Proteomic analysis of chick retina during early recovery from lens-induced myopia

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Abstract. Myopia development has been extensively studied from different perspectives. Myopia recovery is also considered important for understanding the development of myopia. However, despite several previous studies, retinal proteomics during recovery from myopia is still relatively unknown. Therefore, the aim of the present study was to investigate the changes in protein profiles of chicken retinas during early recovery from lens-induced myopia to evaluate the signals involved in the adjustment of this refractive disorder. Three-day old chickens wore glasses for 7 days (-10D lens over the right eye and a plano lens as control over the left eye), followed by 24 h without lenses. Protein expression in the retina was measured by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). Pro-Q Diamond phosphoprotein staining 2D gel electrophoresis was used to analyze phosphoprotein profiles. Protein spots with significant differences (P<0.05) were analyzed by mass spectrometry. The minus lens-treated eye became myopic, however following 24 h recovery, less myopia was observed. 2D-DIGE proteomic analysis demonstrated that three identified protein spots were upregulated at least 1.2-fold in myopic recovery retinas compared with those of the controls, Ras related protein Rab-11B, S-antigen retina and pineal gland and 26S proteasome non-ATPase regulatory subunit 14. Pro-Q Diamond images further revealed three protein spots with significant changes (at least 1.8-fold): β-tubulin was downregulated, while peroxiredoxin 4 and ubiquitin carboxyl-terminal hydrolase-L1 were upregulated in the recovery retinas compared with the control eye retinas. The present study detected previously unreported protein changes in recovering eyes, therefore revealing their potential involvement in retinal remodeling during eye ball reforge.

Introduction

Refractive errors are the result of an incorrect pairing between the axial length of an eye and its refractive power. Optical defocus inflicted to the retina results in refractive errors. Indeed, the use of negative lenses triggers hyperopic defocus and consequent axial myopia, while positive lenses on myopia defocus result in axial hyperopia (1-3). Visual signals are used to develop an emmetropization mechanism, which controls the axial elongation rate in order to locate the retina close to the focal plane (4). An emmetropic eye is one in which distant objects' images are sharply focused on the photoreceptors as a result of the perfect match between optical strengthand axial length. In contrast, a myopic eye has an axial length longer than the focal plane, resulting in an image focused in front of the retina, whilst an axial length shorter than the focal plane is typical of a hyperopic eye, in which the image is focused behind the retina. Hyperopia or myopia development is detected by the retina, triggering a signaling cascade to produce biochemical modifications via the choroid and retinal pigment epithelium (5).

Certain biomarkers could be characterized by important protein modifications such as folding change and post-translational modifications (PTM). Organisms' complexity is mainly due to PTM, mostly considered as the addition of a functional group to one or more amino acids, or the proteolytic cleavage of one or more groups (6). These

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modifications can result in several changes in protein properties (7,8). The most common PTM types are glycosylation and phosphorylation, both resulting in protein function modifications. During the life cycle of the proteins, one in every three is phosphorylated (9). Phosphorylation is characterized by enzyme activity and protein signaling modifications, while glycosylation can modify regulatory functions and cell-cell identification/signaling (10). However, these important PTMs are rarely studied in myopia.

Myopia development was extensively studied from different perspectives (1,2,4-11). Myopia recovery is also considered relevant in understanding myopia and indeed several studies were performed to evaluate this aspect. For example, studies describing sclera protein profiles during development of myopia and recovery are already available (12,13). Recovery from negative lens induced myopia was analyzed through the evaluation of axial elongation (14). However, as far as we know, retinal proteomics during early recovery from lens-induced myopia has not yet been reported. Therefore, in the present study we examined and mapped the proteins involved in early recovery from lens-induced myopia in chickens using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE) and phosphoproteins using Pro-Q Diamond staining 2D-difference gel images. Our results underlined the validity of our approach on finding proteins which have never been previously detected and described in the retina, which could be considered as potential biomarkers in the adjustment of myopia, suggesting potential strategies to counteract it.

