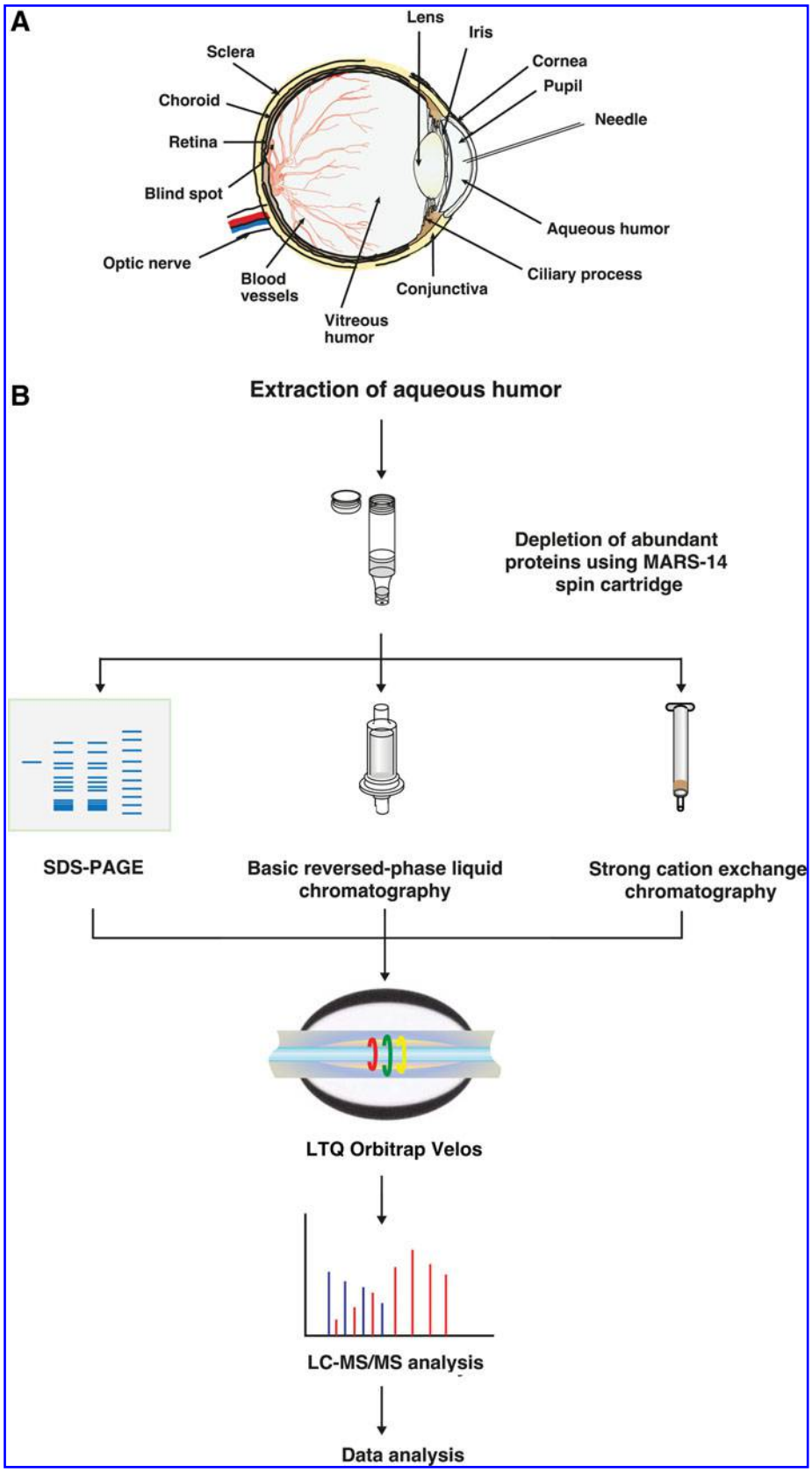

FIG. 1. A sagittal view of the human eye and the workflow employed in this study. **(A)** The aqueous humor bathes the crystalline eye lens and the cornea. For this study, aqueous humor samples were collected by paracentesis performed on patients undergoing cataract surgery. **(B)** After depletion by MARS-14 spin cartridge, aqueous humor samples were subjected to SDS-PAGE, SCX, and bRPLC fractionation. The fractions were then analyzed on the LTQ-Orbitrap Velos mass spectrometer. The data obtained were searched against human RefSeq database using SequestHT and Mascot search algorithms.

properties (Mark, 2010). Apart from supplying nutrients to the avascular ocular structures and maintaining the intraocular pressure, the aqueous humor also has antioxidant properties. Further, it plays an important role in the immune response of the eye (Apte et al., 1996; Kramer et al., 2005).

It has been previously suggested that the eye and the brain are immune privileged (Medawar, 1948). The phenomenon in which an antigen-specific systemic immunologic tolerance is induced to an antigen that has been introduced into the anterior chamber of the eye is called anterior chamber associated immune deviation (ACAID). Here, the antibody responses in the anterior chamber of the eye are preserved however, the delayed type hypersensitivity and the cytotoxic T cell responses are suppressed (Hori, 2008). The aqueous humor that fills the anterior chamber is thus a treasure house of information about this unique immune deviation in the human body.

The constituents of the aqueous humor are controlled and the large molecules are excluded due to presence of the blood–aqueous barrier. The blood–aqueous barrier is contributed by the tight junctions between the nonpigmented epithelial cells and nonfenestrated iris vessels. Several studies suggest that the protein levels in the aqueous humor vary in different ocular disorders (Duan et al., 2008; 2010; Jan-ciauskiene et al., 2006; Knepper et al., 2002; Ohguro et al., 2002; Sakamoto et al., 2001; Tong et al., 2006). However, only a limited number of proteomic studies have been carried out because of which, the baseline proteome of aqueous humor is yet to be defined (Chowdhury et al., 2010; Richardson et al., 2009). A comprehensive proteomic profiling of normal aqueous humor would serve as an invaluable template for future studies that focus on protein dynamics in pathological conditions of the eye. As the aqueous humor can be easily obtained for analysis, studying the changes occurring in the aqueous humor could give us valuable insights into some of the disease processes such as glaucoma, corneal dystrophies, uveitis, and myopia.

Therefore, in this study employing high-resolution mass spectrometry, we carried out an in-depth analysis of the aqueous humor proteome. We identified 763 proteins, of which 386 proteins have been detected for the first time in the aqueous humor. From this study, we confirm the annotated translational start sites of 47 proteins based on the N- termini peptides identified. Concurrently, we also obtained peptide evidence suggesting alternative translational start sites in 2 proteins. Further, this study also provides evidence of signal peptide cleavage sites in 33 proteins and identified novel signal peptide cleavage sites in 13 proteins.

