2014

Gene Expression Signatures In Tree Shrew Retina, Rpe, And Choroid During Experimental Myopia And Recovery

Li He
University of Alabama at Birmingham

GENE EXPRESSION SIGNATURES IN TREE SHREW RETINA, RPE, AND CHOROID DURING EXPERIMENTAL MYOPIA AND RECOVERY

by

LI HE

THOMAS T. NORTON, COMMITTEE CHAIR
CHRISTINE A. CURCIO
MICHAEL R. FROST
PAUL D. GAMLIN
CHRISTIANNE E. STRANG
SHU-ZHEN WANG

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2014

GENE EXPRESSION SIGNATURES IN TREE SHREW RETINA, RPE, AND CHOROID DURING EXPERIMENTAL MYOPIA AND RECOVERY

LI HE

VISION SCIENCE

ABSTRACT

During the development of induced myopia and recovery, the emmetropization mechanism is stimulated to modulate ocular growth. Emmetropization signals originate in the retina, are transmitted and transformed by retinal pigment epithelium (RPE) and choroid, and reach sclera to induce tissue remodeling. Induced myopia occurs in response to myopiagenic stimuli – minus lens, form deprivation, and darkness – (three GO conditions) which increase ocular elongation; recovery from lens-induced myopia (STOP) occurs when minus-lens wear is discontinued after complete compensation, slowing ocular elongation. This dissertation examined gene expression signatures in the retina, RPE, and choroid under GO and STOP conditions to examine the signaling that occurs in these compartments of the direct emmetropization pathway.

Specific Aim 1 examined gene expression signatures in the retina and RPE in early GO conditions and tested two hypotheses: a) gene expression in the RPE is hidden in the combined retina+RPE; b) the gene expression signature in the combined retina+RPE predominantly resembles retina alone. Specific Aim 2, examined gene expression in the choroid, testing two hypotheses: a) the gene expression signatures in the choroid in response to three GO visual conditions (minus-lens wear, form deprivation, and continuous darkness) will have some mRNA changes that are common to all three conditions and may be essential parts of the choroidal GO signals. b) the GO expression pattern will differ from the STOP pattern. In Specific Aim 1, mRNA levels for 44

candidate genes were measured in treated eyes and compared with mRNA levels in control eyes in the retina, RPE, and combined retina+RPE in early GO. It was found that gene expression signatures in the retina and combined retina+RPE were similar, while the RPE gene expression was hidden in the combined retina+RPE. In Specific Aim 2, mRNA levels for 77 candidate genes were measured in treated eyes compared with control eyes in the choroid. It was found that a) three different GO conditions produced similar gene expression signatures; b) the GO and STOP conditions produced very different gene expression signatures.

This project elucidated gene expression signatures in different compartments during myopia development and recovery, and enhanced the knowledge of emmetropization signaling.

Key words: myopia, emmetropization, gene expression, retina, RPE, choroid

DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my family: loving wife, daughter, parents, and parents-in-law. Thanks for support and accompany from all of you, which make my life more meaningful.

I also dedicate this dissertation to my many friends who have supported me within last six years. I will always appreciate all they have done, which make my life more colorful.

ACKNOWLEDGEMENTS

I would never have been able to finish my dissertation without the guidance of my committee members.

I would like to express my deepest gratitude to my advisor, Dr. Thomas Norton, for his excellent guidance, patience, caring, and providing me with an excellent atmosphere for doing research. I would like to thank Dr. Michael Frost, for his outstanding training on my scientific experiments, and significant efforts on improving my manuscripts. I would like to thank Dr. Christine Curcio, Dr. Paul Gamlin, Dr. Christianne Strang, and Dr. Shu-Zhen Wang, for their unconditional supports, valuable suggestions and concise comments on this research. I would like to thank Dr. John Siegwart for his help and academic support throughout this research. I would also like to thank my lab mates Lin Guo and Alexander Ward for helping me collect refractive data and tissue samples. My research would not have been possible without their help.

I would like to thank the chairs and directors of Vision Science Graduate Program, Dr. Keyser, Dr. Srivastava, and Dr. Fullard, for their support and confidence on me. I would also like to thank the program administrators, Ramona Hart, and Dr. Varghese, for their help and encouragement.

TABLE OF CONTENT

Page

ABSTRACT……ii

DEDICATION……...iv

ACKNOWLEDGEMENTS……....v

LIST of TABLES ……..……..……viii

LIST OF FIGURES…….……...……..x

INTRODUCTION...…….……1

Refractive State - Myopia, Hyperopia and Emmetropia …….……..1

Emmetropization Mechanism ……...2

Significance of Myopia Research ..……...3

Tree Shrew as an Animal Model……..4

Manipulating the Emmetropization Mechanism ……..……6
Minus Lens Treatment (GO_{ML})…….6
Form Deprivation Treatment (GO_{FD})……...……..8
Continuous Darkness Treatment (GO_{DK})……...……9
Recovery from Minus-lens Induced Myopia (STOP)……..10

Direct Emmetropization Pathway …….....11

Emmetropization Related Signaling ……12

Retina – Initiator of Signaling ……...13

RPE – a Signaling Compartment ……...15

Choroid – the Next Signaling Compartment ……15

Sclera – Effector of Emmetropization ……17

Summary and Specific Aims ……18

ALTERED GENE EXPRESSION IN TREE SHREW RETINA AND RPE PRODUCED BY SHORT PERIOD OF MINUS-LENS WEAR ...…….……20

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID IN RESPONSE TO THREE MYOPIAGENIC CONDITIONS …….56

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID DURING LENS-INDUCED MYOPIA AND RECOVERY……....95

SUMMARY AND DISCUSSION ……..147

Emmetropization Related Signaling ……...……149

Retina – Origin of Signaling ……...……150

RPE – a Signaling Compartment ……....……152

Choroid – Another Signaling Compartment ……153

Limitation of This Study ……..……154

Future Directions…….……...156

LIST OF GENERAL REFERENCES…….…….157

APPENDIX A…….171

IACUC Approval Form ……...……...172

LIST OF TABLES

Table *Page*

ALTERED GENE EXPRESSION IN TREE SHREW RETINA AND RPE PRODUCED BY SHORT PERIOD OF MINUS-LENS WEAR

1 Genes examined by functional category, with cellular location of the protein encoded by the gene, and its UniProt accession ID ……....32

2 Gene expression differences comparing treated vs. control in all treatment groups ……...…….……...…...37

S1 Primers used: Sequences, amplicon sizes, and efficiencies ……..…...…...33

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID IN RESPONSE TO THREE MYOPIAGENIC CONDITIONS

1 Genes examined by functional category, with cellular location of the protein encoded by the gene and its UniProt accession ID ……..69

2 Gene expression differences comparing right vs. left, treated vs. control, treated vs. normal, and control vs. normal eyes ……..……..……75

3 Genes that were significantly regulated under ML and FD (treated vs. control) or DK (vs. ML control, FD control, or Normal) conditions ……86

S1 Primers used: Sequences, amplicon sizes, and efficiencies ……..…...…...70

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID DURING LENS-INDUCED MYOPIA AND RECOVERY

1 Genes examined by functional category, with cellular location of the protein encoded by the gene and its UniProt accession ID ……..……....107

2 Gene expression differences comparing treated vs. control, treated vs. normal, and control vs. normal eyes ……...……...……114

3 Genes that were significantly regulated in treated vs. control eyes under GO, STAY, or STOP conditions …….……..129

S1 Primers used: Sequences, amplicon sizes, and efficiencies ……...….....108

LIST OF FIGURES

Figure *Page*

INTRODUCTION

1 Three refractive states ……..…….....1

2 Normal refractive development of tree shrew eyes …….5

3 Minus lens compensation ……..……6

4 Refractive development in minus-lens treated animal …….……8

5 Refractive development in recovery from induced myopia ……...…….11

6 Emmetropization mechanism related signaling cascade ……...…….13

7 Summary of emmetropization signaling cascade in response to four different visual conditions ……..18

ALTERED GENE EXPRESSION IN TREE SHREW RETINA AND RPE PRODUCED BY SHORT PERIOD OF MINUS-LENS WEAR

1 Compartments of the direct emmetropization pathway …….....…….23

2 Gene expression fold differences. (A) Treated eyes vs. control eyes in the retina in ML-6 group. (B) Treated eyes vs. control eyes in the retina in ML-24 group ……..……35

3 Comparison of the gene expression differences (treated eye vs. control eye) after 6 and 24 h in retina and in RPE ……..….36

4 Gene expression fold differences. (A) Treated eyes vs. control eyes in the RPE in ML-6 group. (B) Treated eyes vs. control eyes in the RPE in ML-24 group ……...……...……..……38

5 Gene expression fold differences in the treated eyes vs. control eyes in the combined retina+RPE in ML-24C group ……39

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID IN RESPONSE TO THREE MYOPIAGENIC CONDITIONS

1 Experimental groups and duration of treatments ……....….64

2 End-of-treatment refractive measures for the normal, ML, FD, and DK groups ……....73

3 Gene expression fold differences between right and left eyes for the (A) 26N group and (B) DK group ……...…74

4 Gene expression fold differences between the treated and control eyes for the (A) ML group and (B) FD group ……77

5 Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4A (ML) with the differences in Fig. 4B (FD)……..78

6 Gene expression fold differences. (A) DK group (mean of the R and L eyes) vs. ML control eyes. (B) DK group vs. FD control eyes. (C) DK group vs. the 26N group (mean of the R and L eyes)……...80

7 Comparison of the gene expression differences for (**A**) Fig. 4A (ML treated eyes vs. control eyes) with the differences in Fig. 6A (DK vs. ML control eyes; MLC) and (**B**) Fig. 4B (FD treated eyes vs. control eyes) with the differences in Fig. 6B (DK vs. FD control eyes; FDC)……...82

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID DURING LENS-INDUCED MYOPIA AND RECOVERY

1 Experimental groups and duration of treatments ……....….102

2 End-of-treatment refractive measures for the normal, minus lens (LIM-2 & LIM-11), and recovery (REC) groups ……..110

3 Daily refractive measurements of the treated and control eyes of the LIM-11 group showing that the lens-induced refractive hyperopia dissipated as the eyes compensated for the −5 D lens …….……..……111

4 Gene expression fold differences. (A) Normal eyes (right eyes vs. left eyes) in the 26N group. (B) Normal eyes (right eyes vs. left eyes) in the 37N group. (C) Comparison of expression levels at 26N and 37N…….….113

5 Gene expression fold differences. (A) Treated eyes vs. control eyes after 2 days of minus-lens wear. (B) Treated eyes vs. normal (26N) eyes. (C) Control eyes vs. normal (26N) eyes ……...……...……...117

6 Gene expression fold differences. (A) Treated eyes vs. control eyes after 11 days of minus-lens wear. (B) Treated eyes vs. normal (37N) eyes. (C) Control eyes vs. normal (37N) eyes ……..…….…..…..119

7 Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 5A (LIM-2) with the differences in Fig. 6A (LIM-11) showing the similar differential expression patterns in GO and STAY.....121

8 Gene expression fold differences. (**A**) Treated eyes vs. control eyes after 2 days of recovery from full compensation to minus-lens wear. (**B**) Treated eyes vs. normal (37N) eyes. (**C**) Control eyes vs. normal (37N) eyes.....123

9 Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 5A (LIM-2) with the differences in Fig. 8A (REC-2) showing that the gene expression patterns in GO and STOP were very different.....125

10 Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 6A (LIM-11) with the differences in Fig. 8A (REC-2) showing that the gene expression patterns in STAY and STOP were different.....126

INTRODUCTION

Refractive State - Myopia, Hyperopia and Emmetropia

A major function of the eye, as the visual transduction organ, is to refract and focus the input light onto the retina. The light rays reflected from environmental objects are refracted by cornea and lens, pass through the vitreous chamber, and finally are focused at the focal plane (the position of the focus of input light). Three refractive states exist, depending on the relationship between focal plane and axial length (the distance between cornea and retina). When the focal plane matches axial length, the focus of input light lies on the photoreceptors exactly, producing a clear image and an emmetropic refractive

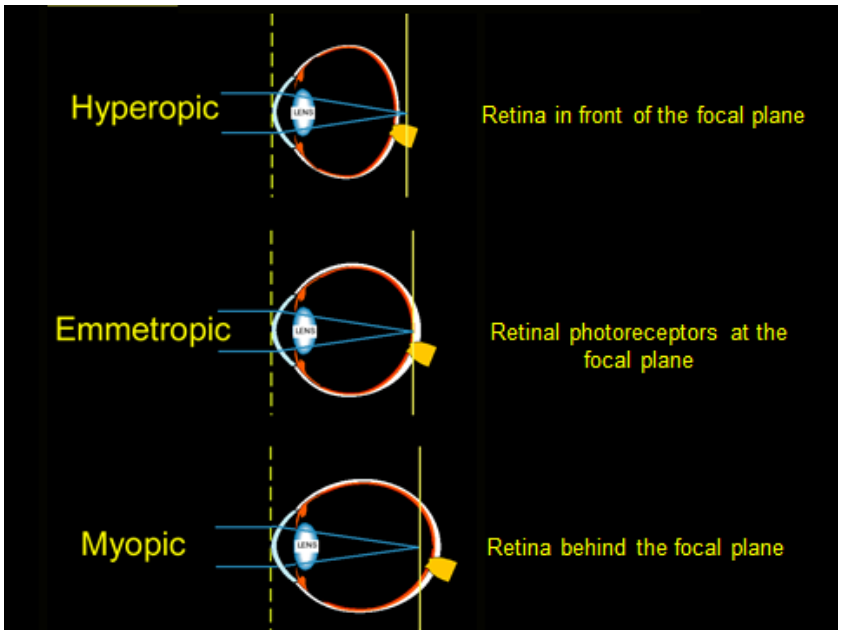

Figure 1. Three refractive states. Hyperopia occurs when axial length is relatively short – retina lies in front of the focal place. Emmetropia occurs when axial length matches the focal plane. Myopia occurs when axial length is relatively longer – retina lies behind the focal plane. Source: Norton lab library

state. The other two refractive states, or refractive errors, are caused by mismatch between focal plane and axial length. When focal plane exceeds axial length, the focal plane of input light lies behind retina, producing a hyperopic refractive state. In contrast, when axial length exceeds focal plane, the focus of input light lies in front of retina, producing a myopic refractive state.

Emmetropization Mechanism

The emmetropization mechanism is a biological feedback mechanism in the juvenile eye, which uses cues from the visual environment to match the axial length to the focal plane (Lauber, McGinnis, & Boyd, 1965; Sherman, Norton, & Casagrande, 1977; Wiesel & Raviola, 1977; Wallman, Turkel, & Trachtman, 1978; Troilo & Judge, 1993; Mutti et al., 2005). Because of maturation of ocular refractive components – cornea, anterior chamber, lens, and vitreous chamber, the focal plane moves gradually away from the cornea. Meanwhile, the emmetropization mechanism responds to the environmental visual cues, and functions to modulate the axial elongation in order to match the axial length with the focal plane (Bradley, Fernandes, Lynn, Tigges, & Boothe, 1999). In animal models treated with optical manipulations - minus lens (ML) or form deprivation (FD) - the emmetropization mechanism is stimulated to increase the axial elongation to compensate for the visual stimulus (Schaeffel, Glasser, & Howland, 1988; Siegwart & Norton, 1993; Troilo & Wallman, 1991; Wiesel & Raviola, 1977; Sherman et al., 1977; Wallman et al., 1978). The response can be abbreviated as “retina detects blur, establishing a GO condition”. In the normal ocular development of human beings, when the emmetropization mechanism is interrupted, myopia may occur as a result. Thus, studies on animal

models can provide information about the detailed functioning of the emmetropization mechanism that can be used to help treat or prevent refractive errors from developing in human.

Significance of Myopia Research

Myopia research has drawn more and more attention in recent years due to the high prevalence and rising incidence of myopia. In the Caucasian population in North America, Europe and Australia, myopia prevalence is about 25 – 40% (Sperduto, Seigel, Roberts, & Rowland, 1983; Fledelius, 1988; Wang, Klein, Klein, & Moss, 1994; Wensor, McCarty, & Taylor, 1999; Attebo, Ivers, & Mitchell, 1999; Vitale, Ellwein, Cotch, Ferris, III, & Sperduto, 2008). More shockingly, in urban Asian populations in South-East Asia, the prevalence of myopia reaches 85 – 96.5% (Goh & Lam, 1994; He et al., 2004; Lin et al., 1999; Quek et al., 2004; Lu et al., 2009; Jung, Lee, Kakizaki, & Jee, 2012). Myopia is not simply excessive axial elongation. High myopia is a risk factor for glaucoma, retinal detachment, cataract, etc (Leske, Chylack, Jr., & Wu, 1991; Marcus, de Vries, Junoy Montolio, & Jansonius, 2011; Algvere, Jahnberg, & Textorius, 1999). Thus, high myopia is considered as a risk factor of blindness. Considering myopia is the consequence of altered function of the emmetropization mechanism, a comprehensive understanding of emmetropization mechanism may lead to potential ways to prevent and/or treat myopia.

Tree Shrew as an Animal Model

Different vertebrate animal models, including fish, chick, mouse, guinea pig, tree shrew, macaque and marmoset monkeys, have been developed to study myopia and the

emmetropization mechanism (Wiesel & Raviola, 1977; Sherman et al., 1977; Wallman et al., 1978; Irving, Sivak, & Callender, 1992; Schaeffel, Hagel, Kohler, & Zrenner, 1992; Hung, Crawford, & Smith, 1995; McBrien & Norton, 1987; Graham & Judge, 1999; Shen & Sivak, 2007; Howlett & McFadden, 2009). Choosing tree shrew as an animal model has several advantages compared with other species. Evolutionarily tree shrews are mammals closely related to primates; the genome sequence is mostly known; it has acute vision; it has binocular vision; it is diurnal animal with a fibrous sclera that is similar to primates; its reproduction period and breeding expense is reasonable; most importantly, tree shrew is sensitive to different myopia-inducing treatments. Tree shrews' eyes are closed at birth, and open about 3 weeks after birth. The first day when both eyes are open is defined as the first day of visual experience (DVE). At the time of eye opening, the tree shrew eyes exhibit high hyperopia of around 25 diopters, with axial length equal to about 85% of the adult value (McBrien & Norton, 1992; Norton & McBrien, 1992). After eye opening, the eyes develop from hyperopia toward emmetropia, as shown in Figure 2. During the first 15 DVE the initial hyperopia declines quickly (over 20 diopters), this process is mainly mediated by increases in the vitreous chamber (Norton & McBrien, 1992). After 15 DVE, the hyperopia declines slowly toward emmetropia. Along with the maturation of cornea and lens, the focal plane moves away from the cornea continually, the axial length changes correspondingly in order to maintain emmetropia (Norton, Amedo, & Siegwart, Jr., 2010). So there is a baseline rate of axial elongation in eyes during normal development.

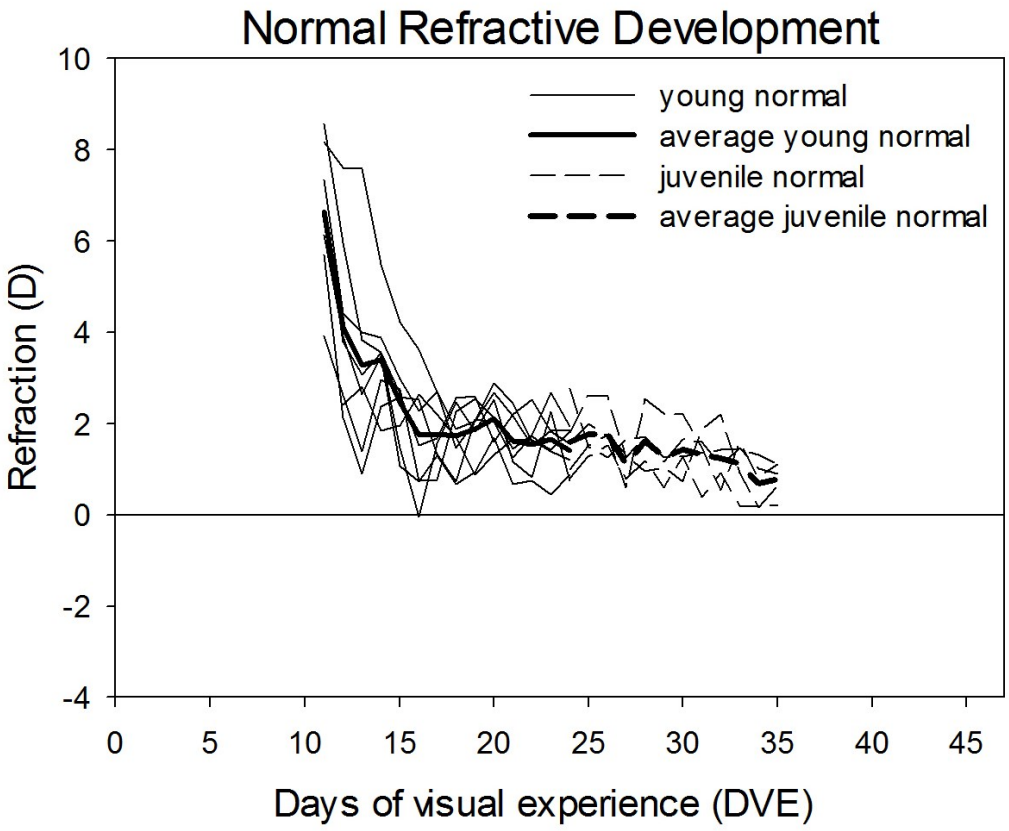

Figure 2. Normal refractive development of tree shrew eyes. Source: Norton lab library

Manipulating the Emmetropization Mechanism

Minus Lens Treatment (GO_{ML})

Wearing a monocular concave (minus power) lens in front of the treated eye produces a compensatory axial elongation and leads to a minus-lens induced myopia. The process of minus lens compensation is shown in Figure 3.

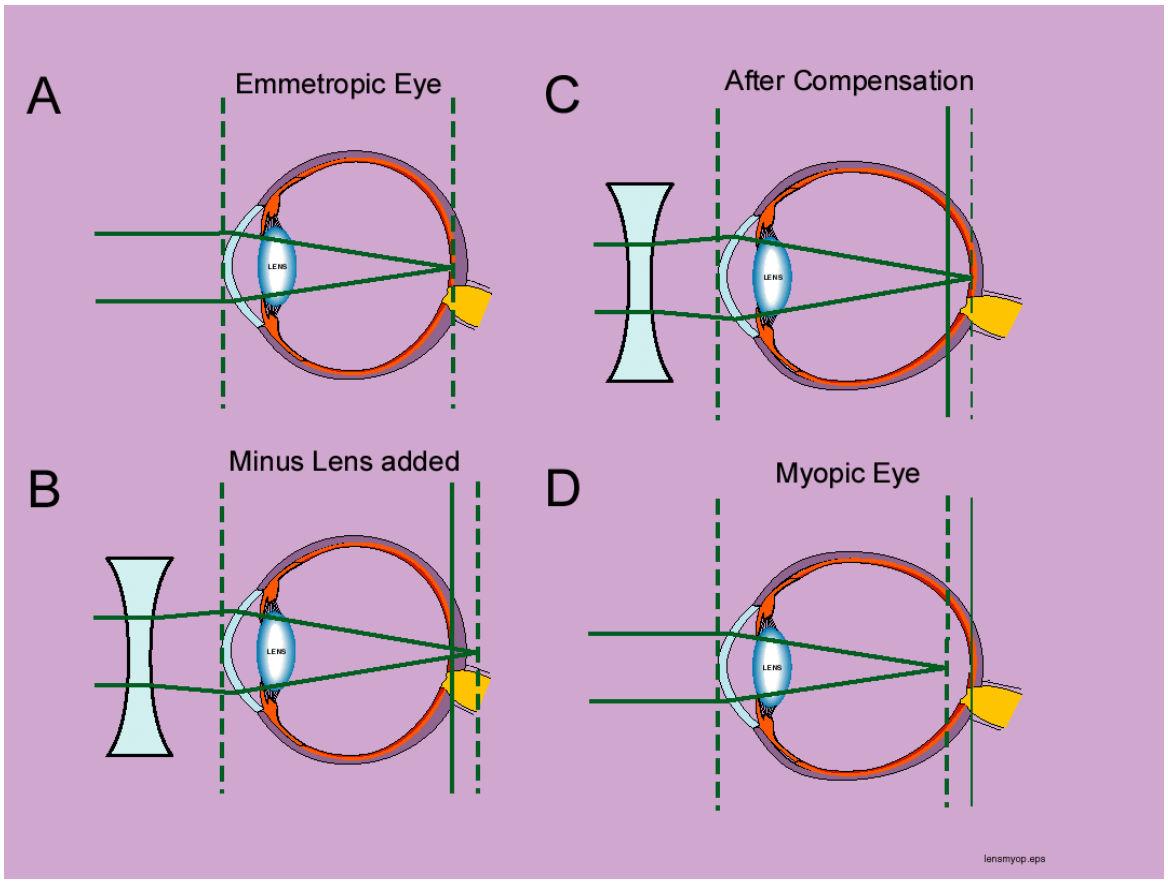

Figure 3. Minus lens compensation. ***A.*** *In an emmetropic eye, the position of focal plane matches the retina.* ***B.*** *A minus lens is held in front of the treated eye, which shifts the focal place behind the retina.* ***C.*** *The emmetropization mechanism is activated, and the axial elongation rate is increased. The eye elongates to match the retina with the shifted focal plane.* ***D.*** *The minus lens is removed, the compensated eye becomes myopic. Source: Norton lab library*

Minus lens treatment has been used to stimulate and study the emmetropization mechanism in chick, guinea pig, tree shrew, and monkey (Schaeffel et al., 1988; Siegwart & Norton, 1993; Troilo & Wallman, 1991; Howlett & McFadden, 2009). Minus-lens induced myopia is a closed-loop feedback: the minus lens creates a hyperopic blur and sets up a refractive error. The emmetropization mechanism increases the axial elongate rate to eliminate the refractive error. When the elongated axial length matches altered focal plane, the eye wearing minus lens has "re-emmetropized"; it has compensated for the lens. It is hypothesized that neurons in the minus-lens treated retina generate a GO_{ML} signal, which, through a signaling cascade (through retinal pigment epithelium [RPE] and choroid to the sclera), produces a GO response (increased elongation rate) of the eye.

In tree shrews, as well as the other animal models, the axial elongation is mainly contributed by elongation of vitreous chamber, while the cornea, anterior chamber, and lens are barely affected (Schaeffel et al., 1992; Irving, Callender, & Sivak, 1995; Irving et al., 1995; Irving, Callender, & Sivak, 1991; Hung et al., 1995; Shaikh, Siegwart, & Norton, 1999). A reliable method to monitor the myopia progression is the daily refractive measures, as shown in Figure 4.

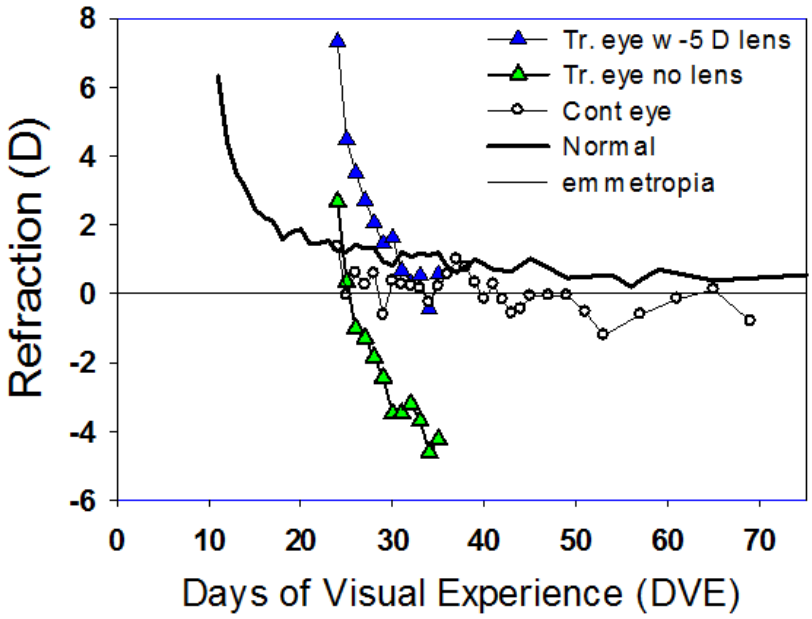

Figure 4. Refractive development in minus-lens treated animal. The black line represents the average refractive development of normal animals. The blue triangles represent the refractive development of minus-lens treated eyes in a juvenile tree shrew measured with lens in place. The green triangles represent the refractive development of minus-lens treated eyes measured without the lens, the treated eye is in the progress of developing myopia. Open circles denote the untreated control eye. Source: Norton lab library

Form Deprivation Treatment (GO_{FD})

Form deprivation, produced by using a monocular translucent diffuser, is another common tool used to induce axial elongation and study the emmetropization mechanism in chick, guinea pig, tree shrew, monkey, and mouse (Wiesel & Raviola, 1977; Sherman et al., 1977; Wallman et al., 1978; Howlett & McFadden, 2006; Schaeffel, Burkhardt, Howland, & Williams, 2004). Form deprivation removes high spatial frequency information from the retina, and reduces the contrast of images. In response, the retina gener-

ates a GO_{FD} signal that causes the eye to elongate, creating form-deprivation myopia. Form deprivation treatment is an open-loop situation, it does not set a refractive error to eliminate, so there is no target when a clear image can be obtained. Therefore, the treated eye continues elongating rapidly at first and then more slowly, until reaching an anatomical limit or removal of the diffuser. In form-deprivation induced myopia, the axial elongation is also mainly caused by an increase in vitreous chamber depth (Marsh-Tootle & Norton, 1989; McBrien & Norton, 1992).

Continuous Darkness Treatment (GO_{DK})

A third visual condition which can increase ocular elongation rate has been found to occur in tree shrews: continuous darkness treatment (DK). If infant tree shrews emmetropize in normal lighted colony conditions (100 – 300 lux, 14:10 light:dark cycle) and then are placed in continuous darkness for 11 days, the eyes elongate (vitreous chamber) and become myopic (Norton, Amedo, & Siegwart, Jr., 2006). Corneal curve and lens thickness are unaffected. It appears that a pre-treatment experience of normal lighting is important, since tree shrews raised from birth in complete darkness do not develop elongated, myopic eyes (McKanna, Casagrande, Norton, & Marsh, 1983). Darkness induced axial elongation also occurs in chicks (Troilo & Wallman, 1991). Initially, chick eyes become myopic, but over time, the cornea becomes flattened and the eyes become severely hyperopic. Darkness treatment is binocular, so there is no possibility of an untreated control eye.

Recovery from Minus-lens Induced Myopia (STOP)

When a minus lens is removed from an eye fully compensated, the eye is elongated and myopic (Figure 4). If the myopic eye is still within juvenile period and active growing phase, it has chance to recover from myopia (Norton & Siegwart, Jr., 1995; Amedo & Norton, 2012). After the minus lens is permanently removed, the emmetropization mechanism uses the myopic refractive error, coupled with its elongated size, to slow the axial elongation rate. Based on data on axial length measures, during recovery, the axial elongation rate is below normal, while the optical power of the eye continues to mature (Siegwart, Jr. & Norton, 2005; Moring, Baker, & Norton, 2007). When the axial length matches the focal plane again, the recovery is achieved. Thus, recovery (REC) is also a "re-emmetropization" process, from induced myopia back to emmetropia.

It is hypothesized that the retina in a recovering eye generates a STOP signal, which, through the signaling cascade (RPE, choroid), produces the STOP effect (slowed elongation of the eye), as retina STOP, eye STOP condition. This STOP signal, like the GO signal, is a hypothetical concept based on the response of the eye. The details of the retinal circuits and responses that underlie this signal are mostly unknown, as are the changes in RPE and choroid that communicate this signal to the sclera.

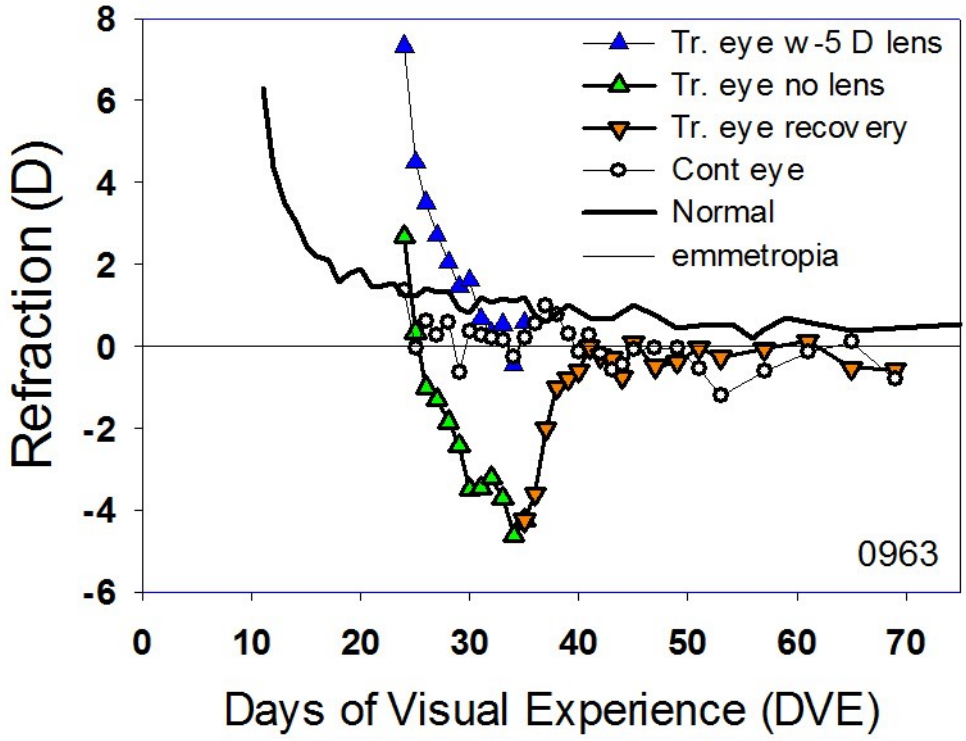

Figure 5. Refractive development in recovery from induced myopia. The green triangles represent the refractive development of minus-lens treated eyes measured without the lens, the treated eye is in the progress of developing myopia. After 11 days of minus lens treatment, the treated eye completed the compensation, and the minus lens was removed. The brown triangles represent the refractive development of eyes recovering from myopia back to emmetropia. Source: Norton lab library

Direct Emmetropization Pathway

Although visual responses leave the eye in the optic nerve and reach central visual areas of the brain, it does not appear that these signals, nor the output to the eye from the central nervous system, are critical for the functioning of the emmetropization mechanism. Basic emmetropization can occur if the optic nerve is cut or if the output from the ganglion cells is functionally blocked. In tree shrews intravitreal injection of TTX (tetro-

dotoxin), which blocked action potentials from leaving the retina, did not block the elongation of form deprived eyes (Norton, Essinger, & McBrien, 1994). In chicks, form deprived eyes still developed myopia following optic nerve section (Troilo, Gottlieb, & Wallman, 1987; Wildsoet & Pettigrew, 1988). These findings imply that signals can move directly from the retina, through the RPE and choroid, to remodel the sclera, without the need for central communication.

If a minus lens, or translucent diffuser, is designed to only cover half of the visual field, the treated half of the eye elongates and develops myopia, while the untreated half is mostly unaffected. This "half-eye myopia" has been observed in tree shrews, chicks, and macaque monkeys (Kang & Norton, 1996; Wallman, Gottlieb, Rajaram, & Fugate-Wentzek, 1987; Smith, III et al., 2009). This finding can only occur if the signaling cascade is not only direct, but also is local, so that the refractive error produces a local retinal GO signal that, through the signaling cascade, affects only the sclera that is immediately adjacent to the affected retina.

Emmetropization Related Signaling

The emmetropization related signaling cascade is summarized in Figure 6. Although it is clear that neurons in the retina are sensitive to defocus (blur) caused by refractive error, it is uncertain how it determines whether the error is due to hyperopia or myopia (Bitzer & Schaeffel, 2002), or why it seems to interpret form deprivation and darkness as hyperopic defocus. In chick and tree shrew, there is evidence that glucagon-containing amacrine cells may be critically involved (Stell, Tao, Karkhanis, Siegwart, Jr.,

& Norton, 2004; Fischer, McGuire, Schaeffel, & Stell, 1999; Vessey, Rushforth, & Stell, 2005; Ashby, Kozulin, Megaw, & Morgan, 2010).