Materials and methods

Animals. White Leghorn chicks (*Gallus gallus domesticus*), hatched in an incubator, were raised in temperature-controlled brooders with water and food freely available under a 12 h light/12 h dark cycle (7:00 a.m.-7:00 p.m.) from the age of one day. Animal care and use were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The experimental procedure and protocol were approved by the animal ethics approval committee of the Hong Kong Polytechnic University (Hong Kong, China).

Lenses and ocular measurements. All chicks were subjected to the same lens treatment and recovery conditions as described below. Initially, 34 chicks were used to monitor biometric data changes. At the end of the recovery period, four chicks were used to perform 2D-DIGE experiments, while six of them were used for Pro-Q Diamond staining 2D gel electrophoresis. Three-day old chickens wore a-10D lens in front of their right eyes and a plano lens in front of their left eyes for 7 days. These lenses were mounted on Velcro rings and glued to the feathers around the eyes. Lenses were cleaned twice every day. At the end of the 7-day period, lenses were removed for 24 h to allow the recovery of the right eyes. Ocular dimensions and refractive errors were measured before and after 7 days of lens wear, and again after 24 h recovery. Axial length is defined as the distance from the front of the cornea to the front of the retina. Ocular parameters were examined by a high-frequency A-scan ultrasound system (Manually Controlled Pulser-Receiver) with a 30 MHz transducer (Immersion Transducers; both Olympus

Scientific Solutions Americas, Waltham, MA, USA) sampled at a rate of 100 MHz, while refractive errors were measured using a streak retinoscope (KJ6A; KangJie Co. Ltd, Jiangyan, China) as previously described (15).

Sample preparation. Once the measurements were completed, the retinal tissue was collected as previously described and frozen in liquid nitrogen (16). The frozen retinal tissue was ground in liquid nitrogen using a Teflon freezer mill (Micro-dismembrator; Braun Biotech, Melsungen, Germany) until a fine powder was obtained, which was solubilized in 300 μl DIGE compatible lysis buffer containing 7 M urea, 2 M thiourea, 40 mM tris, 2% CHAPS, 1% ASB14 and 1 tablet of Complete Mini protease (buffer for 2D-DIGE samples) or phosphatase (buffer for Pro-Q Diamond staining 2D gel electrophoresis samples) inhibitor cocktail in 10 ml buffer (16). After centrifugation, only the supernatant was collected. The solution was then concentrated by cold (-20°C) acetone precipitation over 4 h, followed by centrifugation at 16,000 g at 4°C for 15 min. The pellet obtained was resuspended in lysis buffer and the protein concentration was determined using a 2-D Quant kit (GE Healthcare Life Sciences, Uppsala, Sweden).

2D-DIGE. Fifty micrograms of the minus lens sample and control sample were labelled with 400 pmol dye (GE Healthcare Life Sciences) on ice for 30 min in the dark. An internal standard (a pool made by 50 μg retinal proteins from each sample) was labelled with Cy2 (Table I). All the procedures were performed in the dark, according to the protocols previously described (17-19). Isoelectric focusing (IEF) was achieved using linear immobilized pH gradient strips (IPG strips at a pH between 5 and 8 and 17 cm size; Bio-Rad Laboratories, San Diego, CA, USA). IPG strips were rehydrated at 50 V using Protean IEF cell (Bio-Rad Laboratories) for 12 h under a temperature of 20°C to increase protein uptake. Next, protein samples underwent IEF at 30 k voltage/h (Vh). Next, strips were incubated in equilibration buffer (6 M urea, 30% glycerol, 50 mM tris and 2% SDS, containing 0.5% DTT) for 10 min and they were subsequently incubated in 2% iodoacetamide for other 10 min. Second dimension electrophoresis was then performed using 12% polyacrylamide gels between low fluorescence Pyrex glass plates in Protean II XL (Bio-Rad Laboratories) tank. Image analysis was performed using DeCyder Differential Analysis Software (DeCyder; GE Healthcare Life Sciences). The differential in-gel analysis mode was used for spot detection and quantification, and images from different gels were combined using the biological variance analysis (BVA) mode. Protein spot matching was manually confirmed for all the gels. Gels were fixed and stained with MS compatible silver stain to visualize MS spots as previously described (17) after gel analysis. The details were the following: fixation (10% acetic acid, 40% methanol, 50% double distilled water) overnight, sensitization (0.2% sodium thiosulfate, 30% methanol, 70% double distilled water) 30 min, washing (double distilled water) 5 min x 3 times, silver impregnation (0.25% silver nitrate, 100% double distilled water) 20 min, washing (double distilled water) 1 min x 2 times, developing (2.5% sodium carbonate, 100% double distilled water, 0.04% formaldehyde) until each protein point clearly appeared,