Materials and Methods

Sample preparation

The aqueous humor samples were collected from 250 patients undergoing cataract surgery at the Vittala International Institute of Ophthalmology, Bangalore. The patients with any intercurrent ocular pathology were excluded from the study. The study was carried out after obtaining ethical approval from the Institutional Review Board of the Vittala International Institute of Ophthalmology, Bangalore, and written informed consent from each patient. The aqueous humor samples were pooled and abundant proteins were depleted using human multiple affinity removal system spin cartridge 14 (Agilent Technologies Inc., Cat# 5188-6560). The protein concentration of depleted aqueous humor sample was estimated using Lowry's assay (Lowry et al., 1951). The workflow employed in this study is represented in Figure 1B.

Sample fractionation

SDS-PAGE and in-gel digestion. Two hundred and fifty micrograms of protein was resolved by SDS-PAGE and stained with Coommassie Brilliant Blue. Twenty-six gel bands were excised and in-gel digestion was carried out as described previously (Goel et al., 2013; Harsha et al., 2008). Briefly, the excised bands were destained using 40 mM ammonium bicarbonate in 50% acetonitrile (ACN) solution. The destained gel bands were then subjected to reduction using 5mM dithiothreitol (DTT) (60°C for 45 min), followed by alkylation using 10 mM iodoacetamide (IAA). The gel pieces were dehydrated with 100% ACN, followed by digestion with trypsin (modified sequencing grade; Promega, Madison, WI) at 37°C for 12–16 h. The peptides were extracted from the gel fragments by treating gel bands twice with 0.4% formic acid, 3% ACN solution, once with 0.4% formic acid, 50% ACN solution and finally with 100% ACN. The extracted peptides were vacuum-dried and stored at −80°C until LC-MS/MS analysis.

In-solution digestion. In-solution digestion was carried out as previously described (Harsha et al., 2008). Briefly, 800 μg of protein was reduced with 5 mM DTT and alkylated using 10 mM IAA. The proteins were then digested with trypsin (1:20) (modified sequencing grade; Promega, Madison, WI) at 37°C for 16 h. The reaction was stopped with 0.1% formic acid. The peptides were dried, reconstituted with 0.1% trifluoroacetic acid.

Strong cation exchange (SCX) chromatography

The digested peptides were reconstituted using 10 mM potassium phosphate buffer containing 30% ACN, pH 2.7 (Solvent A). SCX fractionation of peptides equivalent to 250 μg of proteins was carried out on a Polysulfoethyl A column (PolyLC, Columbia, MD) (300 Å, 5 μm, 100 × 2.1 mm) using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) with a binary pump, UV detector, and a fraction collector (Chaerkady et al., 2008; Yang et al., 2010). Peptides were eluted across a linear salt gradient (0 to 35%) of solvent B (10 mM potassium phosphate buffer containing 30% ACN, 350 mM KCl, pH 2.7) at a flow rate of 200 μL/min. The fractions obtained after chromatography were pooled to get 18 total fractions. The samples were dried, reconstituted in 0.1% TFA and desalted using StageTips (Rappsilber et al., 2003). The desalted samples were vacuum-dried and stored at −80°C until further analysis.

Basic pH reversed phase liquid chromatography (bRPLC)

bRPLC fractionation was carried out for peptides equivalent to 250 μg of proteins, with a XBridge C18, 5 μm 250 × 4.6 mm analytical column (Waters Corporation, MA, USA) with a flow rate of 1 mL/min using an Agilent 1200 series HPLC system with a binary pump, autosampler, UV detector, and a fraction collector. Column equilibration was carried out using 7 mM triethyl ammonium bicarbonate solution (TEABC) (Solvent A). Peptides were eluted using a

solution of 7 mM TEABC in 90% ACN (Solvent B). Sample separation was carried out with the following gradient: 1% B for 0–5 min, 10% B for 5–10 min, 35% B for 10–40 min, and 100% B for 40–45 min. The 96 fractions obtained were concatenated to obtain 24 fractions, which were subjected to LC-MS/MS analysis.

LC-MS/MS analysis

Tryptic peptides extracted from gel bands, SCX and bRPLC fractions were analyzed on LTQ-Orbitrap Velos (Thermo Electron, Bremen, Germany) mass spectrometer coupled with Proxeon Easy nLC system (Thermo Scientific, Bremen, Germany). The nanospray source was fitted with an emitter tip (inner diameter 10 μm) (New Objective, Woburn, MA) and a voltage of 2 kV was applied. Peptide enrichment was carried out on a trap column (2 cm $\times$ 75 μm, Magic C_{18}AQ, 5 μm, 100 Å, Michrom Biosciences Inc.) at a flow rate of 3 μL/min. Peptides were resolved on an analytical column (10 cm $\times$ 75 μm, Magic C_{18}AQ, 3 μm, 100 Å, Michrom Biosciences Inc.) at a flow rate of 350 nL/min employing a linear gradient of 7%–30% ACN over 80 min.

MS and MS/MS scan acquisition was carried out in the Orbitrap mass analyzer at a mass resolution of 60,000 and 15,000 at 400 m/z, respectively. MS spectra were acquired in a data-dependent manner targeting twenty most abundant ions with charge state ≥ 2 in each survey scan in the m/z range of 350 to 1,800. Fragmentation was carried out using higher energy collisional dissociation mode with normalized collision energy of 39. Isolation width was set to 2 m/z. Precursor ions selected for MS/MS fragmentation were dynamically excluded for 60 seconds. The automatic gain control for full MS and MS/MS was set to 1×10^6 and 5×10^4 ions, respectively. Internal calibration was carried out by enabling lock mass option using polydimethylcyclosiloxane (m/z, 445.1200025) ions (Olsen et al., 2005).

Data analysis

The mass spectrometry data were searched against NCBI RefSeq human protein database (version 65 containing 34,453 protein sequences and known contaminants) using Mascot (version 2.2.0, Matrix Science, London, UK) and SequestHT search algorithms through the Proteome Discoverer software (Version 1.4.0.288, Thermo Fisher Scientific). The search criteria included oxidation of methionine and protein N-terminal acetylation as variable modifications and carbamidomethyl modification of cysteine as fixed modification. A precursor mass tolerance of 20 ppm, fragment mass tolerance of 0.05 Da was allowed. Up to two missed cleavages were allowed. Peptide spectral matches (PSMs) were filtered using a false discovery rate (FDR) of 1% at the peptide level. The unassigned spectra arising from these searches were exported as a single .mgf file and searched using semi-tryptic search criterion to identify peptides that suggested confirmation of protein start sites or alternative start sites. Possible signal peptide cleavage sites were also confirmed with data analysis.