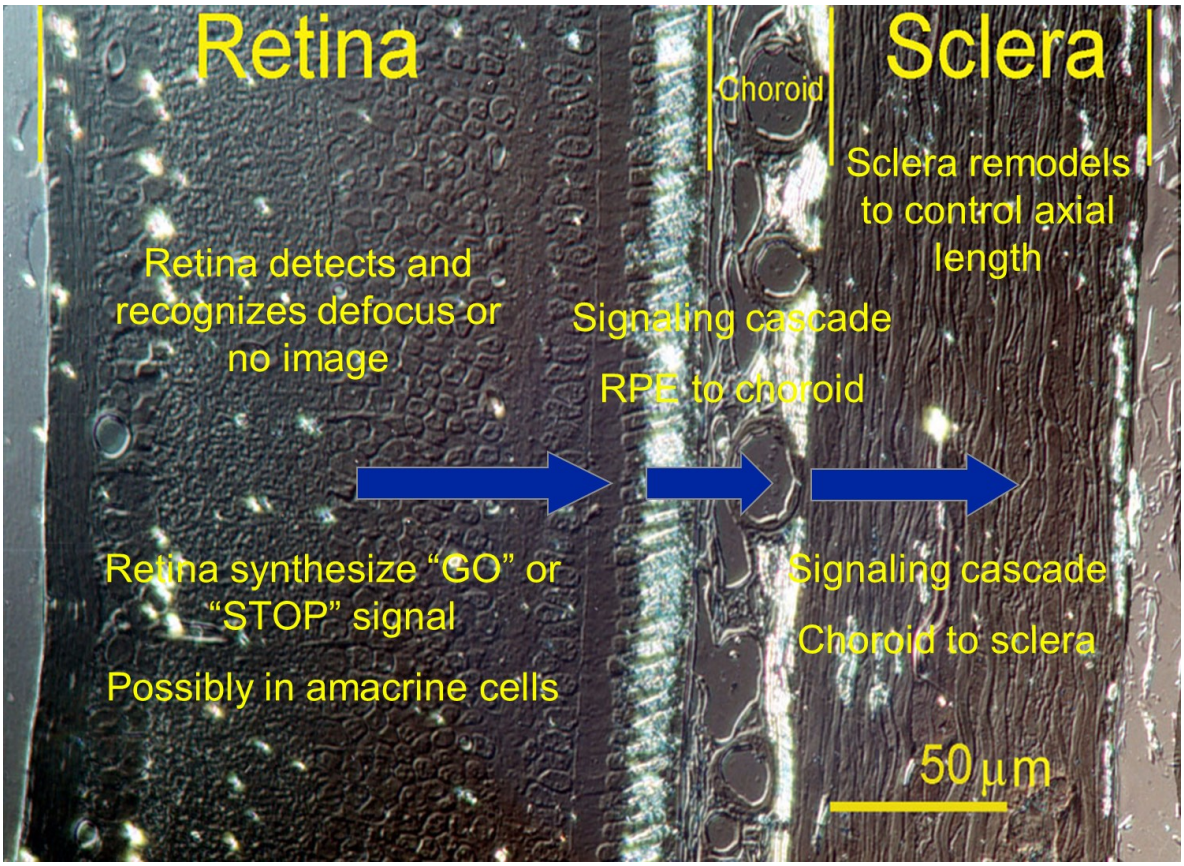

Figure 6. Emmetropization mechanism related signaling cascade. The retina works as a sensor of refractive error, which detects and recognizes defocus or no images. The retina then produces GO or STOP signals according to the sign of the refractive error (hyperopic error/form deprivation/darkness = GO, myopic error = STOP). These signals are transmitted through the RPE and choroid, where signal modification or transformation may occur.

Retina – Initiator of Signaling

As the only tissue in the eye which directly perceives environmental visual signals, it is undoubted the retina is the initiator of emmetropization related signaling. The cellular composition of retina is a complex, and the primary function of retina is starting visual

perception, rather than emmetropization. Emmetropization related signaling may only be a small portion of retinal activity, thus making it difficult to detect in the retina.

Despite the "noise" from retinal visual processing, labs in the myopia field have been working hard to decode the initiating signals in the retina. Multiple techniques (qPCR, microarray, western blot, immunohistochemistry, etc) were used to search for genes, proteins, and molecules which are regulated during minus-lens wear, form deprivation, or other visual treatments. By examining combined retina+RPE in chick treated by form deprivation with microarray technique, 15 genes at 6 hours and 280 genes at 3 days were altered between treated and control eyes (McGlinn et al., 2007). A similar study examining combined retina+RPE in chick treated by minus lens with microarray technique report a longer list of genes showing significant regulation: approximately 1300 transcripts were differentially expressed at 6 hours or 3 days (Stone et al., 2011). Gene expression within amacrine cells were examined with microarray technique in chick treated by minus lens for 24 hours, and 128 genes were differentially expressed (Ashby & Feldkaemper, 2010). In mammals the retina is less extensively studied compared with in chick, but VIP is the gene showing differential expression on both mRNA level and protein level under form deprivation treatment (Stone, Laties, Raviola, & Wiesel, 1988; Tkatchenko, Walsh, Tkatchenko, Gustincich, & Raviola, 2006). There are several genes and molecules that have been reported by more than one lab, making them more likely candidates to be involved in the emmetropization mechanism: VIP, EGR1, BMP2, dopamine, etc (Stone et al., 1988; Tkatchenko et al., 2006; Stone et al., 2011; McGlinn et al., 2007; Feldkaemper & Schaeffel, 2013). However, a lot more work needs to be done to fully understand the signaling in the retina during myopia development.

RPE – a Signaling Compartment

How the retinally-generated signals pass from the retina into the RPE is unknown, in part because the nature of the signaling molecules that must leave the retina is unknown. The presence of tight junctions between adjacent RPE cells would suggest that diffusion through the RPE is difficult. However, there is considerable trafficking, back and forth, of proteins between the photoreceptor out segments and the RPE, raising the prospect that the retinally-generated signaling molecules are actively transported into RPE where, perhaps, they may produce a signaling response by RPE.

RPE transforms the signals from retina and generates new signals. Previous studies on separate RPE tissues reported a defocus-dependent regulation of gene expression of BMP2, BMP4, and BMP7 (Zhang, Liu, Ho, & Wildsoet, 2013; Zhang, Liu, & Wildsoet, 2012). Also considering that the RPE is actively secreting VEGF, and other molecules into the choroid (Strauss, 2005), it is possible that the emmetropization-related signals are included in the RPE secretion. Although there are not many studies on the the role of RPE in the emmetropization, it is important to learn what is happening in the RPE as an important emmetropization signaling compartment.

Choroid – the Next Signaling Compartment

Choroid functions primarily as a vascular structure supplying the outer retina. It is composed of blood vessels, lymphatic ducts, melanocytes, fibroblasts, resident immunocompetent cells, and supporting collagenous and elastic connective tissues (Nickla & Wallman, 2010). In addition to vascular functions, the choroid contains secretory cells, which may secrete signal molecules to modulate choroidal vascularization and scleral

growth (Hu et al., 2009). It is clear that the choroid is a substantial intermediary involved in relaying and/or processing the emmetropization signals, but it is not known how retina/RPE-generated signals reach, and pass through, the choroid or what the nature of the signaling is in the choroid. In the chick, the choroid is very thick because there is no internal retinal vasculature, so the choroid is the entire vascular supply for the retina. In chick, the thickness of the choroid is dramatically changed in response to hyperopic or myopic defocus to move the retina toward the focal plane (Wallman et al., 1995; Wildsoet & Wallman, 1995). The regulation of choroidal thickness is very apparent in the chick, but much less in mammals, where it is thinner. The choroidal thickness of the tree shrew is approximately 60 μm, and the range of thickness change is approximately 5-10 μm (Norton lab, unpublished data).

How the choroid participates in the emmetropization process and signaling is largely unknown. Several studies have found changes in mRNA and/or protein levels in choroid during minus lens, form deprivation treatment and/or recovery. Indeed, each study has found something different. The synthesis of all-trans-retinoic acid is decreased during myopia development, vice versa during recovery; *in vivo*, treating the cultured sclera with retinoic acid inhibited scleral proteoglycan production (Mertz & Wallman, 2000). Hyaluronic acid synthase 2 (HAS2) shows a significant increase on the mRNA expression of in the chick choroid, accompanied by rapid accumulation of hyaluronic acid which may be responsible for the choroidal stromal thickening during REC (Rada, Wiechmann, Hollaway, Baggenstoss, & Weigel, 2010).

The choroid may also play a direct role in controlling ocular growth by synthesizing growth factors and matricellular enzymes. Treatment with a negative lens in chicks

resulted in down-regulation of TGFβ-2 and RALDH2 (Retinal dehydrogenase 2) mRNA concentrations in the choroid (Rada, Hollaway, Lam, Li, & Napoli, 2012; Simon, Feldkaemper, Bitzer, Ohngemach, & Schaeffel, 2004). Several growth factors involved in angiogenesis are synthesized in the choroid, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) (Hu et al., 2009). In addition, several members of matrix metalloproteinase (MMP1, MMP2, MMP3, and MMP9) and their tissue inhibitors (TIMP3) are found to be synthesized in the choroid (Janssen et al., 2008). Part of the problem with many of the previous studies may be that they each have examined only one or a few genes or proteins. Our data from the sclera (Guo, Frost, He, Siegwart, Jr., & Norton, 2013; Gao, Frost, Siegwart, Jr., & Norton, 2011; He, Frost, Siegwart, Jr., & Norton, 2010) suggest that many genes are involved. If a larger number of carefully-selected genes is measured, the signals in the choroid may become clearer.

Sclera – Effector of Emmetropization

The signaling cascade eventually reaches the sclera, where tissue remodeling occurs that alters scleral viscoelasticity and regulates axial elongation. Changes in the biochemical and biomechanical properties of the sclera during the emmetropization process have been well studied in the tree shrew. During minus-lens induced myopia, there are changes in the expression of many genes and proteins which are related to tissue remodelling (Guo et al., 2013; Frost & Norton, 2012; Gao et al., 2011; Frost & Norton, 2007). Minus lens, form deprivation and darkness treatment all produce very similar gene expression changes (Guo et al., 2013). The viscoelasticity of the sclera increases, apparently

because the biochemical changes make the scleral layers (lamellae) slip more easily across each other, so the sclera becomes more "extensible"; in contrast, during recovery from induced myopia, the viscoelasticity of the sclera decreases, making the sclera less "extensible" (Siegwart, Jr. & Norton, 1999). Although the sclera physically controls eye size, its remodeling is controlled by the signaling cascade in preceding tissues, which will be the focus of this dissertation project.

Summary and Specific Aims

As summarized in Figure 7, there are three conditions in which the retina generates GO signals that are passed through each signaling compartment (RPE, choroid, sclera) using unknown signals. With improved dissection technique, we are able to obtain separated retina, RPE, and choroid tissues. Thus we will examine the signals originating in the retina, then transmitted and transformed in the RPE and choroid. There also is a retinal STOP condition. In recovery, the eye (and the sclera) responds with STOP. It is of

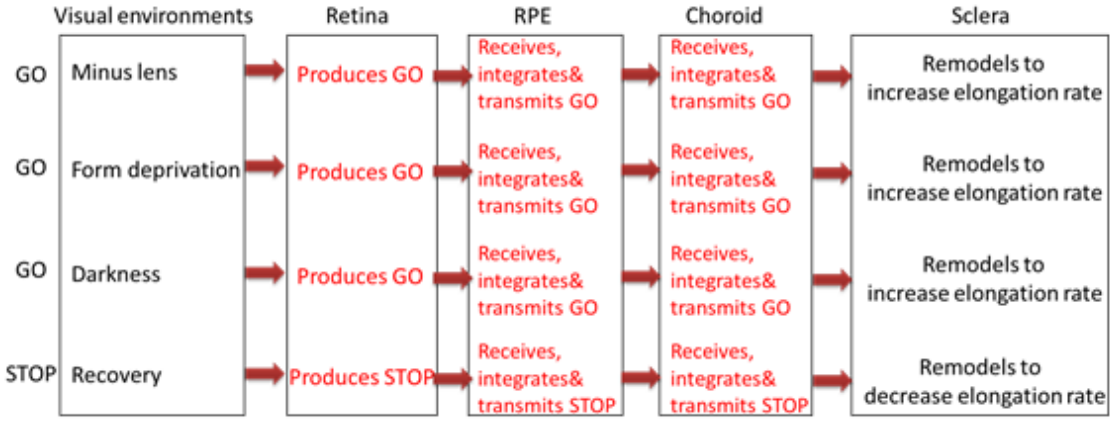

Figure 7. Summary of emmetropization signaling cascade in response to four different visual conditions.

interest to examine signaling in the choroid during the GO conditions as well as the STOP condition.

Specific aim 1 will compare mRNA expression signatures in retina, RPE, and combined retina+RPE in the GO condition. This will test two hypotheses: a) the gene expression signature in RPE is hidden in the combined retina+RPE; b) the gene expression signature in the combined retina+RPE predominantly resembles retina alone.

Specific aim 2 will ask, in the choroid, what are the gene expression signatures in the different GO and STOP conditions? This will test two hypotheses: a) the gene expression signatures in the choroid in three GO visual conditions (minus lens, form deprivation, and continuous darkness) will have some mRNA changes that are common to all three conditions and may be essential parts of the choroidal GO signal. b) the GO expression pattern will differ from the STOP (recovery) condition.

ALTERED GENE EXPRESSION IN TREE SHREW RETINA AND RPE PRODUCED BY SHORT PERIOD OF MINUS-LENS WEAR

by

LI HE, MICHAEL R. FROST, JOHN T. SIEGWART, JR., THOMAS T. NORTON

In preparation for *Molecular Vision* 2014

Format adapted for dissertation

ABSTRACT

Purpose: To examine gene expression in the retina and retinal pigment epithelium (RPE) of tree shrews after short periods of minus lens treatment. To compare gene expression in these two tissues with gene expression in combined retina+RPE.

Methods: Starting 24 days after normal eye opening, three groups of tree shrews (n = 7 each) wore a monocular –5 D lens. The untreated fellow eye served as a control. Group ML-6 was treated for 6 hours; the ML-24 group wore the lens for 24 hours; each group provided separate retina and RPE tissues. Group ML-24C also wore a –5 D lens for 24 hours and provided combined retina+RPE tissue. Quantitative PCR was used to measure the relative differences (treated eye vs. control eye) in mRNA levels for 44 candidate genes.

Results: After 24 hours of lens wear, the refractive state of treated eyes in the ML-24C group did not differ significantly from the control eyes (0.1 ± 0.1 D, mean difference ± SEM). In the ML-24 group, the treated eyes were slightly, but significantly, myopic (–0.4 ± 0.2 D). In the retina of the ML-6 group, mRNA for four genes was significantly regulated: EGR1, BMP2, and CTGF were down-regulated in the treated eyes and SST was up-regulated. In the retina of the ML-24 group, only BMP2 was still down-regulated; IGF2 and VIP were up-regulated. In the RPE of the ML-6 group, mRNA for eight genes was down-regulated; mRNA for three genes (GJA1, IGF2R, and LRP2) was up-regulated. In the RPE of the ML-24 group, mRNA for the same eight genes was still down-regulated along with mRNA for six additional genes. Only LRP2 remained up-regulated.

In the combined retina+RPE of the ML-24C group, mRNA for four genes was significantly regulated: BMP2 was down-regulated in the treated eyes while IGF2, RARB, and VIP were up-regulated. Gene expression in the combined retina+RPE was very similar to that of the retina alone.

Conclusions: Differential gene expression occurs in the retina and RPE early in the development of lens-induced myopia and evolves over time. Gene expression in the RPE is very different from that in the retina. RPE gene expression is masked in combined retina+RPE tissue.

Key Words: myopia, animal models, refractive error, emmetropization, axial elongation, gene expression, retina, RPE

INTRODUCTION

The emmetropization mechanism plays a critical role in modulating axial elongation during normal postnatal eye development. It uses refractive error to adjust the axial elongation rate so that the photoreceptors come to lie very near the focal plane. Two components of this mechanism have been identified. One is a direct emmetropization pathway that functions locally via a direct pathway from retina, into the retinal pigment epithelium (RPE), then into choroid, and finally into the sclera (Wallman & Winawer, 2004; Wildsoet, 2003; Norton et al., 1994). The other component involves connections through central brain structures that control accommodation and send efferent connections to the globe (Gamlin, 1999; Gamlin, Zhang H., Harlow A., & Barbur, 1998; Dillingham, Guggenheim, & Erichsen, 2013). Although the emmetropization mechanism functions less well without communication to central structures, the direct emmetropization pathway is able to adjust axial elongation when central communication is interrupted (Schaeffel, Troilo, Wallman, & Howland, 1990; Troilo, 1990; Wildsoet, 2003).

Imposing refractive error with lenses stimulates a response from the emmetropi-

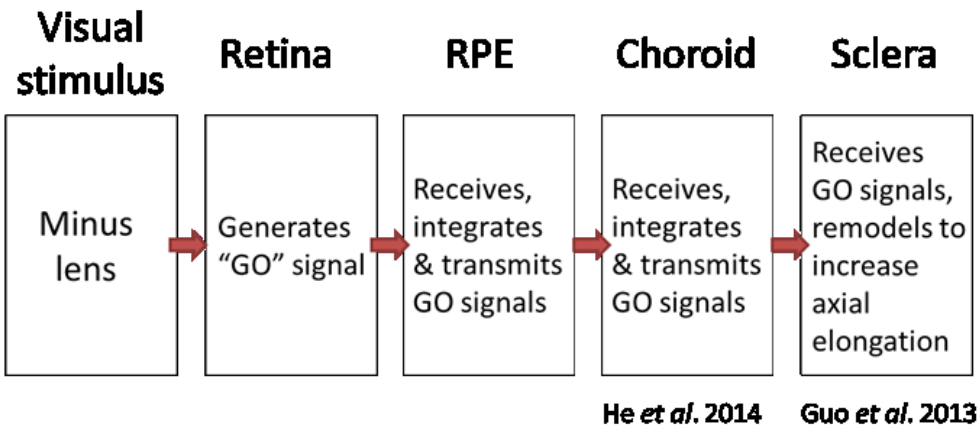

Fig. 1. Compartments of the direct emmetropization pathway.

zation mechanism. A minus-power (concave) lens, held in place in front of a postnatal eye with a goggle frame, produces a hyperopic shift in the eye's refractive state. In response, the retina produces what have been described as "GO" signals (Rohrer & Stell, 1994; Schaeffel & Howland, 1988). The precise nature of the retinal signals remains unknown but appears to involve bipolar and amacrine cells and the retinal dopaminergic pathways (Stone, Lin, Iuvone, & Laties, 1990; Iuvone, Tigges, Stone, Lambert, & Laties, 1991; Young, Raviola, Russell, & Wiesel, 1994; Fischer, Seltner, & Stell, 1997; Stell et al., 2004). As illustrated in Fig. 1, the signals then pass into a separate compartment, the RPE, which appears to receive, integrate, and then transmit the GO signals to the choroid. How retinal GO signals move from the retina to the RPE is still unknown, but it seems likely that they are transformed in some manner in the RPE so that different signaling molecules and pathways are involved in the retina vs. the RPE. The choroid, in turn, also receives, integrates, and then transmits the GO signals to the final compartment, the sclera. The sclera serves as an effector that responds to the GO signals by remodeling the extracellular matrix (Summers Rada, Shelton, & Norton, 2006; Moring et al., 2007; Gao et al., 2011; Guo et al., 2013). In tree shrews, this increases the viscoelasticity of the sclera and appears to allow normal intraocular pressure to expand the vitreous chamber, increasing the axial elongation rate (Siegwart, Jr. & Norton, 1999; Phillips, Khalaj, & McBrien, 2000). Over time, as the eye elongates, the lens-induced hyperopia diminishes. When the elongation of the globe returns the eye to its age-normal refractive state, scleral remodeling returns toward normal axial elongation slows and the eye becomes emmetropic while wearing the lens (Norton et al., 2010).

In tree shrews, small mammals closely related to primates, this lab has examined alterations in gene expression produced in the sclera and in the choroid in response to myopiagenic stimuli and during recovery from lens-induced myopia (Siegwart, Jr. & Norton, 2005; Gao et al., 2011; Guo et al., 2013; Guo, Frost, Siegwart, & Norton, 2014; He et al., 2014; He, Frost, Siegwart, & Norton, 2014). Minus-lens wear produces a pattern ("signature") of change in the expression of many genes in each tissue. The present study is an extension to the RPE. Because of difficulties involved in separating the tissues while also preserving RNA integrity, most previous studies of emmetropization-related gene expression have not examined the RPE as a separate tissue. Rather, RPE typically has been examined, either together with the retina (Stone et al., 2011; McGlinn et al., 2009; He et al., 2011) or with the choroid (Shelton et al., 2008). In either combination, one might suspect that detecting mRNA changes specific to the monolayer RPE might be difficult when it is examined in conjunction with other tissues containing a multiplicity of cell types and functions. Recent studies in chick have examined expression of several genes in RPE alone (Zhang et al., 2013; Zhang et al., 2012).The present study examined alterations in mRNA expression in tree shrews produced by minus-lens wear in the retina, in the RPE, and in the two tissues combined.

One consideration was the timing: how long after the onset of minus-lens wear should the tissues be sampled? The GO signals produced by minus-lens wear travel rapidly, but not instantaneously, through the compartments of the direct emmetropization pathway. In the tree shrew sclera, few changes in gene expression have been found after 1 day of minus-lens wear, but many genes are affected after two days (Gao et al., 2011; Guo et al., 2013). Similarly, after two days or minus-lens wear or form deprivation, the

expression of many genes is altered in the choroid (He et al., 2014). One day of minus lens treatment induced a much weaker response in the choroid (unpublished data). In tree shrew retina, altered expression of early intermediate genes has been found after 1 hour (Stell et al., 2004). Similarly, minus lens-induced gene expression changes have been found after one hour (Ashby et al., 2010), two and six hours (Stone et al., 2011). In chick RPE, gene expression changes have been found after 2 hours of lens treatment (Zhang et al., 2012; Zhang et al., 2013). We elected to examine gene expression after 6 hours, a moderately early delay after the onset of lens wear but one that we expected to produce altered gene expression in the retina and RPE. We also examined these tissues after 24 hours of minus-lens wear, at a time before substantial refractive changes occur but a time at which GO signals should be well-developed in both retina and RPE. Our hypothesis was that we would find changes in gene expression in both tissues at both times and that the expression of different genes would be affected in each tissue. We also suspected that RPE gene-expression changes would be difficult to detect in the combined retina+RPE tissue.

MATERIALS AND METHODS

Experimental Groups

The juvenile tree shrews (*Tupaia glis belangeri*) used in this study were produced in our breeding colony and raised by their mothers on a 14 hr light/10 hr dark cycle. Tree shrew pups open their eyes about three weeks after birth (Norton & McBrien, 1992). The day both eyes are open is the first day of visual experience (DVE). 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 were balanced to include both males and females, and avoided pups from the same parents wherever possible.

Three groups of animals (n = 7 per group) were used in this study. Starting at 24 ± 1 DVE, the ML-6 group wore a monocular –5 D (spherical power) lens for 6 hours and provided separate retina and RPE tissues; the ML-24 group wore the monocular –5 D lens for 24 hours, also providing separate retina and RPE tissues. The ML-24C group also wore a –5 D lens for 24 hours and provided combined retina+RPE tissues.

Lens Treatment

Animals in all groups were anesthetized (17.5 mg ketamine, 1.2 mg xylazine; supplemented with 0.5 - 2.0% isoflurane as needed) and received a dental acrylic pedestal (Siegwart & Norton, 1994) at 21 ± 1 DVE, except for one animal in the ML-24C group that received the pedestal at 10 DVE, and had normal visual experience until ML treatment began. After pedestal installation, all animals were placed in individual cages with standard colony fluorescent lighting, 100 - 300 lux on the floor of the cage. At 24 ± 1

DVE, in all groups, a goggle frame holding a –5 D lens (12 mm diameter PMMA contact lens; Conforma Contact Lenses, Norfolk, VA) was clipped to the pedestal in the morning (approximately 9:30 – 10:00 am), firmly holding the lens in front of the randomly selected treated eye. The untreated fellow control eye had unrestricted vision through an open goggle frame. For the ML-6 group, the animals wore the lens continuously in their home cage until tissue dissection and were prevented from entering their dark nest boxes to ensure that they would receive ample visual stimulation. Animals in the 24 h groups were allowed to enter and exit their nest boxes at will. For the ML-24 and ML-24C groups, the goggles were briefly (< 3 min) removed in the late afternoon to clean the lens under dim illumination. During goggle cleaning, animals were kept in a darkened nest box to minimize exposure to visual stimuli.

Refractive and Axial Measures

For the ML-6 group, non-cycloplegic refractive measures were made, in awake animals, at the start of treatment. Because refractive changes and axial elongation were not expected in such a short treatment period post-treatment refractive measures were omitted. This also minimized the period of vision with the goggle removed just before euthanasia. For the ML-24 and ML-24C groups, awake non-cycloplegic refractive measures were made at the start and end of treatment period with a Nidek ARK-700A infrared autorefractor (Marco Ophthalmic, Jacksonville, FL). Cycloplegic refractive measures were omitted to prevent any interference by atropine on retino-scleral signaling. However, previous studies have shown that non-cycloplegic measures provide a valid estimate of the refractive state and of induced myopia in tree shrews (Norton, Wu, &

Siegwart, Jr., 2003; Amedo & Norton, 2003). All refractive values were calculated as spherical equivalent at the corneal plane and were corrected for the small eye artifact, previously shown to be approximately +4 D in tree shrews (Norton et al., 2003).

At the time the pedestal was attached, ocular component dimensions were measured. In the ML-24C group, A-scan ultrasound was used while the animals were anesthetized to receive the pedestal. In the ML-6 and ML-24 groups, ocular components were measured in awake animals with a Lenstar LS-900 optical biometer (Haag-Streit USA, Mason, OH). These measures were made before the start of minus lens treatment to ensure that the two eyes did not differ significantly in axial length before treatment began.

Tissue Dissection

On completion of the minus lens treatment, animals were terminally anesthetized (17.5 mg ketamine and 1.2 mg xylazine, followed by 50 mg xylazine); both eyes were enucleated and placed into RNAlater solution (Life Technologies, Carlsbad, CA). Extraocular muscles, conjunctiva, and orbital fat were trimmed from the exterior surface of the eye and the cornea dissected away just behind the corneoscleral junction. While viewing through a surgical microscope, the lens and vitreous humor were removed; the retina and RPE, which were tightly bound to each other, were then lifted from the eyecup. The combined retina+RPE tissue was collected in an Eppendorf tube, transferred into liquid nitrogen, and stored in –80°C until homogenization and RNA extraction. To separate retina from the RPE, the combined retina+RPE tissue was transferred into ice-cold PBS buffer. In the PBS, the combined tissue was gently shaken for ~20 minutes. During this process, the RPE separated from the retina and remained suspended in the buffer. The

retina, without RPE attached, was transferred to a clean tube and collected. The RPE pieces left in the PBS buffer were centrifuged and collected after removing the supernatant.

Gene Expression Analysis

The frozen combined retina+RPE, and the separate retina and RPE that were freshly collected were homogenized with a disposable pellet pestle (Fisher Scientific, Pittsburgh, PA) before RNA extraction. Total RNA was isolated using a RiboPure kit (Life Technologies) according to the manufacturer's instructions, with the addition of an on-filter DNase treatment. The purified RNA was quantified (NanoDrop Technologies, Wilmington, DE), with an average yield per retina of 18.5 ± 2.4 μg (mean ± SD), RPE of 2.7 ± 0.7 μg, retina+RPE of 38.2 ± 3.5 μg. RNA quality was confirmed by denaturing gel electrophoresis (RNA FlashGel; Lonza, Rockland, ME). cDNA was synthesized from 1 μg of total RNA in a final reaction volume of 20 μl using a Superscript III RT kit (Life Technologies) with minor modifications (2.5 μM anchored oligo (dT)20 primers and DTT omitted). The resultant cDNA was diluted 5-fold and stored at –20°C until use.

Tree shrew-specific quantitative PCR (qPCR) primers were designed for 44 genes of interest (Table 1) and the reference gene RNA polymerase II (POLR2A) using Beacon Designer 7 (Premier Biosoft International, Palo Alto, CA). Based on the Ct values, none of the treatment conditions affected the expression of the reference gene. Primer sequences, amplicon size, and efficiencies are listed in Supplementary Table S1. The selected candidate genes included representatives whose protein products represented four major groupings: cell surface interaction, intracellular processing, transcriptional regula-

tion, and secreted proteins. Most of the candidate genes were selected since they are located at nodes in an IPA analysis network seeded from previous literature (Zhang et al., 2010; Stone et al., 2011), and the others were chosen based on previous lab data. All primers were designed to work under the same cycling conditions. All amplicons were located within the coding region and most spanned at least one intron; amplicon identity was verified by gel electrophoresis and sequencing.

Relative gene expression was measured by qPCR on a StepOnePlus Real-Time PCR System using Power SYBR Green PCR Master Mix (both, Life Technologies). Reactions were performed in triplicate in a 15 µl volume containing 300 nM each primer and 0.4 µl cDNA template. Cycling parameters were the same for all assays: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 62°C for 60 sec. Single gene products were obtained for all reactions as assessed by melt curve analysis. Relative gene expression was calculated using the $^{\Delta\Delta}$Ct method to first normalize the expression level of the target gene to that of the reference gene, and then to compare the relative expression of the target gene for treated vs. control eyes. The geometric group mean (for the 7 biological replicates) of these expression ratios was used to calculate the fold change in gene expression for each of the target genes.

Statistical Analysis

For refractive and gene expression data, paired t-tests were used to assess treated-eye vs. control-eye differences. In all cases, $p<0.05$ was considered significant and no adjustment for possible false discovery rate was applied. Linear regressions between expression differences were made in SigmaPlot (Systat Software, San Jose, CA).

Gene symbol	Protein name	Location	UniProt
Cell surface interaction			
AQP4	Aquaporin 4	Cell surface	P55087
BMPR1B	Bone morphogenetic protein receptor 1B	Cell surface	O00238
BMPR2	Bone morphogenetic protein receptor 2	Cell surface	Q13873
DRD1	Dopamine receptor D1	Cell surface	P21728
DRD2	Dopamine receptor D2	Cell surface	P14416
FGFR2	Fibroblast growth factor receptor 2	Cell surface	P21802
GJA1	Gap junction α1	Cell surface	P17302
GRM5	Metabotropic glutamate receptor 5	Cell surface	P41594
IGF2R	Insulin-like growth factor 2 receptor	Cell surface	P11717
KCNJ2	Inward rectifier potassium channel 2	Cell surface	P63252
KDR	Vascular endothelial growth factor receptor 2	Cell surface	P35968
LRP2	Low-density lipoprotein receptor-related protein 2	Cell surface	P98164
OPN4	Melanopsin	Cell surface	Q9UHM6
P2RY1	P2Y purinoceptor 1	Cell surface	P47900
SLC18A2	Synaptic vesicular amine transporter	Cell surface	Q05940
SSTR2	Somatostatin receptor 2	Cell surface	P30874
STX2	Syntaxin 2	Cell surface	P32856
VIPR1	Vasoactive intestinal polypeptide receptor 1	Cell surface	P32241
Intracellular processing			
CRABP1	Cellular retinoic acid-binding protein 1	Intracellular	P29762
NOS1	Nitric oxide synthase 1	Intracellular	P29475
RPE65	Retinoid isomerohydrolase	Intracellular	Q16518
TH	Tyrosine 3-hydroxylase	Intracellular	P07101
TYR	Tyrosinase	Intracellular	P14679
Transcriptional regulation			
EGR1	Early growth response protein 1	Intracellular	P18146
HIF1A	Hypoxia-inducible factor 1α	Intracellular	Q16665
RARB	Retinoic acid receptor β	Intracellular	P10826
Secreted proteins			
APOE	Apolipoprotein E	Extracellular	P02649
BMP2	Bone morphogenetic protein 2	Extracellular	P12643
BMP4	Bone morphogenetic protein 4	Extracellular	P12644
CTGF	Connective tissue growth factor	Extracellular	P29279
FGF1	Fibroblast growth factor 1	Extracellular	P05230
FIGF	Vascular endothelial growth factor D	Extracellular	O43915
IGF1	Insulin-like growth factor I	Extracellular	P05019
IGF2	Insulin-like growth factor 2	Extracellular	P01344
MMP2	Matrix metalloproteinase-2	Extracellular	P08253
NOV	Protein NOV homolog	Extracellular	P48745
NRG1	Neuregulin 1	Extracellular	Q02297
PENK	Proenkephalin A	Extracellular	P01210
SERPINF1	Pigment epithelium-derived factor	Extracellular	P36955
SOSTDC1	Sclerostin domain-containing protein 1	Extracellular	Q6X4U4
SST	Somatostatin	Extracellular	P61278
TGFB1	Transforming growth factor β1	Extracellular	P01137
TGFB2	Transforming growth factor β2	Extracellular	P61812
VIP	Vasoactive intestinal peptide	Extracellular	P01282

Table 1. Genes examined by functional category, with cellular location of the protein encoded by the gene, and its UniProt accession ID

Gene	*Forward sequence*	*Reverse sequence*	*Amplicon (bp)*	*Efficiency (%)*
APOE	GGTGCAGACGCTGTCTGACCA	CCTCCAACTCCGCCTTGTAGG	122	90
AQP4	CGACATGGTTCTCATCTCCCTTTG	GAAGACAGACTTGGCGATGCT	148	94
BMP2	GGACACCAGGTTAGTGAATCAGAACA	TGCTTCTCTTCCAAATGAGCCACT	135	97
BMP4	AAAGTCGCCGAGATTC	ATTGTGCTCTTGCTAGG	134	86
BMPR1B	GGAGGTTGCCAGGAGATGTG	CAGCGGTTGGGGAATGAGG	147	95
BMPR2	CAGTCCTGATGAGCATGAACCTT	GCCACCTTCTAATGGTCGCT	109	95
CRABP1	GTGAACGCCATGCTGAG	TCTCCGACCTTGAAGTTGA	137	100
CTGF	CCAACTATGATTCGAGCCAACTG	TTCTCTTCCAGGTCTGCTTCA	173	96
DRD1	CTCAACCTCTGCGTGAT	ATGAAGGAGATGAGGACAG	137	100
DRD2	CACGCACATCCTGAACATACA	GTTGAAGGTGGTGTAGATGATGG	118	91
EGR1	TCCCTGACTACCTGTTTCC	AGTGGAGTGAGCGAAGG	112	90
FGF1	CCAAGAAGCACGCAGAGAAGAA	TCAGAGGAGACTGGCAGGG	124	98
FGFR2	TGAAGGAAGGACACAGGATGGATAAG	GAGGCTGACTGAGGTCCAAGTATTC	180	93
FIGF	GAGAGTGGGCAGTGGTGAATA	AGCGAGAATCCAGACTGGTTAG	231	94
GJA1	CCTAGCCATTGTGGACCA	ATCTCCAGGTCATCAGGC	81	99
GRM5	GCATCGCCCACTCTTACAAA	CCATCAGCAGACCTCTCACT	144	93
HIF1A	ACTGGTTGCATCTCCGTCTCCTACTCA	TCCTGCTCTGTTTGGTGAGGCTGTC	109	94
IGF1	GTCCTCCTCACATCTCTTCTACC	CAGCACCGCAGAGTGTCTC	89	99
IGF2	TGGCATTGTTGAAGAGTGTTG	GAAGCACGGTCGGAGAG	113	100
IGF2R	GTACTCTGACGGAGACCTCACC	TGATTGCACTCAAAGTTTATGACACTCA	96	102
KCNJ2	GCGGTGGATGTTGGTTATCTTCTG	GGTCGTCTGGGTCTCAATGGA	184	97
KDR	ATCTGAAACGCCGCTTAGAC	GCGATGCCAAGAACTCCAT	192	97
LRP2	CAACGGATTGAGGTGGCTAAACTTGA	CCCCATTCATCCAGGCAGATTCG	163	95
MMP2	TGACCTTGACCAGAACACCATCG	GAGCGAAGGCATCATCCACTGT	176	95
NOS1	ACATCACCACGCCACCAA	CCTCCAACACCTCCACGAT	159	94
NOV	CGAACAGACTACAGAGTGGAGTG	GTGATTTCTTGGTGCGGAGACA	191	97
NRG1	CGTGTCCAAGAATGTCATCTCC	TGTGTGCCCATTGCTCCA	145	97
OPN4	CCCTTCCCCACGGTCG	TGCAGAAGGTGTAGATGACC	115	94
P2RY1	ACGGCAGCATCTTGTTC	GGAGATCGCTACCACCA	146	97
PENK	TCCTTGCCAAGCGATACG	TCTTGCTCATTTCTTCGTCGTT	162	98
RARB	CGGCTTGACCATCGCAGAC	GCTGGTTGGCAAAGGTGAACA	197	97
RPE65	CACCCAGATGCCTTGGAAGAAGATGA	CTTCACTCAAGTCCTTGGCGTTCAGA	115	99
SERPINF1	CCTGAAAGCAACCCAGAACTTGA	GACTTGGTAACTTCGCCTTCGTAAC	147	90
SLC18A2	CCTGTTCATCGTGTTCCT	TGGTGGAGTTGTCATAATAGG	194	97
SSTR2	GGCATCAATCAGTTCACCAGCATT	ATGATAGGCAAGATGACCAGCAGAG	167	94
SOSTDC1	GCGGTCCACCAAGTACATCT	ATAGCCTCCTCCGATCCAGTTA	121	97
SST	CCAGACTCCGTCAGTTTCT	CCAGGGCATCGTTCTCA	120	93
STX2	CGAGACCCAGGGCGAGATG	CCTTCTTGCTTTGCTGTGGTATTTGA	121	95
TH	TCCAGTACAAGCATGGCGACC	TGGGTGGCATAAAGGCTCTTCAG	106	93
TGFB1	ACCAGAAATACAGCAACAATTCC	AACCCGTTGATGTCCACTTG	205	91
TGFB2	GCAGAGTTTAGGGTCTTTCGTTTG	CTCGTGAACAGCATCAGTTACATC	189	93
TYR	CCAAGAACCTGATGGAGAAG	AGACGGAAGGCCAAGAT	171	96
VIP	AACGAGTGAGCACCAACATCTC	TCTTGACAGCCATTTGTTTCCTAAGG	108	94
VIPR1	TCCACGCTGCTGCTGATTC	CCCACGACCAGCTCAAAGAC	107	99
POLR2A	CTACCAGCCCCAAGTATTC	GGTGAGTAAGTAGGAGACG	106	98

Table S1. Primers used: Sequences, amplicon sizes, and efficiencies

RESULTS

Refraction

When treated with a minus lens, groups of tree shrews typically do not develop a significant myopia in the treated eyes relative to the control eyes until 2 days of minus lens wear (Norton et al., 2010). In the ML-6 group, pre-treatment measures showed that the refractions of the to-be-treated eyes (1.4 ± 0.5 D; mean ± SEM) were not significantly different from those of the control eyes (0.9 ± 0.4 D). The eyes were not re-measured at the end of the 6 h treatment period. In the ML-24 group, the post-treatment refractive difference (treated eyes – control eyes) showed the treated eyes to have developed a small, significant myopia –0.4 ± 0.2 D. In the ML-24 C group, the difference (0.1 ± 0.1 D) was not significant.

Gene Expression

Retina after 6 & 24 Hours

Six hours of minus lens wear produced significant differences in mRNA expression levels in the treated vs. control eyes for four genes (Fig. 2A). The mRNA expression values are listed in Table 2. mRNA levels for EGR1, BMP2 and CTGF were down-regulated and SST was up-regulated. In the ML-24 group, the pattern of significantly regulated genes had evolved (Fig. 2B, Table 2). BMP2 was still down-regulated, but EGR1, CTGF and SST were not significantly affected. mRNA levels for two other genes, IGF2 and VIP were up-regulated in the treated eyes.