Table I. CyDyes minimal labelling experiment design of retinal samples in 2D-DIGE.

Gel no.	Cy2 (pool internal control)	Cy3 (treated/control)	Cy5 (treated/control)
Gel 1	Pool of all 8 eyes (6.25 μg from both eyes of each animal, total 50 μg)	No. 1 treated (50 μg)	No. 1 control (50 μg)
Gel 2	Pool of all 8 eyes (6.25 μg from both eyes of each animal, total 50 μg)	No. 2 control (50 μg)	No. 2 treated (50 μg)
Gel 3	Pool of all 8 eyes (6.25 μg from both eyes of each animal, total 50 μg)	No. 3 treated (50 μg)	No. 3 control (50 μg)
Gel 4	Pool of all 8 eyes (6.25 μg from both eyes of each animal, total 50 μg)	No. 4 control (50 μg)	No. 4 treated (50 μg)

No. 1, no. 2, no. 3 and no. 4 represent four individual chicks. 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis.

stopping (5% acetic acid, 95% double distilled water) at least for 15 min. The differential protein expression was taken into consideration, if present, in all samples and each should show the same up- or downregulation.

Pro-Q Diamond staining 2D gel electrophoresis. One hundred microgram chick retinas samples were mixed with an equal volume of buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 1% ASB14, 2% DTT, 0.4% Biolytes and a trace amount of bromophenol blue and left on ice for 10 min. IPG strips of 11 cm, at a pH 4-7 were passively rehydrated with the samples in 200 μl rehydration buffer for 12 h. Next, IEF was performed at 100 V for 2 h, 500 V for 1 h, 1,000 V for 1 h, 4,000 V for 2 h and 8,000 V for 5 h under linear voltage ramp using a BioRad PROTEAN IEF Cell. Subsequently, IPG strips were incubated in equilibration buffer I (6 M urea, 30% glycerol, 50 mM tris, 2% SDS, 0.5% DTT) for 10 min, and then in equilibration buffer II (6 M urea, 30% glycerol, 50 mM tris, 2% SDS, 2% iodoacetamide) for 10 min. The strips were loaded onto 12% homogenous SDS PAGE gels of 1.5 mm thickness and sealed with 0.5% agarose. Protein separation in the second dimension was performed at 20 mA per gel using an Ettan DALT (Bio-Rad Laboratories, San Diego, CA, USA).

Gels were stained with Pro-Q Diamond (Pro-Q® Diamond Phosphoprotein Gel Destaining Solution; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to detect phosphoproteins and imaged using 532 nm excitation wavelength and 560 nm band pass filter using Typhoon 9400 Variable Mode Imager (Amersham Biosciences; GE Healthcare Life Sciences). The image analysis was performed by Image Master Platinum, version 5 (Bioinformatics, Uppsala, Sweden). Differential protein expression was taken into consideration if present in at least 5 samples and they should show the same up- or downregulation. Silver was used to visualize the protein spots for mass spectrometry.