Bioinformatics analysis

Protein lists acquired from the tryptic and semi-tryptic searches using Mascot and SequestHT were compiled. Subcellular localization, molecular class, molecular function, biological process, and domain-related information were fetched for the identified proteins from the Human Protein Reference Database (HPRD; http://www.hprd.org) (Prasad et al., 2009). Data were compared against the Plasma Proteome Database (Nanjappa et al., 2014) to identify proteins also known to be secreted in serum or plasma.

Data availability

The data generated in the course of this study was deposited to publicly available databases to make it accessible to the scientific community. The lists of proteins and peptides identified in this study were submitted to Human Proteinpedia http://www.humanproteinpedia.org) and can be visualized using the link: http://www.humanproteinpedia.org/data_display?exp_id=00853. We have also submitted the proteome data to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaino et al., 2013) with the dataset identifier PXD000787.

Results and Discussion

LC-MS/MS analysis of 66 fractions resulted in the generation of 56,481 peptide spectrum matches, which led to the identification of 4,581 peptides corresponding to 763 proteins. Of these, 386 proteins were found to be uniquely identified in aqueous humor in this study in comparison to previous high throughput studies on the aqueous humor (Table 1). Complete lists of proteins and peptides identified in this study have been provided in Supplementary Tables 1 and 2, respectively (Supplementary material is available online at www.liebertpub.com/omi). We also compared this list of 763 proteins with a previous proteomic study of human adult and fetal tissues and purified primary hematopoietic cells by our group (Kim et al., 2014). Fifteen proteins were unique to our study compared to this study. Of these 15 proteins, 7 proteins were also not identified in any of the previous high throughput studies on the aqueous humor. Comparison with the proteins annotated in the Plasma Proteome Database

Table 1. High Throughput Proteomic Studies Published on Human Aqueous Humor

Study	*Method used*	*Number of proteins identified*
1. Yao J et al, 2013b	MALDI-TOF/TOF MS	49
2. Yao J et al, 2013a	MALDI-TOF/TOF MS	68
3. Taube et al, 2013	LC-MS/MS; CE-MS/MS	501
4. Pollreisz et al, 2012	LC-MS/MS	323
5. Kim et al,2012	LC-MS/MS	154
6. Sacca et al, 2012	Antibody microarrays	13
7. Chiang et al, 2012	MALDI-TOF MS	11
8. Bennett et al, 2011	LC-MS/MS	198
9. Anshu et al, 2011	LC-MS/MS	135
10. Chowdhury et al, 2010	LC-MS/MS	676
11. Richardson et al, 2009	LC-MS/MS	152

(http://www.plasmaproteomedatabase.org/) confirmed that 640 proteins have been previously reported in the human serum or plasma.

Classification of aqueous humor proteins using Gene Ontology

All identified proteins were classified based on their domain information and subcellular localization (Fig. 2A). Of the 763 proteins identified in our study, 316 have signal peptides, 39 contain transmembrane domain, and 76 proteins contain both signal peptide and transmembrane domain. Thirty-five percent of the identified proteins were localized in the extracellular matrix. Cytoplasmic proteins also constitute a similar percentage (~31%) of the total proteins identified. The remaining proteins were distributed between the various organelles, including the cellular membranes (17%), the nucleus (10%), the endoplasmic reticulum, Golgi, and mitochondria (~2%–3% each). The proteins were also classified based on their biological processes. A majority of the proteins were found to be involved in cell communication and signal transduction, followed by cell growth, differentiation, proliferation, and energy pathways as depicted in Figure 2B.

Protein N-termini and signal peptide cleavage sites

Peptides identified from semi-tryptic searches using N-term acetylation modification, aided in the confirmation of the annotated translational start sites of 47 proteins. Further, we also identified 2 proteins where peptides mapped downstream of currently annotated translation start sites (Supplementary Table 3A). Thirty-three putative signal peptide cleavage sites were confirmed and 13 possible novel signal peptide cleavage sites were identified by analysis using HPRD information on signal peptide length and SignalP prediction tool (Version 4.1) (Petersen et al., 2011) (Supplementary Tables 3B and 3C).

Discussion

Proteomic analysis of the aqueous humor has previously been carried out by other groups; a list of high-throughput studies on the human aqueous humor proteome is provided in Table 1. However, out of the 763 proteins identified in our study, 386 have not been reported in other proteomic studies. We discuss some of the previously reported and uniquely identified proteins, including some of the proteins that have a role in the glycolysis–gluconeogenesis pathway, in the ensuing sections.

Proteins previously identified in other studies

Among the proteins identified in our study, 377 of them have been described in previous studies on the aqueous humor. The representative MS/MS spectra of one of the previously identified proteins, vitamin D-binding protein (GC) is shown in Figure 3A. We identified several proteins, including transforming growth factor beta 2 (TGFB2), components of the complement pathway such as complement components 3–7 (C3, C4B, C5, C6, C7), and others in our study. Complement factors seem to have an antimicrobial activity within the eye. As previously mentioned, the aqueous humor has an important role to play in the immune responses within the eye and in the phenomenon of ACAID. This immune deviation is thought to be due to the fact that the cornea has no blood supply or lymphatics, the epithelial cells, keratocytes, and endothelial cells of the cornea do not express class II MHC (major histocompatibility complex) molecules while expressing very low levels of class I MHC molecules (Streilein et al., 1979; Wang et al., 1987).

The eye and the spleen seem to have a major role in the induction of this immune tolerance in the presence of various immunomodulators to help the CD4+ and CD8+ cells to differentiate into the ACAID inducing regulatory T cells (ACAID Treg) (Hori, 2008; Wang et al., 2001; Wilbanks et al., 1991; 1992). The CD4+ ACAID Treg cells inhibit the differentiation of Th1 cells in the lymph nodes and the CD8+ ACAID Treg inhibits the function of the effector T cells (Th1 and Th2) at the local site (Ji et al., 2011; Wang et al., 2001). This interplay of immune cells makes corneal transplantation one of the most successful organ transplantations in the body. TGFB2, thrombospondin 1, interferon gamma, interleukin 10, and macrophage inflammatory protein 2 are some of the immunomodulators that have been reported to have specific roles in the induction of ACAID (Ghasemi et al., 2012; Paunicka et al., 2012).

We also identified a number of proteins that have an established role in vasculogenesis such as osteopontin (SPP1), platelet derived growth factor D (PDGFD), vasorin (VASN), and pigment epithelium derived factor (SERPINF1). Osteopontin (Cui et al., 2007; Dai et al., 2009; Du et al., 2009; Wang et al., 2011) and platelet-derived growth factor D are vasculogenic factors, which promote the growth of new blood vessels, whereas vasorin (Ikeda et al., 2004), vitamin D-binding protein (Kalkunte et al., 2005), and pigment epithelium derived factor (Dawson et al., 1999; Haurigot et al., 2012) are anti-angiogenic factors that inhibit blood vessel formation. The presence of these factors in the aqueous humor again emphasizes the fine interplay between the angiogenic and anti-angiogenic factors that probably helps to maintain the avascular nature of both the cornea and the lens which is crucial for the transparency of these tissues.

