Alterations in Protein Expression in Tree Shrew Sclera during Development of Lens-Induced Myopia and Recovery

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PURPOSE. During the development of, and recovery from, negative lens-induced myopia there is regulated remodeling of the scleral extracellular matrix (ECM) that controls the extensibility of the sclera. Difference gel electrophoresis (DIGE) was used to identify and categorize proteins whose levels are altered in this process.

METHODS. Two groups of five tree shrews started monocular lens wear 24 days after eye opening (days of visual experience [VE]). The lens-induced myopia (LIM) group wore a -5 D lens for 4 days. The recovery (REC) group wore a -5 D lens for 11 days and then recovered for 4 days. Two normal groups (28 and 39 days of VE; $n = 5$ each) were also examined, age-matched to each of the treatment groups. Refractive and A-scan measures confirmed the effect of the treatments. Scleral proteins were isolated and resolved by DIGE. Proteins that differed in abundance were identified by mass spectrometry. Ingenuity pathway analysis was used to investigate potential biological pathway interactions.

RESULTS. During normal development (28–39 days of VE), eight proteins decreased and one protein increased in relative abundance. LIM-treated eyes were myopic and longer than control eyes; LIM-control eyes were slightly myopic compared with 28N eyes, indicating a yoking effect. In both the LIM-treated and the LIM-control eyes, there was a general downregulation from normal of proteins involved in transcription, cell adhesion, and protein synthesis. Additional proteins involved in cell adhesion, actin cytoskeleton, transcriptional regulation, and ECM structural proteins differed in the LIM-treated eyes versus normal but did not differ in the control eyes versus normal. REC-treated eyes were recovering from the induced myopia. REC-control eye refractions were not significantly different from the 39N eyes, and few proteins differed from age-matched normal eyes. The balance of protein expression in the REC-treated eyes, compared with normal eyes and REC-control eyes, shifted toward upregulation or a return to normal levels of proteins involved in cell adhesion, cell division, cytoskeleton, and ECM structural pro-

teins, including upregulation of several cytoskeleton-related proteins not affected during myopia development.

CONCLUSIONS. The DIGE procedure revealed new proteins whose abundance is altered during myopia development and recovery. Many of these are involved in cell-matrix adhesions, cytoskeleton, and transcriptional regulation and extend our understanding of the remodeling that controls the extensibility of the sclera. Reductions in these proteins during minus lens wear may produce the increased scleral viscoelasticity that results in faster axial elongation. Recovery is not a mirror image of lens-induced myopia—many protein levels, decreased during LIM, returned to normal, or slightly above normal, and additional cytoskeleton proteins were upregulated. However, no single protein or pathway appeared to be responsible for the scleral changes during myopia development or recovery. (*Invest Ophthalmol Vis Sci.* 2012;53:322–336) DOI:10.1167/iops.11-8354

The sclera, along with the cornea, constitutes the outer coat of the eye. In most vertebrates the sclera is composed of two layers, an inner layer of cartilage and an outer fibrous layer. In humans and other eutherian mammals, only the outer fibrous layer is present¹ and is composed largely of type I collagen along with lower amounts of type III and type V collagen, elastin, proteoglycans, and other structural proteins.^{2–12} This extracellular matrix (ECM) is produced by the scleral fibroblasts and is arranged in interwoven layers, or lamellae. The sclera controls the size of the eye, the location of the retina relative to the focal plane, and, hence, the refractive state. If the axial length matches the focal plane (which is created by the cornea and lens), the eye is emmetropic (distant objects are in focus without accommodation).

Many studies in animal models and humans over the past 35 years have led to the understanding that, during postnatal development, an emmetropization mechanism uses the eye's refractive error to modulate the postnatal biochemistry of the sclera and achieve a close match of the axial length to the focal plane so that eyes are nearly emmetropic.^{11–17} Tree shrews (*Tupaia glis belangeri*) are a well-established animal model used to study the emmetropization mechanism.¹⁸ They are small mammals, closely related to primates.^{19,20}

If a negative-powered lens is held in place in front of one eye with a goggle frame,²¹ it shifts the focal plane away from the cornea, making the eye optically hyperopic. The retina detects the increased hyperopia and initiates a signaling cascade that passes through the retinal pigment epithelium (RPE) and choroid to produce biochemical changes in the sclera. The viscoelasticity of the sclera, measured as the creep rate—the rate of increase in the length of a strip of sclera while under constant tension—is increased.^{22,23} This increased viscoelasticity may allow normal intraocular pressure to expand the globe in a posterior direction.²⁴ The lens-wearing eye rapidly elongates until, after 11 days or less, the retina is moved to the

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shifted focal plane. While wearing the lens, the refractive state of the eye comes to match that of the untreated fellow control eye or normal eyes¹⁸; thus, this process is also referred to as "compensation" for the imposed hyperopia. The scleral creep rate increases during the early phase of compensation, reaching a peak after 4 days of lens wear, and then declines toward normal as the eye completes its compensation.²²

After negative lens compensation, if the lens is removed the eye is myopic. In juvenile and young adult tree shrews, recovery from the induced myopia occurs in most cases.¹⁸ The retina detects the myopic refractive state and, through the signaling cascade, alters the biochemistry and biomechanics of the sclera. The creep rate is rapidly reduced (within 2 days) to below normal values,²⁵ and the axial elongation rate of the growing eye slows below normal.^{26,27} The optics of the eye continue to mature so that the focal plane continues its normal movement away from the cornea, and the retina once again becomes located at the focal plane, removing the imposed myopia.

During negative lens-induced myopia and recovery in tree shrews, there is selective tissue remodeling that, based largely on evidence from examination of mRNA changes, involves alterations in both the degradation and the synthesis of ECM components. Most studies that have investigated how the emmetropization mechanism alters the sclera to produce myopia or recovery, both at the level of proteins and of their mRNAs, have measured changes in specific "candidate" proteins.^{6,10,11,25,27-34} However, examining candidate proteins may overlook important alterations in other proteins that were not selected for investigation. To overcome this, we undertook proteomic studies using two-dimensional gel electrophoresis (2DGE) and mass spectrometry to identify additional scleral proteins whose abundance is altered during myopia development and recovery. In 2DGE, proteins extracted from the sclera are first separated by their isoelectric point and then by molecular weight using a standard SDS-PAGE gel, producing a unique pattern of protein spots. Comparison of gels from treated and control eyes reveals changes in the abundance of individual proteins that are subsequently collected from the gel and identified using mass spectrometry.

In our first effort,³⁵ proteins from treated and control eyes were run on separate gels, which were then aligned with software. The process of aligning the separate gels, on which protein locations differed slightly, limited the sensitivity of that study, and only four proteins (collagen 1 α 1, thrombospondin 1, pigment epithelium derived factor [PEDF], and GRP 78) were identified as having different abundance in the treated versus control eyes. In addition, sclera from normal animals was not included.

We expected that numerous protein changes had gone undetected; therefore, in the present study, we moved to using difference gel electrophoresis (DIGE), which provides an improved ability to detect biological variation.³⁶ DIGE uses three spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) to label three different protein samples that are combined and run on the same gel, so that protein spots from all three are colocalized on the gel, eliminating the need to align separate gels for the treated and control eyes. The inclusion on each gel of an internal standard containing all the proteins present in the experimental samples, representing their average profile, provides improved confidence in spot matching between gels, allowing comparison across conditions (myopia development, recovery, and normal). The significant reduction of inter-gel variability gained from sample multiplexing alongside an internal standard increases the likelihood of definitively measuring treatment effects with increased statistical confidence and quantitation accuracy. The DIGE technique is very sensitive, capable of detecting 0.5 fmol protein over a 10^4 -fold linear

dynamic range, where a 15% change in abundance is >2 SD above normal variation.³⁷ We hoped this technical improvement would allow us to discover additional scleral proteins that change during myopia development and recovery but could not be found in the previous study.