LC-MS/MS. The differentially expressed protein spots were excised and processed as previously described (17-19). Extracts were dried by Speedvac Savant (Thermo Fisher Scientific, Inc.) and dissolved in 20 μl 0.1% formic acid. Peptides were separated using an Ultimate 3000 nano liquid chromatography system (LC Packings; Dionex, San Francisco, CA, USA) and analyzed by HCT Ultra ion trap mass spectrometer (Bruker Daltonics, Ettlingen, Germany) equipped with an online nanospray source. Samples were injected onto a reversed-phase pre-column (300 μm i.d.; 5 mm; C18 PepMap; LC Packings; Dionex) and then eluted and separated for 10 min on nano reversed phase column (75 μm i.d.; 150 mm; C18 PepMap; LC Packings; Dionex) with linear gradient from 96% mobile phase A/4% mobile phase B to 50% mobile phase A/50% mobile phase B. Mobile phase A contained 0.1% (v/v) formic acid in water and mobile phase B contained 0.08% formic acid in water-ACN (20:80, v/v%). The column was connected to an electrospray emitter, distal coating, 20 mm i.d. with 10 mm opening (New Objective, Woburn, MA, USA). Peptides were detected in the positive ion mode and fragmented by collision-induced dissociation using helium as the collision gas. The voltage applied to the capillary cap was -1,500 V and capillary temperature was set at 150˚C. Precursor selection was set at 300-1,500 mass-to-charge ratio (m/z). Two most abundant precursor ions were selected for MS/MS. Three scans were averaged to obtain an MS/MS mass spectrum. MS and MS/MS data were searched using NCBInr protein database (NCBIni_20081017) by MASCOT search engine, according to the following settings: trypsin was designated as the digestion enzyme and one missed cleavage was allowed. Carbamidomethylation of cysteines was set as fixed modification and oxidation of methionine residues as variable modification. The mass tolerances were 1.2 Da for MS and 0.6 Da for MS/MS. Proteins were considered identified when at least two peptides met the confident threshold of 0.05.

Statistical analysis. 2D-DIGE protein spots with an expression change >1.2-fold and a value of P<0.05 by Student's paired t-test, were defined as differentially expressed proteins and considered statistically significant (20,21). Pro-Q Diamond staining 2D-difference gel images protein spots with an expression change >1.8-fold and a value of P<0.05 by Student's Paired t-test were defined as differentially expressed proteins and considered statistically significant. Results were considered statistically significant when confirmed in four (2D-DIGE) or five (Pro-Q Diamond staining) separated gels. Furthermore, a visual check on the significant spots was performed by two separated experts to
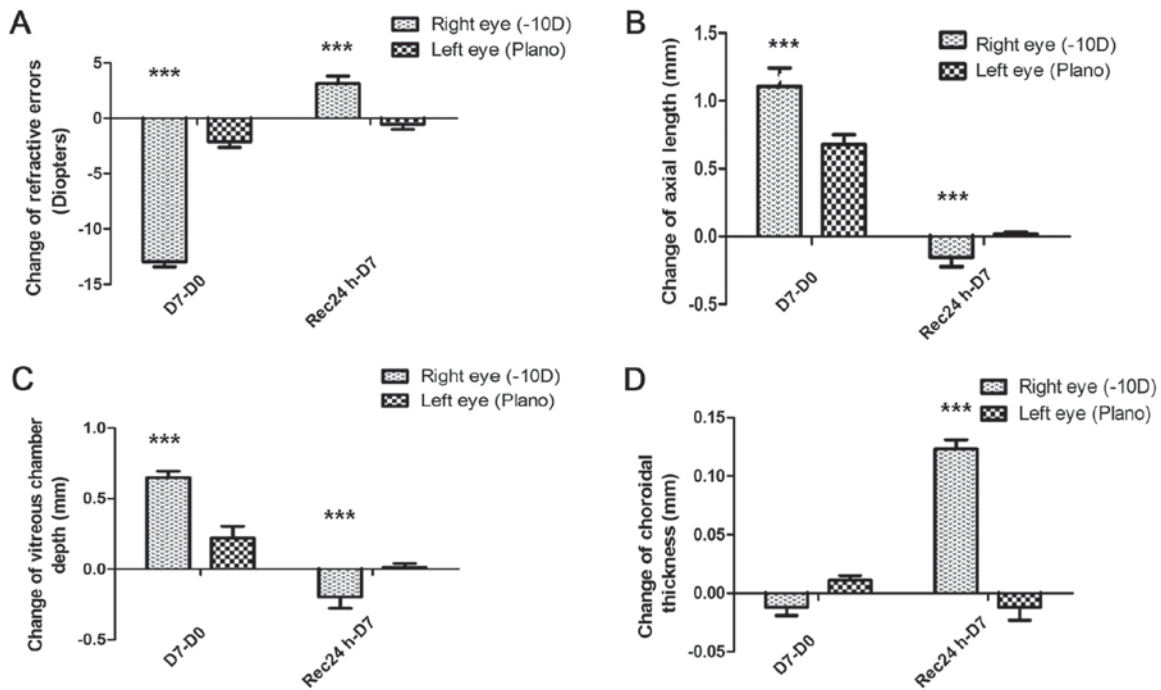
Figure 1. Effect of lens wear and recovery on (A) refractive errors; (B) axial length; (C) vitreous chamber depth; (D) choroidal thickness. Parameters' changes after 7 days lens wear (D7-D0). Parameter changes after 24 h of recovery (Rec24 h-D7). ***P<0.001 vs left eye, n=34.