Fig. 3A compares the differential gene expression in the retina in ML-6 with that in ML-24 for all 44 genes examined. BMP2 was the only significantly down-regulated at both time points, but the fold-difference was smaller in ML-24 than in ML-6. EGR1,

CTGF, and SST were significantly regulated in ML-6 but not ML-24, IGF2 and VIP were significantly regulated in only ML-24. It thus appears that the retinal pattern gene expression was changing over this time period.

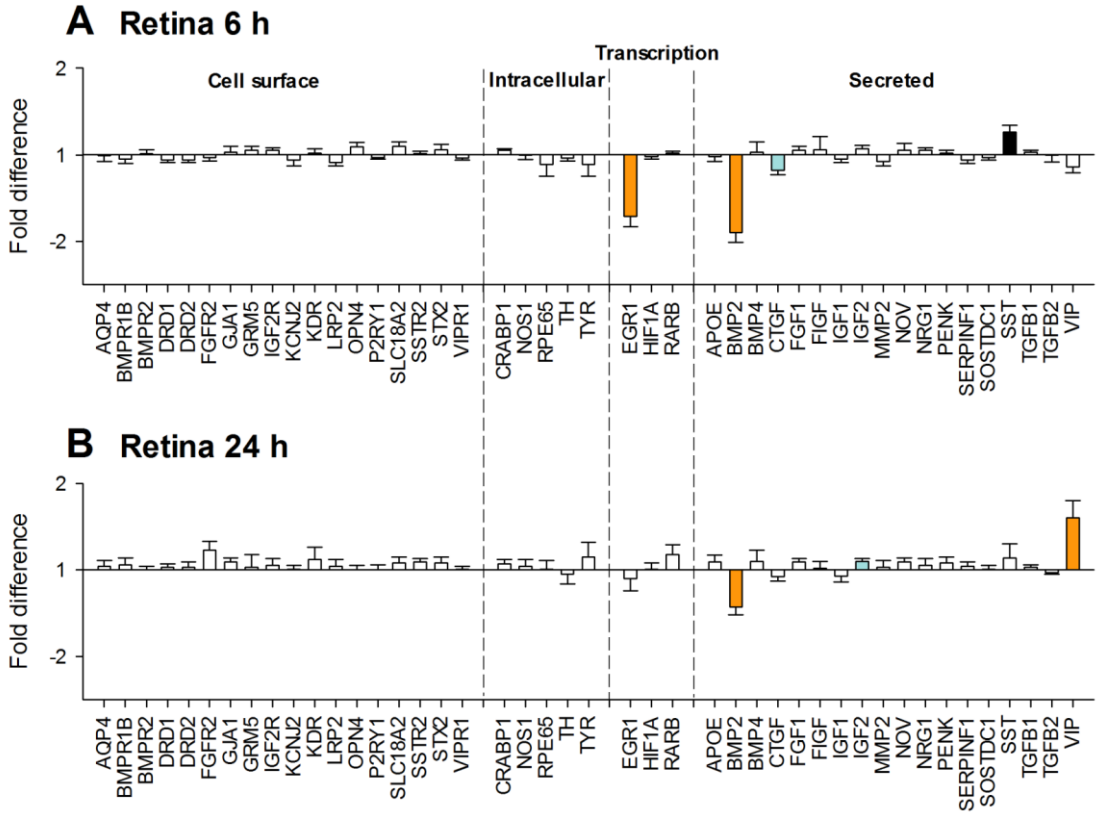

Figure 2. Gene expression fold differences. (A) Treated eyes vs. control eyes in the retina in ML-6 group. (B) Treated eyes vs. control eyes in the retina in ML-24 group. Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Filled bars represent statistically significant differences between the treated and control eyes ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the three different conditions. Error bars = SEM.

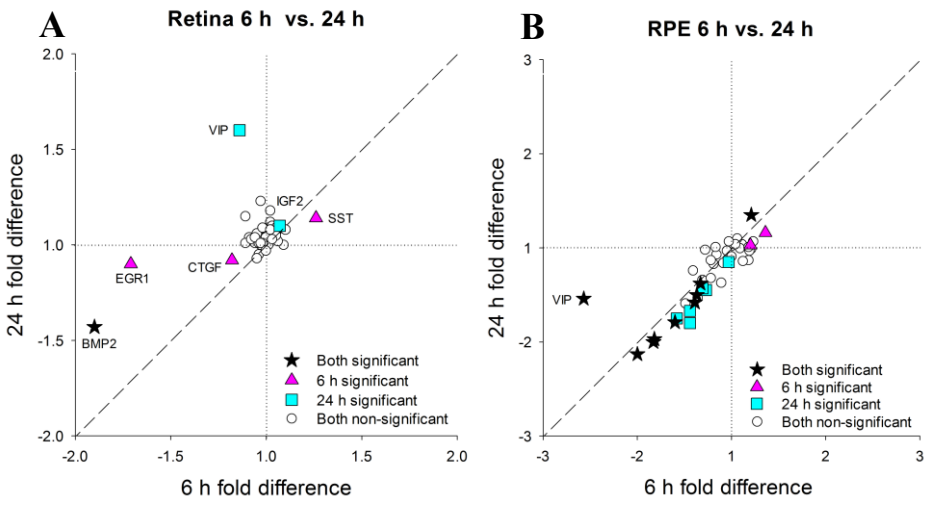

Figure 3. Comparison of the gene expression differences (treated eye vs. control eye) after 6 and 24 h in retina and in RPE. Fig. 3A compares retina 6 h (Fig. 2A) with the retina 24 h (Fig. 2B) showing the slightly different gene expression patterns at two time points. Fig. 3B compares RPE 6 h (Fig. 4A) with RPE 24 h (Fig. 4B). Values near the dashed line indicate genes that responded similarly in the two time points. Stars = significant fold differences for both 6 h and 24 h; triangles = significant fold differences only for 6 h; squares = significant fold differences only for 24 h.

	RET		RPE		Combined
	6 hours	*24 hours*	*6 hours*	*24 hours*	*24 hours*
Cell surface interaction					
AQP4	-1.01	1.04	-1.31	-1.34	1.08
BMPR1B	-1.05	1.06	1.00	1.02	1.03
BMPR2	1.01	1.00	-1.05	-1.03	1.04
DRD1	-1.06	1.03	**-1.60**	**-1.79**	1.04
DRD2	-1.06	1.03	-1.36	-1.53	-1.00
FGFR2	-1.03	1.23	1.11	1.04	1.33
GJA1	1.03	1.09	**1.36**	1.16	1.13
GRM5	1.05	1.03	-1.44	**-1.80**	1.09
IGF2R	1.05	1.05	**1.20**	1.03	1.06
KCNJ2	-1.06	1.01	-1.49	-1.59	1.02
KDR	1.02	1.12	-1.09	-1.16	1.09
LRP2	-1.09	1.04	**1.21**	**1.35**	1.08
OPN4	1.09	1.00	**-1.39**	**-1.58**	-1.03
P2RY1	-1.03	1.00	-1.58	**-1.75**	1.03
SLC18A2	1.10	1.08	**-1.82**	**-1.97**	1.06
SSTR2	1.01	1.09	**-2.00**	**-2.13**	1.06
STX2	1.06	1.08	1.21	-1.01	1.00
VIPR1	-1.04	1.01	-1.44	**-1.67**	-1.03
Intracellular processing					
CRABP1	1.05	1.07	-1.22	-1.32	-1.01
NOS1	-1.00	1.04	**-1.37**	**-1.50**	1.00
RPE65	-1.11	1.01	1.18	-1.13	-1.15
TH	-1.04	-1.05	-1.11	-1.37	-1.01
TYR	-1.11	1.15	1.23	1.07	1.15
Transcriptional regulation					
EGR1	**-1.71**	-1.10	-1.16	-1.07	-1.07
HIF1A	-1.02	1.01	-1.19	-1.17	1.04
RARB	1.02	1.18	1.00	-1.09	**1.14**
Secreted proteins					
APOE	-1.02	1.09	**-1.33**	**-1.38**	1.10
BMP2	**-1.90**	**-1.43**	1.12	-1.14	**-1.17**
BMP4	1.03	1.10	1.19	-1.03	1.11
CTGF	**-1.18**	-1.08	1.09	-1.00	1.04
FGF1	1.05	1.09	-1.27	**-1.45**	1.03
FIGF	1.06	1.02	1.06	1.10	-1.03
IGF1	-1.05	-1.07	-1.33	-1.46	1.03
IGF2	1.07	**1.10**	-1.22	-1.13	**1.07**
MMP2	-1.08	1.03	1.04	1.04	1.06
NOV	1.05	1.09	-1.28	-1.02	-1.03
NRG1	1.05	1.05	-1.03	**-1.15**	1.02
PENK	1.02	1.08	**-1.83**	**-2.00**	-1.00
SERPINF1	-1.06	1.04	1.18	1.01	1.07
SOSTDC1	-1.03	1.01	-1.03	1.07	1.10
SST	**1.26**	1.14	-1.41	-1.24	1.00
TGFB1	1.03	1.03	-1.31	**-1.43**	-1.03
TGFB2	-1.00	-1.03	-1.17	1.01	1.02
VIP	-1.14	**1.60**	**-2.57**	**-1.54**	**1.37**

Table 2. Gene expression differences comparing treated vs. control in all treatment groups. Red text = significant down-regulation, blue = significant up-regulation, grey = expression difference not statistically significant.

RPE 6 hours & RPE 24 hours

Six hours of minus-lens wear produced significant differences in mRNA expression levels for 11 genes in the treated vs. control eyes (Fig. 4A, Table 2). The mRNA expression levels of DRD1, OPN4, SLC18A2, SSTR2, NOS1, APOE, PENK, and VIP were down-regulated; GJA1, IGF2R and LRP2 were up-regulated. In ML-24 group (Fig. 4B, Table 2) the same eight genes remained significantly down-regulated and LRP2 remained up-regulated. In most cases, the fold-difference in ML-24 was larger than in ML-

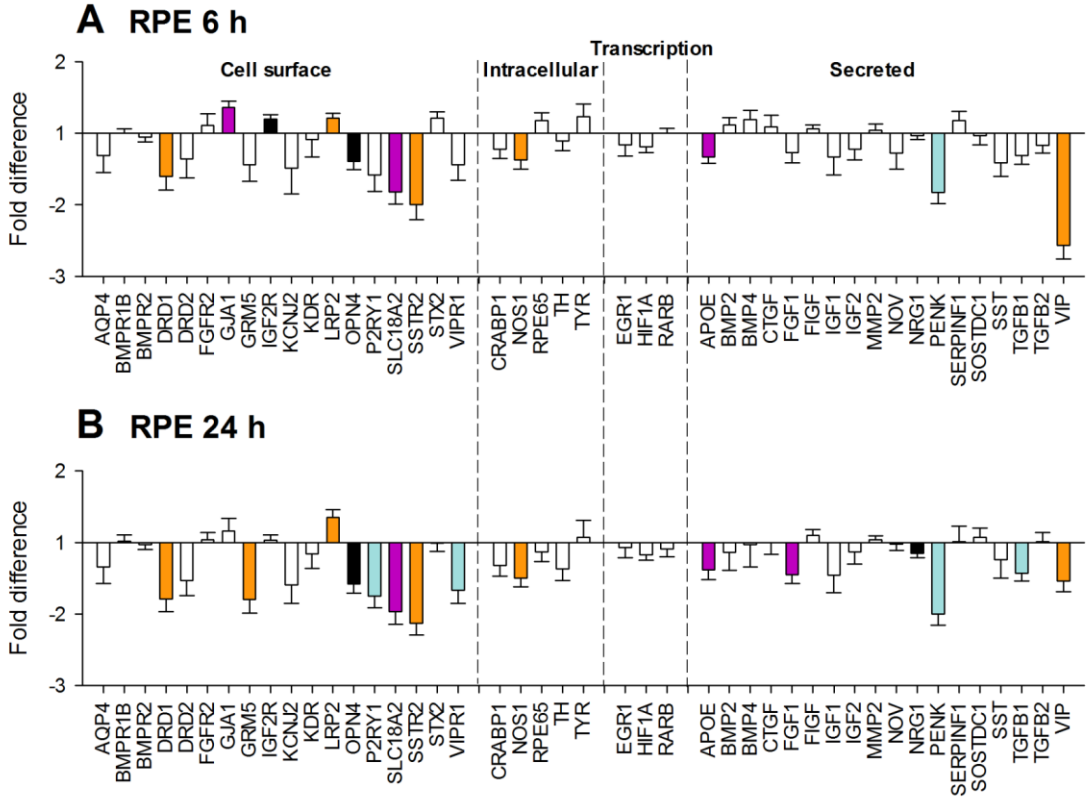

Figure 4. Gene expression fold differences. (A) Treated eyes vs. control eyes in the RPE in ML-6 group. (B) Treated eyes vs. control eyes in the RPE in ML-24 group. Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Filled bars represent statistically significant differences between the treated and control eyes ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the three different conditions. Error bars = SEM.

6. Five additional genes were significantly down-regulated in ML-24: GRM5, P2RY2, VIPR1, FGF1 and NRG1.

The mRNA expression patterns in RPE also evolved between ML-6 and ML-24 as shown in Fig. 3B. However, unlike the changes in retina gene expression, mostly the same genes showed differences in mRNA levels, and by similar amounts, as indicated by the close proximity of most genes to the 1:1 line in Fig. 3B. An exception was mRNA levels for VIP, which was more strongly down-regulated in ML-6 than in ML-24.

Combined Retina+RPE after 24 Hours

In the combined retina + RPE, 24 hours of minus lens wear produced significant differences in mRNA expression levels for 4 genes in the treated vs. control eyes (Fig. 5, Table 2). The mRNA expression level of BMP2 was down-regulated; RARB, IGF2, and VIP were up-regulated. Comparing combined tissue in ML-24C (Fig. 5) with separate

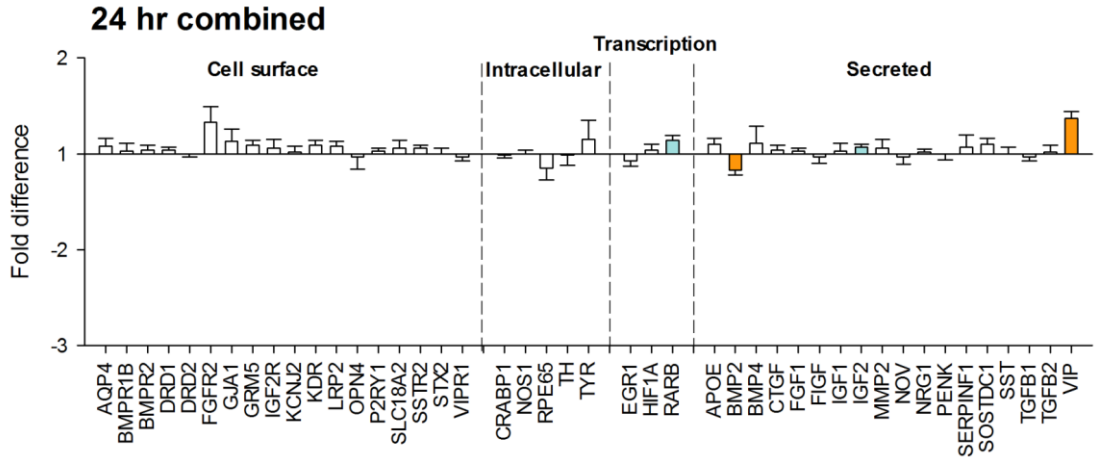

Figure 5. Gene expression fold differences in the treated eyes vs. control eyes in the combined retina+RPE in ML-24C group. Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Filled bars represent statistically significant differences between the treated and control eyes ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the three different conditions. Error bars = SEM.

retina (Fig. 2B) or RPE (Fig. 4B) in ML-24, it is evident that the gene expression pattern of the combined retina+RPE strongly resembled that of separate retina, In both, mRNA for BMP2 was down-regulated in the treated eyes; mRNA for IGF2, and VIP was up-regulated. Although mRNA for RARB was not significantly up-regulated in retina in ML-24, the fold difference (1.18) was very similar to the significant up-regulation (1.14 fold) in the combined retina+RPE in ML-24C.

DISCUSSION

This study had three main results: (1) mRNA levels for a few of the genes in our sample showed differential expression in the retina. Even amongst these few, the pattern evolved between ML-6 and ML-24. (2) When the RPE was examined as a separate tissue, many genes in our sample showed differential expression in ML-6. In ML-24, mostly the same genes were differentially expressed and were joined by others, suggesting that the RPE signal is generally the same, but stronger in ML-24. (3) In tree shrews, differential gene expression of the combined retina+RPE shows mostly the responses of the retina; gene expression in the RPE is masked.

Retinal Gene Expression

The retina is the only tissue in the direct emmetropization pathway that responds directly to visual stimuli. Thus, the genes and proteins regulated in the retina are the initiators of the emmetropization signaling cascade. Primary functions of the retina are to transduce light into neural activity and, through intricate retinal circuitry, to encode characteristics of the visual scene into numerous parallel channels that transmit information through central visual pathways to produce visual perception, guide accommodation, produce eye movements, entrain circadian rhythms and other functions. Thus, the retina contains a large number of neurons that are continually involved with multiple vision-related tasks. The number of retinal neurons involved with emmetropization signaling is unknown, but is likely a small fraction of the total. As a result, emmetropization-related signaling may be submerged in a much larger pattern of activity requiring high levels of gene expression for many of the genes in our sample. This would make it difficult to de-

tect emmetropization-related mRNA changes produced by the sub-population of emmetropization-related neurons and may account for the relatively few genes in our sample that showed differential expression related to minus-lens wear. It also may be a reason that we found small the fold differences in the differentially expressed genes. If, for instance, a gene is active in many cells and its product is utilized in multiple retinal functions, such as ACHE, even large fold differences in a few emmetropization-related neurons would scarcely alter the overall amount of mRNA produced. Interestingly, VIP is one gene whose expression has consistently been found to change during myopia development in combined retina+RPE in chicks (down-regulated) (Stone et al., 2011; McGlinn et al., 2007), in monkey retina (Stone et al., 1988; Tkatchenko et al., 2006) and tree shrews (both up-regulated). This gene is expressed by only a small population of retinal amacrine cells. The absence of "competition" from mRNAs produced by other retinal neurons that are not involved in emmetropization signaling may allow changes in this gene to be more readily detected even though (based on the Ct value) mRNA for VIP is less abundant in retina than most of the other genes in our sample. mRNA for EGR1 whose protein has been localized to a sub-population of bipolar and amacrine cells (Fischer et al., 1999; Bitzer & Schaeffel, 2002), was found to be down-regulated in tree shrews in ML-6, but not in ML-24.

Previous study also reported down-regulation of EGR1 in chick and mouse retina under form deprivation treatment (Brand, Schaeffel, & Feldkaemper, 2007; Ashby et al., 2010). This is consistent with a possible role for EGR1 in the very early phases of GO signaling. However, to the extent that the minus lens reduced retinal illuminance, this also could account for a reduction on EGR1 mRNA.

mRNA for BMP2 was down-regulated in both ML-6 and ML-24, with a slightly larger fold difference in ML-6. This might suggest that, in tree shrews, BMP2 signaling acts as a negative ocular growth regulator in retina so that reduction in BMP2 mRNA is related to increased axial elongation. Somatostatin, the peptide product of SST, is secreted by amacrine cells (Buckerfield, Oliver, Chubb, & Morgan, 1981). It serves as a neurotransmitter and participates in multiple modulatory functions In the retina, including modulating dopamine release and nitric oxide/cGMP signaling (Cervia, Casini, & Bagnoli, 2008). Our data showed an up-regulation of SST mRNA in the retina in ML-6, and also a down-regulation of SSTR2 mRNA in the RPE in ML-6 and ML-24. Somatostatin thus seems likely to be involved in the emmetropization-related GO signaling communicating between retina and RPE. IGF2 showed small but significant up-regulation in both separate retina in ML-24 and combined retina+RPE in ML-24C. The protein product of IGF2, insulin-like growth factor-2, possesses similar growth-promoting activity with insulin, or IGF1 (Chao & D'Amore, 2008). Given that exogenous insulin can exert powerful myopiagenic effect in chick (Feldkaemper, Neacsu, & Schaeffel, 2009), and also the report that insulin and IGF1 were not regulated in chick retina with hyperopic defocus (Penha, Schaeffel, & Feldkaemper, 2011), insulin-like growth factor-2 may serve as a growth promoter made by retina during myopia development.

Despite the small number of genes showing differential expression in our retinal sample, the different genes with altered repression in ML-6 vs. ML-24 suggests that the retinal GO signaling evolves in the first hours after the start of minus-lens wear. BMP2, which was regulated at both time points, may participate in a continuing signal, while the

expression of other genes be participate in either the early or slightly later part of retinal GO signaling.

RPE Gene Expression

The RPE is a polarized cell layer located adjacent to the photoreceptors. In addition to its roles in light-cycle trafficking, in photoreceptor disk phagocytosis, and in establishing and maintaining the blood-retina barrier, emmetropization-related signals enter the RPE, are processed, and passed on to the choroid. To the best of our knowledge, this is the first report of altered differential mRNA expression in the RPE of a mammal that was developing monocular myopia. Perhaps not surprisingly, we found altered expression in many genes. Several of these genes are of interest because their altered mRNA levels are consistent with previous findings or theories: DRD1 (receptor of dopamine signaling), LRP2 (lrp2 mutant zebrafish become myopic) (Veth et al., 2011), NOS1 (involved in nitric oxide production), SSTR2 (receptor of somatostatin), and VIP and VIPR1 (both down-regulated in the RPE, but VIP up-regulated in the retina). In chick RPE, decreased expression of mRNA for BMP2, has been found after 2 hours of minus-lens wear, along with decreased mRNA expression for BMP4 and BMP7. Expression of BMP2 was not significantly reduced in chick after 48 hours. In tree shrews RPE, expression of mRNA for neither BMP2 nor BMP4 was significantly altered in either ML-6 or ML-24. This difference may be due to the differing durations of minus-lens wear in the chick and tree shrew studies, or species difference.

As was shown in Fig. 3B, the gene expression patterns in tree shrew RPE in ML-6 and ML-24 are extremely similar. The eight genes whose mRNA was down-regulated in

ML-6 were still significantly down-regulated in ML-24. The additional six genes whose mRNA was significantly down-regulated in ML-24 showed negative, non-significant fold-differences in ML-6. Possibly, these genes may already have been starting to change their mRNA expression in ML-6. The RPE GO signaling may involve a relatively constant group of genes whose altered expression increases over time, at least within the first day of minus-lens wear.

Gene Expression in Combined Retina+RPE

When we examined the gene expression pattern of 44 target genes in both combined retina+RPE in ML-24C and in the separate retina and RPE in ML-24, the pattern of altered gene expression in the combined retina+RPE (Fig. 5) very closely resembled the pattern found in retina (Fig. 2B). In the combined retina+RPE there were only 4 genes showing significant regulation. Three of these (BMP2, IGF2, and VIP) showed similar, significant regulation in separate retina. Although mRNA for RARB was significantly up-regulated only in the combined tissue, the fold difference in the retinal sample (1.18) was very similar to that of the combined tissue (1.14). In addition, for those genes without significant regulation, their fold differences and direction were also similar (Table 2). In contrast, there were many more genes whose expression was significantly regulated in the separate RPE. However, these significantly-regulated genes were not detected in the combined retina+RPE. Thus, we conclude that the genes expression signature in the combined retina+RPE predominantly represents that in the retina, and the signature in the RPE was mostly masked. We conclude that, in tree shrews, the mRNA from the combined retina+RPE reflect gene expression in the retina. In tree shrews and, possibly, in

other species, if one wishes to study expression changes in RPE, it must be examined as a separate tissue.

It is not surprising that the relatively low fold-changes in the RPE were not detectable in the combined tissue. The mRNA extracted from the monolayer of RPE cells was around one-seventh of the amount extracted from the retina. In addition, based on the Ct value, there are very few genes in RPE (i.e., RPE65) whose expression is greater than in the retina. Thus, in the combined tissue, the modest mRNA changes in the RPE were undetectable.

Previous studies have examined mRNA expression in combined retina+RPE in chick exposed to minus lens or form deprivation. Using gene arrays, Stone and colleagues (2007; 2011) reported more than 1,000 genes with significant regulation, including several genes tested in our study.

In response to minus-lens wear, the mRNA level of EGR1 in chick combined retina+RPE was decreased at 3 days. In our study, mRNA for EGR1 showed significant down-regulation in separate retina in ML-6, but not in combined retina+RPE in ML-24C.

BMP2, in chick combined retina+RPE showed down-regulation of its mRNA both during minus-lens wear and form deprivation at 6 hours and 3 days. Similarly, we found down-regulation of mRNA for BMP2 in tree shrew combined retina+RPE in ML-24C. Our analysis of the tissues showed that this change in BMP2 differential expression occurred in the retina, but not in RPE.

GRM5, LRP2, and OPN4 exhibited significant down-regulation in chick combined retina+RPE after six hours of minus lens wear (Stone et al., 2011). We did not detect similar regulation of these genes in tree shrew combined retina+RPE. However, we

detected the significant down-regulation of mRNA for GRM5 and OPN4, along with significant up-regulation of LRP2 in separate RPE. Whether this is a species difference, or if these changes occurred in chick RPE and were detected in the combined retina+RPE remains to be discovered but further underscores the usefulness of examining the two tissues separately.

Limitations

The design of this study was to compare mRNA levels in the minus-lens treated eyes with those in the untreated fellow "control" eyes because treated eyes develop myopia but the untreated fellow eyes do not. However, when compared with normal eyes, the untreated eyes can show altered mRNA levels. In a preliminary study of combined retina+RPE after 4 day of minus-lens wear (He, Frost, Siegwart, Filios, & Norton, 2011) four genes (APOE, IGF2, FGFR2, and VIP) from a sample of 44 were significantly different between treated eyes and control eyes at mRNA levels. When we then compared control eyes with normal eyes at 28 DVE (age-matched), none of these four genes show significant regulation (He, unpublished results). Moreover, for only 3 of the 44 genes, the control eye mRNA levels differed from the normal mRNA levels. Thus, although in the present study we do not know if there were control-eye changes, it appears from the preliminary study that this was not a major issue.

It also is of interest that, despite selecting genes that we thought might show altered expression in the retina, we found very few whose mRNA levels were significantly regulated. When one compares the low "hit rate" in this tree shrew study with the hundreds of genes found to change in chicks (McGlinn et al., 2007; Stone et al., 2011), and in

RPE/choroid of marmosets (Shelton et al., 2008) seems somewhat surprising that so few genes showed altered expression in this study, despite the relatively small number whose expression was examined. However, excluding the 4 genes with significant regulation in combined retina+RPE (IGF2, RARB, BMP2, and VIP) and the 4 genes with significant regulation in separate retina or RPE (EGR1, LRP2, GRM5, and OPN4), the other 36 genes in our sample in combined retina+RPE were not regulated in chick combined retina+RPE (Stone et al., 2011). Thus, the absence of altered mRNA levels in tree shrew is consistent with the results in chick.

We recognize that 44 genes is a relatively small sample and that there may be other genes, that we did not examine, whose expression may be regulated in retina and/or in RPE and may be important parts of the signaling in the direct emmetropization pathway. However, the sample was large enough to show that gene expression is very different in the retina and RPE and to show the importance of examining each tissue separately.

ACKNOWLEDGMENTS

This study was supported by NIH grants EY005922 and EY003039 (P30). Li He was supported in part by a supplement to EY005922 and by funds from the Department of Vision Sciences. This work was performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Alabama at Birmingham (Li He). Preliminary results were presented in abstract form.

Reference List

Amedo, A. O. & Norton, T. T. (2003). Comparison of Infrared photoretinoscope and autorefractor in tree shrews with and without induced myopia. Optometry and Vision Science 80 (suppl.), 120.

Ref Type: Abstract

Ashby, R., Kozulin, P., Megaw, P. L., & Morgan, I. G. (2010). Alterations in ZENK and glucagon RNA transcript expression during increased ocular growth in chickens. *Mol.Vis., 16,* 639-649.

Bitzer, M. & Schaeffel, F. (2002). Defocus-induced changes in ZENK expression in the chicken retina. *Invest Ophthalmol.Vis.Sci., 43,* 246-252.

Brand, C., Schaeffel, F., & Feldkaemper, M. P. (2007). A microarray analysis of retinal transcripts that are controlled by image contrast in mice. *Mol.Vis., 13,* 920-932.

Buckerfield, M., Oliver, J., Chubb, I. W., & Morgan, I. G. (1981). Somatostatin-like immunoreactivity in amacrine cells of the chicken retina. *Neuroscience, 6,* 689-695.

Cervia, D., Casini, G., & Bagnoli, P. (2008). Physiology and pathology of somatostatin in the mammalian retina: a current view. *Mol Cell Endocrinol., 286,* 112-122.

Chao, W. & D'Amore, P. A. (2008). IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev, 19,* 111-120.

Dillingham, C. M., Guggenheim, J. A., & Erichsen, J. T. (2013). Disruption of the centrifugal visual system inhibits early eye growth in chicks. *Invest Ophthalmol Vis Sci, 54,* 3632-3643.

Feldkaemper, M. P., Neacsu, I., & Schaeffel, F. (2009). Insulin acts as a powerful stimulator of axial myopia in chicks. *Invest Ophthalmol Vis Sci, 50,* 13-23.

Fischer, A. J., McGuire, J. J., Schaeffel, F., & Stell, W. K. (1999). Light- and focus-dependent expression of the transcription factor ZENK in the chick retina. *Nat.Neurosci., 2,* 706-712.

Fischer, A. J., Seltner, R. P., & Stell, W. K. (1997). N-methyl-d-aspartate-induced excitotoxicity causes myopia in hatched chicks. *Canadian Journal of Ophthalmology - Journal Canadien d Ophtalmologie, 32,* 373-377.

Gamlin, P. D. (1999). Subcortical neural circuits for ocular accommodation and vergence in primates. *Ophthalmic and Physiological Optics, 19,* 81-89.

Gamlin, P. D. R., Zhang H., Harlow A., & Barbur, J. L. (1998). Pupil responses to stimulus color, structure and light flux increments in the rhesus monkey. *Vision Research, 38,* 3353-3358.

Gao, H., Frost, M. R., Siegwart, J. T., Jr., & Norton, T. T. (2011). Patterns of mRNA and protein expression during minus-lens compensation and recovery in tree shrew sclera. *Mol.Vis., 17,* 903-919.

Guo, L., Frost, M. R., He, L., Siegwart, J. T., Jr., & Norton, T. T. (2013). Gene expression signatures in tree shrew sclera in response to three myopiagenic conditions. *Invest Ophthalmol Vis Sci, 54,* 6806-6819.

Guo, L., Frost, M. R., Siegwart, Jr. J. T., & Norton, T. T. (2014). Scleral gene expression signatures in tree shrew sclera during lens-induced myopia and recovery. *Experimental Eye Research, submitted for publication*.

He, L., Frost, M. R., Siegwart, J. T., & Norton, T. T. (2014). Gene expression sigatures in tree shrew choroid in response to three myopiagenic conditions. *Vision Research, submitted for publication*.

He, L., Frost, M. R., Siegwart, J. T., Jr., & Norton, T. T. (2014). Gene expression signatures in tree shrew choroid during lens-induced myopia and recovery. *Exp Eye Res, 123,* 56-71.

He, L., Frost, M. R., Siegwart, Jr. J. T., Filios, S., & Norton, T. T. (2011). Retinal gene expression signatures in tree shrew in response to three myopiagenic visual conditions: minus lens, form deprivation, and darkness. Invest Ophthalmol Vis Sci 52, ARVO E-Abstract 6301.

Ref Type: Abstract

Iuvone, P. M., Tigges, M., Stone, R. A., Lambert, S., & Laties, A. M. (1991). Effects of apomorphine, a dopamine receptor agonist, on ocular refraction and axial elongation in a primate model of myopia. *Invest Ophthalmol Vis Sci, 32,* 1674-1677.

McGlinn, A. M., Baldwin, D. A., Tobias, J. W., Budak, M. T., Khurana, T. S., & Stone, R. A. (2007). Form-deprivation myopia in chick induces limited changes in retinal gene expression. *Invest Ophthalmol.Vis.Sci., 48,* 3430-3436.

Moring, A. G., Baker, J. R., & Norton, T. T. (2007). Modulation of glycosaminoglycan levels in tree shrew sclera during lens-induced myopia development and recovery. *Invest Ophthalmol.Vis.Sci., 48,* 2947-2956.

Norton, T. T., Amedo, A. O., & Siegwart, J. T., Jr. (2010). The effect of age on compensation for a negative lens and recovery from lens-induced myopia in tree shrews (*Tupaia glis belangeri*). *Vision Research, 50,* 564-576.

Norton, T. T., Essinger, J. A., & McBrien, N. A. (1994). Lid-suture myopia in tree shrews with retinal ganglion cell blockade. *Visual Neuroscience, 11,* 143-153.

Norton, T. T. & McBrien, N. A. (1992). Normal development of refractive state and ocular component dimensions in the tree shrew (*Tupaia belangeri*). *Vision Research, 32,* 833-842.

Norton, T. T., Wu, W. W., & Siegwart, J. T., Jr. (2003). Refractive state of tree shrew eyes measured with cortical visual evoked potentials. *Optom.Vis.Sci., 80,* 623-631.

Penha, A. M., Schaeffel, F., & Feldkaemper, M. (2011). Insulin, insulin-like growth factor-1, insulin receptor, and insulin-like growth factor-1 receptor expression in

the chick eye and their regulation with imposed myopic or hyperopic defocus. *Mol.Vis., 17,* 1436-1448.

Phillips, J. R., Khalaj, M., & McBrien, N. A. (2000). Induced myopia associated with increased scleral creep in chick and tree shrew eyes. *Invest Ophthalmol.Vis.Sci., 41,* 2028-2034.

Rohrer, B. & Stell, W. K. (1994). Basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-b) act as stop and go signals to modulate postnatal ocular growth in the chick. *Experimental Eye Research, 58,* 553-561.

Schaeffel, F. & Howland, H. C. (1988). Mathematical model of emmetropization in the chicken. *Journal of the Optical Society of America, 5,* 2080-2086.

Schaeffel, F., Troilo, D., Wallman, J., & Howland, H. C. (1990). Developing eyes that lack accommodation grow to compensate for imposed defocus. *Vis Neurosci, 4,* 177-183.

Shelton, L., Troilo, D., Lerner, M. R., Gusev, Y., Brackett, D. J., & Rada, J. S. (2008). Microarray analysis of choroid/RPE gene expression in marmoset eyes undergoing changes in ocular growth and refraction. *Mol.Vis, 14,* 1465-1479.

Siegwart, J. T. & Norton, T. T. (1994). Goggles for controlling the visual environment of small animals. *Laboratory Animal Science, 44,* 292-294.

Siegwart, J. T., Jr. & Norton, T. T. (1999). Regulation of the mechanical properties of tree shrew sclera by the visual environment. *Vision Research, 39,* 387-407.

Siegwart, J. T., Jr. & Norton, T. T. (2005). Selective regulation of MMP and TIMP mRNA levels in tree shrew sclera during minus lens compensation and recovery. *Invest Ophthalmol.Vis.Sci., 46,* 3484-3492.

Stell, W. K., Tao, J., Karkhanis, A., Siegwart, J. T., Jr., & Norton, T. T. (2004). Amacrine Cells responsive to optical conditions in the tree shrew, *Tupaia glis belangeri*. Invest Ophthalmol Vis Sci 45, E-abstract 1159.

Ref Type: Abstract

Stone, R. A., Laties, A. M., Raviola, E., & Wiesel, T. N. (1988). Increase in retinal vasoactive intestinal polypeptide after eyelid fusion in primates. *Proc.Natl.Acad.Sci U.S.A, 85,* 257-260.

Stone, R. A., Lin, T., Iuvone, P. M., & Laties, A. M. (1990). Postnatal control of ocular growth: dopaminergic mechanisms. In *Myopia and the Control of Eye Growth* (155 ed., pp. 45-57). New York: Wiley & Sons.

Stone, R. A., McGlinn, A. M., Baldwin, D. A., Tobias, J. W., Iuvone, P. M., & Khurana, T. S. (2011). Image defocus and altered retinal gene expression in chick: Clues to the pathogenesis of ametropia. *Invest Ophthalmol Vis Sci, 52,* 5765-5777.

Summers Rada, J. A., Shelton, S., & Norton, T. T. (2006). The sclera and myopia. *Experimental Eye Research, 82,* 185-200.

Tkatchenko, A. V., Walsh, P. A., Tkatchenko, T. V., Gustincich, S., & Raviola, E. (2006). Form deprivation modulates retinal neurogenesis in primate experimental myopia. *Proc.Natl.Acad.Sci.U.S.A, 103,* 4681-4686.

Troilo, D. (1990). Experimental studies of emmetropization in the chick. In *Myopia and the Control of Eye Growth* (pp. 89-114). New York: Wiley & Sons.

Veth, K. N., Willer, J. R., Collery, R. F., Gray, M. P., Willer, G. B., Wagner, D. S. et al. (2011). Mutations in zebrafish lrp2 result in adult-onset ocular pathogenesis that models myopia and other risk factors for glaucoma. *PLoS.Genet., 7,* e1001310.

Wallman, J. & Winawer, J. (2004). Homeostasis of eye growth and the question of myopia. *Neuron, 43,* 447-468.

Wildsoet, C. (2003). Neural pathways subserving negative lens-induced emmetropization in chicks--insights from selective lesions of the optic nerve and ciliary nerve. *Current Eye Research, 27,* 371-385.

Young, T. L., Raviola, E., Russell, M. E., & Wiesel, T. N. (1994). Upregulation of vasoactive intestinal polypeptide (VIP) gene expression in the retina of myopic eyes following lid fusion in monkeys [ARVO Abstract]. Investigative Ophthalmology and Visual Science 35[suppl.], S2069.

Ref Type: Abstract

Zhang, Y., Liu, Y., Ho, C., & Wildsoet, C. F. (2013). Effects of imposed defocus of opposite sign on temporal gene expression patterns of BMP4 and BMP7 in chick RPE. *Exp Eye Res, 109,* 98-106.