Protein levels in two groups of normal eyes were also examined, age-matched to the animals with lens-induced myopia and to the animals with 4 days of recovery. This was important because studies in tree shrew and other species have reported changes in control eyes compared with normal eyes (Rucker FJ, et al. *IOVS* 2009;50:ARVO E-Abstract 3931).^{18,22,34,38} Specifically, during lens-induced myopia development, control eyes often become slightly myopic compared with normal eyes after a short period (1–4 days) of -5 D lens wear. Thus, although differences between the treated and control eyes are important, comparison of treated eyes and of control eyes with normal eyes is also necessary to understand how altered protein levels may contribute to the increased creep rate and axial elongation during negative lens-induced myopia and the decreased creep rate and slowed elongation during recovery. In addition, because the eyes of normal juvenile tree shrews in the ages studied have not reached a stable adult size, it was important to learn whether there are normal baseline changes in protein abundance across the time period covered by lens-induced myopia and recovery.

MATERIALS AND METHODS

Experimental Groups

The 20 juvenile tree shrews (*T. glis belangeri*) used in this study were produced in our breeding colony and raised by their mothers on a 14-hour light/10-hour dark cycle. All procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Experimental groups included both males and females, with care taken to avoid pups from the same parents wherever possible.

Two groups of animals ($n = 5$) wore a monocular -5 D (spherical power) lens starting 24 ± 1 days after natural eye opening (days of visual experience [VE]).¹⁸ The lens-induced myopia (LIM) group wore the lens for 4 days to induce rapid axial elongation and myopia, with the treated eye partially compensating for the lens. The recovery (REC) group wore the lens for 11 days, producing full compensation while the lens was worn. The lens was then removed, and the now myopic eye was allowed to recover for 4 days to allow partial recovery and a greatly slowed axial elongation rate. The durations of the lens wear and recovery periods were designed to ensure that the sclera would be undergoing maximal remodeling in each direction at the end of the treatment or recovery periods. In both groups, the untreated fellow eye served as a control. Two normal groups ($n = 5$) were also used, age-matched to the LIM (28N; 28 ± 1 days of VE) and REC (39N; 39 ± 1 days of VE) groups.

Lens Wear

Animals in all groups were anesthetized (17.5 mg ketamine, 1.2 mg xylazine; supplemented with 0.5%–2.0% isoflurane as needed) at 21 ± 1 days of VE (except for 24 days of VE for two 39N animals) and received a dental acrylic pedestal following procedures described by Siegwart and Norton.²¹ Three days later, a goggle frame with a monocular -5 D lens (12-mm diameter PMMA contact lens; Conforma Contact Lenses, Norfolk, VA) was clipped to the pedestal of the LIM and REC groups, firmly holding the lens in front of the randomly selected treated eye. The control eye had unrestricted vision through an open (no lens) goggle frame. Normal animals received a pedestal but wore no goggle. Lenses were cleaned twice daily (approximately 9:00 AM and 4:30 PM). Animals were kept in a darkened nest box to

minimize exposure to visual stimuli while the goggle was briefly (< 3 minutes) removed.

Axial and Refractive Measures

To ensure that the treated and control eyes did not differ in axial length before the lens compensation began, initial ocular component dimensions were measured with A-scan ultrasound, as described by Norton and McBrien,³⁹ while the animals were under anesthesia for the pedestal attachment. A-scan measures were repeated in the animals in the LIM group at the end of treatment; the REC group (except for one animal) was remeasured at the end of the lens-wearing period and again at the end of the recovery period. Terminal A-scan measures were made in the normal animals.

At the start and end of the lens compensation and recovery periods, noncycloplegic refractive measures were made in awake animals with an infrared autorefractor (Nidek ARK-700A; Marco Ophthalmic, Jacksonville, FL).⁴⁰ Normal animals were measured just before euthanasia. Potential interference by atropine on retinoscleral signaling⁴¹ precluded the use of cycloplegic refractive measures. However, previous studies have shown that noncycloplegic awake measures provide a valid estimate of the refractive state and the amount of induced myopia in tree shrews. When compared, cycloplegic (atropine sulfate) refractions are approximately 0.8 D hyperopic compared with noncycloplegic refractions in myopic, normal, and control eyes.^{40,42} Further, treated eye relative to control eye differences are essentially identical between noncycloplegic and cycloplegic measures.⁴²

Sample Preparation

On completion of the final refractive measures, scleral tissue was collected according to published procedures³⁵ and frozen in liquid nitrogen. While still frozen, scleral samples were pulverized to a fine powder in a freezer mill (B. Braun Biotech, Allentown, PA) and the protein content was solubilized in 500 μ L standard extraction buffer (7 M urea, 2 M thiourea, 2% pharmalyte 3–10, 4% CHAPS). Samples were treated simultaneously with 5 mM tributylphosphine and 20 mM 4-vinyl pyridine for 90 minutes at room temperature to reduce and alkylate, respectively, before centrifuging at 21,000g for 20 minutes at 4°C to pellet the remaining cellular debris. Supernatants (~400 μ L) were collected, and the standard extraction buffer was exchanged for DIGE-compatible solubilization buffer (7 M urea, 2 M thiourea, 30 mM Tris base, 4% CHAPS; pH 8.5) using centrifugal ultrafiltration columns with a 10-kDa MWt cutoff (Biomax Ultrafree; Millipore, Billerica, MA).⁴³ In total, the buffer was exchanged three times to ensure removal of any residual pharmalyte 3–10, tributylphosphine, or 4-vinyl pyridine. The amount of recovered protein was determined (2-D Quant Kit; GE Healthcare, Piscataway, NJ), with typical yields in the 1400–1800- μ g range; there was no consistent difference in yield between treated, control, and normal eyes. An equal amount of protein from the right and left eyes was mixed for each normal animal. Samples were subsequently diluted to 5 μ g/ μ L with DIGE-compatible solubilization buffer.

Difference Gel Electrophoresis

Forty micrograms of protein was minimally labeled with 200 pmol fluorescent dye (CyDye; GE Healthcare). Reciprocal labeling (dye-swapping) was used whereby some of the scleras in each treatment group were labeled with Cy3 and the remainder with labeled with Cy5. A mixture comprising equal amounts of all experimental samples was labeled with Cy2 to provide a pooled internal standard sample.³⁶ Internal standard, treated, and control samples were combined appropriately for a total of 120 μ g labeled protein, diluted in rehydration buffer (7 M urea, 2 M thiourea), and supplemented with pharmalyte 3–10 (to a final concentration of 2%).

Combined samples were separated in the first dimension by isoelectric focusing (IEF) on 24-cm, pH 3–10NL IPG strips (PROTEAN IEF cell; Bio-Rad, Hercules, CA). Samples were loaded onto the IPG strip by active rehydration at 20°C for 16 hours, electrode wicks were put into place, and the sample was desalted for 3 hours at 300 V, followed by

focusing at 3500 V for a total of 60 kVh. After IEF, IPG strips were equilibrated (6 M urea, 50 mM Tris-HCl [pH 8.8], 30% glycerol, 2% SDS) for 30 minutes, and the proteins were resolved in the second dimension on large format 12% SDS-polyacrylamide gels (DALTSx; GE Healthcare) at 1 W/gel for 1 hour, followed by 13 W/gel for 5 hours, at 20°C.

The 2D gels were scanned at 100- μ m resolution (Typhoon Trio+ imager; GE Healthcare). The resultant protein profiles were compared with analysis software (SameSpots; Nonlinear Dynamics, Durham, NC) to identify protein spots that significantly differed in abundance among treated, control, and normal eyes during normal development, LIM, or REC (ANOVA, $P < 0.05$; Tukey's HSD post hoc test). No adjustment was made for false discovery rate. A spot picker (Ettan; GE Healthcare) was used to excise spots of interest from duplicate preparative gels that resolved 500 μ g of normal scleral protein visualized with fluorescent gel stain (Flamingo; Bio-Rad). In addition, gel pieces from spot-free regions were collected and run in parallel with the spots of interest as blanks to monitor potential contamination during sample handling.