confirm that they were real protein spots, instead of stripes or artifacts.

Results

Refractive and axial changes. Refractive values and axial ocular dimensions are shown in Fig. 1. After 7 days of lens wear, the treated eyes partially compensated to the presence of the lens, with the minus lens-treated eye becoming myopic (treated vs. control: -9.44±0.54 D vs. 1.48±0.50 D, P<0.001, n=34, Fig. 1A). After 24 h recovery, less myopia was observed in the treated eyes (treated vs. control: -6.31±0.82 D vs. 0.94±0.39 D, P<0.001, n=34, Fig. 1A). The axial length of the minus lens-treated eye was significantly longer than that of the control eye (treated vs. control: 1.32±0.22 mm vs. 0.70±0.23 mm, P<0.001, n=34, Fig. 1B) due to the vitreous chamber enlargement (Fig. 1C). The choroid was significantly thinner in the minus lens-treated eyes than in the control eyes (Fig. 1D). During the recovery time, axial length and vitreous chamber length partly recovered their original values, while the choroid was significantly thicker than the choroid in the control eyes (Fig. 1B-D).

Differential protein expression between myopia recovery retina and control retina by 2D-DIGE. After gel analysis, 1,864±142 protein spots were resolved and compared on the 2D-DIGE gels (Fig. 2). Five spots differed between the right myopia recovery group and the left control group. Five spots showed upregulation in myopic recovery retinas, and three of them were successfully identified by mass spectrometry: Ras related protein Rab-11B, S-antigen retina and pineal gland (arrestin) and 26S proteasome non-ATPase regulatory subunit 14 (PSMD14) (Table II). The other two proteins were not identified because of their very low concentration in chick retina.
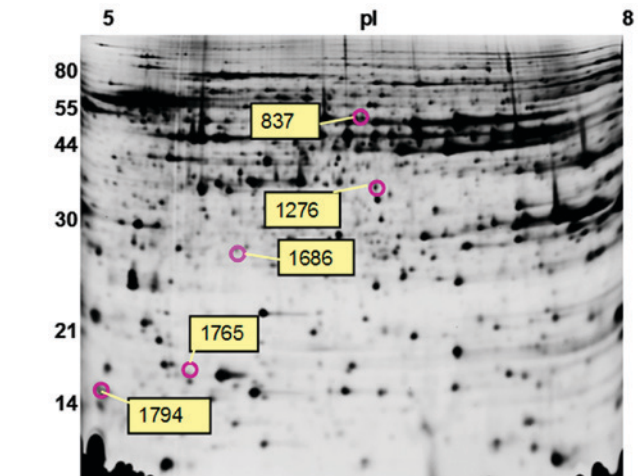

Figure 2. Identification of differential protein expression in chick retinas after minus lens wear and early recovery by 2D-DIGE. The gel image is a representative DIGE gel. Approximate molecular weights and isoelectric points are indicated. All spots represent upregulated proteins in myopic recovery retinas compared to control retinas. 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis.