Zhang, Y., Liu, Y., & Wildsoet, C. F. (2012). Bidirectional, optical sign-dependent regulation of BMP2 gene expression in chick retinal pigment epithelium. *Invest Ophthalmol Vis Sci, 53,* 6072-6080.

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID IN RESPONSE TO THREE MYOPIAGENIC CONDITIONS

by

LI HE, MICHAEL R. FROST, JOHN T. SIEGWART, JR., THOMAS T. NORTON

Accepted by *Vision Research* 2014 July

ABSTRACT

We examined gene expression in tree shrew choroid in response to three different myopiagenic conditions: minus lens (ML) wear, form deprivation (FD), and continuous darkness (DK). Four groups of tree shrews (n = 7 per group) were used. Starting 24 days after normal eye opening (days of visual experience [DVE]), the ML group wore a monocular –5 D lens for 2 days. The FD group wore a monocular translucent diffuser for 2 days. The DK group experienced continuous darkness binocularly for 11 days, starting at 17 DVE. An age-matched normal group was examined at 26 DVE. Quantitative PCR was used to measure the relative (treated eye vs. control eye) differences in mRNA levels in the choroid for 77 candidate genes. Small myopic changes were observed in the treated eyes (relative to the control eyes) of the ML group (−1.0 ± 0.2 D; mean ± SEM) and FD group (−1.9 ± 0.2 D). A larger myopia developed in the DK group (−4.4 ± 1.0 D) relative to Normal eyes (both groups, mean of right and left eyes). In the ML group, 28 genes showed significant differential mRNA expression; eighteen were down-regulated. A very similar pattern occurred in the FD group; twenty-seven of the same genes were similarly regulated, along with five additional genes. Fewer expression differences in the DK group were significant compared to normal or the control eyes of the ML and FD groups, but the pattern was similar to that of the ML and FD differential expression patterns. These data suggest that, at the level of the choroid, the gene expression signatures produced by "GO" emmetropization signals are highly similar despite the different visual conditions.

Key Words: myopia; choroid; emmetropization pathway; gene expression; animal models; refractive error.

INTRODUCTION

It is well established that a visually-guided emmetropization mechanism operates during post-natal visual development in a wide range of vertebrate species, including humans (Schaeffel & Howland, 1988; Smith, III, Hung, & Harwerth, 1999; Norton, 1999; Wallman & Winawer, 2004; Mutti et al., 2005). This mechanism uses refractive error, detected by the retina, to adjust the axial elongation rate of the growing eye to achieve a match between the location of the retina and that of the focal plane, reducing the refractive error. Studies that either cut the optic nerve (Troilo et al., 1987; Raviola & Wiesel, 1985) or suppressed retinal output (Norton et al., 1994) have found that there is a direct pathway within the eye: emmetropization signals originate in the retina, pass into the retinal pigment epithelium (RPE) and then into the choroid, finally reaching the sclera. In tree shrews (mammals closely related to primates), stimulation of this pathway produces remodeling of the scleral extracellular matrix that alters its biomechanical properties, increasing the viscoelasticity and the axial elongation rate. We will refer to this as the "direct emmetropization pathway" because it can operate, albeit less well, in the absence of an "indirect" pathway comprised of connections from the retina, through central visual structures, that controls accommodation and other potential outputs to the eye and can affect refractive development (Dillingham, Guggenheim, & Erichsen, 2013; Wildsoet, 2003; Schaeffel et al., 1990; McFadden & Wildsoet, 2009).

Minus lens (ML) wear and form deprivation (FD) are two treatments often used to stimulate the emmetropization mechanism (Wallman & Winawer, 2004). Wearing a minus lens, held in place in front of the eye in a goggle frame, shifts the focal plane away from the cornea, creating an artificially hyperopic refractive state. This produces what has

been described as a "GO" condition (Rohrer, Negishi, Tao, & Stell, 1993; Schaeffel & Howland, 1988). In response, the lens-wearing eye increases its axial elongation rate, moving the retina to the shifted focal plane at which point the hyperopia is eliminated and the GO condition has dissipated. When the minus lens is removed, the treated eye is myopic. Form deprivation with a translucent diffuser provides ample retinal illuminance but removes the possibility that sharply-focused images can occur on the retina. This also is a GO condition that causes an increase in the axial elongation rate and myopia in the treated eye. However, because elongation cannot restore clear retinal images, the GO condition continues and the elongation rate remains elevated throughout the treatment period.

A third procedure, treatment with a period of continuous darkness (DK), also produces increased axial elongation in tree shrews and in chicks. Tree shrews that were first raised in standard colony lighting with light-on and light-off periods, and then transferred to a completely dark environment, develop an increased axial elongation rate and become myopic compared with age-matched normally-raised animals (Norton et al., 2006). Chicks placed in DK also exhibit increased axial elongation (Troilo & Wallman, 1991). However, prolonged DK treatment also produces flattening of the cornea so that the birds eventually become refractively hyperopic despite having elongated eyes (Lauber, 1991). The retinal mechanism by which darkness produces a GO condition is still unclear.

Based on behavioral and electrophysiological studies, ML and FD treatments produce different, distinct patterns of excitation and inhibition in the retina that are communicated through the geniculostriate visual pathway to produce differing visual responses. Several studies have suggested that the retinal emmetropization-related signaling produced by these two GO conditions can be distinguished (Kee, Marzani, & Wallman, 2001;

Schaeffel, Hagel, Bartmann, Kohler, & Zrenner, 1994; Bitzer, Feldkaemper, & Schaeffel, 2000; Bartmann, Schaeffel, Hagel, & Zrenner, 1994; Fujikado, Kawasaki, Suzuki, Ohmi, & Tano, 1997; Yew & Wildsoet, 2003; Wildsoet, 2003). However, in the sclera, it has been found that ML and FD produce nearly identical gene expression signatures; DK treatment also produces a similar gene expression signature (Guo et al., 2013). It appears that the different retinal activity produced by these three myopiagenic conditions may be converted into a common set of emmetropization signals as it passes through the direct RPE-choroid-sclera emmetropization pathway. Has this consolidation into a common signal occurred at the level of the choroid, or does the choroidal "compartment" of the emmetropization pathway still distinguish amongst the visual conditions that produce a retinally-generated GO condition?

Although changes in levels of proteins or other molecules presumably are key to actually transmitting signals from choroid to sclera, it has been found that changes in mRNA levels can identify the responses of the cells in tissues and are useful in identifying pathways of interest (Gao et al., 2011; Siegwart, Jr. & Norton, 2005; Guo et al., 2013; Schippert, Brand, Schaeffel, & Feldkaemper, 2006; Shelton et al., 2008; Stone et al., 2011; Zhang et al., 2012). In a previous paper in tree shrew choroid (He et al., 2014), we examined the gene expression signatures produced by ML wear (GO) and by recovery from induced myopia (a STOP condition). Short-term ML treatment produced a GO gene expression signature that was distinct from the STOP gene expression signature. These results, involving altered gene expression in many genes, have shown that emmetropization-related signaling can be detected in the choroidal compartment of the direct emmetropization pathway. The goal of the present study was to examine alterations in gene ex-

pression in the choroid after two days of ML wear, two days of FD, and after eleven days of DK treatment. The question asked was whether the three GO conditions would produce the same, or very similar, gene expression signatures?

MATERIALS AND METHODS

Experimental Groups

The methods employed in this study were generally identical to those in our previous paper (He et al., 2014). The juvenile tree shrews (*Tupaia glis belangeri*) used in this study were produced in our breeding colony and raised by their mothers on a 14 h light/10 h dark cycle. Tree shrew pups open their eyes about three weeks after birth. The first day both eyes are open is day one of visual experience (DVE). 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 were balanced to include both males and females, and avoided pups from the same parents wherever possible.

There were four groups of animals (n = 7 per group) (Fig. 1). Starting at 24 ± 1 DVE, the ML group wore a monocular –5 D (spherical power) lens for 2 days; the FD group wore a monocular translucent diffuser for 2 days; the DK group was housed in continuous darkness for 11 days, starting at 17 ± 1 DVE. In the ML and FD groups, the untreated fellow eye served as a control. A normal group (26N) was also examined at 26 DVE. Data from the ML and 26N groups were reported in the previous study (He et al., 2014) and are shown here for direct comparison with the FD and DK group results.

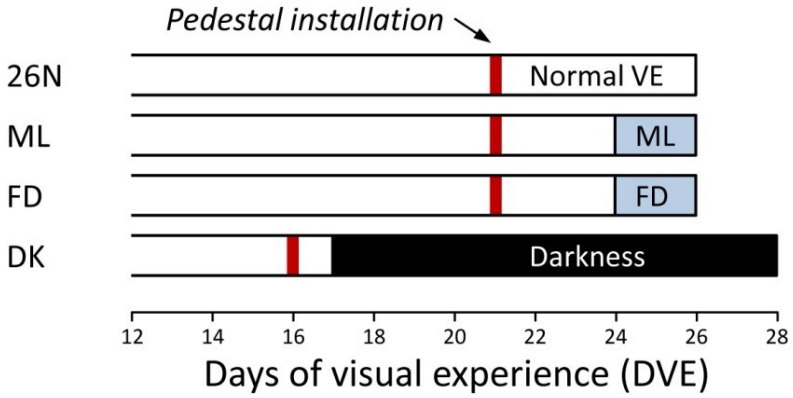

Figure 1. Experimental groups and duration of treatments. The red vertical bar indicates the point when a dental acrylic pedestal was installed under anesthesia. Filled regions

Visual Treatments

Animals in all groups were anesthetized (17.5 mg ketamine, 1.2 mg xylazine; supplemented with 0.5 - 2.0% isoflurane as needed) and received a dental acrylic pedestal. For the ML and FD groups, this occurred at 21 ± 1 DVE; in the DK group, the pedestal was installed at 16 ± 1 DVE. After pedestal installation, all animals were placed in individual cages with standard colony fluorescent lighting (GE F34CW WM ECO cool white or F32T8/25W/SPX41/ECO), 100 - 300 lux on the floor of the cage. In the ML and FD groups, three days after pedestal installation, a goggle frame holding a –5 D lens (12 mm diameter PMMA contact lens; Conforma Contact Lenses, Norfolk, VA) or a translucent diffuser was clipped to the pedestal, firmly holding the lens or diffuser in front of the randomly selected treated eye. The untreated fellow control eye had unrestricted vision through the open goggle frame. Lenses were cleaned twice daily (approximately 9:30

AM and 4:30 PM) while diffusers were cleaned only in the morning. During cleaning, goggles were briefly (< 3 min) removed under dim illumination and animals were kept in a darkened nest box to minimize exposure to visual stimuli. Animals in the DK group were transferred to continuous darkness one day after pedestal installation (at 17 ± 1 DVE) and checked daily with night-vision goggles and infrared illumination; DK treatment ended after 11 days. The 26N group received a pedestal at 21 ± 1 DVE but did not wear a goggle.

Refractive and Axial Measures

Non-cycloplegic refractive measures were made, in awake animals, at the start and end of the treatment period with a Nidek ARK-700A infrared autorefractor (Marco Ophthalmic, Jacksonville, FL). Normal animals were measured just before euthanasia. Cycloplegic refractive measures were omitted to prevent any interference by atropine on retino-scleral signaling (McKanna & Casagrande, 1981). However, previous studies have shown that non-cycloplegic measures provide a valid estimate of the refractive state, and of induced myopia, in tree shrews (Norton, Siegwart, German, Robertson, & Wu, 2000; Norton, Siegwart, Jr., & Amedo, 2006). All refractive values were corrected to the corneal plane and for the small eye artifact (Glickstein & Millodot, 1970), previously shown to be approximately +4 D in tree shrews (Norton et al., 2003).

At the time the pedestal was attached, ocular component dimensions were measured in most of the animals with a Lenstar LS-900 optical biometer (Haag-Streit USA, Mason, OH) to ensure that the two eyes did not differ significantly in axial component dimensions before treatment began. Post-treatment axial component measures were also

taken, except for one DK animal. The Lenstar optical biometer allowed these measures to be made quickly, in awake animals, just before euthanasia.

Choroid Dissection

On completion of the final refractive measures, approximately 2 - 4 hours into the light phase, animals were terminally anesthetized (17.5 mg ketamine and 1.2 mg xylazine, followed by 50 mg xylazine). DK animals were euthanized at similar times. Both eyes were enucleated and placed into RNAlater solution (Life Technologies, Carlsbad, CA). Extraocular muscles, conjunctiva, and orbital fat were trimmed from the exterior surface of the eye and the cornea dissected away just behind the corneoscleral junction. While viewing through a surgical microscope, the lens and vitreous humor were removed; the retina and RPE, which were tightly bound to each other, were then lifted from the eyecup. While still immersed in RNAlater, choroid was teased from the scleral inner surface using the rounded ends of forceps, collected, and then frozen in liquid nitrogen. Because the dissection was extremely gentle, it is possible that small portions of the *lamina fusca*, the outermost layer of the choroid, may have adhered to the sclera in some cases and, thus, not been included in our choroidal sample. Because the retina/RPE separated cleanly from the choroid without dissection, and because the inner surface of the sclera was not disrupted by forceful scraping of the surface (and most scleral fibroblasts are within the layered matrix, not on the inner surface), there is no reason to expect significant contamination of the choroidal samples from either retina/RPE or sclera. As a further precaution, RPE65 mRNA expression was measured in a group of one-day ML animals for both the choroid and RPE, collected, as above, from the same eye. Whilst RPE65 mRNA abun-

dance was approximately 100 fold greater in RPE than in choroid, its differential (treated vs. control eye) expression was not significantly altered in RPE but was significantly up-regulated in the treated eyes of the choroid sample (He et al., 2014). Thus, the RPE cannot be the source of the mRNA for RPE65 in the choroid. We think it is therefore reasonable to conclude that there was no significant contamination of our choroid sample with RPE.

Gene Expression Analysis

Each frozen choroid was homogenized with a disposable pestle (Fisher Scientific, Pittsburgh, PA) from which total RNA was isolated using a RiboPure kit (Life Technologies) according to the manufacturer's instructions, with the addition of an on-filter DNase treatment. The purified RNA was quantified (NanoDrop Technologies, Wilmington, DE), with an average yield per choroid of 4.5 ± 1.2 μg (mean ± SD). RNA quality was confirmed by denaturing gel electrophoresis (RNA FlashGel; Lonza, Rockland, ME). cDNA was synthesized from 1 μg of total RNA in a final reaction volume of 20 μl using a Superscript III RT kit (Life Technologies) with minor modifications (2.5 μM anchored oligo (dT)20 primers and DTT omitted). The resultant cDNA was diluted 5-fold and stored at –20°C until use.

Tree shrew-specific quantitative PCR (qPCR) primers were designed for 77 genes of interest (Table 1) and the reference gene RNA polymerase II (POLR2A) using Beacon Designer v7.7 (Premier Biosoft International, Palo Alto, CA). None of the treatment conditions affected the expression of the reference gene. Primer sequences, amplicon size, and efficiencies are listed in Supplementary Table S1. The selected candidate genes in-

cluded representatives of three major groupings: signaling, metallopeptidases & TIMPs, and extracellular matrix (ECM) proteins. They were selected from genes that were found to change in preliminary studies of tree shrew choroid during ML along with additional genes that were suggested by a whole-transcriptome analysis of three of the ML animals. All primers were designed to work under the same cycling conditions. All amplicons were located within the coding region and most spanned at least one intron; amplicon identity was verified by gel electrophoresis and sequencing.

Relative gene expression was measured by qPCR on a StepOnePlus Real-Time PCR System using Power SYBR Green PCR Master Mix (both, Life Technologies). Reactions were performed in triplicate in a 15 µl volume containing 300 nM each primer and 0.4 µl cDNA template. Cycling parameters were the same for all assays: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 62°C for 60 sec. Single gene products were obtained for all reactions as assessed by melt curve analysis. Relative gene expression was calculated using the $^{\Delta\Delta}$Ct method (Livak & Schmittgen, 2001) to first normalize the expression level of the target gene to that of the reference gene, and then to compare the relative expression of the target gene for treated vs. control eyes, treated vs. normal eyes, and control vs. normal eyes. For DK animals the average of the right and left eyes was compared with normal eyes, and with the control eyes of the ML and FD groups. The geometric group mean (for the 7 biological replicates) of these expression ratios was used to calculate the fold change in gene expression for each of the target genes.

Gene symbol	Protein name	Location	UniProt ID
Signaling – Cell surface			
ADORA2A	Adenosine receptor A2a	Cell surface	P29274
AQP4	Aquaporin 4	Cell surface	P55087
CHRNA7	Cholinergic receptor, nicotinic α7	Cell surface	P36544
DRD2	Dopamine receptor D2	Cell surface	P14416
EPHA1	EPH receptor A1	Cell surface	P21709
FGFR1	FGF receptor 1	Cell surface	P11362
GFRA1	GDNF family receptor α1	Cell surface	P56159
GRM5	Metabotropic glutamate receptor 5	Cell surface	P41594
IGF2R	Insulin-like growth factor 2 receptor	Cell surface	P11717
INSR	Insulin receptor	Cell surface	P06213
OPN1LW	Opsin 1, long-wave-sensitive	Cell surface	P04000
P2RY1	Purinergic receptor P2Y, G-protein coupled, 1	Cell surface	P47900
SCUBE3	Signal peptide, CUB and EGF-like domain-containing protein 3	Cell surface	Q8IX30
TNMD	Tenomodulin	Cell surface	Q9H2S6
VIPR1	VIP receptor 1	Cell surface	P32241
VIPR2	VIP receptor 2	Cell surface	P41587
Signaling – Intracellular			
BCO2	Beta-carotene oxygenase 2	Intracellular	Q9BYV7
CABP5	Calcium binding protein 5	Intracellular	Q9NP86
CAMP	Cathelicidin antimicrobial peptide	Intracellular	P49913
CDC42	Cell division cycle 42	Intracellular	P60953
CHAT	Choline O-acetyltransferase	Intracellular	P28329
CYP26B1	Cytochrome P450 26B1	Intracellular	Q9NR63
NOS1	Nitric oxide synthase 1	Intracellular	P29475
RASGRF1	Ras-specific guanine nucleotide-releasing factor 1	Intracellular	Q13972
RLBP1	Retinaldehyde binding protein 1	Intracellular	P12271
RPE65	Retinoid isomerohydrolase	Intracellular	Q16518
S100A12	Protein S100-A12	Intracellular	P80511
ZNF185	Zinc finger protein 185	Intracellular	O15231
Signaling – Transcription regulators			
EGR1	Early growth response protein 1	Intracellular	P18146
HIF1A	Hypoxia-inducible factor 1α	Intracellular	Q16665
PER2	Period circadian clock 2	Intracellular	O15055
RXRB	Retinoid X receptor β	Intracellular	P28702
VDR	Vitamin D receptor	Intracellular	P11473
Signaling – Secreted			
ANGPTL7	Angiopoietin-related protein 7	Extracellular	O43827
APOE	Apolipoprotein E	Extracellular	P02649
BMP2	Bone morphogenetic protein 2	Extracellular	P12643
BMP4	Bone morphogenetic protein 4	Extracellular	P12644
CILP	Cartilage intermediate layer protein 1	Extracellular	O75339
EGF	Epidermal growth factor	Extracellular	P01133
FAM180A	Family with sequence similarity 180, member A	Extracellular	Q6UWF9
IGF2	Insulin-like growth factor 2	Extracellular	P01344
IL1B	Interleukin 1β	Extracellular	P01584
LTBP1	Latent TGFβ binding protein 1	Extracellular	Q14766
LTF	Lactotransferrin	Extracellular	P02788
MEST	Mesoderm specific transcript	Extracellular	Q5EB52
NRG1	Neuregulin 1	Extracellular	Q02297
NTS	Neurotensin	Extracellular	P30990
PENK	Proenkephalin A	Extracellular	P01210
PI15	Peptidase inhibitor 15	Extracellular	O43692
PTX3	Pentraxin 3	Extracellular	P26022
SOSTDC1	Sclerostin domain-containing protein 1	Extracellular	Q6X4U4
SST	Somatostatin	Extracellular	P61278
TAC1	Protachykinin 1	Extracellular	P20366
TGFB2	Transforming growth factor β2	Extracellular	P61812
TGFB3	Transforming growth factor β3	Extracellular	P10600
TGFBI	TGFβ-induced protein	Extracellular	Q15582
VIP	Vasoactive intestinal peptide	Extracellular	P01282
Signaling – Matricellular			
CYR61	Protein CYR61	Extracellular	O00622
NOV	Nephroblastoma overexpressed gene	Extracellular	P48745
THBS1	Thrombospondin 1	Extracellular	P07996
THBS2	Thrombospondin 2	Extracellular	P35442
TNC	Tenascin C	Extracellular	P24821
MP / TIMP			
ADAMTS4	ADAM metallopeptidase with thrombospondin motif, 4	Extracellular	O75173
ADAMTS5	ADAM metallopeptidase with thrombospondin motif, 5	Extracellular	Q9UNA0
ADAMTSL3	ADAMTS-like 3	Extracellular	P82987
MMP14	Matrix metallopeptidase 14	Cell surface	P50281
TIMP2	TIMP metallopeptidase inhibitor 2	Extracellular	P16035
TIMP3	TIMP metallopeptidase inhibitor 3	Extracellular	P35625
Extracellular matrix			
COL12A1	Collagen type XII, α1	Extracellular	Q99715
COL6A6	Collagen type VI, α6	Extracellular	A6NMZ7
DCN	Decorin	Extracellular	P07585
FMOD	Fibromodulin	Extracellular	Q06828
MXRA5	Matrix remodeling associated protein 5	Extracellular	Q9NR99
NYX	Nyctalopin	Extracellular	Q9GZU5
OGN	Mimecan	Extracellular	P20774
PRELP	Prolargin	Extracellular	P51888
SERPINH1	Serpin H1	Intracellular	P50454

Table 1. Genes examined by functional category, with cellular location of the protein encoded by the gene and its UniProt accession ID. The data shown in this table are re-produced from (He et al., 2014)

Gene	Forward sequence	Reverse sequence	Amplicon (bp)	Efficiency (%)
ADAMTS4	CTACAACCATCGCACGGA	GCCTGGCAGGTGAGTT	108	88
ADAMTS5	TCTTCCATCCTAACCAGCATTG	GGTGGCATCATAAGTCTGTCC	165	98
ADAMTSL3	CCAAGGACCCAAGGCATCGT	TCTGACACACCTGCTTTCTTCTCTG	134	96
ADORA2A	CGTGGTCCCTATGAACTAC	CCGCCAGGAAGATGC	101	100
ANGPTL7	GCTGTCACTTGGCTCTG	AGTCCTTCTCCTGCTTCTTG	193	94
APOE	GGTGCAGACGCTGTCTGACCA	CCTCCAACTCCGCCTTGTAGG	122	90
AQP4	CGACATGGTTCTCATCTCCCTTTG	GAAGACAGACTTGGCGATGCT	148	94
BCO2	GCCCATTGTCCTATTCTTCAGCCAGT	TGCCGAAAGCCACAGCCATAGA	181	95
BMP2	GGACACCAGGTTAGTGAATCAGAACA	TGCTTCTCTTCCAAATGAGCCACT	135	97
BMP4	AAAGTCGCCGAGATTC	ATTGTGCTCTTGCTAGG	134	86
CABP5	AAATGCGAGATGCCTTCAAAGA	CTGCCTCCTGGACAACCT	138	96
CAMP	GAGGATGAGGACCCAGAC	TCTCCAACCCGTTCTTCTT	118	98
CDC42	CCCGTGACCTGAAGGCTGTC	ACACACCTGCGACTCTTCTTGGG	130	98
CHAT	AGGCACTGACTTTCGTGAGAG	CGGTCTGGGCTCGGATG	99	96
CHRNA7	GTACTTCGCCAGCATCAT	ACACCAGTTCAGCAGGA	133	95
CILP	GCCTTCTGTGACGACCAGTC	GTAGGGCTGAGGGACACCAATA	132	97
COL12A1	CAGGTGTCCCGATCAAAGAG	GCCTGGTGGAATGGTGG	95	97
COL6A6	ATCAGCCACATCAATCAAGAG	GATGAGGATGAGGAACTTCTT	195	94
CYP26B1	GCACTACCTGGACTGTGTCA	CGCTGTGTCGTGGGTATCTC	157	95
CYR61	TGGAACCTCGCATTCTGTATAACC	GCCACAAGGTCGCACTTCAC	176	97
DCN	CTGCGTGCCCATGAGAACGAGATC	GGTATCAGCAATGCGGATGTACGAAAGC	168	96
DRD2	CACGCACATCCTGAACATACA	GTTGAAGGTGGTGTAGATGATGG	118	91
EGF	GGAGACGCCAGATAGAATAACG	GACGATGTGCTCACCGAA	193	95
EGR1	TCCCTGACTACCTGTTTCC	AGTGGAGTGAGCGAAGG	112	90
EPHA1	GCCTCCTGGACAACTTC	CACATCACAATCCCAAAGC	136	94
FAM180A	AGACATTCGTCGCCTGAGT	CTTCTGATACCCGTGGGACAA	130	94
FGFR1	CCTGGAGGTCATCATCTACTGC	AGAGTTCATGGAAGCACTGGAG	196	99
FMOD	TCCTGCTGGACCTGAG	TTGTTGGTGAGGCTGTTAT	172	96
GFRA1	CGGCAAGGAGACCAACTT	TTGTAGAGCGACTTCTGCTTC	96	96
GRM5	GCATCGCCCACTCTTACAAA	CCATCAGCAGACCTCTCACT	144	93
HIF1A	ACTGGTTGCATCTCCGTCTCCTACTCA	TCCTGCTCTGTTTGGTGAGGCTGTC	109	94
IGF2	TGGCATTGTTGAAGAGTGTTG	GAAGCACGGTCGGAGAG	113	100
IGF2R	GTACTCTGACGGAGACCTCACC	TGATTGCACTCAAAGTTTATGACACTCA	96	102
IL1B	CAAGGAAGAAGATGGAGAAGCGATT	GGCAGGTAGCTTGGTGAAGTC	182	97
INSR	AACGAGGCGTCGGTCATG	ATCATCTCTTGCAGGGTAGGG	194	92
LTBP1	GGACGAGTGCCTGGAACC	TGCTTGTGGTCTGGAGTTGG	111	103
LTF	GGCTCCAAGTCACGCTGT	GGGCAGTCAACACCGTTTCTT	105	97
MEST	CAAAGATGGAGGTGTGCTGTC	ACTGTAAGAGACTGTCGATGACTAAG	179	94
MMP14	CCCTGGAACCTGGCTACCC	ATAGGTCTTTCCATTGGGCATCC	104	96
MXRA5	GGAAGGAAGGAGTGGATG	GAGGCTGGAAGGTTACA	195	93
NOS1	ACATCACCACGCCACCAA	CCTCCAACACCTCCACGAT	159	94
NOV	CGAACAGACTACAGAGTGGAGTG	GTGATTTCTTGGTGCGGAGACA	191	97
NRG1	CGTGTCCAAGAATGTCATCTCC	TGTGTGCCCATTGCTCCA	145	97
NTS	AATGTGAACAGCCAACCCGAG	AACTCCCAGTGTTGAAAGATCCTG	164	95
NYX	GTGAGCAGGTTCAGCAG	GACAGCGAGGTGTTAGC	101	96
OGN	CTCTCCTTCCTCTACCTGGACCACAAC	CTGAAGATGGATCACGCGCAGACTC	84	96
OPN1LW	ATGGTCACCTGCTGTATC	GGTGGAAGGCGTAGC	232	98
P2RY1	ACGGCAGCATCTTGTTC	GGAGATCGCTACCACCA	146	97
PENK	TCCTTGCCAAGCGATACG	TCTTGCTCATTTCTTCGTCGTT	162	98
PER2	ACGACAACACATACACCGAAT	GCACAGGCGTCTGGATC	100	92
PI15	CCACTGACTCATCCCCGCCAACC	GTTTGCTGCTGGTGGGAACACTTTGC	176	96
PRELP	TTCGGCTGAACTACAACAAGC	GATCTGGGTCCCGTTGATTTTCT	182	99
PTX3	CTGTGTGGGCGGTGGCTTTG	TCCATGTGGCTGAATCTCTGTGACTC	175	94
RASGRF1	GACTCTGACCCAGGAGGA	AGCCTTGTCCGAAGAACTC	185	93
RLBP1	CGTGCCAGGAAGTTCAA	TGAAGAGCATGACCACTC	184	93
RPE65	CACCCAGATGCCTTGGAAGAAGATGA	CTTCACTCAAGTCCTTGGCGTTCAGA	115	99
RXRB	CCTGACCTACTCGTGCC	TACCGCCTCCCTCTTCA	118	91
S100A12	GAGCAAGATGGAAGATCACATGGAGG	TGAGGGTGTTTGGAAGTTCCTTTGTT	134	100
SCUBE3	CCGTCCTCCATTACCACTTAC	CTCGCACGATGTCTTCTACCA	184	95
SERPINH1	ACAAGAACAAGGCAGAC	GCACCAGGAAGATGAAG	186	103
SOSTDC1	GCGGTCCACCAAGTACATCT	ATAGCCTCCTCCGATCCAGTTA	121	97
SST	CCAGACTCCGTCAGTTTCT	CCAGGGCATCGTTCTCA	120	93
TAC1	CGGAGCCCTTCGAGCATCTTCTG	CCCGTTTGCCCATTAATCCAAAGAATTGC	81	96
TGFB2	GCAGAGTTTAGGGTCTTTCGTTTG	CTCGTGAACAGCATCAGTTACATC	189	93
TGFB3	ATCACCATAACCCGCATCTAATCC	CGCACACAGCAGTTCTCCTC	139	90
TGFBI	CCTCGGCACTCATCTCTCC	GCAAATTCTTCATCTTGGCATCG	107	94
THBS1	CTGTCAGAACTCAGTCACCATC	CCACGGAGACCAGCCATC	136	91
THBS2	GAGACCGACTTCAGGAACTTC	CGAAACCCACTGCGATGC	142	100
TIMP2	AGATGGGCTGTGAATGC	CCGTCGCTTCTCTTGAT	151	96
TIMP3	CCGTGTCTATGATGGCAAGATG	ACAAAGCAAGGCAGGTAATAGC	153	99
TNC	AGACGCCAAGACTCGCTACAG	CAGGTTGACACGGTGACAGTTC	184	94
TNMD	GCTGGATGAGAGAGGTTATTGT	TGATGACACGACAGATGACC	137	100
VDR	AACTTGCACGAGGAGGAAC	CTTCTGGATCATCTTGGCATAG	189	93
VIP	AACGAGTGAGCACCAACATCTC	TCTTGACAGCCATTTGTTTCCTAAGG	108	94
VIPR1	TCCACGCTGCTGCTGATTC	CCCACGACCAGCTCAAAGAC	107	99
VIPR2	CCACGCACACCTCCTC	CACTTCCTCTTCAGCTCACT	127	90
ZNF185	GGAGGCGTCTGTACCTACTG	GCAAATCCCACACCTAAAGCAAT	108	97
POLR2A	CTACCAGCCCCAAGTATTC	GGTGAGTAAGTAGGAGACG	106	98

Table S1. Primers used: Sequences, amplicon sizes, and efficiencies. The data shown in this table are reproduced from (He, Frost, Siegwart, Jr., & Norton, 2014)

Statistical Analysis

One-way analysis of variance (ANOVA; Statistica, Statsoft, Tulsa, OK) was used to compare control and normal eye refractive data across groups of animals; paired t-tests were used to determine if significant myopia (treated eye vs. control eye, DK vs. normal) had developed. For gene expression data, paired t-tests were used to assess treated eye vs. control eye differences; unpaired t-tests were used to test for gene expression differences between all independent groups. For both t-tests and ANOVA, $p < 0.05$ was considered significant and no adjustment for possible false discovery rate was applied. Linear regressions between expression differences were made in SigmaPlot (Systat Software, San Jose, CA).

RESULTS

Refraction

The final refractive values of the normal, treated, and control eyes in the four groups are shown in Fig. 2. As expected in tree shrews at 26 DVE, both eyes of the normal group were slightly hyperopic (right eyes, 0.8 ± 0.4 D; left eyes, 1.3 ± 0.4 D; mean ± SEM). After two days of treatment, the ML treated eyes showed a small, statistically-significant myopic shift; the treated eyes were −1.0 ± 0.2 D myopic in comparison to the control eyes. After 2 days of form deprivation, the treated eyes in the FD group also were significantly myopic (−1.9 ± 0.2 D) relative to control eyes. After 11 days of dark treatment, the DK group exhibited a statistically-significant myopic shift (−4.4 ± 1.0 D) compared with the 26N group (both groups, mean of right and left eyes). The control eyes in the ML and FD groups did not differ significantly from the 26 DVE normal eyes (one-way ANOVA, $p = 0.79$).

Ocular component dimensions, measured with the Lenstar, confirmed that the vitreous chamber of the treated eyes had elongated, relative to the control eyes. In the ML group, the vitreous chamber of the treated eyes (measured to the front of the retina) was 0.016 ± 0.004 mm (mean ± SEM) larger than in the control eyes. In the FD group, the treated eye vitreous chamber was 0.038 ± 0.011 mm longer than in the control eyes. The vitreous chamber in the DK group (2.94 ± 0.03 mm) was significantly larger than the vitreous in the 26N group (2.86 ± 0.02 mm). It also was significantly longer than the vitreous chamber in the control eyes of the ML (2.83 ± 0.02 mm) and FD (2.86 ± 0.02 mm) groups. Corneal thickness, anterior chamber depth, and lens thickness did not differ between treated vs. control eyes or between the 26N and DK groups. The choroid was

slightly thinner in the treated eyes of the ML group (0.60 ± 0.005 μm vs. 0.65 ± 0.005 μm) and FD group (0.58 ± 0.002 μm vs. 0.062 ± 0.003 μm) but the differences were not statistically significant. Thus, the refractive myopia was primarily the result of a larger vitreous chamber depth.

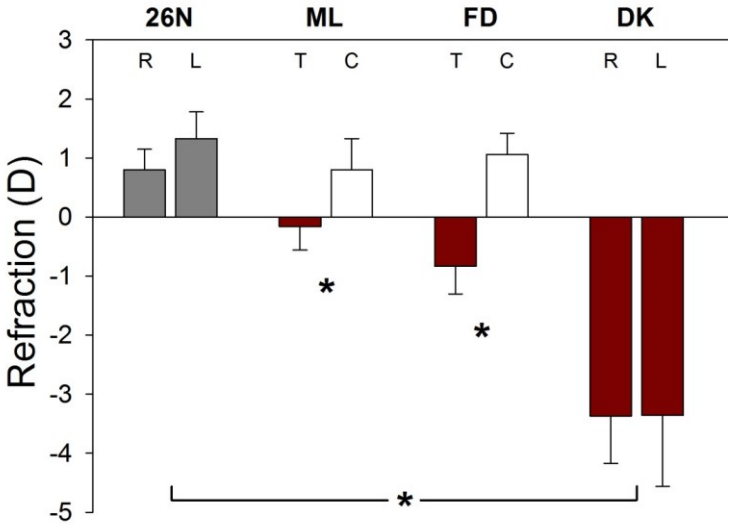

Figure 2. End-of-treatment refractive measures for the normal, ML, FD, and DK groups. Values are the mean refraction ± SEM for the right (R) and left (L) eyes of the 26N and DK groups or for the treated (T) and control (C) eyes of the ML and FD groups. ML and FD treated eyes were significantly different relative to control eyes; the DK eyes were

Gene Expression

Normal and DK Groups

The mRNA levels in normal eyes, and the variability between normal left and right eyes, can provide a basis for comparison with the levels and variability found in treated and control eyes. Fig. 3A compares gene expression in the left and right eyes of the 26N group reported previously (He et al., 2014). Expression values for all 77 genes

are provided in Table 2. Expression levels did not differ significantly between left and right eyes for any of the 77 genes. Fig. 3B compares gene expression in the left and right eyes of the DK group. There were no significant differences between left and right eye mRNA levels for any of the 77 genes. Thus, the differences found in the treatment groups can be attributed to the experimental conditions.

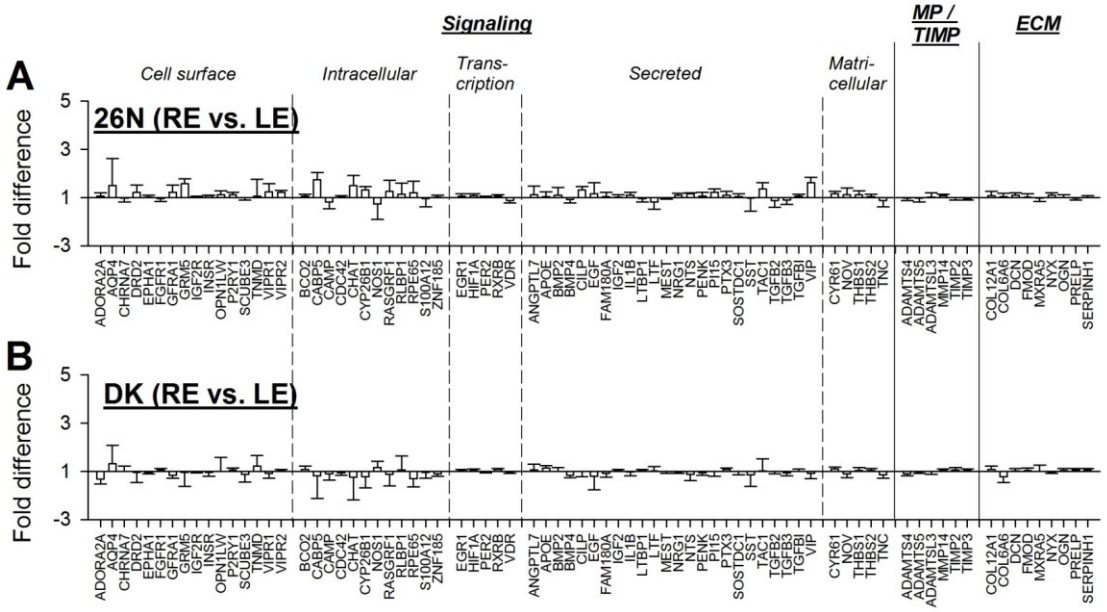

Figure 3. Gene expression fold differences between right and left eyes for the (A) 26N group and (B) DK group. Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Error bars = SEM. No gene showed significant regulation in either group. The data shown in A are reproduced from (He et al., 2014).