Mass Spectrometry and Data Analysis

Excised gel spots were processed according to published procedures.³⁵ Briefly, spots were digested with 10 ng/ μ L trypsin in 25 mM ammonium bicarbonate, 10% acetonitrile (Trypsin Gold; Promega, Madison, WI) at 37°C for 16 hours. Supernatants were collected and then concentrated to near dryness in a vacuum centrifuge. Peptide samples were resuspended in 10 μ L 0.1% formic acid, desalted, and concentrated to 5 μ L (ZipTipsC₁₈; Millipore). Liquid chromatography-electrospray ionization tandem mass spectrometry analysis was carried out by the UAB Mass Spectrometry and Proteomics Shared Facility as previously reported, with minor modifications.⁴⁴ The resultant MS/MS data were converted to mzXML format and submitted to Mascot (www.matrixscience.com). These data were then analyzed (Protein Prophet; Institute for Systems Biology, Seattle, WA) to determine a high confidence "best fit" for a specific peptide fragmentation pattern.

Most of the investigated spots could be assigned a single protein identity; however, some contained up to four different proteins. Individual protein abundance levels in these mixtures were estimated using exponentially modified protein abundance index (emPAI) values to calculate a molar fraction for each protein.⁴⁵ Where the same protein was present in more than one spot, the mean of the normalized expression ratios for all spots was calculated and then converted to fold change. The identified proteins were mapped to human orthologs, and the resultant list of Entrez Gene IDs was uploaded to IPA software (Ingenuity Pathway Analysis; Ingenuity Systems, Redwood City, CA) to visualize potential biological pathway interactions. Gene ontology classification of molecular function and biological process were extracted from the UniProt (www.uniprot.org) and Panther (www.pantherdb.org) databases.

RESULTS

Refractive and Axial Changes

Right and left eyes of the two normal groups (28N and 39N) did not differ significantly on any measure; thus, the right and left eye values were averaged. Similar to animals examined in previous studies, the eyes of these two normal groups were still approaching emmetropia from hyperopia.^{18,34,39} Their refractions (corrected for the small eye "artifact")^{40,46} were slightly hyperopic at 1.5 ± 0.2 D for the 28N group and 0.7 ± 0.4 D for the 39N group (Fig. 1). The axial lengths of these two groups differed slightly, with the older 39N group (7.64 ± 0.05 mm) slightly longer than the younger 28N group (7.54 ± 0.04 mm). However, this 100- μ m axial enlargement (1.3%) was not statistically significant (t -test for independent samples; $P > 0.05$).

The refractive values for the treated and control eyes of the LIM group are shown in Figure 1A. After 4 days of lens wear,

FIGURE 1. (A) Refractive values of treated (T), control (C), and age-matched normal eyes (28N and 39N) for the LIM and REC groups (mean $\pm$ SEM). The average refraction of the LIM-control eyes was significantly myopic compared with the 28N group. After 11 days of lens wear (REC start), the REC-treated eyes had fully compensated for the -5 D lens. During the 4 days of recovery, the amount of myopia decreased in the recovering eyes (RT). The REC-control eyes (RC) did not differ significantly from the 39N group. Refractive values are corrected for the 4 D small eye "artifact" of retinoscopy.^{40,46} (B) Axial length values for the same groups. * $P < 0.05$; ** $P < 0.01$.

the treated eyes of all animals partially compensated to the -5 D lens and became myopic. As a group, the treated eyes were -2.8 ± 0.5 D myopic in comparison with the fellow untreated control eyes and -3.4 ± 0.6 D myopic in comparison with their refraction at the start of lens wear. The axial lengths of the LIM-treated eyes (7.71 ± 0.05 mm) were significantly longer than the lengths of the LIM-control eyes (7.65 ± 0.06 mm) because of enlargement of the vitreous chamber (Fig. 1B). Thus, as intended, the animals in the LIM group had partially compensated for the lens. Based on daily refractive measures made in other studies,^{18,47} the treated eyes were actively elongating, and their scleras presumably were more extensible than normal because of continued signals from the emmetropization mechanism.

Refractions of the control eyes of the LIM group declined by -0.7 ± 0.2 D (to 0.2 ± 0.3 D) during the 4 days of treatment and were significantly less hyperopic (by 1.5 D) than the eyes of the 28N group (unpaired *t*-test, $P < 0.01$), indicating that monocular lens wear had a small effect on the untreated control eyes (Fig. 1A). These eyes were also longer than the 28N eyes. Yoking of the control eye refractive state, similar to that

found in the present study, has been reported after 1 to 4 days of negative lens wear in other studies of tree shrews.^{18,34} The presence of a small, but significant, yoking effect indicated that there might be altered protein levels in the control eyes so that the protein levels of the LIM-treated and LIM-control eyes should each be compared with 28N eyes.

As also shown in Figure 1A, the animals in the REC group had compensated for the -5 D lens after 11 days of lens wear so that, measured with the lens removed at the start of recovery, the REC-treated eyes were -5.5 ± 0.6 D myopic in comparison with the REC-control eyes, which were not significantly different in refraction from the age-matched 39N group. After 4 days of recovery, the myopia of the REC-treated eyes had decreased by 2.7 ± 0.2 D. Based on daily measures in previous studies of recovery,^{18,48} rapid recovery was under way. The axial lengths of the REC-treated eyes ($n = 4$), measured at the end of lens wear, were 0.11 ± 0.01 mm longer than those of the REC-control eyes; this difference decreased to 0.08 ± 0.03 mm after 4 days of recovery (Fig. 1B), indicating that the rate of axial elongation had slowed to below a normal level. Based on previous studies, the creep rate of the recovering eye sclera should have been less extensible than normal because of signals from the emmetropization mechanism.²⁵ The control eyes of this group did not differ from those of the 39N group in either refraction or axial length.

Protein Expression Changes

Overall, 1226 protein spots were resolved and compared in the DIGE gels, covering a relative abundance range of more than 3 orders of magnitude. In total, 82 spots differed in at least one comparison between groups, with fold changes ranging from -2.81 to $+1.96$ -fold. Of the 82 spots of interest, 79 were successfully examined by mass spectrometry (the other three failed, presumably because of their very low abundance), identifying 60 distinct proteins that were primarily involved in cell adhesion, cytoskeleton, and transcriptional regulation, along with ECM structural proteins. In 22 cases, a protein was present in more than one spot, and the mean fold change for all spots was calculated to determine the overall change in that protein. All fold changes across spots for a given protein were in the same direction and of very similar magnitude, with the only exception being peroxiredoxin 1, which was upregulated in one spot but downregulated in another. Although most spots contained a single protein (58 spots), some contained two (18 spots), three (one spot), or four (two spots) proteins (see Discussion). Molar fractions calculated from emPAI values were used to suggest the predominant protein that was likely behind the change in spot abundance; "secondary" proteins found in the same spot are shown indented in Tables 1 and 2 and in Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8354/-DCSupplemental>). The gel locations of the protein spots whose abundances differed are shown in Figure 2.

Four types of differences in abundance were found: between the scleras of younger (28N) and older (39N) normal animals, apparently reflecting slowed growth in the older group of animals; between the scleras of LIM-treated and age-matched normal eyes or REC-treated eyes and normal eyes; between LIM- or REC-control eyes and normal eyes; between treated eyes and control eyes during LIM or REC. These changes are summarized in Tables 1 and 2; in which blue indicates upregulation, red indicates downregulation, and gray indicates no significant expression difference. The proteins in these tables are arranged in clusters with similar functions.