Differential protein expression between myopia recovery retina and control retina by Pro-Q Diamond staining 2D gel electrophoresis. After gel analysis, 4 spots showed a 1.8-fold difference between the two groups. One spot was downregulated in the myopia recovery group and 3 were upregulated (Fig. 3).

Of these 4 spots of interest, 3 were successfully identified by mass spectrometry: β-tubulin, peroxiredoxin 4 and ubiquitin carboxyl-terminal hydrolaseL1 (UCHL1) (Table III). The remaining protein was not identified because of its very low concentration in chick retina.

Table II. Differential protein expression between myopia early recovery retina and control retina in 2D-DIGE.

Spot no.	IPI no.	Mascot protein name	Mowse score	Sequence coverage (%)	Mascot Pi	Mascot Mw (kDA)	Peptides no.	Fold difference
837	596741	Arrestin	81.45	5.58	6.33	46.64	4	1.20
1686	573563	Rab-11B	192.46	33.94	5.57	46.64	8	1.24
1276	604055	PSMD14	225.45	16.45	6.00	34.72	8	1.23

IPI, International Protein Index. 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis.

Table III. Differential protein expression between myopia early recovery retina and control retina in Pro-Q Diamond staining 2D gel electrophoresis.

Spot no.	NCBI GI no.	Mascot protein name	Mowse score	Sequence coverage (%)	Mascot Pi	Mascot Mw (kDA)	Peptides no.	Fold difference
s11	118766754	β-tubulin	332.729	18.01	5.62	41.48	12	-1.91
s29	118084001	Peroxiredoxin 4	7.72	13.21	6.28	29.62	3	1.96
s30	115391986	UCHL1	88.54	9.40	5.81	25.21	2	1.91

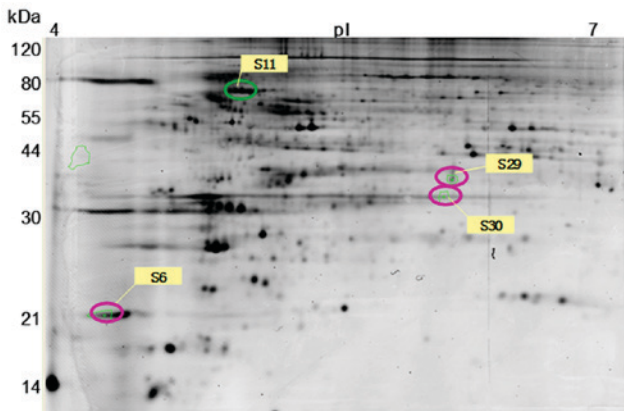UCHL1, ubiquitin carboxyl-terminal hydrolaseL1

Figure 3. Identification of differential protein expression in myopia early recovery retinas and control retinas by Pro-Q Diamond staining 2D gel electrophoresis. The gel image is a representative Pro-Q Diamond staining gel. Approximate molecular weights and isoelectric points are indicated. Spots in red indicate upregulated proteins, while those in green are downregulated proteins in myopia recovery retinas.

Discussion

Our results indicated that short-term myopia was reversible after minus lens removal, as shown by the axial vitreous chamber length, although choroidal thickness changed in an opposite direction to axial length. These results were in accordance with previous reports (22-27).

DIGE has introduced an internal control, and has a relatively high resolution. The internal standard paradigm in DIGE allows a precise comparison within and across treatment groups, thus displaying new proteins despite their quantity alterations as a consequence of the comparison (28). Samples

are labelled using different fluorescent dyes, mixed and resolved on a single 2D gel. The fluorescence-based technique has also an advantage of a broad linear dynamic range of 10^4, allowing the detection of slight changes in protein expression as low as 10%, with a confidence level of 95% (29-31).

Previous studies revealed that Rab family proteins possess a regulatory role in membrane transport, which is essential for developmental processes (32,33). Indeed, Rab11 is essential for Drosophila eye development and embryogenesis, since Rab11 endosomes control vesicle exocytosis and membrane growth in the cellularization process (34,35). Rab11 family-interacting protein 4 (Rab11-FIP4) is mainly expressed in neural tissues, playing a role in zebra fish and mouse retinal development (36,37). Rab11 is also important in the transport of other rhabdomeric proteins, such as transient receptor potential (TRP) channel, which is a light-activated Ca^{2+} channel working in phototransduction (38). From the above reports, we might conclude that Rab11 is required for the formation of endosomal multivesicular bodies in rhabdomeres, and might have a role in the traffic of rhabdomeric membranes.