Oxidative damage can result in a number of molecular changes in the lens, resulting in the development of cataract. Thus, the oxidative reduction mechanisms possess special importance in the lens. Glutathione, a tripeptide, has a central role to play in protecting the lens from oxidative damage (Olofsson et al., 2009). Superoxide dismutase, glutathione synthase, and glutathione reductase are enzymes involved in the redox reaction in the lens and were identified in the aqueous humor in our study.

Proteins uniquely identified in this study

We have identified 386 proteins that have not been previously identified in other high throughput studies on the aqueous humor to the best of our knowledge. A partial list of proteins identified uniquely in this study is provided in Table 2. This list includes proteins crystalline, beta B3 (representative MS/MS spectra depicted in Fig. 3B), sorbitol dehydrogenase (SORD), filensin (BFSP1), phakinin (BFSB2), platelet-derived growth factor subunit A isoform 2 (PDGFA), and platelet-derived growth factor beta polypeptide (PDGFB). Sorbitol dehydrogenase is an enzyme that plays an important role in the metabolism of glucose in the lens, especially in patients with diabetes.

The human lens is almost entirely dependent on glucose for its energy requirements. Glucose enters the lens by

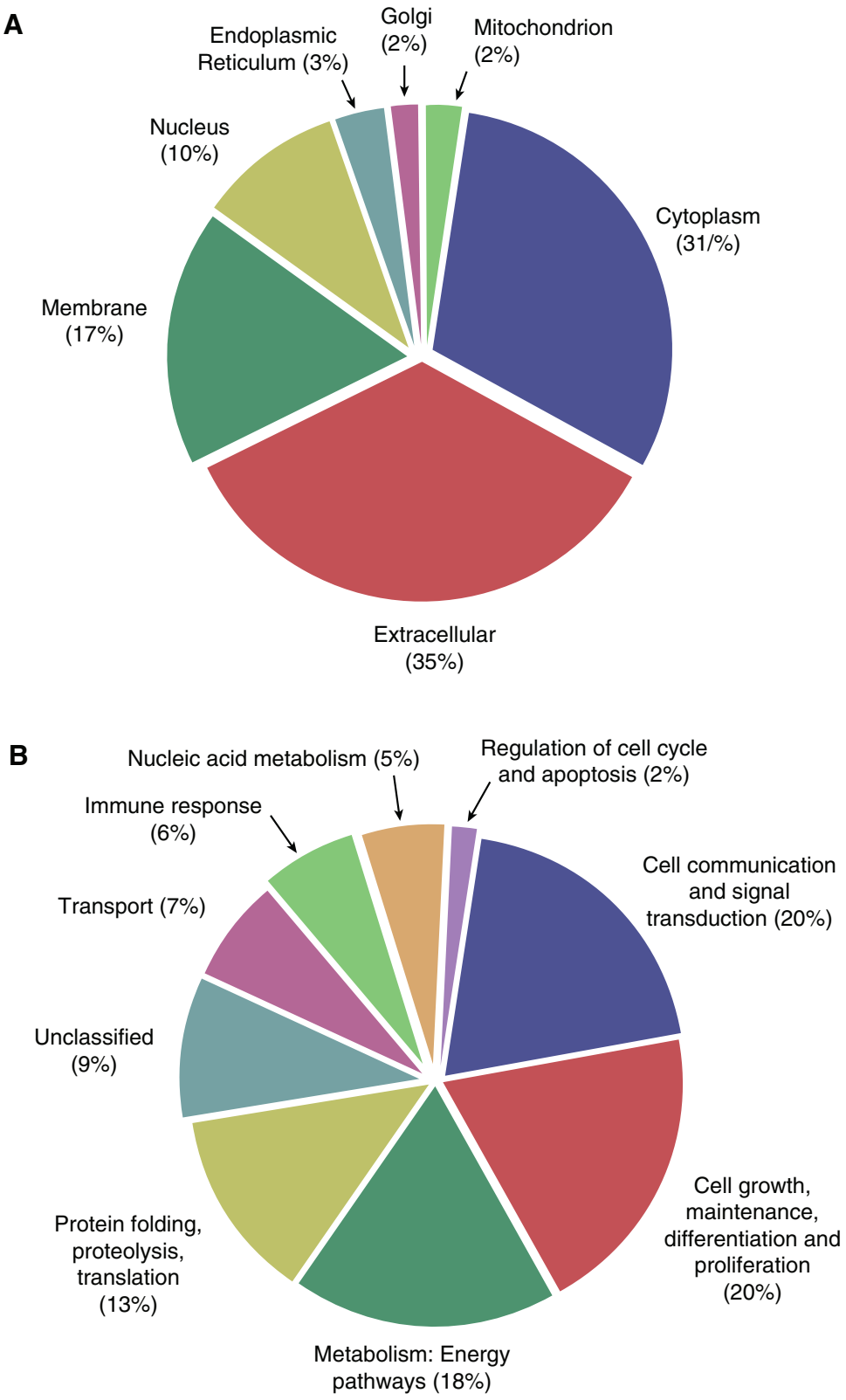

FIG. 2. Subcellular localization and functional annotation of identified proteins. **(A)** Gene Ontology-based analysis for subcellular localization of proteins identified in the aqueous humor. **(B)** Distribution of identified proteins based on their biological processes. All depicted data were obtained from Human Protein Reference Database (http://www.hprd.org).