TABLE 1. Summary of Protein Expression Differences during Lens-Induced Myopia

Protein	Spot No.	Gene Symbol	Fold Change		Biological Function or Process*
			T vs. N	C vs. N	
<i>Expression Differences in Treated Eyes Relative to either Normal or Control</i>					
Collagen 1 $\alpha 2$	47, 49	COL1A2	-1.49	-1.21	ECM constituent
Collagen 12 $\alpha 1$	47, 49	COL12A1	-1.49	-1.21	Modifies interaction between collagen fibrils and surrounding ECM
Colligin	2, 32, 33	SERPINH1	-1.18	1.02	Chaperone—involved in collagen biosynthetic pathway
Thrombospondin 1	44, 45	THBS1	-1.52	1.07	Cell adhesion—mediates both cell-cell and cell-ECM interactions
Keratocan	24, 25, 29	KERA	-2.37	-1.28	Cell-ECM interaction—collagen fibril turnover
Myocilin	24, 25, 29	MYOC	-2.37	-1.28	Cell migration—via integrin signaling pathway; actin cytoskeleton organization
hnRNP K	24, 25	HNRNPK	-2.37	-1.29	Transcription regulation
Protein disulfide isomerase A3	24, 25	PDISA3	-2.37	-1.29	Protein folding; regulated by MMP2
Fortilin	52	TPT1	-1.23	-1.09	Cytoskeleton biosynthesis, calcium homeostasis
Apolipoprotein A1	52	APOA1	-1.23	-1.09	Signal transduction—CDC42 pathway; transporter protein
Gelsolin	20	GSN	-1.22	-1.14	Cytoskeleton—actin binding, key regulator of filament remodeling
Collagen 14 $\alpha 1$	20	COL14A1	-1.22	-1.14	Cell adhesion—integrates collagen fibrils into bundles
Vimentin	26, 60, 61	VIM	-1.39	-1.25	Cytoskeleton
Tropomyosin $\alpha 3$	50	TPM3	-1.12	-1.11	Cytoskeleton—binds to, and stabilizes, actin filaments
CDC42	4, 5	CDC42	-1.76	-1.14	Rho GTPase—regulates integrin signaling and actin cytoskeleton
Angiopoietin-related protein 7	1, 14, 38, 41	ANGPTL7	-1.17	1.10	Signal transduction (response to oxidative stress); cell adhesion
PPEDF	34–36, 77	SERPINF1	-1.51	-1.13	Signal transduction (reported to bind hyaluronan)
Peroxiredoxin 1	13	PRDX1	-1.25	-1.15	Signal transduction—regulates growth factor and TNF α signaling
TAR DNA-binding protein 43	37	TARDBP	-1.17	1.15	Transcription regulation
α -Centractin	37	ACTR1A	-1.17	1.15	Cytoskeleton—microtubule based vesicle motility
RuvB-like 1	28	RUVBL1	-1.28	-1.14	Transcription regulation
β -Lactoglobulin	38	PAEP	-1.17	1.04	Transporter activity—retinol binding
Cellular retinoic acid-binding protein 1	57	CRABP1	-1.44	-1.22	Lipid metabolism; role in retinoic acid-mediated processes
Adenosylhomocysteinase	14	AHCY	-1.08	1.24	Hydrolase activity—involved in purine metabolism?
ATP synthase, subunit alpha	31	ATP5A1	-1.16	-1.05	ATP synthesis
Procollagen C-endopeptidase	28, 31	PCOLCE	-1.22	-1.09	ECM—enhances the activation of type I procollagen; may also have MMP inhibitory activity
Aldehyde dehydrogenase 3 A1	31, 75, 76	ALDH3A1	-1.16	-1.05	Glycolysis
Glutamate dehydrogenase 1	30	GLUD1	-1.20	-1.10	Glutamate turnover

(continues)

TABLE 1. (continued). Summary of Protein Expression Differences during Lens-Induced Myopia

Protein	Spot No.	Gene Symbol	Fold Change			Biological Function or Process*
			T vs. N	C vs. N	T vs. C	
<i>Expression Differences in Bobb Treated and Control Eyes Relative to Normal</i>						
Collagen 1 $\alpha 1$	3, 8, 15–18, 62, 63	COL1A1	-1.76	-1.82	-1.22	ECM constituent
Tetranectin	55	CLEC3B	-2.00	-2.03	1.02	Matricellular protein that may play a role in tissue remodeling
SPARC (osteonectin)	6	SPARC	-2.81	-2.70	-1.04	Cell-ECM interaction—collagen and thrombospondin binding
Collagen 6 $\alpha 1$	19	COL6A1	1.46	1.49	-1.02	Cell adhesion—mediates fibronectin organization
Galectin 1	58	LGALS1	-1.61	-1.71	1.06	Cell adhesion; signal transduction
tRNA-splicing ligase RtcB homolog	27	C22orf28	-1.31	-1.16	-1.13	Cell adhesion—binds to vinculin (focal adhesion component)
β -Actin	9, 39, 40	ACTB	-1.29	-1.31	-1.11	Cytoskeleton—integrin signaling; cell motility, structure, and integrity
Cofilin 1	54	CFI1	-1.15	-1.14	-1.01	Cytoskeleton—actin remodeling; regulated by Rho GTPases
Glia maturation factor beta	56	GIMF	-1.29	-1.19	-1.09	Cytoskeleton—actin depolymerizing factor
Tropomyosin $\alpha 4$	11	TMP4	-1.16	-1.11	-1.05	Cytoskeleton—binds to, and stabilizes, actin filaments
14-3-3 protein epsilon	11	YWHAE	-1.16	-1.11	-1.05	Signal transduction—chaperone
Annexin A1	12, 46	ANXA1	1.03	1.29	1.01	Signal transduction; regulation of cellular growth
Receptor of activated PKC 1	12	GNB2L1	1.35	1.34	1.00	Signal transduction—focal adhesions
Peroxiredoxin 1	53	PRDX1	1.33	1.39	-1.04	Signal transduction—regulates growth factor and TNF- α signaling
Peroxiredoxin 4	10	PRDX4	-1.30	-1.21	-1.07	Signal transduction—regulates activation of NF- κ B
Eukaryotic translation initiation factor 4H	51	EIF4H	-1.54	-1.41	-1.10	Translation regulation
Myosin 8	51	MYH8	-1.54	-1.41	-1.10	Cytoskeleton—myosin subunit
GAPDH	42	GAPDH	1.47	1.56	-1.06	Transcription regulation; glycolysis
hnRNP E1	7	PCBP1	-1.70	-1.52	-1.12	Transcription regulation
hnRNP L	23	HNRNPL	-1.24	-1.16	-1.07	Transcription regulation
Acyl-CoA dehydrogenase, very long chain	23	ACADVL	-1.24	-1.16	-1.07	Lipid metabolism
Albumin	21, 22, 72	ALB	1.40	1.59	-1.18	Chaperone

Blue indicates upregulation, red indicates downregulation, and gray indicates no significant expression difference. The proteins are arranged in clusters with similar functions.
 * Taken from the UniProt (www.uniprot.org) and Panther (www.pantherdb.org) databases.

TABLE 2. Summary of Protein Expression Differences during Recovery

Protein	Spot No.	Gene Symbol	Fold Change			Biological Function or Process*
			T vs. N	C vs. N	T vs. C	
Expression Differences in Treated Eyes Relative to either Normal or Control						
Collagen 1 α1	3, 8, 15-18, 62, 63	COL1A1	1.30	1.04	1.18	ECM constituent
Colligin	2, 32, 33	SERPINF1	1.17	1.02	1.16	Chaperone—involved in collagen biosynthetic pathway
Thrombospondin 1	44, 45	THBS1	-2.08	-1.17	-1.77	Cell adhesion—mediates both cell-cell and cell-ECM interactions
Keratocan	24, 25, 29	KERA	1.96	1.29	1.23	Cell-ECM interaction—collagen fibril turnover
Myocilin	24, 25, 29	MYOC	1.96	1.29	1.23	Cell migration—via integrin signaling; actin cytoskeleton organization
hnRNP K	24, 25	HNRNPK	1.42	1.19	1.19	Transcription regulation
Protein disulfide isomerase A3	24, 25	PDIA3	1.42	1.19	1.19	Protein folding; regulated by MMP2
Fortilin	52	TPT1	1.34	1.10	1.22	Cytoskeleton biosynthesis, calcium homeostasis
Apolipoprotein A1	52	APOA1	1.34	1.10	1.22	Signal transduction—CDC42 pathway; transporter protein
CDC42	4, 5	CDC42	-2.03	-1.39	-1.41	Rho GTPase—regulates integrin signaling and actin cytoskeleton
F-actin-capping protein, subunit beta	59	CAPZB	1.33	1.14	1.17	Cytoskeleton—actin binding, involved in cytoskeletal organization
Annexin A4	59	ANXA4	1.33	1.14	1.17	Signal transduction; regulation of cellular growth
T-complex protein 1, subunit theta	64	CCT8	1.59	1.33	1.19	Chaperone—actin folding
T-complex protein 1, subunit epsilon	64	CCT5	1.59	1.33	1.19	Chaperone—actin folding
PEPDF	34-36, 77	SERPINF1	1.22	1.14	1.07	Signal transduction (reported to bind hyaluronan)
Phosphatidylethanolamide-binding protein 1	71	PEBP1	-1.22	-1.14	-1.07	Signal transduction—regulates MAP kinase and NF-κB signaling
Eukaryotic translation initiation factor 4H	51	EIF4H	-1.34	-1.30	-1.03	Translation regulation
Myosin 8	51	MYH8	-1.34	-1.30	-1.03	Cytoskeleton—myosin subunit
Triosephosphate isomerase	70, 78, 79	TP11	-1.27	-1.24	-1.02	Glycolysis