	26N	ML			FD			DK			DK
	RE vs. LE	*T vs. C*	*T vs. N*	*C vs. N*	*T vs. C*	*T vs. N*	*C vs. N*	*T vs. MLC*	*T vs. FDC*	*T vs. 26N*	*RE vs. LE*
Signaling – Cell surface											
ADORA2A	1.06	-1.24	-1.88	-1.52	-1.01	1.57	1.58	1.37	-1.74	-1.10	-1.32
AQP4	1.50	1.46	1.65	1.12	1.38	-1.24	-1.72	-1.74	1.11	-1.55	1.31
CHRNA7	-1.03	1.15	1.42	1.23	1.02	1.19	1.17	1.57	1.50	1.94	1.00
DRD2	1.21	1.68	1.70	1.01	1.07	1.00	-1.06	-1.18	-1.10	-1.17	-1.04
EPHA1	1.01	**-1.55**	**-1.75**	-1.13	**-1.85**	**-1.81**	1.02	-1.84	**-2.13**	**-2.09**	-1.05
FGFR1	-1.05	-1.68	-1.87	-1.11	-1.14	**-1.43**	-1.25	-1.47	-1.30	-1.63	1.05
GFRA1	1.22	-1.12	-1.18	-1.05	1.05	-1.00	-1.06	-1.44	-1.44	-1.52	-1.17
GRM5	1.57	1.34	1.50	1.12	-1.36	1.34	1.83	-1.26	-2.06	-1.13	-1.05
IGF2R	1.01	-1.16	1.01	1.17	1.03	1.34	1.31	-1.07	-1.19	1.10	-1.01
INSR	1.07	-1.49	-1.76	-1.18	1.10	1.21	1.10	-1.24	-1.45	-1.46	-1.05
OPN1LW	1.12	1.13	1.07	-1.06	1.80	1.00	-1.79	-1.59	1.06	-1.69	1.00
P2RY1	1.12	**1.29**	-1.24	-1.60	**1.31**	1.24	-1.06	1.24	-1.22	-1.29	1.06
SCUBE3	-1.00	**-1.62**	-1.28	1.27	**-1.70**	-1.13	1.50	**-2.53**	**-3.00**	-2.00	-1.13
TNMD	1.06	-1.27	-2.23	-1.76	-1.69	-2.59	-1.53	-1.06	-1.22	-1.86	1.22
VIPR1	1.24	1.11	1.03	-1.07	1.37	-1.60	-2.19	-1.03	1.98	-1.11	-1.08
VIPR2	1.22	-1.55	-1.63	-1.05	**-1.23**	-1.26	-1.02	1.06	1.03	1.01	1.02
Signaling – Intracellular											
BCO2	1.06	**-1.48**	-1.33	1.11	**-1.60**	-1.10	1.46	-1.37	-1.79	-1.23	1.08
CABP5	1.74	2.14	2.14	1.00	-1.27	-1.38	-1.09	-1.15	-1.05	-1.15	-1.19
CAMP	-1.19	2.25	4.00	1.78	-1.39	1.63	2.27	1.05	-1.22	1.86	-1.11
CDC42	1.02	-1.25	-1.46	-1.16	-1.05	1.02	1.07	1.18	-1.05	1.02	-1.07
CHAT	1.49	1.22	1.70	1.39	-1.36	1.36	1.84	-1.33	-1.76	1.05	-1.24
CYP26B1	1.31	**-4.05**	**-3.28**	1.24	**-2.32**	**-2.16**	1.07	**-5.34**	**-4.64**	**-4.32**	-1.22
NOS1	-1.27	1.53	-1.62	-2.46	1.25	-2.44	**-3.07**	**-2.19**	-1.76	**-5.39**	1.16
RASGRF1	1.26	1.06	1.43	1.34	-1.29	1.57	2.03	1.29	-1.17	1.73	-1.13
RLBP1	1.14	**2.76** x	-1.22	**-3.37**	**4.10** x	**-2.80**	**-11.47**	-1.51	2.26	**-5.08**	1.06
RPE65	1.20	**2.28**	1.59	-1.43	**4.80** x	1.56	**-3.08**	1.40	**3.02**	-1.02	-1.31
S100A12	-1.04	1.62	2.34	1.45	-1.50	1.60	2.40	-1.39	-2.31	1.04	-1.05
ZNF185	1.01	**-1.35**	**-1.62**	-1.20	**-1.36**	-1.22	1.12	-1.75	**-2.35**	**-2.10**	-1.11
Signaling – Transcription											
EGR1	1.05	-1.33	-1.42	-1.07	**-1.31**	-1.53	-1.17	1.23	1.33	1.14	1.04
HIF1A	1.07	**1.20**	1.07	-1.12	**1.11**	1.09	-1.02	1.22	1.12	1.09	1.03
PER2	1.02	-1.35	-1.36	-1.01	-1.06	1.27	1.35	-1.52	**-2.09**	**-1.54**	-1.00
RXRB	1.05	-1.22	-1.28	-1.05	1.01	1.13	1.11	1.17	-1.13	-1.01	1.07
VDR	-1.12	-1.69	-1.86	-1.10	-1.03	-1.12	-1.09	-1.27	-1.28	-1.40	-1.03
Signaling – Secreted											
ANGPTL7	1.11	1.27	1.17	-1.09	1.49	1.16	-1.29	1.44	1.70	1.32	1.06
APOE	1.04	1.49	**2.83**	**1.90**	1.20	-1.74	**-2.09**	-1.18	**3.36**	1.61	1.13
BMP2	1.09	**1.54**	1.18	-1.30	**1.91** x	1.13	**-1.70**	1.40	**1.83**	1.08	1.00
BMP4	-1.08	**1.48**	1.48	1.00	**1.58**	**2.02**	1.28	**3.41**	**2.68**	**3.42**	-1.16
CILP	1.31	**-3.00**	**-2.52**	1.19	**-3.03**	**-2.11**	1.43	-2.39	-2.88	-2.01	-1.01
EGF	1.16	2.09	1.38	-1.51	**4.13**	1.49	-2.77	**-8.47**	**-4.62**	**-12.79**	-1.21
FAM180A	1.03	**-1.60**	-1.58	1.01	**-1.49**	-1.34	1.11	-1.78	-1.96	-1.76	-1.08
IGF2	1.01	**1.24**	1.44	1.16	**1.32**	1.41	1.07	1.14	1.23	1.32	1.02
IL1B	1.10	1.05	1.16	1.11	1.09	1.40	1.28	1.43	1.24	1.58	-1.03
LTBP1	-1.05	1.03	-1.15	-1.19	1.01	-1.08	-1.09	-1.02	-1.12	-1.22	1.02
LTF	-1.19	1.36	2.20	1.62	-1.32	1.21	1.59	-1.74	-1.71	-1.08	1.01
MEST	-1.02	**-1.64**	-1.55	1.06	**-2.30**	**-1.94**	1.19	**-2.16**	**-2.41**	-2.03	-1.01
NRG1	1.09	**1.47**	**1.55**	1.06	**1.52**	**1.76**	1.16	**2.38**	2.17	**2.52**	-1.02
NTS	1.15	1.41	1.03	-1.36	1.37	-1.01	-1.39	1.63	1.67	1.20	-1.12
PENK	1.06	**-1.73**	-1.90	-1.09	**-1.60**	-1.13	1.41	-1.96	**-3.03**	-2.14	-1.06
PI15	1.22	**3.41**	**2.87**	-1.19	**5.84**	**6.56**	1.12	**3.19**	2.39	**2.68**	-1.00
PTX3	1.10	**-3.48**	**-2.25**	1.55	**-3.77**	**-2.45**	1.54	**-3.09**	**-3.08**	**-2.00**	1.07
SOSTDC1	1.04	**-1.70**	**-2.22**	-1.31	**-2.30**	-1.74	1.32	-1.01	-1.74	-1.32	-1.01
SST	-1.02	-1.45	-1.17	1.25	-2.12	-3.16	-1.49	**-3.79**	-2.04	**-3.04**	-1.15
TAC1	1.35	1.22	1.28	1.05	-1.03	1.02	1.06	-1.25	-1.26	-1.19	1.01
TGFB2	-1.12	-1.03	**-1.72**	-1.67	1.09	**-1.56**	**-1.71**	1.44	1.47	-1.16	-1.01
TGFB3	-1.10	-1.07	1.03	1.10	1.07	-1.46	-1.56	-1.36	1.26	-1.24	-1.07
TGFBI	1.06	**1.46**	1.48	1.02	**1.61**	1.44	-1.12	1.57	1.78	1.59	1.00
VIP	1.62	1.03	-1.10	-1.13	-1.57	-1.45	1.09	1.14	-1.08	1.00	-1.09
Signaling – Matricellular											
CYR61	1.15	**-1.63**	-1.20	1.35	**-2.15**	-1.75	1.23	-1.41	-1.28	-1.04	1.10
NOV	1.11	**-2.14**	**-2.75**	-1.28	**-1.99**	**-2.68**	-1.34	-1.51	-1.44	-1.93	-1.11
THBS1	1.11	**-2.97**	**-3.08**	-1.04	**-1.90**	-1.96	-1.03	-1.30	-1.31	-1.35	1.04
THBS2	1.04	-1.98	-2.25	-1.14	1.02	1.13	1.10	1.16	-1.08	1.02	1.04
TNC	-1.12	-1.24	1.21	1.49	1.20	-1.19	-1.43	-1.22	1.43	1.22	-1.15
MP / TIMP											
ADAMTS4	-1.03	-1.33	1.08	1.44	-1.00	-1.05	-1.05	-1.08	1.39	1.33	-1.10
ADAMTS5	-1.03	-1.06	-1.20	-1.13	1.03	-1.25	-1.28	1.04	1.17	-1.09	-1.02
ADAMTSL3	1.02	**-1.99**	-1.20	1.65	**-1.85**	-1.19	1.55	-1.69	-1.59	-1.02	-1.01
MMP14	1.07	1.14	1.05	-1.09	**1.27**	1.08	-1.18	-1.05	1.04	-1.14	1.03
TIMP2	-1.01	-1.42	-1.29	1.09	1.12	1.08	-1.04	1.12	1.27	1.23	1.06
TIMP3	-1.05	**-1.28**	**-1.53**	-1.20	-1.07	1.05	1.12	1.03	-1.12	-1.01	1.03
Extracellular matrix											
COL12A1	1.08	-1.90	**-1.97**	-1.04	**-1.49**	-1.40	1.07	1.05	-1.05	1.01	1.08
COL6A6	1.04	**-1.83**	-1.35	1.35	**-1.76**	-1.12	1.58	-1.63	-1.90	-1.21	-1.23
DCN	1.09	1.07	1.08	1.00	1.06	1.16	1.09	1.21	1.11	1.21	1.01
FMOD	1.01	-1.19	**-1.82**	-1.52	-1.15	**-1.87**	-1.63	-1.20	-1.12	**-1.83**	1.03
MXRA5	-1.03	-1.18	-1.04	1.13	-1.02	1.11	1.13	-1.26	-1.26	-1.12	1.00
NYX	1.09	-1.09	-1.26	-1.16	-1.06	1.35	1.43	1.18	-1.27	1.02	-1.02
OGN	1.00	**-1.32**	**-1.70**	-1.29	**-1.37**	-1.26	1.08	-1.08	**-1.50**	-1.38	1.03
PRELP	-1.02	-1.08	-1.29	-1.20	1.10	1.23	1.12	1.13	-1.19	-1.06	1.06
SERPINH1	1.00	-1.12	-1.09	1.03	-1.08	1.39	1.50	1.16	-1.26	1.19	1.05

Table 2. Gene expression differences comparing right vs. left, treated vs. control, treated vs. normal, and control vs. normal eyes. MLC = ML control eye, FDC = FD control eye. Red text = significant down-regulation, blue = significant up-regulation, grey = expression difference not statistically significant. "X" = differential expression likely due to control eye effect.

ML and FD Groups

The ML group (Fig. 4A) had a significant difference in mRNA expression levels between the treated and control eyes for 28 genes; 18 of these genes were down-regulated. Expression values are listed in Table 2.

The FD group (Fig. 4B) had a pattern of differential mRNA expression that was very similar to that in the ML group in terms of which genes did, and did not, show significant differential expression, the direction of the differential expression, and, generally, in the magnitude of that fold difference. Twenty-seven genes were differentially expressed in both; 17 were down-regulated. In addition, mRNA levels for 5 genes were significantly affected in the FD group that were not altered in the ML group: VIPR2, EGR1, and COL12A1 were down-regulated; EGF and MMP14 were significantly up-regulated. One gene (TIMP3) was significantly down-regulated in ML animals but not altered in the FD group treated eyes. In almost all cases in both the ML and FD groups, the control eye gene expression was not significantly different from that in the 26N group, suggesting that treated vs. control differences are due to a change in the treated eye. For one gene (RLBP1) in the ML group the differential effect occurred because control eye, but not treated eye, mRNA levels were significantly different from the 26N group. In the FD group, this was the case for three genes (RLBP1, RPE65, and BMP2), as indicated in Fig. 4 and Table 2.

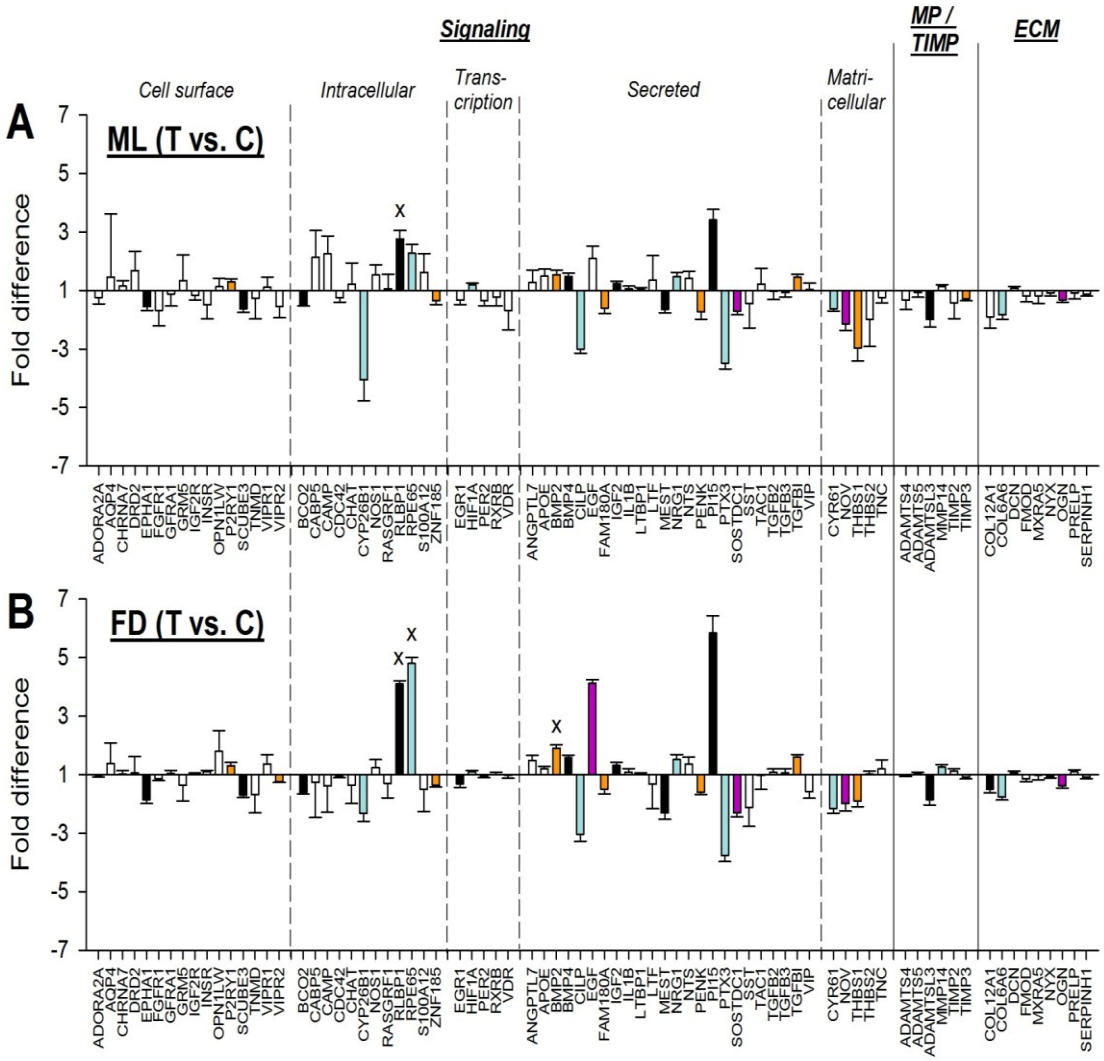

Figure 4. Gene expression fold differences between the treated and control eyes for the (A) ML group and (B) FD group. Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Filled bars represent statistically significant differences between the treated and control eyes ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the two different conditions. Error bars = SEM. The X's indicate genes for which the significant treated vs. control eye difference was a result of the control eye differing from normal (Table 2). The data shown in A are reproduced from (He et al., 2014b).

Comparison between ML and FD GO Signatures

The similarities in the mRNA expression patterns in the ML and FD groups are illustrated in Fig. 5, which compares differential expression in the ML group (Fig. 4A) with the differential expression in the FD group (Fig. 4B) for the 33 genes whose expression was significantly altered in either group. All of the genes were regulated in the same direction in both conditions and generally by very similar amounts. The correlation was

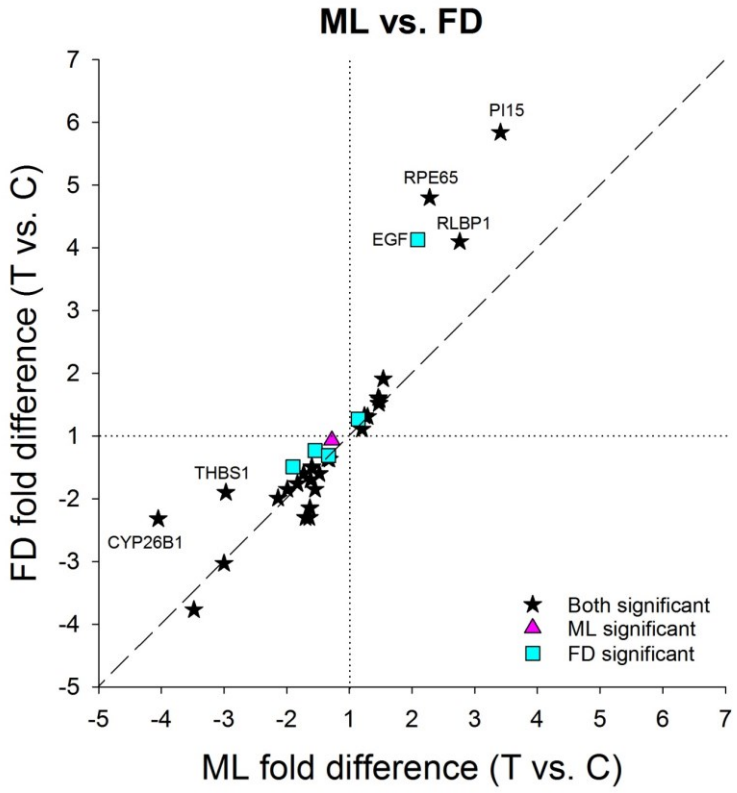

Figure 5. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4A (ML) with the differences in Fig. 4B (FD), showing the similar differential expression patterns in the two GO conditions. Values near the dashed line indicate genes that responded similarly in the two conditions. Stars = significant differences for both ML and FD; triangles = significant differences only for ML; squares = significant differences only for FD.

significant ($p < 0.0001$) with $r^2 = 0.79$. Although the direction of the shared differential expression was the same for these genes, the amplitude (of the fold differences) differed for several genes. PI15, RPE65, RLBP1, and EGF were more strongly up-regulated in the FD group than in the ML group. THBS1 and CYP26B1 were more strongly down-regulated in the ML group than in the FD group.

DK Group

Because the DK group received binocular treatment, there was not a within-animal control for comparison. In Fig. 6, the gene expression in the DK group (mean of right and left eyes) was compared with the three possible control groups: the control eyes of the ML group (Fig. 6A), the control eyes of the FD group (Fig. 6B), and with the 26N group (mean of right and left eyes) (Fig. 6C). Fold differences for these comparisons are given in Table 2. It is evident in Fig. 6 that similar patterns occurred regardless of which eyes were used as a comparison. Fewer fold differences were statistically significant than in the ML and FD groups treated vs. control eye comparisons, presumably because comparisons were made across groups of animals, rather than within animals. However, 4 genes were significantly regulated in the DK group in all three of the comparisons in Fig. 6: CYP26B1, EGF, and PTX3 were down-regulated, while BMP4 was up-regulated. Whilst EGF was strongly down-regulated in the DK group in comparison with the ML control eyes, the FD control eyes, and the 26N eyes, it was significantly up-regulated in the treated vs. control eyes of the FD group.

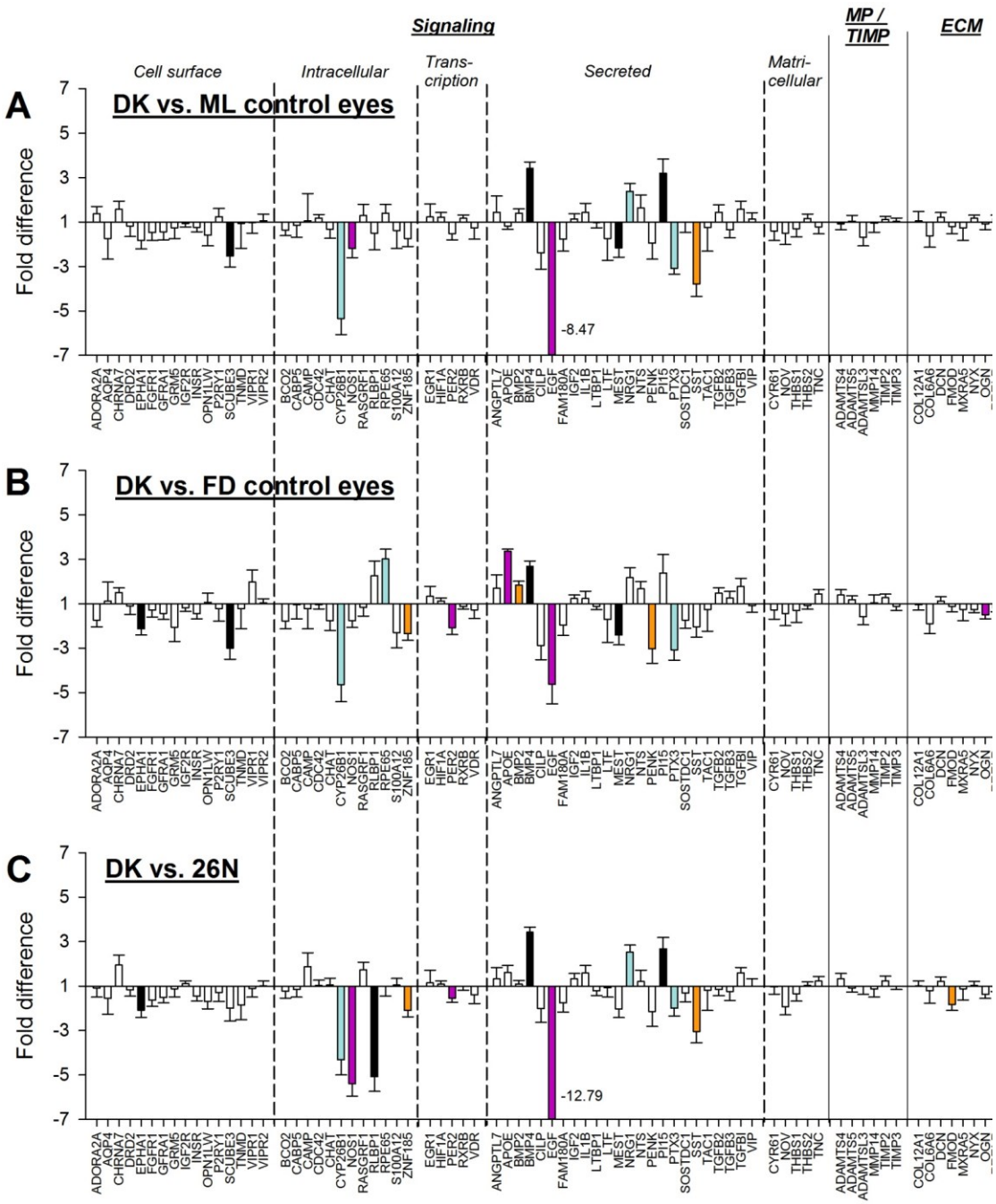

Figure 6. Gene expression fold differences. (A) DK group (mean of the R and L eyes) vs. ML control eyes. (B) DK group vs. FD control eyes. (C) DK group vs. the 26N group (mean of the R and L eyes). Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Filled bars represent statistically significant differences ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the three different conditions. Error bars = SEM. In A and C, the off-scale fold differences for EGF are indicated next to the bar.

Comparison of DK GO Signature with ML and FD GO Signatures

Gene expression in the DK group eyes, compared with either the ML or FD control eyes, was similar to that found for the treated vs. control differences for the ML and FD groups. Fig. 7 is organized similarly to Fig. 5. Fig. 7A plots the DK group (relative to the ML control eyes) (Fig. 6A) against the ML treated vs. control eye fold differences (Fig. 4A). Included in the figure are 31 genes that were significantly regulated in either ML (T vs. C), DK relative to the ML control eyes, or both. Fig. 7B plots the DK group (relative to the FD control eyes) (Fig. 6B) against the FD treated vs. control eye differences (Fig. 4B), including 34 genes which were significantly regulated in either FD (T vs. C), DK relative to the FD control eyes, or both. Figures 7A and 7B are similar. The comparisons of gene expression in the DK group with the ML and FD groups are more variable than was the comparison between the ML and FD groups (Fig. 5). Yet, the fold differences for nearly all genes are in the upper right or lower left quadrant indicating that they were up- or down-regulated in both DK and ML groups (Fig. 7A) or in both the DK and FD groups (Fig. 7B). The notable exception, in Fig. 7B, is EGF, which was significantly up-regulated in the FD group but down-regulated in the DK group. Also in Fig 7A, EGF is strongly down-regulated in the DK group (−8.47) and so is not shown given the figure's scale. The correlations (excluding EGF) were significant in both Fig. 7A ($p < 0.0001$, $r^2 = 0.50$) and Fig. 7B ($p < 0.0001$, $r^2 = 0.51$).

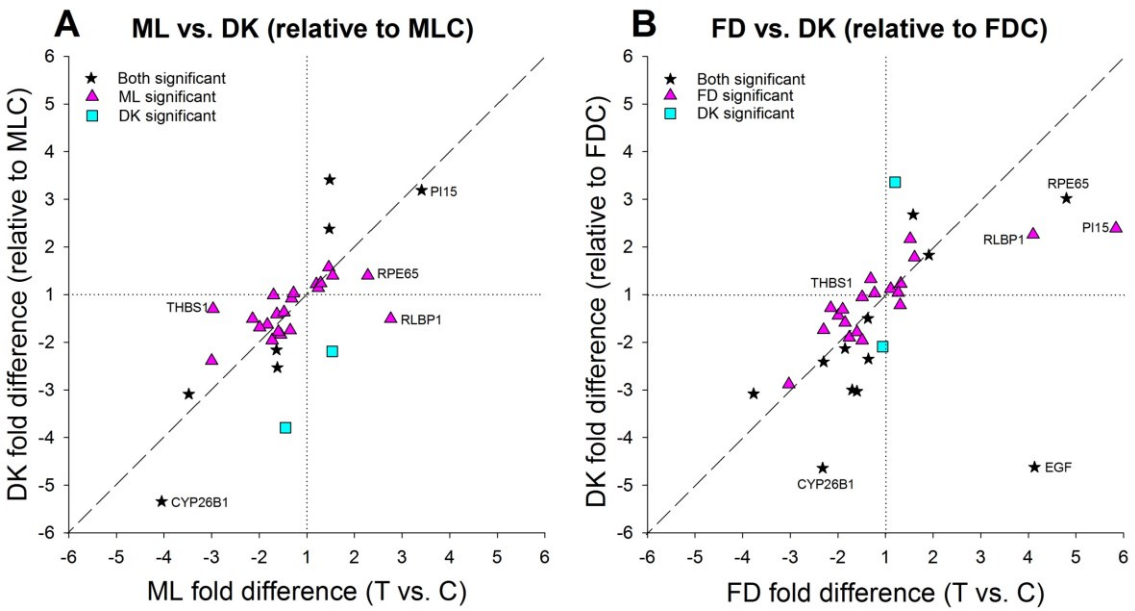

Figure 7. *Comparison of the gene expression differences for (**A**) Fig. 4A (ML treated eyes vs. control eyes) with the differences in Fig. 6A (DK vs. ML control eyes; MLC) and (**B**) Fig. 4B (FD treated eyes vs. control eyes) with the differences in Fig. 6B (DK vs. FD control eyes; FDC). Values near the dashed line indicate genes that responded with similar fold differences in the two conditions. Stars = significant fold differences for both ML and DK (in **A**) or both FD and DK (in **B**); triangles = significant fold differences only for ML or FD; squares = significant fold differences only for DK.*

DISCUSSION

A Common Choroidal GO Signature?

In response to myopiagenic visual stimuli, the retinal compartment of the direct emmetropization pathway generates GO signals that are conveyed into the RPE (He, Frost, & Norton, 2014), thence into the choroid (He et al., 2014), and eventually reach the sclera (Guo et al., 2013) where they modulate the axial elongation rate of the growing postnatal eye (Siegwart, Jr. & Norton, 1998). It seems evident that the three GO conditions used in this study, minus lens wear, form deprivation, and continuous darkness, must produce dramatically different responses in many of the retinal horizontal, bipolar, and amacrine cells and in the patterns of retinal ganglion cell activity that are sent through the optic nerve to central visual structures, yet all three produce a nearly identical response in the scleral fibroblasts (Guo et al., 2013). There is evidence in chicks that the emmetropization-related signaling may differ between ML and FD conditions, but this is a topic of ongoing debate (Morgan, Ashby, & Nickla, 2013). If they do differ, it seems likely that the differences are in the responses of retinal neurons, whose influence is then passed on to the RPE and choroid.

To what degree does the direct emmetropization pathway (as described in the Introduction) distinguish between these different myopiagenic visual conditions after the signals leave the retina? Although efferent influences to the choroid from the "indirect" emmetropization pathway cannot be completely ruled out, the present results suggest that the direct emmetropization mechanism may not distinguish between the different visual stimuli at the level of the scleral fibroblast response. Guo et al. (2013) found in tree shrews that ML and FD produce virtually identical scleral gene expression signatures in a

sample of 55 genes, and that DK also produces a very similar gene expression pattern. It thus seems relevant to assess whether these three GO conditions produce distinct or similar gene expression patterns in the choroid, which presumably is the primary source of the signals that produce the scleral fibroblast response.

The present study found that the three GO conditions produce very similar gene expression signatures in our sample of 77 genes in the choroid. The ML and FD gene expression signatures are extremely similar. These similarities extend not only to which genes are differentially expressed, and the magnitude of the fold differences, but also to which genes in the sample did not show significant differential expression. The DK signature may be less similar, but it is difficult to be certain without having a within-animal control eye for comparison.

We found only limited evidence of genes that respond specifically to one GO condition but not the others. mRNA expression for EGF was significantly up-regulated (4.13 fold) in FD. It was also up-regulated (2.09 fold) in ML, but the up-regulation was not statistically significant. Whether this represents a real difference between these conditions, or was due to variability in the relatively small groups of seven animals is unclear. However, EGF was dramatically down-regulated in DK choroid when compared with the ML control eyes (−8.47 fold), FD control eyes (−4.62 fold), and normal eyes (−12.79 fold). Evidence for other possible differences is less compelling; mRNA levels for three additional genes, NOS1, PER2, and SST, were significantly down-regulated in the DK eyes in two of the three comparisons but not altered in ML or FD. mRNA for APOE was significantly up-regulated in one of the three comparisons (DK vs. the FD control eyes); it was not regulated in the treated eyes vs. control eyes of the ML and FD groups. Thus,

mRNA for EGF, in the DK group, was the only example in the present study of a gene whose regulation might be related to a particular myopiagenic visual condition. However, the overall similarity of the three choroidal GO gene expression signatures suggests that the details of the visual conditions that produce the initial GO signal in the retina are substantially less important in the choroid than they are in the retina.

Components of the GO Signal

A reason to examine the response in choroid to these three myopiagenic stimuli is to learn if there is an essential "core" of key genes that modulate the signaling cascade into the sclera, causing it to remodel, and increasing the rate of axial elongation. The strongest evidence for a single GO response/signal would seem to be the changes that occur in all three myopiagenic conditions. Table 3 shows those genes from our sample whose mRNA levels were significantly modulated during GO. Three genes were consistently modulated in the same direction in all three GO conditions (shaded in Table 3): the treated vs. control eyes of the ML and FD groups, and in the DK treated eyes when compared with all three possible "control" eyes. mRNA for CYP26B1 and PTX3 were down-regulated and mRNA for BMP4 was up-regulated. Using a less stringent criterion, significant differences in mRNA levels in the ML, FD, and two of the three DK group comparisons, six additional genes (italicized in Table 3) could be included in the core of the potential GO signal/response. EPHA1, SCUBE3, ZNF185, and MEST were down-regulated; NRG1 and PI15 were up-regulated.

The suggestion that these genes might be the essential components of a choroidal GO response must be tempered by several limitations. One is that the "essential GO" sig-

Functional category	ML	FD	DK		
			vs. MLC	*vs. FDC*	*vs. 26N*
Signaling - Cell surface	*EPHA1*	*EPHA1*		*EPHA1*	*EPHA1*
	SCUBE3	*SCUBE3*	*SCUBE3*	*SCUBE3*	
		VIPR2			
	P2RY1	P2RY1			
Signaling - Intracellular	BCO2	BCO2			
	CYP26B1	CYP26B1	CYP26B1	CYP26B1	CYP26B1
	ZNF185	*ZNF185*		*ZNF185*	*ZNF185*
			NOS1		NOS1
	RLBP1	RLBP1			RLBP1
	RPE65	RPE65		RPE65	
Signaling - Transcription		EGR1			
				PER2	PER2
	HIF1A	HIF1A			
Signaling - Secreted	CILP	CILP			
	FAM180A	FAM180A			
	MEST	*MEST*	*MEST*	*MEST*	
	PENK	PENK		PENK	
	PTX3	PTX3	PTX3	PTX3	PTX3
	SOSTDC1	SOSTDC1			
			SST		SST
				APOE	
	BMP2	BMP2		BMP2	
	BMP4	BMP4	BMP4	BMP4	BMP4
	IGF2	IGF2			
	NRG1	*NRG1*	*NRG1*		*NRG1*
	PI15	*PI15*	*PI15*		*PI15*
	TGFBI	TGFBI			
		EGF	EGF	EGF	EGF
Signaling - Matricellular	CYR61	CYR61			
	NOV	NOV			
	THBS1	THBS1			
MP / TIMP	ADAMTSL3	ADAMTSL3			
	TIMP3				
		MMP14			
Extracellular matrix	COL6A6	COL6A6			
	OGN	OGN		OGN	
		COL12A1			
					FMOD

Table 3. Genes that were significantly regulated under ML and FD (treated vs. control) or DK (vs. ML control, FD control, or Normal) conditions. Red gene symbols = down-regulation, blue = up-regulation, grey shading = significant regulation in all five comparisons, italics = significant regulation in four of the five comparisons.

nal may change over time and may also reflect the "strength" of the GO signal. The ML and FD groups were examined after two days of treatment, and developed slightly different amounts of myopia (−1.0 ± 0.2 D vs. −1.9 ± 0.2 D). The similarity of the mRNA signatures (Fig. 5) may reflect that very similar alterations in gene expression are important early in the development of induced myopia. The differences in expression levels of a few more strongly-affected genes (Fig. 5) may reflect their importance in the amount or rate of myopia development. For instance, the stronger up-regulation of PI15, RPE65, RLBP1, and EGF in the FD group may suggest that they are involved in the higher rate of axial elongation and myopia development. The lower down-regulation of THBS1 and CYP26B1 during FD may also contribute to the more rapid myopia development. The DK group experienced a longer (11 day) treatment period. As is the case with form deprivation, whatever signal is produced by continuous darkness to cause increased axial elongation did not change over time – the darkness was continuous throughout the treatment period. Thus is seems that the choroids in the DK group likely were in the same "GO" situation after 11 days as they were earlier in the treatment period. However, if the choroidal response to the GO condition changes with the duration of treatment, it might help account for the less similar constellation of differentially expressed genes compared with the FD and ML groups. For instance, the dramatic down-regulation of EGF after 11 days of DK may be a late-developing signal related to alterations in circadian signaling (Nickla, Wildsoet, & Wallman, 1998; Morgan et al., 2013).

It also is possible that some of the shared gene expression changes occur because the choroid physically responds similarly in all three myopiagenic conditions (Nickla & Wallman, 2010; Summers, 2013) in ways that may, or may not, be part of the emme-

tropization signaling cascade. Although the choroid is much thinner in mammals than in chicks, there is evidence in tree shrews (Siegwart, Jr. & Norton, 1998) that, as in chick, it becomes thinner during myopia development (though the thinning was not statistically significant in the present study). In chicks, there is reduced choroidal blood flow and there could be remodeling of the choroidal extracellular matrix (Summers, 2013). A subset of ten genes in our sample that were significantly altered in GO conditions play a role in vascular regulation and/or angiogenesis (BMP2, BMP4, CYR61, EGF, HIF1A, IGF2, NRG1, PTX3, THBS1, and TIMP3); the altered mRNA level for these genes may have been part of a common choroidal response. It is, of course, unknown if such vascular and/or extracellular matrix changes may not only be part of a local choroidal response, but also part of the emmetropization signal as well. To directly test the possibility that the shared gene expression changes may reflect simply changes in choroidal thinning or vascular flow, future studies might examine these genes in treatments in which the eye elongates without choroidal thinning, or slows its elongation without choroidal thickening (Nickla & Wallman, 2010). It also could not be distinguished in this study the degree to which the choroidal signatures represent the response of choroidal cells to incoming signals from the RPE and the degree to which they are involved in generating signals that are transmitted to the sclera.