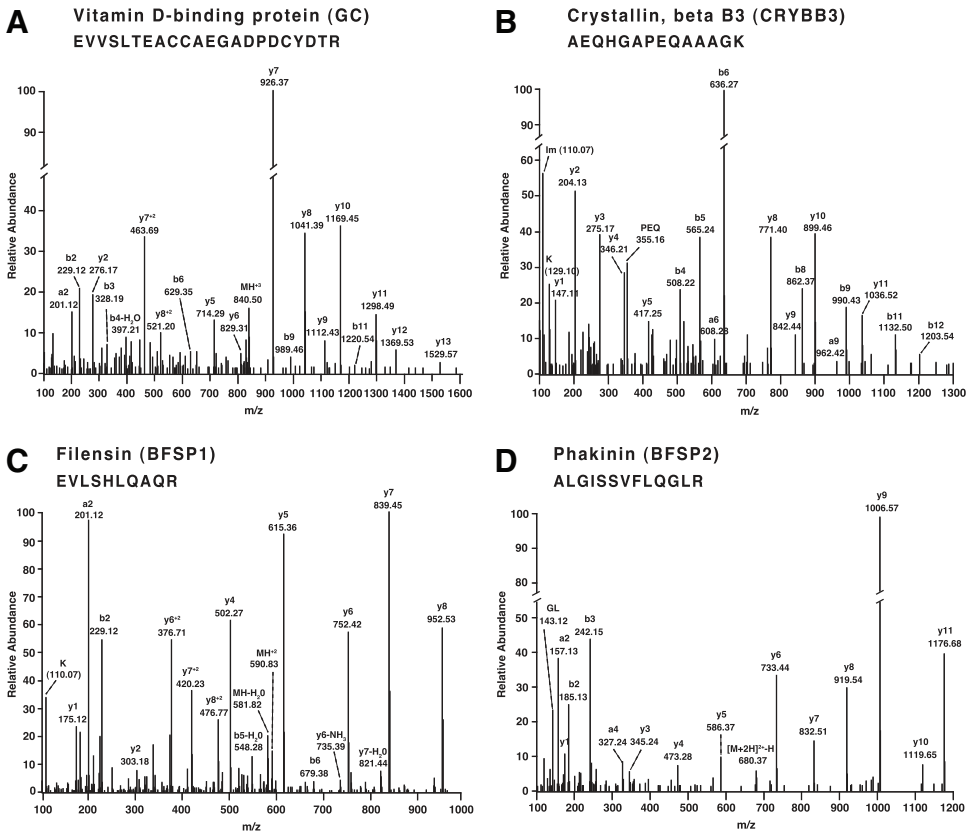

FIG. 3. Representative MS/MS spectra of some proteins identified in this study. **(A)** vitamin D- binding protein (GC), a protein previously described in other studies. Proteins uniquely identified in our study: **(B)** crystalline, beta B3 (CRYBB3)**, (C)** filensin (BFSP1), and **(D)** phakinin (BFSP2).

facilitated diffusion and it is rapidly metabolized such that the level of free glucose in the lens is kept to a minimum. Seventy percent of the energy requirement of the lens is met by the anaerobic glycolysis of glucose, while aerobic metabolism through the Kreb's cycle contributes to 20% of the energy needs. Some of the excess glucose entering the lens enters the sorbitol pathway, and glucose gets converted to sorbitol as a result of the action of aldose reductase (Kim et al., 2011; Yabe-Nishimura, 1998). Normally, the sorbitol formed in this fashion is converted to fructose by SORD which, in turn, can diffuse out of the lens. In diseased condition, however, sorbitol cannot be efficiently converted to fructose leading to its accumulation, osmotic stress, and development of cataract. A similar mechanism is implicated in the development of some of the other complications of diabetes (Carr et al., 1995). Thus, SORD has a crucial role to play in the prevention of sugar-related cataract. BFSP1 and BFSP2 are type III intermediate filament proteins. Representative MS/MS spectra for these two proteins are shown in Figure 3C and 3D. BFSP1 has two isoforms of length 665 and 540 amino acids, respectively. During differentiation of the lens epithelial cells to fiber cells, both these proteins show maximal accumulation towards the end of the elongation process. These two proteins appear to be unique to the differentiated lens fiber cell.

Platelet-derived growth factors are ubiquitous growth factors and are dimers of disulfide-linked polypeptide chains. Platelet-derived growth factors act through two receptors (platelet-derived growth factor receptor alpha, and platelet-derived growth factor receptor beta), which are receptor tyrosine kinases (Andrae et al., 2008). Studies on Xenopus embryos, sea urchins, and zebrafish have shown that platelet-derived growth factor alpha has an important role to play in the gastrulation phase of embryonic development (Ataliotis et al., 1995; Montero et al., 2004; Ramachandran et al., 1995; 1997).

PDGFA also plays a role in cell proliferation, cell migration, organogenesis, and development of the axial skeleton and teeth (Andrae et al., 2008). PDGFB, on the other hand, appears to be essential in the development of the cardiovascular system, the hematopoietic system, and in angiogenesis. Platelet-derived growth factors are key players in disease conditions including cancers (glioblastoma), fibrotic diseases (pulmonary and liver fibrosis), and vascular disorders (atherosclerosis) (Andrae et al., 2008). They have also been implicated in the pathogenesis of proliferative vitreoretinopathies such as diabetic retinopathy and proliferative vitreoretinopathy (Mori et al., 2002). High retinal expression of PDGFA has been shown to promote predominantly glial tissue proliferation as seen in proliferative vitreoretinopathy, whereas high retinal expression of PDGFB promotes proliferation of vascular and glial tissue, leading to tractional retinal detachment, mimicking the pathologic changes occurring in diabetic retinopathy (Mori et al., 2002).

Table 2. A Partial List of Proteins Uniquely Identified in the Aqueous Humor Proteome

	Gene symbol	*Protein name*	*Subcellular localization*	*Biological process*	*Molecular function*
1	*SORD*	Sorbitol dehydrogenase	Plasma membrane	Metabolism; energy pathways	Catalytic activity
2	*BFSP1*	Beaded filament structural protein 1, filensin	Cytoplasm	Cell growth and/or maintenance	Structural constituent of cytoskeleton
3	*BFSP2*	Beaded filament structural protein 2, phakinin	Cytoplasm	Cell growth and/or maintenance	Structural constituent of cytoskeleton
4	*PDGFA*	Platelet-derived growth factor alpha polypeptide	Extracellular	Cell proliferation; cell surface receptor linked signal transduction; cell migration	Growth factor activity
5	*PDGFB*	Platelet-derived growth factor beta polypeptide	Extracellular	Cell communication; signal transduction	Growth factor activity
6	*PPBP*	Pro-platelet basic protein	Extracellular	Cell communication; signal transduction	Chemokine activity
7	*AKR1A1*	Aldo-keto reductase family 1, member A1 (aldehyde reductase)	Cytoplasm	Metabolism	Oxidoreductase activity
8	*CTSA*	cathepsin A	Lysosome	Protein metabolism	Serine-type peptidase activity
9	*MIP*	Major intrinsic protein of lens fiber	Plasma membrane	Transport	Water channel activity
10	*PFKL*	6-Phosphofructokinase, liver type isoform b	Cytoplasm	Metabolism; energy pathways	Catalytic activity
11	*PGM1*	Phosphoglucomutase-1 isoform 3	Cytoplasm	Metabolism; energy pathways	Catalytic activity
12	*RLBP1*	Retinaldehyde binding protein 1	Cytoplasm	Transport	Transporter activity
13	*TRH*	Thyrotropin releasing hormone	Extracellular	Cell communication; signal transduction	Peptide hormone
14	*HSPB1*	Heat shock protein beta-1	Cytoplasm	Protein metabolism	Chaperone activity
15	*WFDC1*	WAP four-disulfide core domain 1	Extracellular	Cell communication; signal transduction	Protease inhibitor activity

Enzymes involved in glycolysis and pentose phosphate pathway identified in this study

In this study, we identified 22 enzymes that are known to be involved in glycolysis, gluconeogenesis, and the pentose phosphate pathway. The enzymes identified are highlighted in blue in Figure 4. Glycolysis is a metabolic process by which a molecule of glucose is catabolized to yield two molecules each of pyruvate, adenosine triphosphate (ATP), and reduced nicotinamide adenine dinucleotide through a series of enzyme-catalyzed reactions. Various glycolytic enzymes and enzymes involved in the pentose phosphate pathway known to be localized in the cytoplasm have been identified in the aqueous humor.