(continues)

(continues)

TABLE 2. (continued). Summary of Protein Expression Differences during Recovery

Protein	Spot No.	Gene Symbol	Fold Change			Biological Function or Process*
			T vs. N	C vs. N	T vs. C	
Expression Differences in Both Treated and Control Eyes Relative to Normal						
Collagen 1 $\alpha 2$	47, 49	COL1A2	1.48	1.31	1.20	ECM constituent
Collagen 12 $\alpha 1$	47, 49	COL12A1	1.48	1.31	1.20	Modifies interaction between collagen fibrils and surrounding ECM
Tetranectin	55	CLEC3B	1.02	1.06	-1.04	Matricellular protein that may play a role in tissue remodeling
Mimectan (ostreoglycin)	48, 65, 68, 69	OGN	-1.44	-1.66	-1.21	Cell-ECM interaction—collagen fibril turnover; growth factor activity
SPARC (osteonectin)	6	SPARC	1.33	1.18	1.13	Cell-ECM interaction—collagen and thrombospondin binding
Collagen 6 $\alpha 1$	19	COL6A1	1.12	1.21	-1.08	Cell adhesion—mediates fibronectin organization
Galectin 1	58	LGALS1	-1.14	-1.18	1.03	Cell adhesion; signal transduction
tRNA-splicing ligase RtcB homolog	27	C22orf28	-1.18	-1.18	-1.00	Cell adhesion—binds to vinculin (focal adhesion component)
Gelsolin	20	GSN	-1.09	-1.08	-1.01	Cytoskeleton—actin binding, key regulator of filament remodeling
Collagen 14 $\alpha 1$	20	COL14A1	-1.09	-1.08	-1.01	Cell adhesion—integrates collagen fibrils into bundles
β -Actin	9, 39, 40	ACTB	-1.01	-1.08	1.07	Cytoskeleton—integrin signaling; cell motility, structure, and integrity
Vimentin	26, 60, 61	VIM	-1.31	-1.37	1.03	Cytoskeleton
Cofilin 1	54	CFL1	-1.09	-1.04	-1.05	Cytoskeleton—actin remodeling; regulated by Rho GTPases
Glia maturation factor beta	56	GMFB	1.02	-1.03	1.05	Cytoskeleton—actin depolymerizing factor
LIM and SH3 domain protein 1	67	LASP1	-1.68	-1.72	1.03	Cytoskeleton—actin binding, cytoskeletal organization
Tropomyosin $\alpha 3$	50	TMP3	-1.07	-1.09	1.02	Cytoskeleton—binds to, and stabilizes, actin filaments
Tropomyosin $\alpha 4$	11	TPM4	1.01	-1.05	1.06	Cytoskeleton—binds to, and stabilizes, actin filaments
14-3-3 protein epsilon	11	YWHAE	1.01	-1.05	1.06	Signal transduction—chaperone
Annexin A1	12, 46	ANXA1	1.35	1.38	-1.02	Signal transduction; regulation of cellular growth
Receptor of activated PKC 1	12	GNB2L1	1.48	1.48	1.00	Signal transduction—focal adhesions
Annexin A2	42, 43, 66, 68	ANXA2	-1.50	-1.79	-1.24	Signal transduction; regulation of cellular growth
Angiopoietin-related protein 7	1, 14, 38, 41	ANGPTL7	-1.04	1.01	-1.04	Signal transduction (response to oxidative stress); cell adhesion
Peroxiredoxin 1	53	PRDX1	1.25	1.25	1.00	Signal transduction—regulates growth factor and TNF α -signaling
Peroxiredoxin 1	13	PRDX1	-1.22	-1.16	-1.05	Signal transduction—regulates growth factor and TNF α -signaling
Peroxiredoxin 4	10	PRDX4	-1.00	-1.04	1.04	Signal transduction—regulates activation of NF- κ B
RuvB-like 1	28	RUVBL1	-1.04	-1.08	1.04	Transcription regulation
TAR DNA-binding protein 43	37	TARDBP	-1.00	1.02	-1.02	Transcription regulation
α -Centractin	37	ACTR1A	-1.00	1.02	-1.02	Cytoskeleton—microtubule based vesicle motility
hnRNP E1	7	PCBP1	-1.31	-1.34	1.03	Transcription regulation
hnRNP L	23	HNRNPL	1.02	-1.01	1.04	Transcription regulation
Acyl-CoA dehydrogenase, very long chain	23	ACADVL	1.02	-1.01	1.04	Lipid metabolism
GAPDH	42	GAPDH	1.14	1.19	-1.04	Transcription regulation; glycolysis
Aldehyde dehydrogenase 3 A1	31, 75, 76	ALDH3A1	-1.27	-1.27	-1.00	Glycolysis
Albumin	21, 22, 72	ALB	-1.16	-1.29	1.11	Chaperone
ATP synthase, subunit alpha	31	ATP5A1	-1.27	-1.27	-1.00	ATP synthesis
Procollagen C-endopeptidase	28, 31	PCOLCE	-1.15	-1.17	1.02	ECM—enhances activation of type I procollagen; may inhibit MMPs
Adenosylhomocysteinase	14	AHCY	-1.01	1.01	-1.02	Hydrolase activity—involved in purine metabolism?
Glutamate dehydrogenase 1	30	GLUD1	-1.25	-1.26	1.00	Glutamate turnover
Cellular retinoic acid-binding protein 1	57	CRABP1	1.01	-1.09	1.10	Lipid metabolism; role in retinoic acid-mediated processes
β -Lactoglobulin	38	PAEP	-1.01	1.05	-1.07	Transporter activity—retinol binding

Blue indicates upregulation, red indicates downregulation, and gray indicates no significant expression difference. The proteins are arranged in clusters with similar functions.

*Taken from the UniProt (www.uniprot.org) and Panther (www.pantherdb.org) databases.

FIGURE 2. Example DIGE gel image (Cy2 channel) showing the different patterns of protein expression in the sclera during myopia development (LIM) and recovery (REC). *Ellipses* indicate changes found during LIM, *rectangles* indicate changes found during REC, and *hexagons* indicate changes between 28N and 39N. *Red*: downregulation; *blue*: upregulation. Spot numbers are also shown in Tables 1 and 2 and Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8354/-DCSupplemental>).