Arrestin inhibits the activated phototransduction cascade, although the inhibitory effect mechanism is still not well-known (39). An accepted hypothesis is that arrestin binds to the phosphorylated and photoexcited rhodopsin, thus quenching the light-dependent cGMP-phosphodiesterase activation, or it may directly act on inhibiting cGMP-phosphodiesterase activation (40,41). Additionally, arrestin exerts an inhibitory activity on rhodopsin phosphatase (42). Our results showed that arrestin was upregulated in the myopic recovery retinas, suggesting that it might play a role in desensitizing the photoactivated transduction cascade and it may be consequently involved in the occurrence and recovery of myopia.

PSMD14, a 2.5-MDa protein, exerts an important role in the ubiquitin proteasome system. Indeed, it removes impaired proteins, as well as those no longer required (43-45). Although, humans possess several non-ATPase subunits, few of them have been cloned and sequenced, but their roles remain unclear. However, the S14 unit is a NIN1 homologue, which is a yeast gene involved in the cell cycle, encoding for a protein necessary for both G1/S and G2/M transitions (46). According to our results, PSMD14 was upregulated in the recovering myopic retinas, suggesting that in the process of myopia development and recovery, tissue metabolism was active, as a remarkable amount of metabolic waste was produced and this protein was acting as a scavenger.

The proteins whose expression was modified, belonged to the categories 'metabolic' and 'protein degradation and apoptosis', suggesting that they might have several different roles in retinal signal processing during the development of myopia and its recovery after lens removal. Although 2D-DIGE studies mostly focused on analyzing native retina protein profiles, PTM results were also crucial in determining several protein functions, such as cell differentiation, cell cycle control, and signal transduction (47,48). More than 200 different PTM types have been described, but not all of them are relevant in the biological process regulation: Protein phosphorylation is one of the most studied PTM (49).

Tubulins have been shown to exert significant roles in eye development. Tubulin α-1 chain may impart a 'GO/GROW' signal for myopia in chicken retinas (50). In contrast, tubulin downregulation, such as tubulin α-2 and tubulin α-6, may correspond to a decreased cell and internal organelle movement during sclera remodeling (3). Furthermore, tubulin α-3 seems to be involved in keratoconus, a human corneal non-inflammatory disease, resulting in myopia and astigmatism and it is considered as a neuromarker due to its presence in developing retina (51-53). Our results revealed a decrease in phosphorylation activity of β-tubulin in the retina of myopia recovery eyes, suggesting a role of the retina in the decreased scleral remodeling activity in myopia recovery group.

Peroxiredoxin 4 is a cytoplasmic or organellar anti-oxidative enzyme involved in intracellular redox signaling and gene transcription (54,55). Guinea pigs' sclera proteomic analysis showed that peroxiredoxin 4 was decreased in the monocular deprivation eyes (3). Tree shrew sclera proteomic study also found a decrease in peroxiredoxin 4 levels during lens-induced myopia (13). In the present study, peroxiredoxin 4 phosphorylation activity increased in the myopia recovery eye, suggesting the presence of many oxidation products. Since peroxiredoxin 4 decreases oxidative stress through hydrogen peroxide reduction in a thiol-dependent catalytic cycle, the increase of this protein might suggest an increased protective effect (56).

UCHL1 is considered a neuron-specific protein (57). A recent report demonstrated that UCHL1 silencing resulted in a reduction of both cell proliferation and migration in human retinal endothelial cells from human corpses (58). Since UCHL1 is a deubiquitinating enzyme, their results suggest that its silencing may inhibit neovascularization, thus indicating UCHL1 involvement in proliferation, migration and neovascularization within the retina. Although they conclude their work

suggesting a role of UCHL1 in neovascular eye diseases, our results showing an UCHL1 increase in the retina of a myopia recovering chick eye might indicate its potential role in regulating retinal rearrangement in the recovery eye.