As mentioned earlier, nearly 70% of the glucose entering the lens is metabolized through the anaerobic glycolytic pathway. Since the lens is an avascular structure, it depends on the aqueous humor for its metabolic activity. Alternatively, glucose is converted to glucose-6-phosphate, which is further oxidized to pentose phosphates by the pentose phosphate pathway. The net result is the production of two molecules of nicotinamide adenine dinucleotide phosphate (NADPH) and ribose-5-phosphate. NADPH is a reducing agent for biosynthetic reactions and ribose-5-phosphate is a precursor for nucleic acid synthesis. The NADPH provided by this pathway helps to protect the cells of the lens and cornea from the oxidative damage of oxygen and the free radicals generated by them (Kinoshita, 1965).

Some of the enzymes we identified include key enzymes catalyzing irreversible reactions of glycolysis such as 6-phosphofructokinase (PFKL) and pyruvate kinase (PKM2). The enzyme PFKL catalyzes the conversion of D fructose-6-phosphate to D fructose-1, 6- bisphosphate—the first irreversible step in the glycolysis pathway. The enzyme PKM2 catalyzes the final step of the glycolytic pathway, the transfer of a phosphoryl group from phosphoenolpyruvate to adenosine diphosphate (ADP) to generate a molecule of ATP and pyruvate.

Conclusions

Our study provides an in-depth analysis of the aqueous humor proteome in humans. The observed enrichment of proteins involved in anaerobic metabolism may cater to the energy needs of avascular structures in the eye such as the lens and cornea. The large number of vasculogenic proteins and immunomodulators identified in this study may play a role in the fine balance between angiogenic and anti-angiogenic factors and the special immune privilege of corneal transplantation, which are not yet fully understood. The data from this

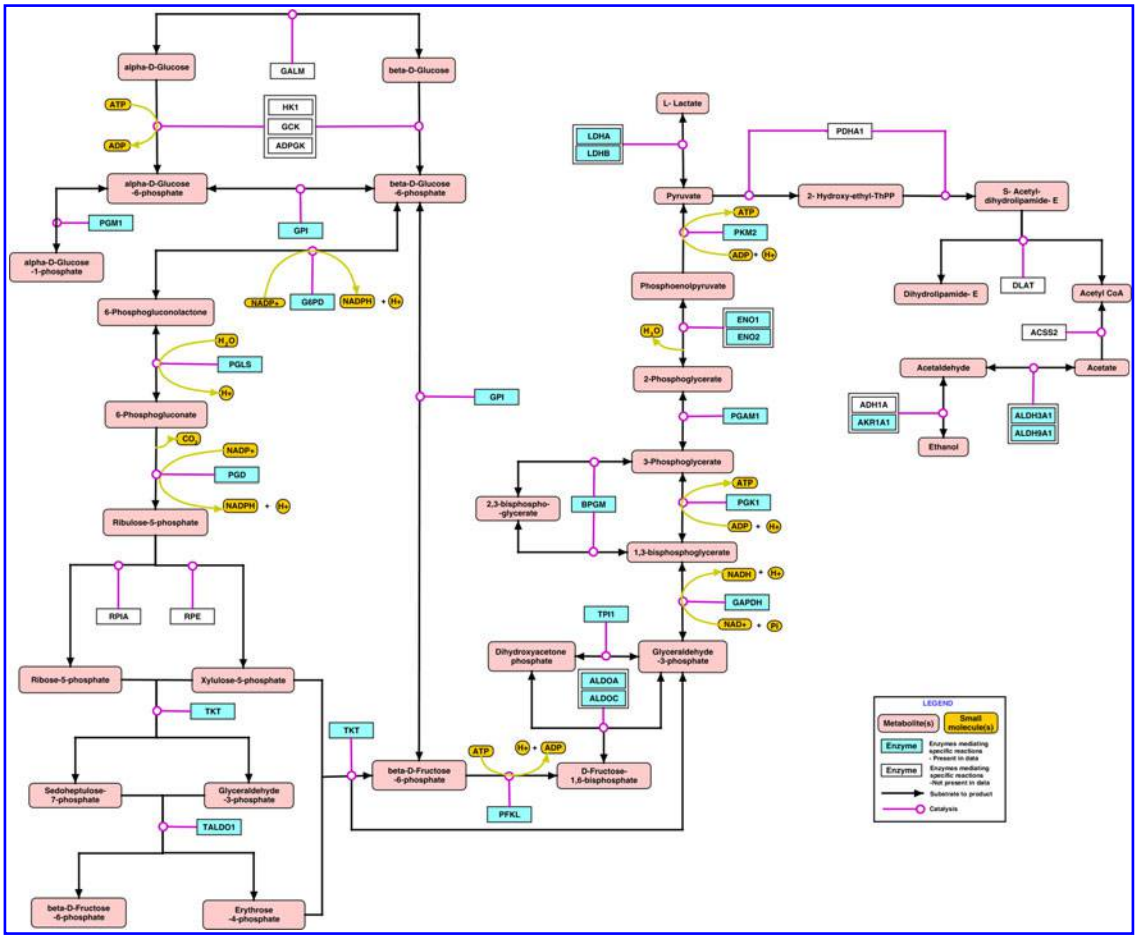

FIG. 4. Pathway representation of identified proteins involved in anaerobic energy metabolism. Twenty-two enzymes involved in glycolysis, gluconeogenesis, and the pentose phosphate pathway were identified in our study. The observed enrichment of these proteins may cater to the energy needs of avascular structures such as the eye lens and cornea that are bathed by the aqueous humor.

study will serve as a baseline for future proteomic studies on pathological conditions such as glaucoma, cataract, uveitis, and pseudoexfoliation syndrome.