Protein Expression at Two Points during Normal Development

Measurements in this study were made during the juvenile period, after the infantile high-growth period ended during which most of the sclera had formed.⁴⁹ Consistent with this slowed growth of normal sclera and the 11-day difference in age, there were few differences in protein abundance found between the 28N and 39N groups. As shown in Supplementary Table S1 (<http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8354/-DCSupplemental>), the abundance of eight proteins decreased significantly in the older compared with younger group, and one protein increased. These proteins (e.g., aldehyde dehydrogenase 3 A1, heat shock 70-kDa protein 1A/1B, and albumin) were generally associated with basic metabolism functions or chaperone activity during protein maturation, as is consistent with a slowed growth rate in older compared with younger animals.¹⁸

Protein Expression during Lens-Induced Myopia

LIM-Treated versus Age-Matched Normal (28N) Scleras. When the abundance of proteins in the LIM-treated eyes was compared with that of age-matched 28N eyes (Table 1, top), 39 proteins were downregulated in the scleras of the LIM-treated eye compared with normal, whereas six proteins were upregulated. To compare the patterns of protein expression during LIM and also during recovery (REC), Figures 3 and 4 show protein expression during LIM on the *x*-axis and the expression

during REC (described in the next section) on the *y*-axis. The downregulated proteins belonged to several functional categories, based on the UniProt (www.uniprot.org) and Panther (www.pantherdb.org) databases. These included cell and focal adhesions, cytoskeleton and transcriptional regulation, and ECM proteins, such as type I collagen.

LIM-Control versus 28N Scleras. The LIM-control eyes, which were slightly, but significantly, myopic compared with the 28N eyes, also showed alterations in abundance of a smaller number of scleral proteins compared with the normal eyes. Sixteen proteins were downregulated and six were upregulated compared with the 28N eyes. All these proteins also differed in abundance, and by similar amounts, between the LIM-treated and 28N eye scleras, indicating that yoking occurred at the protein level (Fig. 3B, *x*-axis; Table 1, bottom) but included a much smaller number of proteins in the LIM-control eye scleras. Decreased in both the treated and control eyes were proteins whose functions involve focal and cell adhesion, such as SPARC; cytoskeletal proteins, including β -actin, tropomyosin $\alpha 4$, and cofilin 1; transcription and protein synthesis proteins (hnRNP E1); and structural ECM proteins, such as collagen 1 $\alpha 1$. The six upregulated proteins, which also were upregulated in the LIM-treated eyes (Table 1, bottom), are generally involved in signal transduction (e.g., annexin A1 and peroxiredoxin 1) and cell adhesion. The similar pattern of altered protein expression, compared with normal, in both the LIM-control and LIM-treated eyes indicated the presence of

FIGURE 3. (A) Plot of treated eye scleral proteins compared with normal eye scleral proteins during LIM (x-axis) and REC (y-axis), showing that the pattern of protein expression differed between the two conditions. A value of 1 indicates no difference in protein abundance. Negative values indicate a decreased abundance in the treated eye compared with the control eye. Most proteins were downregulated during LIM (squares and triangles to the left of the vertical line). During REC, many were no longer downregulated (triangles clustered around the horizontal line), and some were upregulated (squares and circles above the horizontal line). Three types of difference are shown: significant treated versus control differences during both LIM and REC (circles), significant differences during LIM but not REC (triangles), and significant differences during REC but not LIM (squares). (B) Control versus normal eye scleral protein changes during LIM and REC show that there was a similar pattern of altered protein abundance in both eyes that involved fewer proteins in the control eyes of each group.

binocular effects that likely were the cause of the small refractive and axial changes seen in the control eyes. However, these altered control eye protein levels apparently were sufficient to produce only a weak myopic shift in these eyes.

Because of the much stronger refractive and axial response of the LIM-treated eyes, we were interested in the identities of those proteins whose abundance differed from the control eye scleras in either of two ways: those showing differential expression that was significantly different in the treated versus control eyes and those that differed from normal in the LIM-treated eyes but did not differ from normal in the LIM-control

eyes. These proteins are listed in Table 1 (top) and are shown graphically in Figures 4 and 5A.

LIM Differential Expression. As shown on the x-axis of Figure 4 and in Table 1 (top), 18 proteins were downregulated in the LIM-treated versus the control eyes. These included collagens 1 $\alpha 2$ and 12 $\alpha 1$; thrombospondin 1, which mediates cell adhesion and collagen synthesis; CDC42, a Rho GTPase that regulates pathways that control integrin signaling and actin cytoskeleton organization; colligin, a chaperone involved in collagen biosynthesis; and other signaling and adhesion-related proteins.

Figure 5A (and Table 1, top) distinguishes proteins that were downregulated in the LIM-treated eye scleras, compared with normal, but were not significantly downregulated in the control eyes, compared with normal. The proteins that changed similarly in both LIM-treated and LIM-control eyes (circles) are arranged along the 1:1 dashed line. The proteins of interest, whose abundance was significantly different from normal in the LIM-treated eyes but not significantly different from normal in the LIM-control eyes (Table 1, top), are indicated by the triangles. These proteins, like the differentially expressed proteins, distinguish the LIM-treated eyes from the LIM-control eyes and might have contributed to its stronger refractive and axial response. This group included several cell adhesion and cytoskeletal proteins (gelsolin, vimentin, and tropomyosin $\alpha 3$) and others involved in transcriptional regulation, glycolysis, and lipid metabolism.

Protein Expression during Recovery from Induced Myopia

Many of the proteins that were reduced in abundance during LIM returned to normal levels during recovery. As a result, there were fewer proteins that differed significantly between the recovering (REC-treated) eyes and either the REC-control or age-matched normal eyes. Further, most of the proteins that differed significantly in recovery were upregulated in the recovering eyes, compared with the normal or control eyes. As was the case during lens-induced myopia, we compared the recovering eye scleras with both the age-matched normal and the control eye scleras.

REC-Treated versus Age-Matched Normal (39N) Scleras.

The pattern during recovery was for most of the proteins in the recovering eye to return to normal levels or to be upregulated compared with normal (Table 2, top). As shown by the trian-

FIGURE 4. Plot of differential protein expression during development of LIM (x-axis) versus differential expression during REC (y-axis) showing the different patterns in these two conditions. Protein names are located adjacent to the data points. The altered expression of these proteins may be responsible for the greater response of the lens-treated eyes during LIM and during REC. Symbols are the same as in Figure 3.

FIGURE 5. Plots of treated (T) and control eye (C) scleral proteins compared with normal (N) during (A) myopia development (LIM) and (B) recovery (REC). In both graphs, the x-axis plots the fold difference between the treated eye scleras and normal. The y-axis plots the control eye scleras versus normal. Proteins whose abundance differed significantly compared with normal in the same direction in both the treated and the control eyes are indicated by the circles clustered along the 1:1 dashed line. Proteins whose abundance differed in the LIM-treated or REC-treated eyes but not in the control eyes, and therefore are of interest, are indicated by the triangles.

gles in Figure 3A (y-axis), 24 of the proteins that were significantly decreased during myopia development (LIM) were no longer significantly different from normal during recovery. These included gelsolin, vimentin, cofilin 1, SPARC, and tropomyosins $\alpha 3$ and $\alpha 4$. Nine proteins (circles to the left of the vertical line) that were less abundant during LIM now were more abundant compared with normal eye scleras. These were primarily proteins involved in collagen synthesis, such as colligin, keratocan, and collagen 12 $\alpha 1$. Levels of collagen 1 $\alpha 1$ and 1 $\alpha 2$, components of the primary structural collagen in sclera, were also significantly higher than in the normal eyes. Two proteins (annexin A1 and receptor of activated PKC1) that were upregulated during LIM remained upregulated during recovery compared with normal eyes. Four proteins that were unaffected during LIM were upregulated in recovery, all related to cytoskeleton turnover.

REC-Control versus 39N Scleras. Control eyes of the REC group did not differ significantly from normal in their refractions or axial lengths. There were, however, 10 proteins whose levels were significantly different from normal (Table 2, bottom; Fig. 3B, y-axis). All but one (vimentin) of these differences were in the same direction compared with normal as with the REC-treated versus 38N scleras, suggesting a limited amount of protein expression yoking similar to, but smaller in amount than, that seen in the LIM-control eyes. Only three of these "yoked" proteins (annexin A1, receptor of activated PKC1, and tRNA-splicing ligase RtcB homolog) coincided between the LIM and REC groups, and their expression did not appear to follow a consistent pattern.