An additional limitation in the ability to define the key genes involved in the choroidal compartment of the direct emmetropization pathway is that although our sample is large enough to show that there are similarities and, potentially, to have found differences if they exist, this is nonetheless a relatively small sample. A preliminary whole-transcriptome analysis using mRNA from three of the ML animals suggested that over

300 distinct genes (from the just over 14,000 found to be expressed in the tree shrew choroid) may be up- or down-regulated by at least 1.2 fold (Frost, personal communication, 2013). It may be that genes not included in our sample also respond in the same way to ML, FD, and DK and thus are part of a "core" of genes in the choroidal compartment of the direct emmetropization pathway.

If, as the data of this study suggest, there is a consistent group of genes whose expression responds in the same manner to ML, FD, and DK, it would raise the question of whether the incoming signals from RPE do, or do not, distinguish between these conditions? Once information about defocus has been extracted by retinal neurons, how important, for the direct emmetropization pathway, are the details of the visual scene? The emmetropization mechanism seems to function in a wide range of vertebrate species that exist in many different visual environments. The visual system uses channels comprised of differing neurons to respond to light increments and decrements, to color, direction of motion, etc. Neurons in these channels are specialized to respond to stimuli matched to their receptive-field properties; they have limited sensitivity to other characteristics of the visual scene. Similarly, perhaps, the direct emmetropization pathway may retain only the defocus-related signaling and be insensitive to the visual details. Thus far, we only have evidence in tree shrew choroid and sclera suggesting that vision-specific information may not be encoded. It will be interesting to learn if this is also the case in other species and also in the RPE.

ACKNOWLEDGMENTS

This study was supported by NIH grants EY005922 and EY003039 (P30). Li He was supported in part by a supplement to EY005922 and by funds from the Department of Vision Sciences. This work was performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Alabama at Birmingham (Li He). Preliminary results were presented in abstract form (the 14th International Myopia Conference, 2013).

REFERENCES

Bartmann, M., Schaeffel, F., Hagel, G. & Zrenner, E. (1994). Constant light affects retinal dopamine levels and blocks deprivation myopia but not lens-induced refractive errors in chickens. *Vis Neurosci,* 11, 199-208.

Bitzer, M., Feldkaemper, M. & Schaeffel, F. (2000). Visually induced changes in components of the retinoic acid system in fundal layers of the chick. *Exp Eye Res,* 70, 97-106.

Dillingham, C. M., Guggenheim, J. A. & Erichsen, J. T. (2013). Disruption of the centrifugal visual system inhibits early eye growth in chicks. *Invest Ophthalmol Vis Sci,* 54, 3632-3643.

Fujikado, T., Kawasaki, Y., Suzuki, A., Ohmi, G. & Tano, Y. (1997). Retinal function with lens-induced myopia compared with form-deprivation myopia in chicks. *Graefes Arch Clin Exp Ophthalmol,* 235, 320-324.

Gao, H., Frost, M. R., Siegwart, J. T., Jr. & Norton, T. T. (2011). Patterns of mRNA and protein expression during minus-lens compensation and recovery in tree shrew sclera. *Mol Vis,* 17, 903-919.

Glickstein, M. & Millodot, M. (1970). Retinoscopy and eye size. *Science,* 168, 605-606.

Guo, L., Frost, M. R., He, L., Siegwart, J. T., Jr. & Norton, T. T. (2013). Gene expression signatures in tree shrew sclera in response to three myopiagenic conditions. *Invest Ophthalmol Vis Sci,* 54, 6806-6819.

He, L., Frost, M. R. & Norton, T. T. (2014a). Differential gene expression in tree shrew retina compared with retinal pigment epithelium (RPE) in response to six hours of minus-lens wear. *Invest Ophthalmol Vis Sci,* 55, ARVO E-Abstract 3037.

He, L., Frost, M. R., Siegwart, J. T., Jr. & Norton, T. T. (2014b). Gene expression signatures in tree shrew choroid during lens-induced myopia and recovery. *Exp Eye Res,* 123, 56-71.

Kee, C. S., Marzani, D. & Wallman, J. (2001). Differences in time course and visual requirements of ocular responses to lenses and diffusers. *Invest Ophthalmol Vis Sci,* 42, 575-583.

Lauber, J. K. (1991). Three avian eye enlargement protcols as myopia models: effects of pharmacological intervention. *J Ocular Pharmacol,* 7, 65-75.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods,* 25, 402-408.

McFadden, S. A. & Wildsoet, C. (2009). Mammalian eyes need an intact optic nerve to detect the sign of defocus during emmetropisation. *Invest Ophthalmol Vis Sci,* 50, ARVO E-Abstract 1620.

McKanna, J. A. & Casagrande, V. A. (1981). Atropine affects lid-suture myopia development. *Doc Ophthalmol Proc Ser,* 28, 187-192.

Morgan, I. G., Ashby, R. S. & Nickla, D. L. (2013). Form deprivation and lens-induced myopia: are they different? *Ophthalmic Physiol Opt,* 33, 355-361.

Mutti, D. O., Mitchell, G. L., Jones, L. A., Friedman, N. E., Frane, S. L., Lin, W. K., Moeschberger, M. L. & Zadnik, K. (2005). Axial growth and changes in lenticular and corneal power during emmetropization in infants. *Invest Ophthalmol Vis Sci,* 46, 3074-3080.

Nickla, D. L. & Wallman, J. (2010). The multifunctional choroid. *Prog Retin Eye Res,* 29, 144-168.

Nickla, D. L., Wildsoet, C. & Wallman, J. (1998). The circadian rhythm in intraocular pressure and its relation to diurnal ocular growth changes in chicks. *Exp Eye Res,* 66, 183-193.

Norton, T. T. (1999). Animal models of myopia: Learning how vision controls the size of the eye. *ILAR J,* 40, 59-77.

Norton, T. T., Amedo, A. O. & Siegwart, J. T., Jr. (2006a). Darkness causes myopia in visually experienced tree shrews. *Invest Ophthalmol Vis Sci,* 47, 4700-4707.

Norton, T. T., Essinger, J. A. & McBrien, N. A. (1994). Lid-suture myopia in tree shrews with retinal ganglion cell blockade. *Vis Neurosci,* 11, 143-153.

Norton, T. T., Siegwart, J. T., Jr. & Amedo, A. O. (2006b). Effectiveness of hyperopic defocus, minimal defocus, or myopic defocus in competition with a myopiagenic stimulus in tree shrew eyes. *Invest Ophthalmol Vis Sci,* 47, 4687-4699.

Norton, T. T., Siegwart, J. T., German, A. J., Robertson, J., & Wu, W. W. (2000). Comparison of cycloplegic streak retinoscopy with autorefractor measures in tree shrew eyes with, and without, induced myopia. *Invest Ophthalmol Vis Sci,* 41, ARVO Abstract 563

Norton, T. T., Wu, W. W. & Siegwart, J. T., Jr. (2003). Refractive state of tree shrew eyes measured with cortical visual evoked potentials. *Optom Vis Sci,* 80, 623-631.

Raviola, E. & Wiesel, T. N. (1985). An animal model of myopia. *N Engl J Med,* 312, 1609-1615.

Rohrer, B., Negishi, K., Tao, J. & Stell, W. K. (1993). A role for basic fibroblast growth factor (bFGF) in the visually guided regulation of eye growth in the chick. *Invest Ophthalmol Vis Sci,* 34, ARVO Abstract 2489.

Schaeffel, F., Hagel, G., Bartmann, M., Kohler, K. & Zrenner, E. (1994). 6-Hydroxy dopamine does not affect lens-induced refractive errors but suppresses deprivation myopia. *Vision Res,* 34, 143-149.

Schaeffel, F. & Howland, H. C. (1988). Mathematical model of emmetropization in the chicken. *J Opt Soc Am,* 5, 2080-2086.

Schaeffel, F., Troilo, D., Wallman, J. & Howland, H. C. (1990). Developing eyes that lack accommodation grow to compensate for imposed defocus. *Vis Neurosci,* 4, 177-183.

Schippert, R., Brand, C., Schaeffel, F. & Feldkaemper, M. P. (2006). Changes in scleral MMP-2, TIMP-2 and TGF□-2 mRNA expression after imposed myopic and hyperopic defocus in chickens. *Exp Eye Res,* 82, 710-719.

Shelton, L., Troilo, D., Lerner, M. R., Gusev, Y., Brackett, D. J. & Rada, J. S. (2008). Microarray analysis of choroid/RPE gene expression in marmoset eyes undergoing changes in ocular growth and refraction. *Mol Vis,* 14, 1465-1479.

Siegwart, J. T., Jr. & Norton, T. T. (1998). The susceptible period for deprivation-induced myopia in tree shrew. *Vision Res,* 38, 3505-3515.

Siegwart, J. T., Jr. & Norton, T. T. (2005). Selective regulation of MMP and TIMP mRNA levels in tree shrew sclera during minus lens compensation and recovery. *Invest Ophthalmol Vis Sci,* 46, 3484-3492.

Smith, E. L., III, Hung, L. F. & Harwerth, R. S. (1999). Developmental visual system anomalies and the limits of emmetropization. *Ophthalmic Physiol Opt,* 19, 90-102.

Stone, R. A., McGlinn, A. M., Baldwin, D. A., Tobias, J. W., Iuvone, P. M. & Khurana, T. S. (2011). Image defocus and altered retinal gene expression in chick: Clues to the pathogenesis of ametropia. *Invest Ophthalmol Vis Sci,* 52, 5765-5777.

Summers, J. A. (2013). The choroid as a sclera growth regulator. *Exp Eye Res,* 114, 120-127.

Troilo, D., Gottlieb, M. D. & Wallman, J. (1987). Visual deprivation causes myopia in chicks with optic nerve section. *Curr Eye Res,* 6, 993-999.

Troilo, D. & Wallman, J. (1991). The regulation of eye growth and refractive state: an experimental study of emmetropization. *Vision Res,* 31, 1237-1250.

Wallman, J. & Winawer, J. (2004). Homeostasis of eye growth and the question of myopia. *Neuron,* 43, 447-468.

Wildsoet, C. (2003). Neural pathways subserving negative lens-induced emmetropization in chicks--insights from selective lesions of the optic nerve and ciliary nerve. *Curr Eye Res,* 27, 371-385.

Yew, K. & Wildsoet, C. F. (2003). The usual effects of high-power negative lens and diffusers show differential susceptibility to disruption to the diurnal light cycle. *Invest Ophthalmol Vis Sci,* 44, ARVO E-Abstract 1979.

Zhang, Y., Liu, Y. & Wildsoet, C. F. (2012). Bidirectional, optical sign-dependent regulation of BMP2 gene expression in chick retinal pigment epithelium. *Invest Ophthalmol Vis Sci,* 53, 6072-6080.

GENE EXPRESSION SIGNATURES IN TREE SHREW CHOROID DURING LENS-INDUCED MYOPIA AND RECOVERY

by

LI HE, MICHAEL R. FROST, JOHN T. SIEGWART, JR., THOMAS T. NORTON

Experimental Eye Research 2014 Jun;123:56-71

ABSTRACT

Gene expression in tree shrew choroid was examined during the development of minus-lens induced myopia (LIM, a GO condition), after completion of minus-lens compensation (a STAY condition), and early in recovery (REC) from induced myopia (a STOP condition). Five groups of tree shrews (n = 7 per group) were used. Starting 24 days after normal eye-opening (days of visual experience [DVE]), one minus-lens group wore a monocular –5 D lens for 2 days (LIM-2), another minus-lens group achieved stable lens compensation while wearing a monocular –5 D lens for 11 days (LIM-11); a recovery group also wore a –5D lens for 11 days and then received 2 days of recovery starting at 35 DVE (REC-2). Two age-matched normal groups were examined at 26 DVE and 37 DVE. Quantitative PCR was used to measure the relative differences in mRNA levels in the choroid for 77 candidate genes that were selected based on previous studies or because a whole-transcriptome analysis suggested their expression would change during myopia development or recovery. Small myopic changes were observed in the treated eyes of the LIM-2 group (−1.0 ± 0.2 D; mean ± SEM) indicating eyes were early in the process of developing LIM. The LIM-11 group exhibited complete refractive compensation (−5.1 ± 0.2 D) that was stable for five days. The REC-2 group recovered by 1.3 ± 0.3 D from full refractive compensation. Sixty genes showed significant mRNA expression differences during normal development, LIM, or REC conditions. In LIM-2 choroid (GO), 18 genes were significantly down-regulated in the treated eyes relative to the fellow control eyes and 10 genes were significantly up-regulated. In LIM-11 choroid (STAY), 10 genes were significantly down-regulated and 12 genes were significantly up-regulated. Expression patterns in GO and STAY were similar, but not identical. All genes that

showed differential expression in GO and STAY were regulated in the same direction in both conditions. In REC-2 choroid (STOP), 4 genes were significantly down-regulated and 18 genes were significantly up-regulated. Thirteen genes showed bi-directional regulation in GO vs. STOP. The pattern of differential gene expression in STOP was very different from that in GO or in STAY. Significant regulation was observed in genes involved in signaling as well as extracellular matrix turnover. These data support an active role for the choroid in the signaling cascade from retina to sclera. Distinctly different treated eye vs. control eye mRNA signatures are present in the choroid in the GO, STAY, and STOP conditions. The STAY signature, present after full compensation has occurred and the GO visual stimulus is no longer present, may participate in maintaining an elongated globe. The 13 genes with bi-directional expression differences in GO and STOP responded in a sign of defocus-dependent manner. Taken together, these data further suggest that a network of choroidal gene expression changes generate the signal that alters scleral fibroblast gene expression and axial elongation rate.

Key Words: myopia, animal models, refractive error, emmetropization, axial elongation, gene expression, choroid

INTRODUCTION

Studies of postnatal refractive development in both children and in animal models have found that there is a visually-guided emmetropization mechanism that uses refractive error to guide the growth of the eye so that the axial length eventually matches the location of the focal plane, producing visual images that are focused on the photoreceptors (emmetropia) (Mutti et al., 2005; Norton, 1999; Wallman & Winawer, 2004). In animal models, the emmetropization mechanism can be manipulated with lenses, held in front of one (or both) eyes in a goggle frame. Minus-power (negative) lenses shift the focal plane away from the cornea, making the eye hyperopic. This produces retinal GO signals that cause an increase in the axial (vitreous chamber) elongation rate, moving the retina to the shifted focal plane and restoring emmetropia while the lens is in place (Irving et al., 1991; Irving et al., 1995; Norton et al., 2010). In tree shrews, the eye remains elongated as long as the lens is left in place, evidently because some form of STAY signal reaches the sclera (Norton et al., 2010). When the lens is removed, the increased axial length causes the eye to experience lens-induced myopia (LIM). The retina then generates STOP signals that, in juvenile animals where the eyes are still growing, rapidly slow the axial elongation rate to below normal, producing recovery (REC) from the induced myopia (Norton et al., 2010).

Although the emmetropization mechanism performs more effectively in a fully intact animal, eyes can still respond to myopiagenic stimuli if the optic nerve is cut (Troilo, 1990; Wildsoet & McFadden, 2010) or output is functionally blocked with tetrodotoxin (Norton et al., 1994). In addition, covering only half of the visual field with a minus lens produces elongation and myopia only in the affected visual field (Diether & Schaeffel,

1997; Norton & Siegwart, 1991; Smith, III et al., 2010). Thus, there is a direct, spatially-localized signaling cascade from the retina to the sclera that must pass through the retinal pigment epithelium (RPE) and choroid. The choroid, in addition to being the vascular supply to the photoreceptors, RPE, and sclera (Birol et al., 2007; Lutty et al., 2010; Oyster, 1999), plays an important role in the emmetropization mechanism (Nickla & Wallman, 2010; Summers, 2013; Wallman & Winawer, 2004) as a way-station in the signaling cascade that conveys the GO, STAY, and STOP signals to the sclera. The extent to which some signaling molecules generated by the retina or RPE may simply pass through the choroid on the way to the sclera and other, new, signaling molecules are generated by the choroid is not known. Examining gene expression changes in the choroid alone, relative to changes in the retina and RPE, may help clarify the situation.

In species such as chick, the choroid is thick because there are no blood vessels on the vitreal side of the retina and the choroid is the sole retinal vascular supply. Chick choroid displays rapid changes within a few hours after the onset of LIM and REC, thinning during myopia development and thickening during recovery (Wallman et al., 1995). Previous studies of chick choroid have reported changes in gene expression during the development of LIM, form deprivation-induced myopia (FDM), and recovery from FDM, suggesting the choroid plays an active role producing new signaling molecules (Mertz & Wallman, 2000; Nickla et al., 2009; Nickla & Wildsoet, 2004; Nickla et al., 2006; Rada et al., 2012; Rada et al., 2001; Rada & Wiechmann, 2009; Rada et al., 2010; Simon et al., 2004). In mammals (guinea pig, tree shrew, marmoset, and macaque), the choroid is not as thick, proportionally, as in chicks and seems to undergo smaller changes in thickness

during myopia development and recovery (Gentle & McBrien, 1999; Howlett & McFadden, 2009; Hung et al., 2000; Siegwart, Jr. & Norton, 1998; Troilo et al., 2000).

A previous study in the marmoset examined gene expression in the combined RPE and choroid (Shelton et al., 2008). While a number of changes were found, it is not possible to know if the changes occurred in the RPE or choroid. In tree shrews (mammals closely related to primates; Luckett, 1980) and guinea pigs, studies have examined changes of a few genes and proteins in the choroid in form-deprived animals (Cui et al., 2010; Jobling et al., 2009; Liu et al., 2007; McBrien et al., 2009). In the present study, we examined, in the choroid, changes in gene expression of a large sample of genes in animals treated with a minus lens.

During the development of LIM in tree shrews, the GO signals from the choroid produce remodeling of the scleral extracellular matrix that increases the viscoelasticity of the sclera (measured as increased creep rate), allowing normal intraocular pressure to expand the globe (Phillips et al., 2000; Siegwart, Jr. & Norton, 1999). With continued lens wear, STAY signals from the choroid must be present because tree shrew eyes remain in an elongated state until minus lens-wear is discontinued (Norton et al., 2010). During REC, the STOP signals from the choroid cause a rapid reduction the creep rate that slows the axial elongation rate (Siegwart, Jr. & Norton, 1999).

The time-course of remodeling in the tree shrew sclera is rapid, but not instantaneous. After one day of LIM, there is little alteration of mRNA levels (Gao et al., 2011). After 2 days of LIM, scleral gene expression is altered; a scleral GO signature is found (Guo et al., 2013) and creep rate is elevated (Siegwart, Jr. & Norton, 1999). Very similar, somewhat stronger gene expression changes are found after four days of LIM (Frost &

Norton, 2012; Guo et al., 2013) and scleral creep rate reaches a peak at this time (Siegwart, Jr. & Norton, 1999). After 11 days of LIM, the eyes have fully compensated for the minus lens but scleral viscoelasticity remains slightly elevated (Siegwart, Jr. & Norton, 1999) and some gene expression differences remain in the sclera (Guo, personal communication, 2013) suggesting the presence of STAY signals in the choroid at this time point. During recovery, the scleral gene expression after one day is little changed (Guo et al., 2012); after two days of recovery, a scleral STOP remodeling response has developed (Guo, personal communication, 2013) and scleral creep rate has dropped to, or below, normal (Siegwart, Jr. & Norton, 1999). Based on this time-course, it is expected that GO and STOP signals should be detectable in the choroid after two days of LIM and after two days of REC respectively.

The goal of the present study was to examine alterations in gene expression in the choroid, measured as alterations in mRNA levels, after two days of LIM (GO), 11 days of LIM (STAY), and after two days of REC (STOP). Although changes in levels of proteins or other molecules presumably are key to actually transmitting signals from choroid to sclera, it has been found that changes in mRNA can help to identify the responses of the cells in tissues and are useful in identifying pathways of interest (Gao et al., 2011; Guo et al., 2013; He et al., 2011; Schippert et al., 2006; Shelton et al., 2008; Siegwart, Jr. & Norton, 2005; Stone et al., 2011; Zhang et al., 2012). Based on these previous studies, our hypothesis was not only that many of the genes examined would show changes in mRNA expression but also that the pattern of differential gene expression would differ in the GO, STAY, and STOP conditions.

MATERIALS AND METHODS

Experimental Groups

The juvenile tree shrews (Tupaia glis belangeri) used in this study were produced in our breeding colony and raised by their mothers on a 14 hr light/10 hr dark cycle. Tree shrew pups open their eyes about three weeks after birth. The day both eyes are open is the first day of visual experience (DVE). 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 were balanced to include both males and females, and avoided pups from the same parents wherever possible.

Five groups of animals (n = 7 per group) were used in this study (Fig. 1). Starting at 24 ± 1 DVE, a minus-lens wear group (LIM-2) wore a monocular –5 D (spherical power) lens for 2 days; the animals in this group also provided scleral mRNA for another

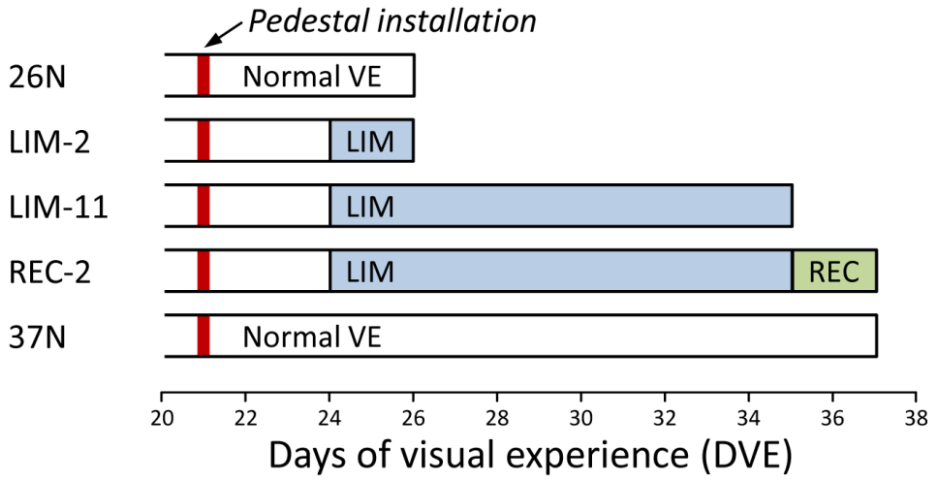

Figure 1. Experimental groups and duration of treatments. The red vertical bar indicates the point when a dental acrylic pedestal was installed under anesthesia. Filled regions indicate the type and duration of visual treatment. The right end of each bar indicates the time point when mRNA levels were measured.

study (Guo et al., 2013). A second minus-lens group (LIM-11) wore a monocular –5 D lens for 11 days and fully compensated for the lens. A recovery group (REC-2) recovered for 2 days starting at 35 ± 1 DVE, after 11 days of minus-lens wear that produced full compensation to a –5 D lens. In the LIM and REC groups, the untreated fellow eye served as a control. Two age-matched (26 DVE and 37 DVE) normal groups were also studied.

Lens Treatment

Animals in all groups were anesthetized (17.5 mg ketamine, 1.2 mg xylazine; supplemented with 0.5 - 2.0% isoflurane as needed) and received a dental acrylic pedestal (Siegwart & Norton, 1994) at 21 ± 1 DVE (Fig. 1). After pedestal installation, all animals were placed in individual cages with standard colony fluorescent lighting, 100 - 300 lux on the floor of the cage. Three days later, in the LIM and REC groups, a goggle frame holding a –5 D lens (12 mm diameter PMMA contact lens; Conforma Contact Lenses, Norfolk, VA) was clipped to the pedestal, firmly holding the lens in front of the randomly selected treated eye. The untreated fellow control eye had unrestricted vision through an open goggle frame. Twice daily (approximately 9:30 AM and 4:30 PM), the goggles were briefly (< 3 min) removed to clean the lens under dim illumination. During goggle cleaning, animals were kept in a darkened nest box to minimize exposure to visual stimuli. For the REC group, after 11 days of minus lens compensation, the goggle was removed, and the treated eye was allowed to recover for 2 days. The normal groups received a pedestal but did not wear a goggle.

Refractive and Axial Measures

Non-cycloplegic refractive measures were made daily, in awake animals, throughout the treatment period with a Nidek ARK-700A infrared autorefractor (Marco Ophthalmic, Jacksonville, FL) (Norton et al., 2003). Normal animals were measured just before euthanasia. Cycloplegic refractive measures were omitted to prevent any interference by atropine on retino-scleral signaling (McKanna & Casagrande, 1981). However, previous studies have shown that non-cycloplegic measures provide a valid estimate of the refractive state and of induced myopia in tree shrews (Norton et al., 2006b; Norton et al., 2003). All refractive values were corrected for the small eye artifact (Glickstein & Millodot, 1970), previously shown to be approximately +4 D in tree shrews (Norton et al., 2003).

At the time the pedestal was attached, ocular component dimensions were measured whilst under anesthesia with A-scan ultrasound (Norton & McBrien, 1992) to ensure that the two eyes did not differ significantly in axial length before treatment began. Post-treatment A-scan measures were not made to eliminate any possibility that the time spent under anesthesia, required for the A-scan procedure, might alter gene expression. In all groups, post-treatment axial component measures were made with a Lenstar LS-900 optical biometer (Haag-Streit USA, Mason, OH). This instrument allowed measures to be quickly made, in awake animals, before euthanasia. Comparison of A-scan and Lenstar measures of the vitreous chamber in 32 animals in this laboratory, with between –1 D and –12 D of induced myopia, showed that the axial differences measured with the Lenstar were very similar to those measured with A-scan ultrasound (data not shown).

Choroid Dissection

On completion of the final refractive measures, approximately 2 - 4 hours into the light phase, animals were terminally anesthetized (17.5 mg ketamine and 1.2 mg xylazine, followed by 50 mg xylazine); both eyes were enucleated and placed into RNAlater solution (Life Technologies, Carlsbad, CA). Extraocular muscles, conjunctiva, and orbital fat were trimmed from the exterior surface of the eye and the cornea dissected away just behind the corneoscleral junction. While viewing through a surgical microscope, the lens and vitreous humor were removed; the retina and RPE, which were tightly bound to each other (Malik et al., 2003; Wang et al., 2012), were then lifted from the eyecup. While still immersed in RNAlater, choroid was teased from the scleral inner surface by gentle separation using the rounded ends of forceps, collected, and then frozen in liquid nitrogen. Because the dissection was extremely gentle, it is possible that small pieces of the lamina fusca, the outermost layer of the choroid, may have adhered to the sclera in some cases and, thus, not been included in our choroidal sample. Because the retina/RPE separated cleanly from the choroid without dissection, and because the inner surface of the sclera was not disrupted by forceful scraping of the surface (and most scleral fibroblasts are within the layered matrix, not on the inner surface), there is no reason to expect significant contamination of the choroidal samples from either retina/RPE or sclera.

Gene Expression Analysis

Each frozen choroid was homogenized with a disposable pellet pestle (Fisher Scientific, Pittsburgh, PA) from which total RNA was isolated using a RiboPure kit (Life Technologies) according to the manufacturer's instructions, with the addition of an on-

filter DNase treatment. The purified RNA was quantified (NanoDrop Technologies, Wilmington, DE), with an average yield per choroid of 5.0 ± 1.3 μg (mean ± SD). RNA quality was confirmed by denaturing gel electrophoresis (RNA FlashGel; Lonza, Rockland, ME). cDNA was synthesized from 1 μg of total RNA in a final reaction volume of 20 μL using a Superscript III RT kit (Life Technologies) with minor modifications (2.5 μM anchored oligo (dT)20 primers and DTT omitted). The resultant cDNA was diluted 5-fold and stored at –20°C until use.

Tree shrew-specific quantitative PCR (qPCR) primers were designed for 77 genes of interest (Table 1) and the reference gene RNA polymerase II (POLR2A) using Beacon Designer 7 (Premier Biosoft International, Palo Alto, CA). None of the treatment conditions affected the expression of the reference gene. Fold differences (right eye vs. left eye or treated vs. control; mean ± SEM) for a typical qPCR run were: 26N, 1.11 ± 0.07; LIM-2, 1.07 ± 0.08; LIM-11, −1.01 ± 0.04; REC-2, 1.10 ±0.08; 37N, 1.03 ± 0.14. Primer sequences, amplicon size, and efficiencies are listed in Supplementary Table S1. The selected candidate genes included representatives of three major groupings: signaling, metallopeptidases & TIMPs, and extracellular matrix (ECM) proteins. They were selected from genes that were found to change in preliminary studies of tree shrew choroid during LIM-2 along with additional genes that were suggested by a whole-transcriptome analysis of three of the LIM-2 animals. All primers were designed to work under the same cycling conditions. All amplicons were located within the coding region and most spanned at least one intron; amplicon identity was verified by gel electrophoresis and sequencing.

Gene symbol	Protein name	Location	UniProt ID
Signaling – Cell surface			
ADORA2A	Adenosine receptor A2a	Cell surface	P29274
AQP4	Aquaporin 4	Cell surface	P55087
CHRNA7	Cholinergic receptor, nicotinic α7	Cell surface	P36544
DRD2	Dopamine receptor D2	Cell surface	P14416
EPHA1	EPH receptor A1	Cell surface	P21709
FGFR1	FGF receptor 1	Cell surface	P11362
GFRA1	GDNF family receptor α1	Cell surface	P56159
GRM5	Metabotropic glutamate receptor 5	Cell surface	P41594
IGF2R	Insulin-like growth factor 2 receptor	Cell surface	P11717
INSR	Insulin receptor	Cell surface	P06213
OPN1LW	Opsin 1, long-wave-sensitive	Cell surface	P04000
P2RY1	Purinergic receptor P2Y, G-protein coupled, 1	Cell surface	P47900
SCUBE3	Signal peptide, CUB and EGF-like domain-containing protein 3	Cell surface	Q8IX30
TNMD	Tenomodulin	Cell surface	Q9H2S6
VIPR1	VIP receptor 1	Cell surface	P32241
VIPR2	VIP receptor 2	Cell surface	P41587
Signaling – Intracellular			
BCO2	Beta-carotene oxygenase 2	Intracellular	Q9BYV7
CABP5	Calcium binding protein 5	Intracellular	Q9NP86
CAMP	Cathelicidin antimicrobial peptide	Intracellular	P49913
CDC42	Cell division cycle 42	Intracellular	P60953
CHAT	Choline O-acetyltransferase	Intracellular	P28329
CYP26B1	Cytochrome P450 26B1	Intracellular	Q9NR63
NOS1	Nitric oxide synthase 1	Intracellular	P29475
RASGRF1	Ras-specific guanine nucleotide-releasing factor 1	Intracellular	Q13972
RLBP1	Retinaldehyde binding protein 1	Intracellular	P12271
RPE65	Retinoid isomerohydrolase	Intracellular	Q16518
S100A12	Protein S100-A12	Intracellular	P80511
ZNF185	Zinc finger protein 185	Intracellular	O15231
Signaling – Transcription regulators			
EGR1	Early growth response protein 1	Intracellular	P18146
HIF1A	Hypoxia-inducible factor 1α	Intracellular	Q16665
PER2	Period circadian clock 2	Intracellular	O15055
RXRB	Retinoid X receptor β	Intracellular	P28702
VDR	Vitamin D receptor	Intracellular	P11473
Signaling – Secreted			
ANGPTL7	Angiopoietin-related protein 7	Extracellular	O43827
APOE	Apolipoprotein E	Extracellular	P02649
BMP2	Bone morphogenetic protein 2	Extracellular	P12643
BMP4	Bone morphogenetic protein 4	Extracellular	P12644
CILP	Cartilage intermediate layer protein 1	Extracellular	O75339
EGF	Epidermal growth factor	Extracellular	P01133
FAM180A	Family with sequence similarity 180, member A	Extracellular	Q6UWF9
IGF2	Insulin-like growth factor 2	Extracellular	P01344
IL1B	Interleukin 1β	Extracellular	P01584
LTBP1	Latent TGFβ binding protein 1	Extracellular	Q14766
LTF	Lactotransferrin	Extracellular	P02788
MEST	Mesoderm specific transcript	Extracellular	Q5EB52
NRG1	Neuregulin 1	Extracellular	Q02297
NTS	Neurotensin	Extracellular	P30990
PENK	Proenkephalin A	Extracellular	P01210
PI15	Peptidase inhibitor 15	Extracellular	O43692
PTX3	Pentraxin 3	Extracellular	P26022
SOSTDC1	Sclerostin domain-containing protein 1	Extracellular	Q6X4U4
SST	Somatostatin	Extracellular	P61278
TAC1	Protachykinin 1	Extracellular	P20366
TGFB2	Transforming growth factor β2	Extracellular	P61812
TGFB3	Transforming growth factor β3	Extracellular	P10600
TGFBI	TGFβ-induced protein	Extracellular	Q15582
VIP	Vasoactive intestinal peptide	Extracellular	P01282
Signaling – Matricellular			
CYR61	Protein CYR61	Extracellular	O00622
NOV	Nephroblastoma overexpressed gene	Extracellular	P48745
THBS1	Thrombospondin 1	Extracellular	P07996
THBS2	Thrombospondin 2	Extracellular	P35442
TNC	Tenascin C	Extracellular	P24821
MP / TIMP			
ADAMTS4	ADAM metallopeptidase with thrombospondin motif, 4	Extracellular	O75173
ADAMTS5	ADAM metallopeptidase with thrombospondin motif, 5	Extracellular	Q9UNA0
ADAMTSL3	ADAMTS-like 3	Extracellular	P82987
MMP14	Matrix metallopeptidase 14	Cell surface	P50281
TIMP2	TIMP metallopeptidase inhibitor 2	Extracellular	P16035
TIMP3	TIMP metallopeptidase inhibitor 3	Extracellular	P35625
Extracellular matrix			
COL12A1	Collagen type XII, α1	Extracellular	Q99715
COL6A6	Collagen type VI, α6	Extracellular	A6NMZ7
DCN	Decorin	Extracellular	P07585
FMOD	Fibromodulin	Extracellular	Q06828
MXRA5	Matrix remodeling associated protein 5	Extracellular	Q9NR99
NYX	Nyctalopin	Extracellular	Q9GZU5
OGN	Mimecan	Extracellular	P20774
PRELP	Prolargin	Extracellular	P51888
SERPINH1	Serpin H1	Intracellular	P50454

Table 1. Genes examined by functional category, with cellular location of the protein encoded by the gene and its UniProt accession ID