Acknowledgments

We thank the Department of Biotechnology (DBT), Government of India, for research support to the Institute of Bioinformatics. We thank the Infosys Foundation for research support to the Institute of Bioinformatics. Pavithra Rajagopalan, Sneha M. Pinto, Raja Sekhar Nirujogi, and Gajanan J. Sathe are recipients of Senior Research Fellowships from Council of Scientific and Industrial Research (CSIR), Government of India. Srikanth S. Manda, Gourav Dey, and Yashwanth Subbannayya are recipients of Senior Research Fellowships from University Grants Commission (UGC), Government of India. Dr. Harsha Gowda is a Wellcome Trust/DBT India Alliance Early Career Fellow. Dr. T. S. Keshava Prasad and Dr. Krishna R. Murthy are the recipients of a research grant on ''Proteomics based identification and validation of urinary Biomarkers for Age-related Macular Degeneration'' from DBT. Dr. T. S. Keshava Prasad is the recipient of a research grant on ''Development of infrastructure and a computational framework for analysis of proteomic data'' from DBT.

Author Disclosure Statement

The authors declare that there are no conflicting financial interests.

References

Andrae J, Gallini R, and Betsholtz C. (2008). Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22, 1276–1312.

Apte RS, and Niederkorn JY. (1996). Isolation and characterization of a unique natural killer cell inhibitory factor present in the anterior chamber of the eye. J Immunol 156, 2667–2673.

Ataliotis P, Symes K, Chou MM, Ho L, and Mercola M. (1995). PDGF signalling is required for gastrulation of Xenopus laevis. Development 121, 3099–3110.

Barsotti MF, Bartels SP, Freddo TF, and Kamm RD. (1992). The source of protein in the aqueous humor of the normal monkey eye. Invest Ophthalmol Vis Sci 33, 581–595.

Brubaker RF. (1991). Flow of aqueous humor in humans [The Friedenwald Lecture]. Invest Ophthalmol Vis Sci 32, 3145–3166.

Carr IM, and Markham AF. (1995). Molecular genetic analysis of the human sorbitol dehydrogenase gene. Mamm Genome 6, 645–652.

Chaerkady R, Harsha HC, Nalli A, et al. (2008). A quantitative proteomic approach for identification of potential biomarkers in hepatocellular carcinoma. J Proteome Res 7, 4289–4298.

Chowdhury UR, Madden BJ, Charlesworth MC, and Fautsch MP. (2010). Proteome analysis of human aqueous humor. Invest Ophthalmol Vis Sci 51, 4921–4931.

Cui R, Takahashi F, Ohashi R, et al. (2007). Abrogation of the interaction between osteopontin and alphavbeta3 integrin reduces tumor growth of human lung cancer cells in mice. Lung Cancer 57, 302–310.

Dai J, Peng L, Fan K, et al. (2009). Osteopontin induces angiogenesis through activation of PI3K/AKT and ERK1/2 in endothelial cells. Oncogene 28, 3412–3422.

Dawson DW, Volpert OV, Gillis P, et al. (1999). Pigment epithelium-derived factor: A potent inhibitor of angiogenesis. Science 285, 245–248.

Du XL, Jiang T, Sheng XG, Gao R, and Li QS. (2009). Inhibition of osteopontin suppresses in vitro and in vivo angiogenesis in endometrial cancer. Gynecol Oncol 115, 371–376.

Duan X, Lu Q, Xue P, et al. (2008). Proteomic analysis of aqueous humor from patients with myopia. Mol Vis 14, 370–377.

Duan X, Xue P, Wang N, Dong Z, Lu Q, and Yang F. (2010). Proteomic analysis of aqueous humor from patients with primary open angle glaucoma. Mol Vis 16, 2839–2846.

Freddo TF, Bartels SP, Barsotti MF, and Kamm RD. (1990). The source of proteins in the aqueous humor of the normal rabbit. Invest Ophthalmol Vis Sci 31, 125–137.

Ghasemi H, Ghazanfari T, Yaraee R, Owlia P, Hassan ZM, and Faghihzadeh S. (2012). Roles of IL-10 in ocular inflammations: A review. Ocul Immunol Inflamm 20, 406–418.

Goel R, Murthy KR, Srikanth SM, Pinto SM, Bhattacharjee M, Kelkar DS, et al. (2013). Characterizing the normal proteome of human ciliary body. Clin Proteomics 10, 9.

Green K, and Pederson JE. (1972). Contribution of secretion and filtration to aqueous humor formation. Am J Physiol 222, 1218–1226.

Harsha HC, Molina H, and Pandey A. (2008). Quantitative proteomics using stable isotope labeling with amino acids in cell culture. Nat Protoc 3, 505–516.

Haurigot V, Villacampa P, Ribera A, et al. (2012). Long-term retinal PEDF overexpression prevents neovascularization in a murine adult model of retinopathy. PLoS One 7, e41511.

Hori J. (2008). Mechanisms of immune privilege in the anterior segment of the eye: What we learn from corneal transplantation. J Ocul Biol Dis Infor 1, 94–100.

Ikeda Y, Imai Y, Kumagai H, et al. (2004). Vasorin, a transforming growth factor beta-binding protein expressed in vascular smooth muscle cells, modulates the arterial response to injury in vivo. Proc Natl Acad Sci USA 101, 10732–10737.

Janciauskiene S, Brandt L, Wallmark A, Westin U, and Krakau T. (2006). Secreted leukocyte protease inhibitor is present in aqueous humours from cataracts and other eye pathologies. Exp Eye Res 82, 505–511.

Ji SX, Yin XL, and Yang PZ. (2011). Effect of CD4(+)CD25(+) regulatory T cells in the development of anterior chamber-associated immune deviation. Int J Ophthalmol 4, 19–25.

Kalkunte S, Brard L, Granai CO, and Swamy N. (2005). Inhibition of angiogenesis by vitamin D-binding protein: Characterization of anti-endothelial activity of DBP-maf. Angiogenesis 8, 349–360.

Kim JK, Lee YS, Kim SH, Bae YS, and Lim SS. (2011). Inhibition of aldose reductase by phenylethanoid glycoside isolated from the seeds of Paulownia coreana. Biol Pharm Bull 34, 160–163.

Kim MS, Pinto SM, Getnet D, et al. (2014). A draft map of the human proteome. Nature 509, 575–581.

Kinoshita JH. (1965). Pathways of glucose metabolism in the lens. Invest Ophthalmol 4, 619–628.

Knepper PA, Mayanil CS, Goossens W, et al. (2002). Aqueous humor in primary open-angle glaucoma contains an increased level of CD44S. Invest Ophthalmol Vis Sci 43, 133–139.