REC Differential Expression. During recovery, there were fewer differences in protein levels between the REC-treated and the REC-control eye scleras than were seen between LIM-treated versus LIM-control eye scleras. As shown in Tables 1 (top) and 2 (top) and Figures 3 and 4, many of the proteins whose abundance was significantly lower during myopia development were no longer differentially expressed during recovery (triangles). Two proteins (thrombospondin 1 and CDC42; Fig. 3) that were downregulated during myopia development remained significantly downregulated in the recovering scleras. Two other proteins (annexin A2 and mimecan) that were not differentially expressed during myopia development were significantly lower in abundance in the recovering scleras. Four proteins related to collagen or cytoskeleton biosynthesis (collagen 1 $\alpha 1$, colligin, apolipoprotein A1, and fortillin) that were significantly downregulated during myopia development were significantly upregulated during recovery compared with the REC-control eye scleras.

Figure 5B (and Table 2, top) distinguishes proteins that differed between the scleras of the REC-treated eyes and the scleras of normal (39N) eyes but that did not differ significantly in the control eyes compared with normal. The proteins of interest, whose abundance was significantly different from normal in the REC-treated eyes but not significantly different from normal in the REC-control eyes (Table 2, top), are indicated by the triangles. These proteins, like the differentially expressed proteins, distinguish the REC-treated eyes from the REC-control eyes and might have contributed to its stronger refractive and axial response. There was very little overlap between the proteins shown by triangles in Figure 5A (LIM) and 5B (REC), as may be seen by examining Tables 1 (top) and 2 (top), suggesting that the protein alterations during recovery were not a mirror image of those during myopia development.

Pathway Analysis

The proteins that were of most interest to us were those that had expression differences in the treated eye compared with either fellow control eyes or age-matched normal eyes but no differences in control eyes compared with normal, during either myopia development (LIM) or recovery (REC). IPA was used to determine whether these "proteins of interest" fit into established interaction networks. To the extent that IPA could do this, it would confirm that the altered proteins are involved in an interactive network and not simply a cluster of unrelated proteins. IPA connected all but one of these proteins (angiopoietin-related protein 7) in a direct interaction network (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8354/-/DCSupplemental>).

DISCUSSION

The use of DIGE with its internal standard paradigm, which allows accurate comparisons both within and across treatment groups, coupled with measures of age-matched normal groups, revealed new proteins whose abundance is altered during myopia development and recovery. Many of these are involved in cell-matrix adhesions, cytoskeleton and transcriptional regulation, and extend our understanding of the remodeling that controls the extensibility of the sclera during myopia development and recovery.

As expected, we found few differences between the two normal groups that differed in age by 11 days and whose eyes had undergone only small axial and refractive changes. These differences, predominantly decreases in the abundance of pro-

teins involved in basic metabolism or chaperone functions during protein maturation, seemed indicative of the gradual slowing of scleral growth during this brief period of juvenile eye development.¹⁸ The low number of differences between the two very similarly aged normal groups suggested that the much larger number of differences between the normal eyes and either the treated or control eyes of the LIM and REC groups were real and were not due to inherent variability in the DIGE technique or subsequent analysis (SameSpots; Nonlinear Dynamics).

The addition of the normal groups in this study also allowed detection of a small refractive yoking effect on the LIM-control eyes. The refractive change over the four days of treatment in the LIM-control eyes was small (-0.7 ± 0.2 D) compared with the -3.4 ± 0.6 D myopic change in the LIM-treated eyes during the same period. It is reasonable to think that the refractive change of the control eyes, in the same direction as the LIM-treated eyes, was produced by scleral changes involving the proteins whose abundance changed in parallel in both the LIM-treated and the LIM-control eyes.

In the recovery group, the REC-control eyes were not significantly different from normal eyes, either refractively or axially, and there were fewer control versus normal differences in scleral protein levels. These expression differences from normal suggest that there might be refractive and axial yoking effects during recovery but that they are too small to be detected with the refractive and axial measurement techniques used in this study.

Altered Protein Expression Patterns during Myopia Development and Recovery

An underlying assumption of this and other studies that have examined mRNA or protein changes in sclera during myopia development is that signals generated in the retina by the emmetropization mechanism reach the sclera and cause changes in mRNA and protein abundance that, in turn, cause the eye to elongate and move the retina behind the focal plane. In chicks, this is due to growth of the cartilaginous inner layer of the sclera.⁵⁰ In tree shrew (and perhaps other eutherian mammals) there is decreased DNA synthesis,⁷ loss of scleral dry weight,⁸ reduced collagen abundance⁶ and increased viscoelasticity,^{22,23} which may allow normal intraocular pressure to increase the axial length.²⁴ We thus expected to find an overall decrease in the abundance of at least some scleral proteins during myopia development. The hope was that the specific proteins that were lower in abundance would be related to scleral structure or to other aspects of the sclera and would help us to understand the mechanism by which scleral viscoelasticity, and axial length, increase.

The differential (LIM-treated vs. control) and LIM-treated versus normal expression patterns were overwhelmingly for downregulation. Specific proteins that were downregulated included, as expected, not only structural proteins such as type I collagen^{6,10} but also an extensive collection of proteins involved in cell-matrix interactions and cytoskeleton remodeling along with a few proteins that are implicated in collagen turnover and focal adhesion-related signal transduction.

The downregulation of a broad array of proteins involved in cell adhesion is consonant with the hypothesis, described in previous papers from this laboratory,^{27,34} that the increased creep rate of the tree shrew sclera during myopia development has two causes. One is loss of type I collagen, most likely at the edges of the lamellae where MMPs, such as the active form of MMP-2,²⁸ and membrane-bound MMP-14,³⁴ produced by the fibroblasts, have their highest concentration. The other is change to focal adhesions and other attachments between the fibroblasts and the ECM. Added to these changes are alterations

of components within the interlamellar spaces, including decreased levels of hyaluronan and other GAGs²⁷ and a reduction of aggrecan core protein.²⁹ Taken together, these changes may make it easier for the layers of the sclera to slip across one another, raising the creep rate.

During recovery, when the elongated eyes slow their axial elongation rate and the scleral creep rate decreases below normal in tree shrews,²⁵ the overall pattern was for proteins whose expression had decreased during LIM to return to levels that were either not significantly different from normal or control eyes or that were slightly above normal or control eye levels. It is interesting that these modest increases apparently are sufficient to produce the refractive recovery and slowed axial elongation. This may be mediated by the rebound in type I collagen abundance, which presumably occurred at the edges of the lamellae adjacent to the fibroblasts that make the collagen. This could provide a substrate to which integrin receptors once again could attach, helping to reduce the cross-lamellar slippage and, hence, the creep and axial elongation rates. One might speculate that the formation of adhesions between the fibroblasts and the newly formed collagen matrix, aided by an increase in hyaluronan,²⁷ a possible increase in aggrecan²⁹ and, hence, the creation of a stable water-binding gel in the interlamellar regions, could be sufficient to reduce the viscoelasticity to below normal.

The addition of cell-matrix interaction proteins to the mix of proteins that decrease during myopia development and increase during recovery supports this view, which contrasts with the suggestion that weakening of the scleral collagen is an important factor in the development of induced myopia.^{9,49} Such a view seems inconsistent with the finding that intraperitoneal administration of β -amino propionitrile, which blocks cross-linking of newly formed collagen, does not produce elongation and myopia in the untreated control eyes of form-deprived animals; the effect is restricted to the form-deprived treated eyes.⁵¹ Both treated and control eyes presumably had weakened collagen fibrils, but only the treated eyes developed enhanced myopia and axial elongation, suggesting that collagen fibril strength is not a critical factor in controlling axial elongation in eyes that have normal visual experience and are not subjected to excessive intraocular pressure.

Previous Studies

Although several proteomic studies have examined changes in retina/RPE during myopia development or recovery,⁵²⁻⁵⁴ only two—our previous effort in tree shrew³⁵ and the study by Zhou et al.⁵⁵ using form-deprived guinea pigs—have examined mammalian sclera. The present study replicated the downregulation of the three proteins during myopia development that we reported previously, but we did not reproduce the significant increase during recovery of heat-shock protein GRP 78. The amount and direction of the abundance change was similar: the GRP 78 spot was upregulated in the REC-treated eye compared with the REC-control eye scleras in both studies—1.26-fold in this study compared with 1.29-fold in the 2007 paper—but the difference in the present study was not significant ($P = 0.153$).