Gene	*Forward sequence*	*Reverse sequence*	*Amplicon (bp)*	*Efficiency (%)*
ADAMTS4	CTACAACCATCGCACGGA	GCCTGGCAGGTGAGTT	108	88
ADAMTS5	TCTTCCATCCTAACCAGCATTG	GGTGGCATCATAAGTCTGTCC	165	98
ADAMTSL3	CCAAGGACCCAAGGCATCGT	TCTGACACACCTGCTTTCTTCTCTG	134	96
ADORA2A	CGTGGTCCCTATGAACTAC	CCGCCAGGAAGATGC	101	100
ANGPTL7	GCTGTCACTTGGCTCTG	AGTCCTTCTCCTGCTTCTTG	193	94
APOE	GGTGCAGACGCTGTCTGACCA	CCTCCAACTCCGCCTTGTAGG	122	90
AQP4	CGACATGGTTCTCATCTCCCTTTG	GAAGACAGACTTGGCGATGCT	148	94
BCO2	GCCCATTGTCCTATTCTTCAGCCAGT	TGCCGAAAGCCACAGCCATAGA	181	95
BMP2	GGACACCAGGTTAGTGAATCAGAACA	TGCTTCTCTTCCAAATGAGCCACT	135	97
BMP4	AAAGTCGCCGAGATTC	ATTGTGCTCTTGCTAGG	134	86
CABP5	AAATGCGAGATGCCTTCAAAGA	CTGCCTCCTGGACAACCT	138	96
CAMP	GAGGATGAGGACCCAGAC	TCTCCAACCCGTTCTTCTT	118	98
CDC42	CCCGTGACCTGAAGGCTGTC	ACACACCTGCGACTCTTCTTGGG	130	98
CHAT	AGGCACTGACTTTCGTGAGAG	CGGTCTGGGCTCGGATG	99	96
CHRNA7	GTACTTCGCCAGCATCAT	ACACCAGTTCAGCAGGA	133	95
CILP	GCCTTCTGTGACGACCAGTC	GTAGGGCTGAGGGACACCAATA	132	97
COL12A1	CAGGTGTCCCGATCAAAGAG	GCCTGGTGGAATGGTGG	95	97
COL6A6	ATCAGCCACATCAATCAAGAG	GATGAGGATGAGGAACTTCTT	195	94
CYP26B1	GCACTACCTGGACTGTGTCA	CGCTGTGTCGTGGGTATCTC	157	95
CYR61	TGGAACCTCGCATTCTGTATAACC	GCCACAAGGTCGCACTTCAC	176	97
DCN	CTGCGTGCCCATGAGAACGAGATC	GGTATCAGCAATGCGGATGTACGAAAGC	168	96
DRD2	CACGCACATCCTGAACATACA	GTTGAAGGTGGTGTAGATGATGG	118	91
EGF	GGAGACGCCAGATAGAATAACG	GACGATGTGCTCACCGAA	193	95
EGR1	TCCCTGACTACCTGTTTCC	AGTGGAGTGAGCGAAGG	112	90
EPHA1	GCCTCCTGGACAACTTC	CACATCACAATCCCAAAGC	136	94
FAM180A	AGACATTCGTCGCCTGAGT	CTTCTGATACCCGTGGGACAA	130	94
FGFR1	CCTGGAGGTCATCATCTACTGC	AGAGTTCATGGAAGCACTGGAG	196	99
FMOD	TCCTGCTGGACCTGAG	TTGTTGGTGAGGCTGTTAT	172	96
GFRA1	CGGCAAGGAGACCAACTT	TTGTAGAGCGACTTCTGCTTC	96	96
GRM5	GCATCGCCCACTCTTACAAA	CCATCAGCAGACCTCTCACT	144	93
HIF1A	ACTGGTTGCATCTCCGTCTCCTACTCA	TCCTGCTCTGTTTGGTGAGGCTGTC	109	94
IGF2	TGGCATTGTTGAAGAGTGTTG	GAAGCACGGTCGGAGAG	113	100
IGF2R	GTACTCTGACGGAGACCTCACC	TGATTGCACTCAAAGTTTATGACACTCA	96	102
IL1B	CAAGGAAGAAGATGGAGAAGCGATT	GGCAGGTAGCTTGGTGAAGTC	182	97
INSR	AACGAGGCGTCGGTCATG	ATCATCTCTTGCAGGGTAGGG	194	92
LTBP1	GGACGAGTGCCTGGAACC	TGCTTGTGGTCTGGAGTTGG	111	103
LTF	GGCTCCAAGTCACGCTGT	GGGCAGTCAACACCGTTTCTT	105	97
MEST	CAAAGATGGAGGTGTGCTGTC	ACTGTAAGAGACTGTCGATGACTAAG	179	94
MMP14	CCCTGGAACCTGGCTACCC	ATAGGTCTTTCCATTGGGCATCC	104	96
MXRA5	GGAAGGAAGGAGTGGATG	GAGGCTGGAAGGTTACA	195	93
NOS1	ACATCACCACGCCACCAA	CCTCCAACACCTCCACGAT	159	94
NOV	CGAACAGACTACAGAGTGGAGTG	GTGATTTCTTGGTGCGGAGACA	191	97
NRG1	CGTGTCCAAGAATGTCATCTCC	TGTGTGCCCATTGCTCCA	145	97
NTS	AATGTGAACAGCCAACCCGAG	AACTCCCAGTGTTGAAAGATCCTG	164	95
NYX	GTGAGCAGGTTCAGCAG	GACAGCGAGGTGTTAGC	101	96
OGN	CTCTCCTTCCTCTACCTGGACCACAAC	CTGAAGATGGATCACGCGCAGACTC	84	96
OPN1LW	ATGGTCACCTGCTGTATC	GGTGGAAGGCGTAGC	232	98
P2RY1	ACGGCAGCATCTTGTTC	GGAGATCGCTACCACCA	146	97
PENK	TCCTTGCCAAGCGATACG	TCTTGCTCATTTCTTCGTCGTT	162	98
PER2	ACGACAACACATACACCGAAT	GCACAGGCGTCTGGATC	100	92
PI15	CCACTGACTCATCCCCGCCAACC	GTTTGCTGCTGGTGGGAACACTTTGC	176	96
PRELP	TTCGGCTGAACTACAACAAGC	GATCTGGGTCCCGTTGATTTTCT	182	99
PTX3	CTGTGTGGGCGGTGGCTTTG	TCCATGTGGCTGAATCTCTGTGACTC	175	94
RASGRF1	GACTCTGACCCAGGAGGA	AGCCTTGTCCGAAGAACTC	185	93
RLBP1	CGTGCCAGGAAGTTCAA	TGAAGAGCATGACCACTC	184	93
RPE65	CACCCAGATGCCTTGGAAGAAGATGA	CTTCACTCAAGTCCTTGGCGTTCAGA	115	99
RXRB	CCTGACCTACTCGTGCC	TACCGCCTCCCTCTTCA	118	91
S100A12	GAGCAAGATGGAAGATCACATGGAGG	TGAGGGTGTTTGGAAGTTCCTTTGTT	134	100
SCUBE3	CCGTCCTCCATTACCACTTAC	CTCGCACGATGTCTTCTACCA	184	95
SERPINH1	ACAAGAACAAGGCAGAC	GCACCAGGAAGATGAAG	186	103
SOSTDC1	GCGGTCCACCAAGTACATCT	ATAGCCTCCTCCGATCCAGTTA	121	97
SST	CCAGACTCCGTCAGTTTCT	CCAGGGCATCGTTCTCA	120	93
TAC1	CGGAGCCCTTCGAGCATCTTCTG	CCCGTTTGCCCATTAATCCAAAGAATTGC	81	96
TGFB2	GCAGAGTTTAGGGTCTTTCGTTTG	CTCGTGAACAGCATCAGTTACATC	189	93
TGFB3	ATCACCATAACCCGCATCTAATCC	CGCACACAGCAGTTCTCCTC	139	90
TGFBI	CCTCGGCACTCATCTCTCC	GCAAATTCTTCATCTTGGCATCG	107	94
THBS1	CTGTCAGAACTCAGTCACCATC	CCACGGAGACCAGCCATC	136	91
THBS2	GAGACCGACTTCAGGAACTTC	CGAAACCCACTGCGATGC	142	100
TIMP2	AGATGGGCTGTGAATGC	CCGTCGCTTCTCTTGAT	151	96
TIMP3	CCGTGTCTATGATGGCAAGATG	ACAAAGCAAGGCAGGTAATAGC	153	99
TNC	AGACGCCAAGACTCGCTACAG	CAGGTTGACACGGTGACAGTTC	184	94
TNMD	GCTGGATGAGAGAGGTTATTGT	TGATGACACGACAGATGACC	137	100
VDR	AACTTGCACGAGGAGGAAC	CTTCTGGATCATCTTGGCATAG	189	93
VIP	AACGAGTGAGCACCAACATCTC	TCTTGACAGCCATTTGTTTCCTAAGG	108	94
VIPR1	TCCACGCTGCTGCTGATTC	CCCACGACCAGCTCAAAGAC	107	99
VIPR2	CCACGCACACCTCCTC	CACTTCCTCTTCAGCTCACT	127	90
ZNF185	GGAGGCGTCTGTACCTACTG	GCAAATCCCACACCTAAAGCAAT	108	97
POLR2A	CTACCAGCCCCAAGTATTC	GGTGAGTAAGTAGGAGACG	106	98

Table S1. Primers used: Sequences, amplicon sizes, and efficiencies

Relative gene expression was measured by qPCR on a StepOnePlus Real-Time PCR System using Power SYBR Green PCR Master Mix (both, Life Technologies). Reactions were performed in triplicate in a 15 µL volume containing 300 nM each primer and 0.4 µL cDNA template. Cycling parameters were the same for all assays: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 62°C for 60 sec. Single gene products were obtained for all reactions as assessed by melt curve analysis. Relative gene expression was calculated using the ΔΔCt method (Livak & Schmittgen, 2001) to first normalize the expression level of the target gene to that of the reference gene, and then to compare the relative expression of the target gene for treated vs. control eyes, treated vs. normal eyes, and control vs. normal eyes. The geometric group mean (for the 7 biological replicates) of these expression ratios was used to calculate the fold change in gene expression for each of the target genes.

Statistical Analysis

One-way analysis of variance (ANOVA; Statistica, Statsoft, Tulsa, OK) was used to compare control and normal eye refractive data across groups of animals; paired t-tests were used to determine if significant myopia (treated eye vs. control eye) or recovery had occurred. For gene expression data, paired t-tests were used to assess treated-eye vs. control-eye differences; unpaired t-tests were used to test for gene expression differences between all independent groups. In all cases, $p<0.05$ was considered significant and no adjustment was applied for possible false discovery rate. Linear regressions between expression differences were made in SigmaPlot (Systat Software, San Jose, CA).

RESULTS

Refraction

The final refractive values of the normal, minus-lens treated, control, and recovering eyes in the five groups are shown in Fig. 2. As expected in tree shrews at 26 DVE, both eyes of the normal group were slightly hyperopic (right eyes, 0.8 ± 0.4 D; left eyes, 1.3 ± 0.4 D; mean ± SEM). At 37 DVE both eyes of the normal group also were slightly hyperopic (right eyes, 1.1 ± 0.3 D; left eyes, 0.9 ± 0.3 D; mean ± SEM) as expected in juvenile animals of this age because the emmetropization process is nearly complete

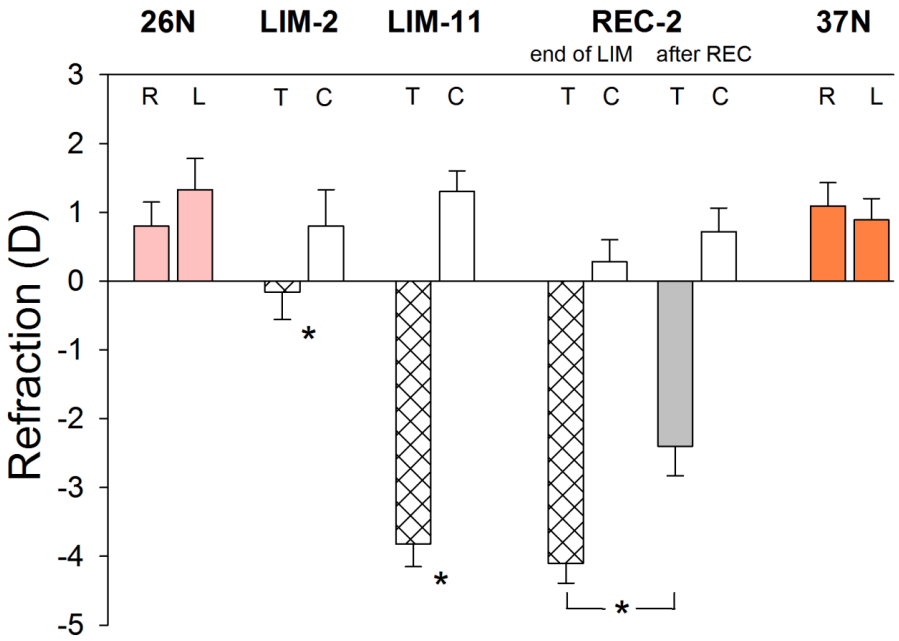

Figure 2. End-of-treatment refractive measures for the normal, minus lens (LIM-2 & LIM-11), and recovery (REC) groups. Values are the mean refraction ± SEM for the right (R) & left (L) eyes of the normal and for the treated (T) & control (C) eyes of the LIM and REC groups. Treated eyes in all groups were significantly different relative to control eyes. Treated eyes in the REC-2 group showed significant recovery from the refractions measured at the end of LIM (bracket).

(Norton et al., 2006a). After two days of treatment, the LIM-2 treated eyes showed a small, statistically-significant myopic shift; the treated eyes were −1.0 ± 0.2 D myopic in comparison to the control eyes. After 11 days of treatment, the treated eyes in the LIM-11 group achieved complete compensation (−5.1 ± 0.2 D, treated eyes – control eyes). Fig. 3 shows the time-course of the compensation for the minus lens. As a group, refractions in the treated eyes, while wearing the lens, achieved and maintained a refractive match with their fellow control eyes for 5 days prior to the final measurements. The REC-2 group also had achieved complete refractive compensation (Fig. 2) at the end of lens wear. After 2 days of recovery from lens compensation, the treated eyes recovered by 1.3 ± 0.3 D.

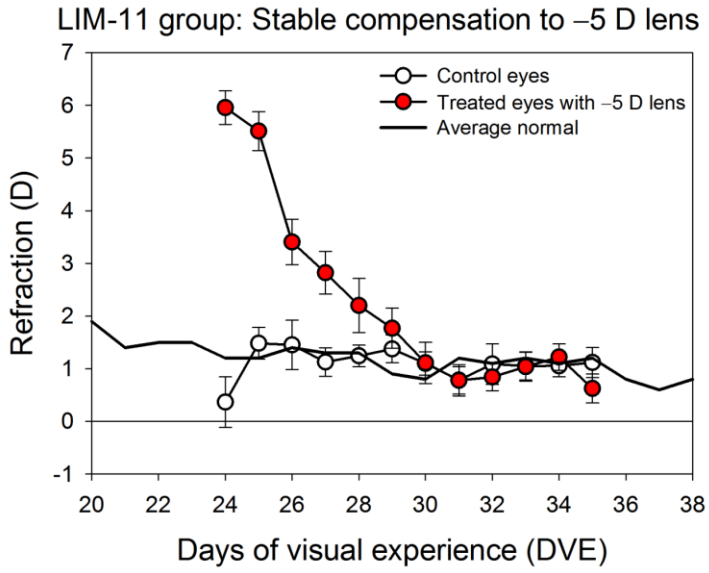

Figure 3. Daily refractive measurements of the treated and control eyes of the LIM-11 group showing that the lens-induced refractive hyperopia dissipated as the eyes compensated for the −5 D lens. Measurements of the treated eyes were taken while the −5 D lens was in place, providing a measure of the refractive state experienced by the treated eyes during treatment. The low control eye value at the beginning of treatment was caused by a low refraction in a single animal, perhaps from accommodation.

The control eyes in the LIM-2, LIM-11, and REC-2 groups did not differ significantly from the 26 DVE and 37 DVE normal eyes (one-way ANOVA, $p = 0.56$). Ocular component dimensions, measured with the Lenstar in the LIM-2 and LIM-11 groups confirmed that the vitreous chamber of the treated eyes had elongated, relative to the control eyes, by 0.016 ± 0.004 mm (LIM-2) and by 0.11 ± 0.01 mm (LIM-11). In the LIM-11 group the choroid thickness, measured with the Lenstar, was slightly, but significantly, thinner (0.058 ± 0.002 vs. 0.064 ± 0.004 mm) than in the control eyes.

Gene Expression

Normal Animals

The mRNA levels in normal eyes, and the variability between normal left and right eyes, can provide a basis for comparison with the levels and variability found in treated and control eyes. Figs. 4A and 4B compare gene expression in the right and left eyes of the 26N and 37N groups. Group expression values for all 77 genes are provided in Table 2. Although considerable variability was occasionally seen for some genes between left- and right-eye mRNA levels in individual animals, for both the 26N and 37N groups, expression levels did not differ significantly between left and right eyes for any of the 77 genes.

As expected in juvenile animals whose eyes are growing more slowly with increasing age, significant differences in the expression of some genes was found between the 26N and 37N groups. Expression levels were lower in the 37N group for 10 genes; five genes were up-regulated. One gene (RLBP1; retinaldehyde binding protein 1) showed a dramatic down-regulation (−13.98 fold). NOS1 (nitric oxide synthase 1)

mRNA levels were −4.07 fold lower in the older normal group. All remaining differences were less than 2-fold.

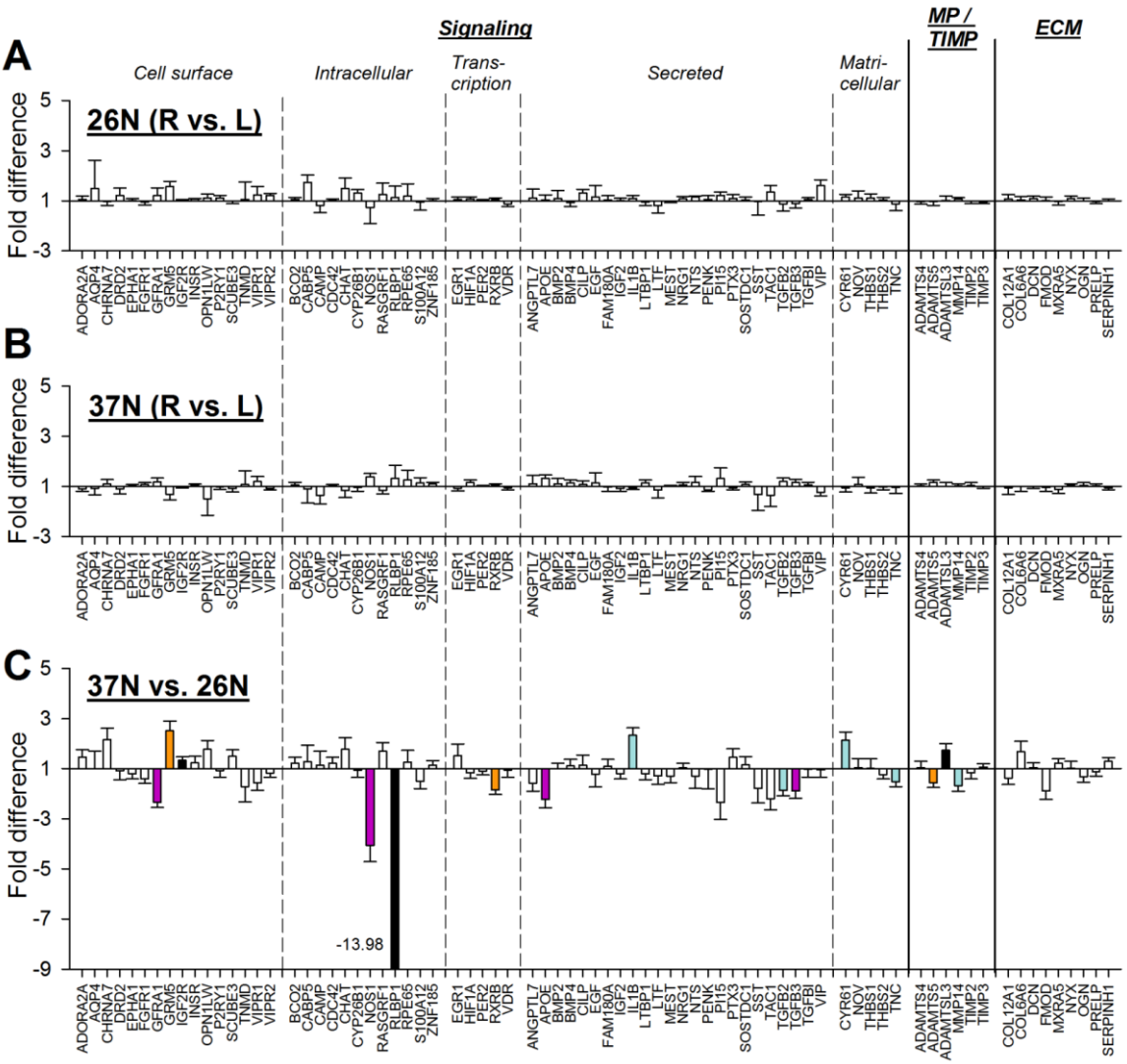

Figure 4. Gene expression fold differences. (A) Normal eyes (right eyes vs. left eyes) in the 26N group. (B) Normal eyes (right eyes vs. left eyes) in the 37N group. (C) Comparison of expression levels at 26N and 37N; down-regulation indicates lower expression in the 37N group. Headings separated by vertical dashed lines indicate functional grouping of the protein products of the genes. Filled bars represent statistically significant differences between the left and right eyes or between the two age groups ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the three different conditions. Error bars indicate SEM. In C, the off-scale fold difference for RLBP1 (−13.98) is indicated next to the bar.

	26 Normal	37 Normal	37N vs. 26N	LIM-2			LIM-11			REC-2		
	RE vs. LE	*RE vs. LE*		*T vs. C*	*T vs. N*	*C vs. N*	*T vs. C*	*T vs. N*	*C vs. N*	*T vs. C*	*T vs. N*	*C vs. N*
Signaling – Cell surface												
ADORA2A	1.06	-1.10	1.46	-1.24	-1.88	-1.52	1.03	1.39	1.36	-1.03	**-2.00**	**-1.94**
AQP4	1.50	-1.08	1.00	1.46	1.65	1.12	-1.48	**-4.20**	-2.84	1.93	-1.11	-2.14
CHRNA7	-1.03	1.09	2.15	1.15	1.42	1.23	1.19	1.26	1.06	1.28	-1.57	**-2.02**
DRD2	1.21	-1.11	-1.09	1.68	1.70	1.01	1.19	-1.03	-1.22	1.95	2.17	1.11
EPHA1	1.01	1.02	-1.20	**-1.55**	**-1.75**	-1.13	**-1.37 x**	1.07	**1.46**	**1.77**	**2.08**	1.18
FGFR1	-1.05	1.07	-1.41	-1.68	-1.87	-1.11	-1.00	**1.42**	**1.42**	**1.22**	1.33	1.10
GFRA1	1.22	1.18	**-2.34**	-1.12	-1.18	-1.05	1.15	-1.03	-1.18	1.48	**2.39**	1.62
GRM5	1.57	-1.32	**2.51**	1.34	1.50	1.12	1.29	-2.13	**-2.74**	2.67	1.02	**-2.61**
IGF2R	1.01	-1.02	**1.33**	-1.16	1.01	1.17	1.05	1.02	-1.03	-1.06	**-1.63**	**-1.53**
INSR	1.07	1.05	1.23	-1.49	-1.76	-1.18	1.04	1.16	1.11	1.01	**-1.93**	**-1.96**
OPN1LW	1.12	-1.50	1.77	1.13	1.07	-1.06	1.04	**-3.74**	**-3.90**	1.75	1.17	-1.49
P2RY1	1.12	-1.02	-1.08	**1.29**	-1.24	-1.60	**1.22**	1.70	1.39	-1.16	**-1.53**	-1.32
SCUBE3	-1.00	-1.09	1.49	**-1.62**	-1.28	1.27	**-1.31 x**	1.29	**1.69**	**1.61**	1.25	-1.29
TNMD	1.06	1.07	-1.72	-1.27	-2.23	-1.76	-1.42	-1.06	1.34	1.37	1.13	-1.21
VIPR1	1.24	1.19	-1.57	1.11	1.03	-1.07	-1.01	-1.75	**-1.74**	1.48	1.48	1.00
VIPR2	1.22	-1.08	-1.18	-1.55	-1.63	-1.05	-1.08	1.17	1.25	1.13	1.22	1.08
Signaling – Intracellular												
BCO2	1.06	1.06	1.21	**-1.48**	-1.33	1.11	-1.26	-1.05	1.20	**1.53**	**1.70**	1.11
CABP5	1.74	-1.10	1.28	2.14	2.14	1.00	-1.01	-1.71	-1.70	3.05	2.62	-1.16
CAMP	-1.19	-1.36	1.13	2.25	4.00	1.78	1.05	-1.73	**-1.81**	-1.30	1.09	1.42
CDC42	1.02	1.03	1.21	-1.25	-1.46	-1.16	1.05	1.02	-1.02	-1.05	-1.60	-1.52
CHAT	1.49	-1.17	1.77	1.22	1.70	1.39	-1.31	-1.61	-1.23	**3.37**	1.78	-1.89
CYP26B1	1.31	-1.04	-1.06	**-4.05**	**-3.28**	1.24	**-1.60**	-1.06	1.51	1.43	1.11	-1.29
NOS1	-1.27	1.37	**-4.07**	1.53	-1.62	-2.46	1.03	-1.89	-1.96	3.34	4.35	1.30
RASGRF1	1.26	-1.17	1.69	1.06	1.43	1.34	-1.03	1.08	1.12	2.20	1.28	-1.72
RLBP1	1.14	1.31	**-13.98**	**2.76 x**	-1.22	**-3.37**	**3.79**	1.83	-2.07	**3.58**	**23.43**	**6.55**
RPE65	1.20	1.26	1.26	**2.28**	1.59	-1.43	**6.36 x**	1.02	**-6.23**	3.64	1.84	-1.98
S100A12	-1.04	1.13	-1.50	1.62	2.34	1.45	1.20	**2.04**	1.71	-1.83	1.21	2.22
ZNF185	1.01	1.09	1.13	**-1.35**	**-1.62**	-1.20	-1.12	-1.03	1.08	**1.37**	-1.07	-1.47
Signaling – Transcription												
EGR1	1.05	-1.07	1.52	-1.33	-1.42	-1.07	-1.14	**-4.28**	**-3.76**	-1.05	-1.17	-1.12
HIF1A	1.07	1.15	-1.17	**1.20**	1.07	-1.12	1.05	**1.54**	**1.47**	1.15	-1.00	-1.15
PER2	1.02	1.01	-1.10	-1.35	-1.36	-1.01	1.06	1.14	1.07	1.04	-1.06	-1.10
RXRB	1.05	1.06	**-1.85**	-1.22	-1.28	-1.05	1.00	1.11	1.11	-1.08	**1.56**	**1.68**
VDR	-1.12	-1.06	-1.06	-1.69	-1.86	-1.10	-1.09	1.19	1.30	-1.12	-1.33	-1.18
Signaling – Secreted												
ANGPTL7	1.11	1.10	-1.58	1.27	1.17	-1.09	**1.40**	**2.44**	1.74	1.15	1.19	1.04
APOE	1.04	1.32	**-2.23**	1.49	**2.83**	**1.90**	1.14	1.07	-1.06	1.13	2.22	**1.98**
BMP2	1.09	1.11	1.00	**1.54**	1.18	-1.30	**1.93 x**	1.10	**-1.75**	**2.16 x**	1.12	**-1.92**
BMP4	-1.08	1.14	1.11	**1.48**	1.48	1.00	**1.58**	**1.90**	1.20	1.18	-1.21	-1.43
CILP	1.31	1.07	1.14	**-3.00**	**-2.52**	1.19	**-1.57**	1.19	1.86	1.17	-1.10	-1.29
EGF	1.16	1.13	-1.23	2.09	1.38	-1.51	**6.83 x**	1.19	**-5.73**	4.06	1.98	-2.06
FAM180A	1.03	-1.03	1.09	**-1.60**	-1.58	1.01	-1.22	1.17	1.43	**1.96**	1.27	-1.54
IGF2	1.01	-1.09	-1.20	**1.24**	1.44	1.16	**1.21**	**1.45**	1.20	**-1.38**	-1.14	1.20
IL1B	1.10	-1.06	**2.33**	1.05	1.16	1.11	-1.03	1.12	1.16	-1.28	**-3.26**	**-2.54**
LTBP1	-1.05	1.14	-1.21	1.03	-1.15	-1.19	1.08	1.28	1.19	1.23	-1.02	-1.25
LTF	-1.19	-1.15	-1.29	1.36	2.20	1.62	1.26	1.07	-1.18	-1.24	1.12	1.39
MEST	-1.02	1.00	-1.30	**-1.64**	-1.55	1.06	**-1.45**	1.26	1.83	**1.88**	**3.03**	1.62
NRG1	1.09	1.06	1.03	**1.47**	**1.55**	1.06	**1.54**	**2.34**	1.52	**-1.87**	-1.36	1.37
NTS	1.15	1.16	-1.30	1.41	1.03	-1.36	1.30	1.66	1.27	2.36	2.35	-1.00
PENK	1.06	-1.14	-1.02	**-1.73**	-1.90	-1.09	**-1.57**	1.13	1.77	-1.28	1.05	1.35
PI15	1.22	1.32	-2.35	**3.41**	**2.87**	-1.19	**4.18**	**10.83**	2.59	**-5.73**	**-6.52**	-1.14
PTX3	1.10	-1.06	1.45	**-3.48**	**-2.25**	1.55	**-2.21**	-1.65	1.34	1.53	1.39	-1.10
SOSTDC1	1.04	1.07	1.15	**-1.70**	**-2.22**	-1.31	-1.39	-1.07	1.30	**2.06**	1.62	-1.27
SST	-1.02	-1.33	-1.79	-1.45	-1.17	1.25	-1.16	-1.67	-1.44	2.92	3.67	1.26
TAC1	1.35	-1.36	-2.20	1.22	1.28	1.05	-1.25	1.48	1.85	1.59	**2.76**	**1.73**
TGFB2	-1.12	1.19	**-1.87**	-1.03	**-1.72**	-1.67	-1.02	-1.17	-1.15	**1.51**	1.60	1.05
TGFB3	-1.10	1.16	**-1.89**	-1.07	1.03	1.10	-1.11	1.39	1.54	**1.61**	**2.60**	1.61
TGFBI	1.06	1.06	-1.03	**1.46**	1.48	1.02	**1.32**	-1.11	-1.46	-1.21	-1.44	-1.19
VIP	1.62	-1.24	-1.05	1.03	-1.10	-1.13	-1.02	1.00	1.02	1.27	-1.14	-1.45
Signaling – Matricellular												
CYR61	1.15	-1.06	**2.14**	**-1.63**	-1.20	1.35	-1.09	**-2.06**	-1.89	1.43	1.29	-1.10
NOV	1.11	1.07	1.03	**-2.14**	**-2.75**	-1.28	-1.55	1.18	1.84	**1.37**	-1.06	-1.45
THBS1	1.11	-1.06	1.01	**-2.97**	**-3.08**	-1.04	**-1.59**	-1.10	1.45	1.11	-1.05	-1.17
THBS2	1.04	-1.03	-1.24	-1.98	-2.25	-1.14	-1.03	1.08	1.11	1.00	1.03	1.03
TNC	-1.12	-1.04	**-1.52**	-1.24	1.21	1.49	1.17	1.46	1.24	**1.44**	1.78	1.24
MP / TIMP												
ADAMTS4	-1.03	1.02	1.04	-1.33	1.08	1.44	1.04	1.30	1.25	-1.07	1.06	1.13
ADAMTS5	-1.03	1.16	**-1.56**	-1.06	-1.20	-1.13	**1.26**	1.29	1.02	1.15	-1.01	-1.16
ADAMTSL3	1.02	1.00	**1.73**	**-1.99**	-1.20	1.65	**-1.35**	1.17	1.57	**1.60**	1.44	-1.11
MMP14	1.07	1.01	**-1.68**	1.14	1.05	-1.09	1.07	**1.73**	**1.62**	-1.06	1.27	1.35
TIMP2	-1.01	1.03	-1.17	-1.42	-1.29	1.09	1.07	1.50	1.40	-1.04	-1.03	1.01
TIMP3	-1.05	-1.00	1.06	**-1.28**	**-1.53**	-1.20	-1.09	1.11	1.21	1.04	-1.18	-1.23
Extracellular matrix												
COL12A1	1.08	-1.06	-1.38	-1.90	**-1.97**	-1.04	**-1.25**	**1.63**	**2.03**	1.22	1.18	-1.03
COL6A6	1.04	-1.00	1.68	**-1.83**	-1.35	1.35	-1.25	1.61	**2.01**	**2.75**	2.01	-1.37
DCN	1.09	-1.00	1.04	1.07	1.08	1.00	1.11	**1.80**	**1.62**	-1.13	-1.20	-1.07
FMOD	1.01	-1.05	-1.89	-1.19	**-1.82**	-1.52	-1.03	-1.00	1.03	1.04	1.57	1.52
MXRA5	-1.03	-1.12	1.21	-1.18	-1.04	1.13	1.10	**2.05**	**1.87**	**1.62**	1.26	-1.29
NYX	1.09	1.04	1.01	-1.09	-1.26	-1.16	-1.02	1.38	1.41	**-1.28**	-1.58	-1.24
OGN	1.00	1.05	-1.33	**-1.32**	**-1.70**	-1.29	-1.13	1.30	1.47	1.14	1.15	1.00
PRELP	-1.02	1.01	-1.13	-1.08	-1.29	-1.20	1.08	1.31	1.22	-1.13	-1.04	1.09
SERPINH1	1.00	-1.06	1.29	-1.12	-1.09	1.03	-1.01	1.19	1.20	-1.01	**-1.52**	**-1.51**

Table 2. Gene expression differences comparing treated vs. control, treated vs. normal, and control vs. normal eyes. Red text = significant down-regulation, blue = significant up-regulation, grey = expression difference not statistically significant, "X" = differential expression likely due to control eye effect.

Two-day Minus Lens Treatments (GO)

Two days of −5 D lens wear (LIM-2) produced significant differences in mRNA expression levels for 28 genes in the treated vs. control eyes (Fig. 5A); expression values are listed in Table 2. Eighteen of the 28 differentially expressed genes were down-regulated, while the other 10 were up-regulated. As may be seen by examining Figs. 5B and 5C, the significant up-regulation of one gene (RLBP1) occurred because the control eye mRNA levels were significantly reduced relative to age-matched normal eyes (26N; mean of right and left eye values).

Previous studies of mRNA and protein levels in tree shrew sclera have found alterations in expression levels not only in the treated eyes, but also in the untreated fellow control eyes when compared with age-matched normal eyes (Frost & Norton, 2012; Gao et al., 2011). Because (unpaired) comparisons of expression between groups of animals are less sensitive than (paired) comparisons between the two eyes within an animal, fewer significant differences are typically detected. In the treated vs. normal comparison (Fig. 5B), 16 genes differed significantly in their expression levels. When comparing control eyes with the 26N group (C vs. N), mRNA levels for 2 genes (RLBP1, down-regulated; APOE [apolipoprotein E], up-regulated) were significantly different from normal levels (Fig. 5C).

When mRNA levels in treated eyes were compared with normal eyes (Fig. 5B), significant differences were found that did not entirely match the differences found between treated and control eyes (Fig. 5A). In three instances (COL12A1 [collagen type XII, α1], FMOD [fibromodulin], and TGFB2 [transforming growth factor β2]), the treated eyes were significantly different from normal eyes but the treated vs. control eye dif-

ferences were not significant. The reason is that the control eye mRNA levels also were different from the normal eyes in the same direction as the treated eyes. For a fourth gene, APOE, both the control eye and treated eye mRNA levels were significantly different from normal so that the treated vs. control eye difference was very small and was not statistically significant.

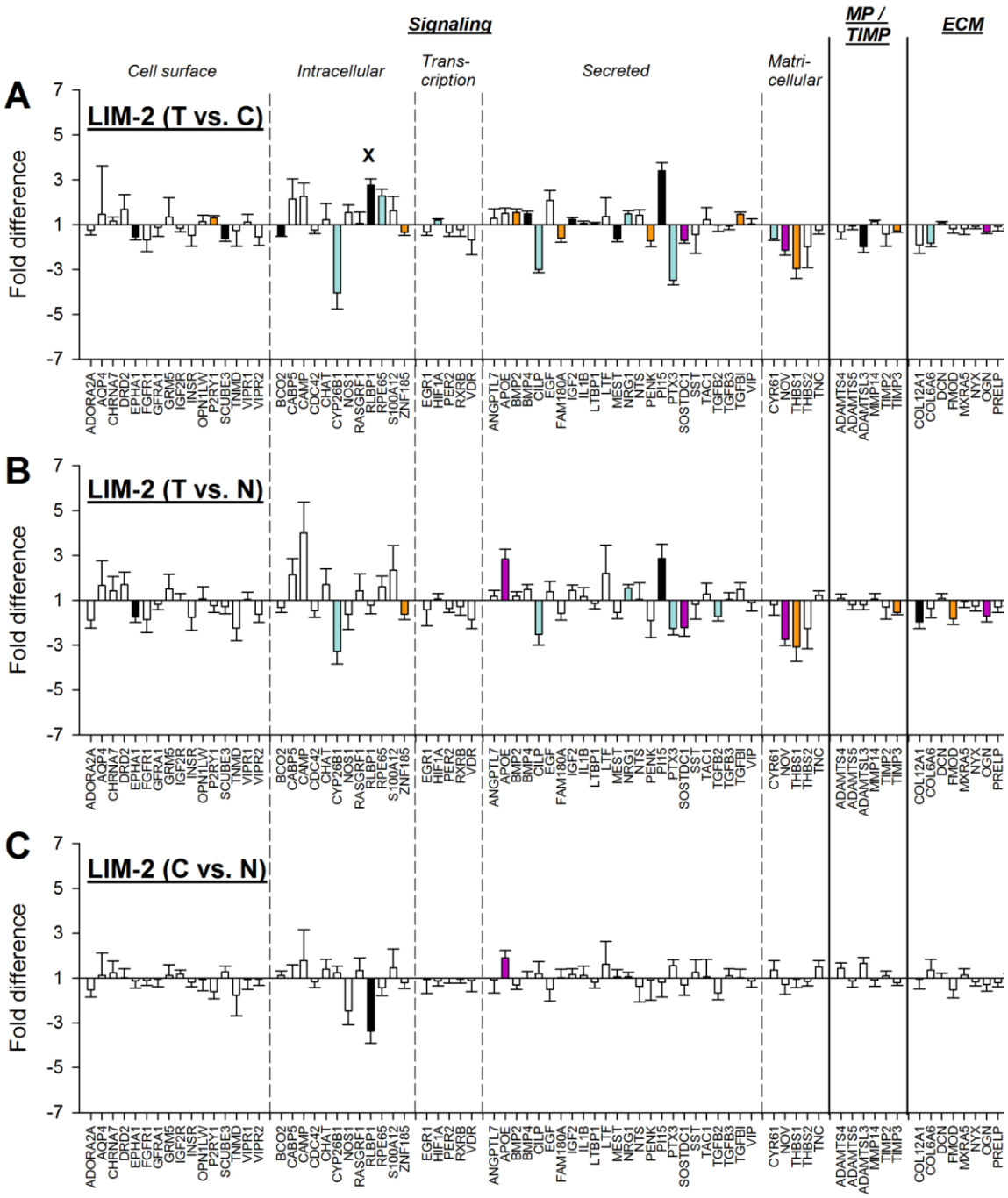

Figure 5. Gene expression fold differences. (A) Treated eyes vs. control eyes after 2 days of minus-lens wear. (B) Treated eyes vs. normal (26N) eyes. (C) Control eyes vs. normal (26N) eyes. Symbols as in Figure 4. In A, the "x" indicates that the significant treated-eye vs. control-eye up-regulation of RLBP1 was produced by decreased mRNA expression in the control eyes (shown in C).

Eleven-day Minus Lens Treatments (STAY)

Although the treated eyes of each animal in the LIM-11 group had refractively compensated fully for the −5 D lens after 11 days of lens wear, significant differences in mRNA expression were found between treated vs. control eyes (Fig. 6A), and also between treated vs. normal (Fig. 6B) and control vs. normal eyes (Fig. 6C). Expression values are listed in Table 2. mRNA levels for 22 genes were significantly different between treated eyes and control eyes. Ten were down-regulated, and the other 12 were up-regulated. Five of these (BMP2 [bone morphogenetic protein 2], EGF [epidermal growth factor], EPHA1 [EPH receptor A1], RPE65 [retinoid isomerohydrolase], and SCUBE3 [signal peptide, CUB and EGF-like domain-containing protein 3]) were significant because the control eye mRNA levels were significantly affected (compared with normal) while the treated eye levels were not.

As shown in Table 2 and in Figs. 6B and 6C, numerous significant differences in gene expression were found both between the treated eyes compared with 37N eyes and between control eyes compared with 37N eyes. mRNA levels of 16 genes differed between treated and normal eyes (Fig. 6B) and 17 genes were significantly different between control eyes and normal eyes (Fig. 6C). Potential reasons for the control eye gene expression to differ from normal will be presented in the Discussion. As in the LIM-2 group, some of the genes in which the treated eye mRNA levels were significantly different from normal were not found to be significantly different when treated eyes were compared with control eyes. This was because treated and control eyes both differed from normal in the same direction.