Although a recent study demonstrated the potential involvement of oxidative stress and lipid metabolism in ocular eye growth (59), no differentially expressed proteins related to these pathways were found in the current results. Apolipoprotein A1 (ApoA1) is suggested to act as a 'STOP' signal in the myopia development (50). ApoA1 also acts as a retinoic acid-binding protein secreted by the choroid and sclera during eye growth (60). However, we did not find any ApoA1 upregulation in the recovering myopic retina. The underlying reason could be that different ocular tissues have been studied and different techniques have been employed. In addition, our study mainly focused on the expression of soluble proteins within the pH 4-7 range (in Pro-Q Diamond phosphoprotein staining 2D gel electrophoresis) and the pH 5-8 range (in 2D-DIGE), in which only part of the retinal proteins can be resolved. This is a very different approach than the ones used in the two cited studies. Moreover, Yu *et al* (59) studied protein regulation in the vitreous instead of in the retina as was done in our work. Hence, the entire retinal proteome in this study could not be visualized. Furthermore, during early active growth, great inter-animal variations in retinal proteins could be present. In order to decrease the inter-animal variations, we used both eyes in each chick and we only chose the differentially expressed proteins detected in all chicks and in the same change direction. Therefore, this study did not describe all the differences in retinal proteins during the myopia recovery process and purposely excluded those proteins that failed to repeat in all chicks. Although this method can minimize false positive results, some potentially important proteins such as ApoA1 can be missed. On the other hand, in this study the control eye is not a myopic eye but a paired 'normal' eye. As a result, some of the proteins which increased (or decreased) during myopia process but decreased (or increased) during the myopia recovery process are missed. The initial protein increase (or decrease) can be partly counteracted by the following protein decrease (or increase), thus, the total change is very small and might not be detected. Our study firstly focused on those most significant and repeatable differences in proteins not only in their abundance but also their PTMs in the myopic recovery retina. Although the actual number of proteins that were regulated was likely to be under-estimated in this study, the differentially expressed proteins reported are helpful for generating hypotheses that can unravel the biochemical mechanisms implicated in myopia early recovery.

In our present study, we did not perform confirmation of the proteomics results by conventional western blot or gene expression because of the relatively low fold changes of differentially expressed proteins we found. Poorer consistency in quantification and limited antibodies for chicks are two typical technical constrains for confirming MS data, which is more robust, accurate, reproducible and achieving low limits of detection (61). As regard gene expression, more recent studies on microarray and RNA sequencing have indicated that mRNA levels may not be accurate as surrogates for corresponding protein levels as mRNA levels do not usually predict the corresponding protein levels (62-64). Hannis weighs PCR

amplicons using MS and obtained accurate protein results to unambiguously determine the base composition of each amplicon, and not the other way around, that is, using PCR to confirm MS results (65). Hence, our further direction is trying to apply multiple reactions monitoring (MRM) MS as an alternative approach to conventional western blot analysis. This targeted approach was reported as a next-generation platform overcoming many of the limitations of western blotting, providing new prospects for protein analysis (20). Once the setup is established, we may run MS-based validation on protein level.

Overall, our results suggested that retinal proteomics and its related characterization could be useful in the discovery of biomarkers. The observed differences in protein expression between groups revealed proteins that are potentially involved in myopia early recovery, which might be important in developing strategies to counteract myopia.

Acknowledgements

Funding

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YYZ wrote the manuscript and performed the experiments. RKMC supervised the entire work and critically revised the manuscript, analyzed and interpreted the data and had final approval of the manuscript. JCW and BZ collected the results and drafted the manuscript. KKL analyzed and interpreted the results and supervised the entire work. TCL analyzed and interpreted the results and critically revised the manuscript. QL and CHT designed the experiments and approved the final version of the manuscript.

Ethics approval and consent to participate

The experimental procedure and protocols were approved by the Animal Ethics Approval Committee of the Hong Kong Polytechnic University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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