The Zhou et al.⁵⁵ study differed in many ways from the present study: the myopia-inducing treatment was 7 weeks in duration compared with 4 days in this study, standard 2DGE was used on pooled samples (the lack of biological replicates precluded statistical analysis), and only protein spots that differed in abundance by more than threefold were examined. They reported 18 proteins that had abundance differences in the deprived eye scleras compared with normal eyes (six up-regulated, 12 downregulated) and 16 proteins that differed from normal during recovery (four upregulated, 12 downregu-

lated). The proteins with altered abundance seemed to involve a wide variety of biological functions, predominantly metabolism and protein maturation. A few protein families were found to change in both studies; these were generally associated with the cytoskeleton, transcriptional regulation, or protein maturation. Although it is somewhat surprising that relatively few proteins were found to be similarly altered in both studies, this might have been due to species differences, methodological differences, or differences in treatment duration or the use of form deprivation versus minus lens wear.

A study in chick that examined primarily retinal changes during LIM and plus lens-induced hyperopia⁵⁴ found altered levels of apolipoprotein A1 in the retina and sclera. Apolipoprotein A1, which was characterized as a "stop" signal by Bertrand et al.,⁵⁴ was also identified in the present study but was colocalized in a single spot with fortilin, which appeared to be the primary protein in this spot based on calculated molar fraction (2:1 ratio compared with apolipoprotein A1). Although a change in the abundance of apolipoprotein A1 in tree shrew sclera cannot be ruled out, it appears more likely that the altered abundance of this particular spot (downregulation during LIM, upregulation during REC) in the present study was caused by altered levels of fortilin.

Relationship to mRNA Changes

The overall pattern of altered mRNA levels found in tree shrew sclera in this laboratory and others, during negative lens-induced myopia and recovery, is similar to that found in the present protein expression study. In our previous studies, mRNA levels for many genes were decreased during LIM; the notable exceptions were TGF- β -induced protein and the degradative enzymes MMP-2 and MMP-14, whose abundance increased during LIM and decreased during REC. During recovery many mRNA levels returned to, or significantly above, the levels in the control eye.

Generally, the reported correlation between mRNA and protein abundances has been notoriously poor.⁵⁶ However, a number of proteins were identified in the present study for which similar changes have been described at the mRNA level (Guo L, et al. *IOVS* 2011;52:ARVO E-Abstract 6299).³⁴ These include colligin, keratocan, and collagen 1 α 1, which were all downregulated during LIM but upregulated in REC, whereas thrombospondin I and PEDF were similarly downregulated during LIM at both the mRNA and the protein levels. Thrombospondin downregulation was confirmed with immunoblot analysis.³⁴

Control Eye Effects

The yoked response of the untreated control eyes during LIM has been reported numerous times in previous studies of tree shrews and other species (Rucker FJ, et al. *IOVS* 2009;50:ARVO E-Abstract 3931)^{18,22,34,38} and underscores the need to compare both treated and control eyes with normal eyes. Although the measures in this study were made without cycloplegia, it is unlikely that the absence of cycloplegia could have produced the yoking. Previous studies have found that, measured with the Nidek autorefractor, cycloplegia shifts all measures (myopic, control, and normal eyes) in the hyperopic direction by the same amount, approximately 0.8 D, so that the refractive differences remain the same.^{40,42}

The cause of the yoking, which occurs at the refractive, axial, mRNA, and protein levels, remains elusive. Its existence implies that, in addition to the emmetropization mechanism that operates within each eye, there is some form of binocular mechanism, presumably of central origin. A possible source of a binocular effect might be choroidal blood flow. Shih et al.⁵⁷ found that monocular form deprivation in chicks can reduce

choroidal blood flow not only in deprived eyes but also in untreated control eyes. If reduced blood flow is part of the signaling process of the emmetropization mechanism or an effect of this signaling, then the binocular effect, if it occurs in tree shrews, might contribute to yoking in the control eyes. It is interesting, but nonetheless mysterious at this juncture, why the yoking effect appears to occur more strongly during myopia development than during recovery, and why it occurs transiently, disappearing after a few days of lens wear.¹⁸

Whatever the reasons for control eye yoking, the effect at the protein level was a shared set of proteins whose abundance differed in the same direction in the LIM-control eyes as it did in the LIM-treated eyes. This shared set of proteins was smaller in number than the proteins that distinguished the LIM-treated eye from normal or from the control eye scleras but seemed similar in biological function, which involved cell-ECM interactions, transcription, and, in a limited amount, ECM proteins. Included in Table 1 are the measured fold differences that did not reach statistical significance in the control eyes (values in gray). The sign (upregulation or downregulation) of many of these were similar in both the LIM-control eyes versus normal and the LIM-treated eyes versus normal, suggesting that there was a very similar pattern of protein changes in both eyes. The smaller axial and refractive shift in the LIM-control eyes was a matter of how much the protein abundance changed rather than of the types of proteins that were involved; there was less upregulation or downregulation of proteins in the control eye, and this resulted in smaller refractive and axial changes. However, there was a distinguishing feature: there were fewer significant differences in the LIM-control eye scleras in proteins involved in cell adhesion.

Limitations

Although the use of DIGE allowed identification of a large number of proteins that were differentially expressed during LIM and REC, technical factors nonetheless limited the types of proteins that could be measured. Although 2DGE provides superior resolution of soluble proteins, most membrane-bound proteins (such as MMP-14) are poorly resolved by this technique and consequently were not examined. Small peptides, which may represent the signals from the choroid into the sclera, or small proteins, such as TIMPs, would not be seen on these 2D gels, which generally resolve in the 20- to 250-kDa range. In addition, proteins of low abundance, below the detection limit of the fluorescent dyes used in DIGE, would have limited representation in this analysis.

Another factor inherent in 2D gels is the presence of more than one protein in a single spot, estimated to be the case in as many as 75% of protein spots.⁵⁸ Given the potential size of a proteome, it should not be too surprising that on a gel with approximately 1200 well-resolved spots, many would be found to contain multiple proteins. This occurred in only 21 of the spots identified in this study, whose abundance was found to differ in either LIM or REC. In such cases, estimates of the relative proportion of each protein in the spot were used to determine which protein might be responsible for the change in abundance. However, it should be noted that emPAI was developed for use with fully sequenced genomes.⁵⁸ Given the incomplete sequencing of the tree shrew genome, the mole fraction values generated by emPAI analysis should be used with caution.

There were nontechnical limitations in the design of the study that also warrant mention. We examined differences in protein expression at only a single time point during myopia development and recovery. Examination of earlier time points would have provided information on the time course of those changes we observed after 4 days of LIM and of REC. We also

did not examine scleral proteins after full compensation to the -5 D lens had occurred. We focused instead on time points when expected changes in the particular proteins that are involved in producing the increased or decreased viscoelasticity, which is strongly affected at the measured time points.

CONCLUSION

IPA (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-8354/-DCSupplemental>) showed that the altered protein expression patterns we observed during LIM and REC fit into established networks of protein interactions. This was reassuring because it suggested that the protein changes were biologically meaningful rather than a random collection of protein changes that might not contribute to our understanding of how protein changes produce altered scleral viscoelasticity. Further, the changes in myopia development and recovery involve the same network of proteins.

The protein changes found in this study, including the interconnected network of proteins found with the IPA, do not provide any direct evidence about the nature of the retinal, RPE, or choroidal changes that constitute either the “go” signals that cause increased axial elongation or the “stop” signals that produce slowed axial elongation. The changes measure the result of those incoming signals but do not reveal their identity. However, the scleral changes, in both protein and mRNA levels, are modest in amount and suggest that the emmetropization mechanism acts through normal physiological processes during the development of minus lens-induced myopia and recovery and that these involve not only structural ECM proteins but also proteins involved in cell adhesion.

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