Kramer M, Goldenberg-Cohen N, Axer-Siegel R, Weinberger D, Cohen Y, and Monselise Y. (2005). Inflammatory reaction in acute retinal artery occlusion: cytokine levels in aqueous humor and serum. Ocul Immunol Inflamm 13, 305–310.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. (1951). Protein measurement with the Folin phenol reagent. J Biol Chem 193, 265–275.

Mark HH. (2010). Aqueous humor dynamics in historical perspective. Surv Ophthalmol 55, 89–100.
McLaren JW, Ziai N, and Brubaker RF. (1993). A simple three-compartment model of anterior segment kinetics. Exp Eye Res 56, 355–366.
Medawar PB. (1948). Immunity to homologous grafted skin; The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. Br J Exp Pathol 29, 58–69.
Montero JA, and Heisenberg CP. (2004). Gastrulation dynamics: Cells move into focus. Trends Cell Biol 14, 620–627.
Mori K, Gehlbach P, Ando A, et al. (2002). Retina-specific expression of PDGF-B versus PDGF-A: Vascular versus nonvascular proliferative retinopathy. Invest Ophthalmol Vis Sci 43, 2001–2006.
Nanjappa V, Thomas JK, Marimuthu A, et al. (2014). Plasma Proteome Database as a resource for proteomics research: 2014 update. Nucleic Acids Res 42, D959–965.
Ohguro H, Maruyama I, Nakazawa M, and Oohira A. (2002). Antirecoverin antibody in the aqueous humor of a patient with cancer-associated retinopathy. Am J Ophthalmol 134, 605–607.
Olofsson EM, Marklund SL, and Behndig A. (2009). Enhanced diabetes-induced cataract in copper-zinc superoxide dismutase-null mice. Invest Ophthalmol Vis Sci 50, 2913–2918.
Olsen JV, de Godoy LM, Li G, et al. (2005). Parts per million mass accuracy on an Orbitrap mass spectrometer via lock mass injection into a C-trap. Mol Cell Proteomics 4, 2010–2021.
Paunicka K, Chen PW, and Niederkorn JY. (2012). Role of IFN-gamma in the establishment of anterior chamber-associated immune deviation (ACAID)-induced CD8+ T regulatory cells. J Leukoc Biol 91, 475–483.
Petersen TN, Brunak S, von Heijne G, and Nielsen H. (2011). SignalP 4.0: Discriminating signal peptides from transmembrane regions. Nat Methods 8, 785–786.
Prasad TSK, Goel R, Kandasamy K, et al. (2009). Human Protein Reference Database–2009 update. Nucleic Acids Res 37, D767–772.
Ramachandran RK, Govindarajan V, Seid CA, Patil S, and Tomlinson CR. (1995). Role for platelet-derived growth factor-like and epidermal growth factor-like signaling pathways in gastrulation and spiculogenesis in the Lytechinus sea urchin embryo. Dev Dyn 204, 77–88.
Ramachandran RK, Wikramanayake AH, Uzman JA, Govindarajan V, and Tomlinson CR. (1997). Disruption of gastrulation and oral-aboral ectoderm differentiation in the Lytechinus pictus embryo by a dominant/negative PDGF receptor. Development 124, 2355–2364.
Rappsilber J, Ishihama Y, and Mann M. (2003). Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem 75, 663–670.
Richardson MR, Price MO, Price FW, et al. (2009). Proteomic analysis of human aqueous humor using multidimensional protein identification technology. Mol Vis 15, 2740–2750.
Sakamoto T, Ito S, Yoshikawa H, et al. (2001). Tissue factor increases in the aqueous humor of proliferative diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol 239, 865–871.
Streilein JW, Toews GB, and Bergstresser PR. (1979). Corneal allografts fail to express Ia antigens. Nature 282, 326–327.
To CH, Kong CW, Chan CY, Shahidullah M, and Do CW. (2002). The mechanism of aqueous humour formation. Clin Exp Optom 85, 335–349.
Tong JP, Chan WM, Liu DT, et al. (2006). Aqueous humor levels of vascular endothelial growth factor and pigment epithelium-derived factor in polypoidal choroidal vasculopathy and choroidal neovascularization. Am J Ophthalmol 141, 456–462.
Vizcaino JA, Cote RG, Csordas A, et al. (2013). The PRoteomics IDEntifications (PRIDE) database and associated tools: Status in 2013. Nucleic Acids Res 41, D1063–1069.
Wang HM, Kaplan HJ, Chan WC, and Johnson M. (1987). The distribution and ontogeny of MHC antigens in murine ocular tissue. Invest Ophthalmol Vis Sci 28, 1383–1389.
Wang Y, Goldschneider I, O'Rourke J, and Cone RE. (2001). Blood mononuclear cells induce regulatory NK T thymocytes in anterior chamber-associated immune deviation. J Leukoc Biol 69, 741–746.
Wang Y, Yan W, Lu X, et al. (2011). Overexpression of osteopontin induces angiogenesis of endothelial progenitor cells via the avbeta3/PI3K/AKT/eNOS/NO signaling pathway in glioma cells. Eur J Cell Biol 90, 642–648.
Wilbanks GA, Mammolenti M, and Streilein JW. (1992). Studies on the induction of anterior chamber-associated immune deviation (ACAID). III. Induction of ACAID depends upon intraocular transforming growth factor-beta. Eur J Immunol 22, 165–173.
Wilbanks GA, and Streilein JW. (1991). Studies on the induction of anterior chamber-associated immune deviation (ACAID). 1. Evidence that an antigen-specific, ACAID-inducing, cell-associated signal exists in the peripheral blood. J Immunol 146, 2610–2617.
Yabe-Nishimura C. (1998). Aldose reductase in glucose toxicity: A potential target for the prevention of diabetic complications. Pharmacol Rev 50, 21–33.
Yang Y, Chaerkady R, Kandasamy K, et al. (2010). Identifying targets of miR-143 using a SILAC-based proteomic approach. Mol Biosyst 6, 1873–1882.

Address correspondence to:
Akhilesh Pandey, MD, PhD
McKusick-Nathans Institute of Genetic Medicine
733 N. Broadway, BRB 527
Johns Hopkins University
Baltimore, MD 21205

E-mail: pandey@jhmi.edu

or

Krishna R Murthy, DO,MRCOphth (Lon)
Institute of Bioinformatics
International Technology Park
Bangalore 560066
India

E-mail: krishna@viio.org

Abbreviations Used

ACAID = anterior chamber-associated immune deviation
ACN = acetonitrile
bRPLC = basic reverse phase liquid chromatography
DTT = dithiothreitol
IAA = iodoacetamide
SCX = strong cation exchange chromatography
SDS-PAGE = sodium dodecyl sulfate—polyacrylamide gel electrophoresis
TFA = trifluoroacetic acid
TEABC = triethyl ammonium bicarbonate