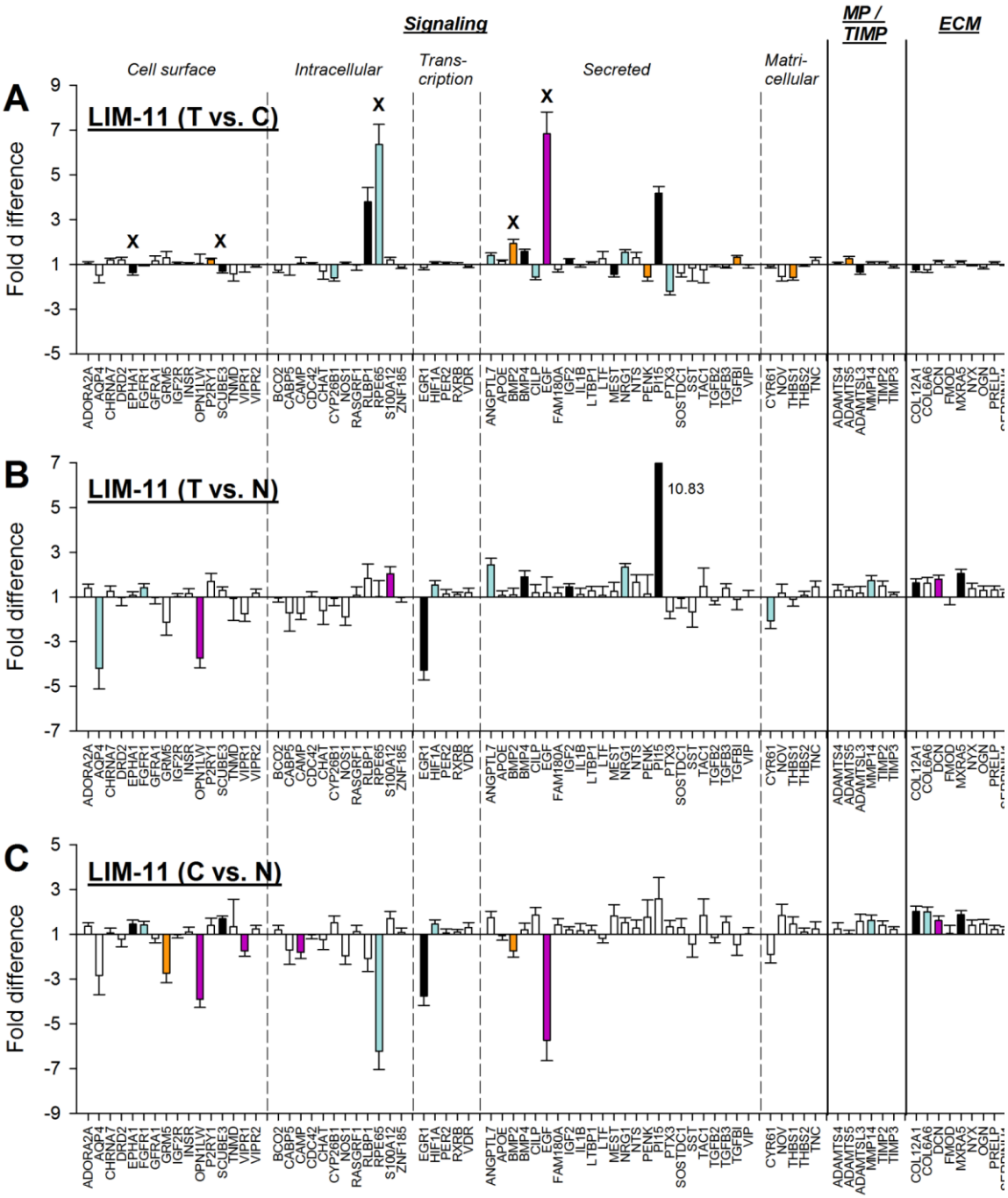

Figure 6. Gene expression fold differences. (A) Treated eyes vs. control eyes after 11 days of minus-lens wear. (B) Treated eyes vs. normal (37N) eyes. (C) Control eyes vs. normal (37N) eyes. Symbols as in Figure 4. In A, the "x" indicates genes in which the significant treated-eye vs. control-eye differences for five genes (EPHA1, SCUBE3, RPE65, BMP2, and EGF) were produced by altered mRNA expression in the control eyes. In B, the off-scale fold difference for PI15 (10.83) is indicated next to the bar.

Comparison between GO and STAY Patterns

The similarities in the mRNA patterns between the LIM-2 group and LIM-11 group are of interest. After 2 days of minus-lens wear, retinally-derived signals must be passing through the choroid to produce the scleral remodeling that results in axial elongation. After 11 days of minus-lens wear, the treated eyes have completed their compensation and are maintaining an elongated state. mRNA differences that are unique to the LIM-2 group may be involved in actively promoting elongation (a GO signal) whereas mRNA differences that are unique to the LIM-11 group may be involved in maintaining the elongated state (a STAY signal). Shared mRNA differences may be involved in both GO and STAY. Fig. 7 compares the significant treated vs. control eye differences at LIM-2 with those at LIM-11. Eighteen genes had significantly different mRNA levels in the treated vs. control eyes at both LIM-2 and LIM-11. All were regulated in the same direction in both conditions; nine were down-regulated in both conditions and nine were up-regulated at both LIM-2 and LIM-11. The correlation was significant ($p < 0.05$) with a slope of 0.88 and $r^2 = 0.58$. Although the direction of the shared differential expression was the same for these genes, the amplitude (fold differences) differed for several genes. Four genes labeled in Fig. 7 (CILP [cartilage intermediate layer protein 1], CYP26B1 [cytochrome P450 26B1], PTX3 [pentraxin 3], and THBS1 [thrombospondin 1]) were more strongly down-regulated in the LIM-2 group than in the LIM-11 group. Three additional genes (PI15 [peptidase inhibitor 15], RLBP1, and RPE65), also labeled in Fig. 7, were more strongly up-regulated in the LIM-11 group than in the LIM-2 group. In addition, there were 10 genes that were significantly affected in LIM-2 but not in LIM-11, and 4 genes that were significantly regulated only in the LIM-11 group.

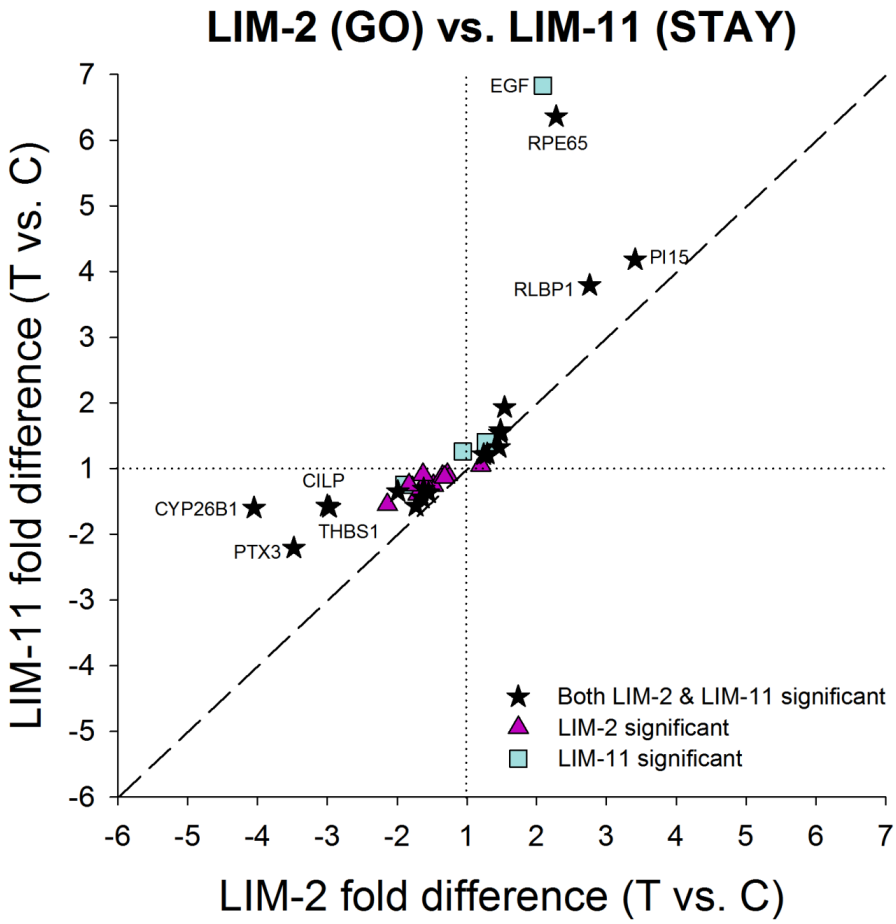

Figure 7. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 5A (LIM-2) with the differences in Fig. 6A (LIM-11) showing the similar differential expression patterns in GO and STAY. All genes that were up- or down-regulated in LIM-2 were regulated in the same direction at LIM-11. Highly-regulated genes are labeled. Values near the dashed line indicate genes that responded similarly in the two conditions. Stars = significant fold differences for both LIM-2 and LIM-11; triangles = significant fold differences only for LIM-2; squares = significant fold differences only for LIM-11.

Two-day Recovery (STOP)

As shown in Fig. 2, the treated eyes of the REC-2 group had begun to recover from the full compensation produced by 11 days of −5 D lens wear. The mRNA expression pattern at REC-2 presumably reflects STOP signals present in the choroid at this time point. Fig. 8A shows the fold differences in gene expression between the treated and control eyes in the REC-2 group. Expression values are also listed in Table 2. Twenty-two genes were significantly different in treated eyes compared with control eyes, 18 were up-regulated, and the other 4 were down-regulated. One of the up-regulated genes (BMP2) was significant because the control eye mRNA levels were significantly down-regulated (compared with normal) while the treated eye levels were not.

When comparing treated eyes with age-matched normal eyes in the 37N group (T vs. N), there were 15 genes significantly different (Fig. 8B). Comparing control eyes with age-matched normal eyes in 37N group (C vs. N), there were 12 genes significantly different (Fig. 8C). As in the LIM-2 and LIM-11 groups, some of the genes in the REC-2 group in which the treated eye mRNA levels were significantly different from normal were not found to be significantly different when treated eyes were compared with control eyes. This was because treated and control eyes both differed from normal in the same direction.

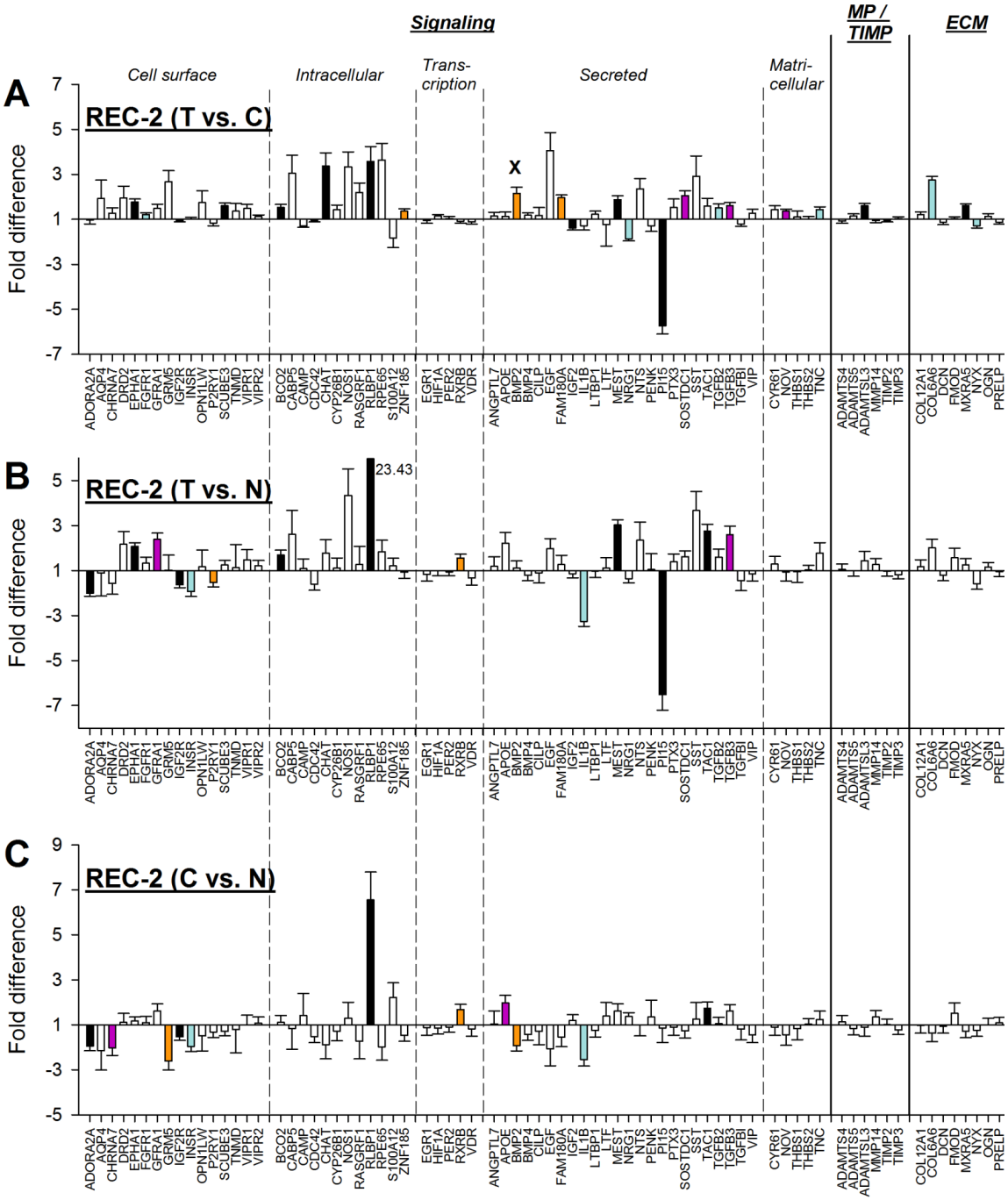

*Figure 8. Gene expression fold differences. (**A**) Treated eyes vs. control eyes after 2 days of recovery from full compensation to minus-lens wear. (**B**) Treated eyes vs. normal (37N) eyes. (**C**) Control eyes vs. normal (37N) eyes. Symbols as in Figure 4. In **A**, the "x" indicates that the significant treated-eye vs. control-eye up-regulation of one gene (BMP2) was produced by decreased mRNA expression in the control eyes. In **B**, the off-scale fold difference for RLBP1 (23.43) is indicated next to the bar.*

Comparison of GO and STOP Patterns

The differences in the mRNA patterns between the LIM-2 group (GO) and REC-2 group (STOP) are of interest. As shown in Fig. 9, the pattern is very different from that in Fig. 7 in that the genes were generally regulated in opposite directions in STOP than they were in GO. Most of the 35 genes that showed significant regulation in one or both conditions either were down-regulated in LIM and up-regulated in REC (top left quadrant of Fig. 9) or the reverse (bottom right quadrant of Fig. 9). Thirteen genes showed bi-directional regulation; 20 additional genes showed significant differences in either LIM or REC, but not both; 2 genes (BMP2 and RLBP1) were up-regulated under both, but regulation of RLBP1 in LIM-2 and BMP2 in REC-2 are likely caused by a control-eye effect.

Comparison of STAY and STOP Patterns

A final relevant comparison is between the treated-eye vs. control-eye differences at LIM-11 (STAY) and those present at REC-2 (STOP); this comparison is shown in Fig. 10. Not surprisingly, the pattern more closely resembles that seen comparing GO and STOP (Fig. 9) than that in Fig. 7, comparing GO vs. STAY. Seven genes showed significant bi-directional regulation, 13 genes were significantly regulated under LIM-11 but not REC-2, 13 other genes were significantly regulated under REC-2 but not LIM-11, and two genes (BMP2 and RLBP1) were significantly up-regulated under both LIM-11 and REC-2.

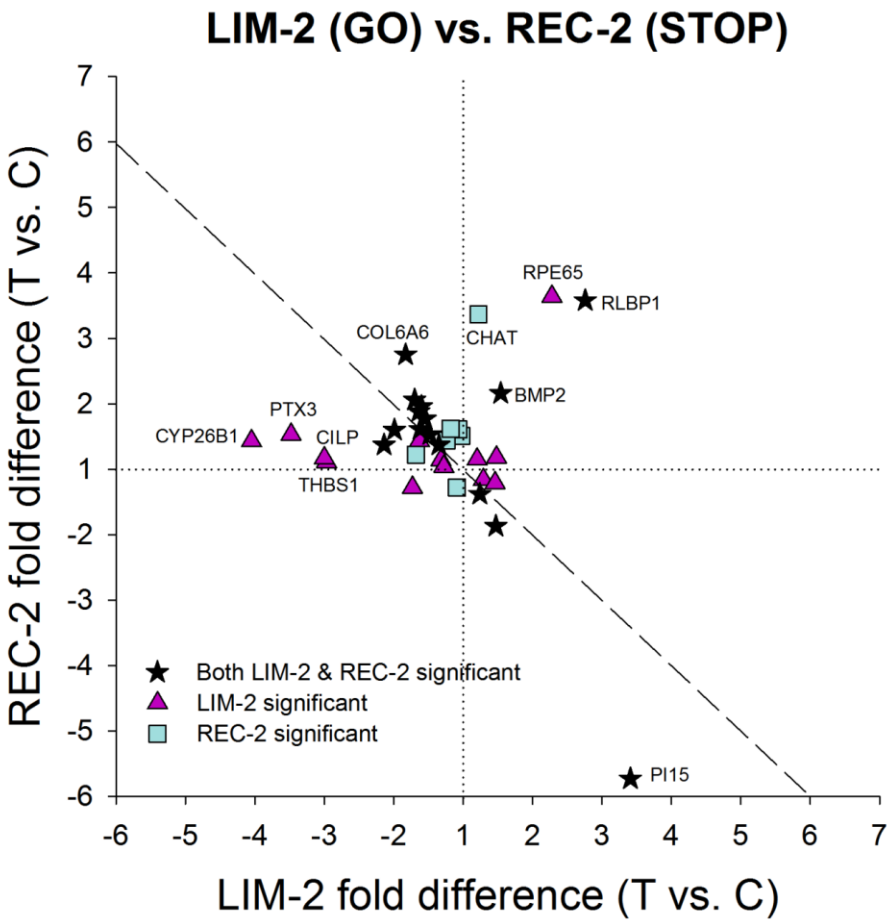

Figure 9. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 5A (LIM-2) with the differences in Fig. 8A (REC-2), showing that the gene expression patterns in GO and STOP were very different. Highly-regulated genes are labeled. Values near the dashed line indicate genes that responded with opposite fold-differences in the two conditions. Stars = significant fold differences for both LIM-2 and REC-2; triangles = significant fold differences only for LIM-2; squares = significant fold differences only for REC-2.

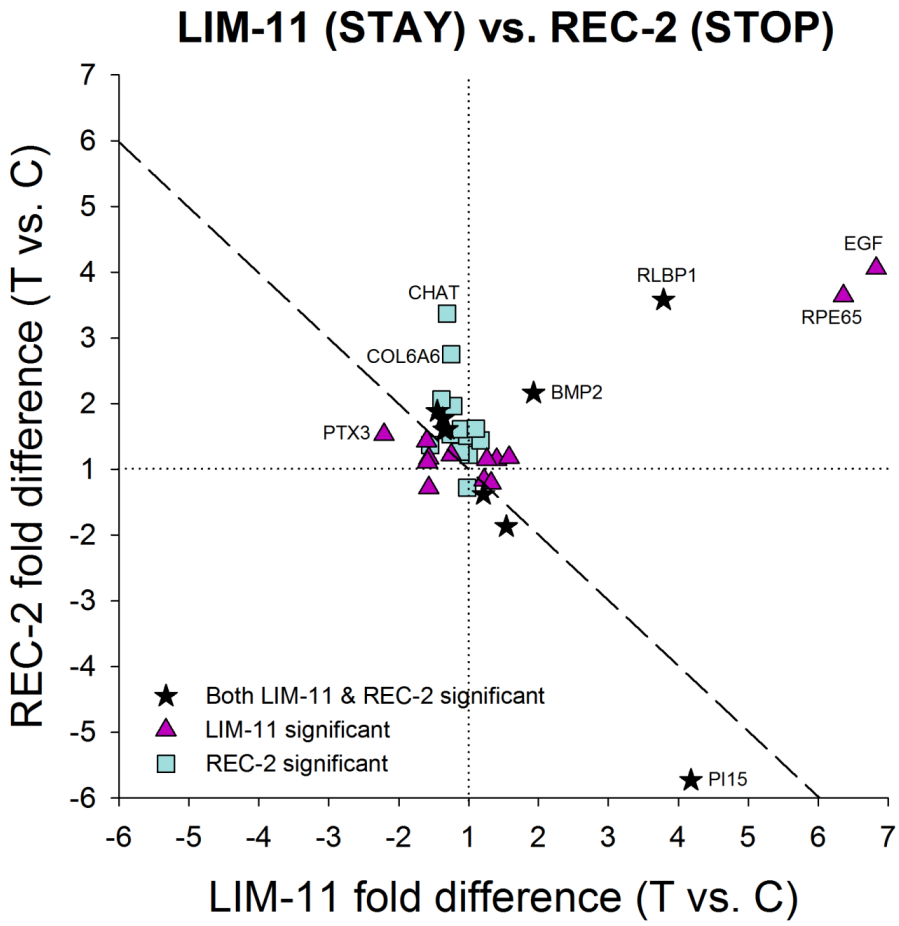

Figure 10. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 6A (LIM-11) with the differences in Fig. 8A (REC-2) showing that the gene expression patterns in STAY and STOP were different. Highly-regulated genes are labeled. Values near the dashed line indicate genes that responded with opposite fold-differences in the two conditions. This correlation was significant ($p = 0.02$; slope $= -0.55$; $r^2 = 0.17$). Stars = significant fold differences for both LIM-11 and REC-2; triangles = significant fold differences only for LIM-11; squares = significant fold differences only for REC-2.

DISCUSSION

Gene Expression Signatures

In addition to its important role as the vascular supply to the RPE and outer retina, the choroid participates in the emmetropization mechanism (Nickla & Wallman, 2010; Summers, 2013). It receives, presumably from RPE, unknown molecules that initiate responses in the choroid that, in turn, generate signals which pass to the sclera where they produce scleral remodeling and regulate the rate of axial elongation in juvenile eyes (Wallman & Winawer, 2004; Norton, Metlapally, & Young, 2008). We examined differential mRNA expression by choroidal cells 1) during normal development; 2) early in the development of LIM, a GO condition in which the eye is increasing its axial elongation; 3) after completing compensation to a minus lens, a STAY condition where the eye remains elongated until lens-wear is discontinued; and 4) early in the recovery process, a STOP condition in which the axial elongation rate slows to produce refractive recovery. We have found that distinct and unique constellations of differential mRNA expression occur amongst the sampled genes in each of these conditions as the cells in the choroid receive, integrate, and generate emmetropization-related signals that are transmitted to the sclera. Sixty of the 77 genes examined in the choroid showed significant mRNA expression differences during normal development, GO, STAY, or STOP conditions, including not only (treated eye vs. control eye) differential expression, but also expression differences between treated or control eyes relative to age-matched normal eyes, and as a function of age in normal development. Not only are the mRNA expression patterns different in each condition, only one of the genes examined (RLBP1) was differentially expressed in all of the conditions (normal growth, GO, STAY, and STOP). Thus, there is selective regula-

tion with gene expression being altered uniquely in each condition. Table 3 lists the genes whose expression differs, exhibiting up- or down-regulation between the treated vs. control eyes in the GO, STAY, and STOP conditions. The patterns of gene expression are unique to each condition, constituting what may be considered gene expression "signatures".

Although the concept of GO and STOP signals was first raised many years ago (Schaeffel & Howland, 1991; Rohrer & Stell, 1994), it has not been clear if they are separate signals, or even if there are two signals. Potentially, STOP could be the absence of GO, or vice versa. The gene expression differences in choroid suggest that there may, indeed, be two different signals at the level of the choroid, one for GO and a different one for STOP; some genes may participate in both, showing bi-directional regulation that is opposite in STOP than it is in GO. Comparing LIM-2 (GO) and REC-2 (STOP), there were 13 bi-directionally regulated genes (Table 3). In addition to the bi-directionally regulated genes, there are others that were significantly regulated in GO but not STOP and vice versa, suggesting that there may be a "core" of genes that act in a push-pull manner and others that participate only in GO or STOP.

The present study also suggests the presence of a STAY signature in the choroid that resembles, but is distinct from, the GO signature (Fig. 7). As shown in Fig. 3, the refractions of the treated eyes in the LIM-11 group, measured with the –5 D lens in place, were no longer hyperopic and, as a group, had been within 1 D of their fellow control eyes for five days. The refractive hyperopia that generated retinal GO signals and stimulated the emmetropization mechanism to produce the elongation had dissipated. The treated eyes, however, were elongated compared to their untreated fellow control eyes. In

Functional category		GO (LIM-2)	STAY (LIM-11)	STOP (REC-2)
Signaling - Cell surface	B	EPHA1	EPHA1 x	EPHA1
	B	SCUBE3	SCUBE3 x	SCUBE3
		P2RY1	P2RY1	
				FGFR1
Signaling - Intracellular	B	BCO2		BCO2
	B	ZNF185		ZNF185
		CYP26B1	CYP26B1	
		RLBP1 x	RLBP1	RLBP1
		RPE65	RPE65 x	
				CHAT
Signaling - Transcription		HIF1A		
Signaling - Secreted	B	IGF2	IGF2	IGF2
	B	NRG1	NRG1	NRG1
	B	PI15	PI15	PI15
	B	FAM180A		FAM180A
	B	MEST	MEST	MEST
	B	SOSTDC1		SOSTDC1
		BMP2	BMP2 x	BMP2 x
		BMP4	BMP4	
		TGFBI	TGFBI	
		CILP	CILP	
		PENK	PENK	
		PTX3	PTX3	
			ANGPTL7	
			EGF x	
				TGFB2
				TGFB3
Signaling - Matricellular	B	NOV		NOV
		THBS1	THBS1	
		CYR61		
				TNC
MPs / TIMPs	B	ADAMTSL3	ADAMTSL3	ADAMTSL3
		TIMP3		
			ADAMTS5	
Extracellular matrix	B	COL6A6		COL6A6
			COL12A1	
		OGN		
				MXRA5
				NYX

Table 3 Genes that were significantly regulated in treated vs. control eyes under GO, STAY, or STOP conditions. "B" = bi-directional regulation between GO and STOP, red gene symbols = down-regulation, blue = up-regulation, "X" = differential expression likely due to control eye effect.

other tree shrews that wore a –5 D lens for 30 days, the eyes remained elongated until lens-wear was discontinued, whereupon refractive recovery commenced (Norton et al., 2010). It seems unlikely that the retina would still generate a GO signal because the eyes had achieved a refractive match to the control eyes, yet some signal evidently existed that kept the treated eye elongated. This may be a representation, at the mRNA level, of a "shape factor" or "eye-size factor" that has been hypothesized previously (Schaeffel & Howland, 1991; Troilo & Wallman, 1991; Nickla, Sharda, & Troilo, 2005; Siegwart, Jr. & Norton, 2013). Perhaps this STAY signal is more evident in the choroid than in the sclera because the scleral remodeling returns toward normal but does not develop a recovery (STOP) pattern because of a continued signal from the choroid. As shown in Table 3 and Fig. 7, this STAY signature includes many of the genes that are differentially expressed in GO (LIM-2) and are similarly expressed in both, but several genes that are significantly differentially expressed in GO are no longer significantly affected in STAY, along with a few additional genes expressed in STAY but not GO. Four genes, strongly up-regulated in STAY, RPE65 and EGF along with RLBP1 and PI15 (both also up-regulated in GO), may be of interest. From Table 3 and Fig. 10, it also is clear that the STOP signature is very different from the STAY signature. Assuming that the REC-2 group also had a STAY signature at the end of lens-wear, the transition between STAY and STOP must be very rapid, occurring over a two-day period.

The presence of mRNA for RPE65 in the choroidal samples raises the question of whether or not there might be RPE tissue intermixed with the choroid. Several lines of evidence suggest that this was not the case. Although RPE65 is highly abundant in the RPE, it is also found in many locations, typically in association with vitamin A metabo-

lism and/or with melanocytes. For instance, RPE65 is also found to be expressed, at mRNA and/or protein levels, in human cones (Tang, Buhusi, Ma, & Crouch, 2011), bovine ciliary epithelial tissue (Salvador-Silva et al., 2005), mouse hypothalamus (Helfer et al., 2012), and in keratinocytes (Hinterhuber et al., 2004) and melanocytes (Amann et al., 2012) in human skin. In tree shrews, preliminary results comparing differential mRNA expression in RPE with that in choroid have found that mRNA for RPE65 is not differentially regulated after one day of −5 D lens wear, whereas it is significantly up-regulated in the choroid. Thus, our finding mRNA for RPE65 in the choroid samples is not indicative of contamination from RPE cells. Although, in fresh tissue, retina is easily separated from RPE, which adheres to the choroid (Bruch's membrane), treatment with RNAlater causes the RPE to adhere tightly to the retina (Wang, Zhang, Aredo, Lu, & Ufret-Vincenty, 2012; Malik, Chen, & Olsen, 2003). These factors, along with our observation during tissue dissection that retina/RPE together cleanly separated from the choroid lead us to conclude that there was little, if any, RPE contamination of the choroidal samples.

Examination of Table 2 shows that the GO, STAY, and STOP signatures included only 7 of the 15 genes whose expression changed in normal animals between 26 DVE and 37 DVE. If these normal changes are related to slowed axial elongation, it might be expected that they would show a similar direction of altered expression during recovery, when the elongation rate also is slowed. However, only one gene did so, ADAMTSL3 [ADAMTS-like 3], up-regulated in both. RLBP1, which was strongly down-regulated (−13.98 fold) at 37 DVE, was significantly up-regulated during STOP. Similarly, it might be expected that the normal change in age-related expression differences would be in the opposite direction to the expression changes found during GO or STAY. This was the case for four

genes (ADAMTS5 [ADAM metallopeptidase with thrombospondin motif, 5], ADAMTSL3, CYR61 [protein CYR61], and RLBP1). Thus, it appears that a subset of the genes whose expression is related to emmetropization signaling are also involved in the normal slowing of eye growth with age.

The differential mRNA expression in the GO, STAY, and STOP conditions contrast sharply with the very similar mRNA levels found in the left and right eyes of the two normal groups. As seen in Fig. 4, mRNA levels are nearly identical in the two eyes of these normal groups. Of the 77 genes examined, only 2 (at 26 DVE) displayed right-eye vs. left-eye differences that exceeded 1.5 fold. The absence of any significant mRNA level differences in the two eyes of normal groups lends confidence to our decision to not use a correction for false discovery.

Components of the Choroidal Signatures

The group of 77 genes examined in this study is a subset of a much larger group of genes that probably show differential expression in normal development and/or in GO, STAY, and STOP. A preliminary whole-transcriptome analysis using mRNA from three of the LIM-2 and REC-2 animals suggests that over 300 distinct genes (of the over 18,000 genes annotated in the low-coverage tree shrew genome) may be up- or down-regulated by at least 1.20 fold, with changes occurring in each direction (Frost, personal communication, 2013). The subset of genes we examined with qPCR is large enough to determine that the GO, STAY, and STOP signatures are distinct from one another, but there undoubtedly are other genes that also show differential expression in these conditions. While the expression of other genes that we did not measure may be as, or more,

important in the receipt and re-transmission of emmetropization-related signals in the choroid, expression changes in this sample of genes provide useful information about the role of the choroid in emmetropization.

It is clear from examination of Table 2, Table 3, and Figs. 5, 6, & 8, that genes belonging to several functional categories are included in the GO, STAY, and/or STOP signatures. Table 2 may be particularly helpful in illustrating that differential expression was selective, involving some, but not all, of the candidate genes whose protein products include cell-surface receptors, intracellular signaling, transcription factors, and numerous secreted signaling proteins. If the protein products of these genes are altered in accordance with the mRNA levels, the defocus-dependent behavior of such genes suggests that their levels may be involved in regulating axial elongation in a bi-directional, push-pull manner. This is consistent with the hypothesis of Nickla and Wallman (2010) that "the choroid contains secretory cells which function in the visual regulation of ocular growth, by influencing the biosynthetic activity of the sclera."

Among the genes for cell-surface signaling, EPHA1 (actin cytoskeleton-related signaling) and SCUBE3 (which activates TGFβ signaling via binding with TGFBR2 [TGFβ receptor 2]) were bi-directionally regulated, down in GO and STAY and up in STOP. The purinergic receptor gene P2RY1 was up-regulated in GO and STAY; however, ADORA2A (adenosine receptor A2a) was not significantly affected, a similar result to that found at the protein level in form-deprived guinea pig choroid (Cui et al., 2010). Genes for several intracellular signaling proteins were differentially expressed. ZNF185 (zinc finger protein 185), an actin cytoskeleton-related protein enriched at focal adhesions, and BCO2 (beta-carotene oxygenase 2), which may play a role in retinoic acid synthesis,

were down-regulated in GO and up-regulated in STOP. Other genes related to retinoid and retinoic acid metabolism and signaling (CYP26B1, RLBP1, and RPE65) also showed differential expression, consistent with the possible involvement of the retinoic acid signaling pathway in the choroidal component of the emmetropization mechanism. However, RXRB (retinoid X receptor β) was not affected in GO, STAY, or STOP. All-trans-retinoic acid (atRA), an important growth regulator, has been reported to be involved in the choroid in the regulation of axial elongation in chick, guinea pig, and primates. The synthesis of atRA was down-regulated in chick choroid during myopia development and may be up-regulated in mammalian choroid during myopia development (Mertz & Wallman, 2000; McFadden, Howlett, & Mertz, 2004; Troilo, Nickla, Mertz, & Summers Rada, 2006). The mRNA expression for RALDH2 (retinal dehydrogenase 2), an enzyme involved in atRA synthesis that we did not examine, was up-regulated in chick choroid during recovery from induced myopia and during plus-lens wear (Simon et al., 2004; Rada et al., 2012). Of the sampled genes involved in transcriptional regulation, only HIF1A (hypoxia inducible factor 1α) was affected, being up-regulated in GO.

Many genes for secreted signaling proteins were differentially expressed, including six that were bi-directionally regulated. The mRNA levels for IGF2 (insulin-like growth factor 2) were up-regulated in GO and STAY and down-regulated in STOP. So, too, was NRG1 (neuregulin 1), which can be involved in inducing acetylcholine receptor expression at neuromuscular junctions, and PI15, a cysteine-rich secretory protein of unknown function. Three other bi-directionally regulated genes (FAM180A [family with sequence similarity 180, member A], MEST [mesoderm specific transcript], and SOSTDC1 [sclerostin domain-containing protein 1]) showed the opposite pattern, down-

regulation in GO and up-regulation in STOP. The down-regulation of SOSTDC1, an inhibitor of BMP activity, combined with the up-regulation of BMP2 and BMP4 (members of the TGFβ superfamily) may significantly increase BMP signaling in GO. In addition, TGFB2 and TGFB3 (also members of the TGFβ superfamily) were up-regulated in the treated eyes in STOP. The absence of significant regulation of these two genes during GO agrees with the results of Jobling et al. (2009) in choroid of form-deprived tree shrews. In the choroid of minus-lens-treated chicks (GO), Simon et al. (2004) found a down-regulation of TGFB2. The up-regulation we found during STOP may be consistent with that report. The combined effect of up-regulation of TGFB2, TGFB3, and SCUBE3 may increase TGFβ signaling in STOP. mRNA for TGFBI (TGFβ-induced protein) was up-regulated in GO and STAY, a finding that is consistent with a report in marmoset (Shelton et al., 2008), showing that TGFBI in RPE/choroid was significantly up-regulated in minus-lens treated eyes compared with plus-lens treated eyes. Other signaling-related genes (ovotransferrin and avian thymic hormone) that have been found to have altered expression in chick choroid (Rada, Huang, & Rada, 2001; Rada & Wiechmann, 2009) were not examined in this study.

mRNA for several matricellular proteins (NOV [nephroblastoma overexpressed gene], THBS1, and CYR61) were down-regulated during GO; one (NOV) was up-regulated in STOP. Down-regulation of THBS1 has been also found in tree shrew sclera along with a reduction in its protein during GO (Gao et al., 2011; Frost & Norton, 2012). In the sample of genes coding for metallopeptidases or TIMPs, the metallopeptidase ADAMTSL3 was bi-directionally regulated, down in GO and STAY and up in STOP.

Of the extracellular matrix genes in our sample, the general pattern was for down-regulation in GO or STAY and up-regulation in STOP. An exception was NYX (nyctalopin), which was down-regulated only in STOP. In chicks, HAS2 (hyaluronan synthase 2) mRNA expression was reported to increase significantly in the choroid of eyes recovering from myopia (Rada et al., 2010). We were unable to design reliable qPCR primers for tree shrew HAS2; preliminary whole-transcriptome analysis data on three animals from the REC-2 group (Frost, personal communication, 2013) suggest that HAS2 may be up-regulated during STOP.

Overall, the signatures for GO, STAY, and STOP in tree shrew choroid are both complex and unique. A better understanding of the roles the affected genes may play will require an improved knowledge of how the genes interact as part of a complex network *in vivo*. However, our results are consistent, during GO, with an up-regulation of the BMP pathway and down-regulation of the TGFβ pathway. During STOP there may be a partial reversal of that pattern along with up-regulation of the TGFβ pathway. Finally, considering that the choroid is a highly vascularized tissue, the involvement of a group of 13 genes, spanning several functional groupings, with roles in vascular regulation and/or angiogenesis (BMP2, BMP4, CYR61, EGF, FGFR1, HIF1A, IGF2, NRG1, PTX3, TGFB2, THBS1, TIMP3, and TNC), may occur due either to blood-flow regulation and/or as part of emmetropization signaling.

Control Eye Effects

As noted in Table 2 and Figs. 5, 6, & 8, there were numerous instances in which the gene expression in the control eyes differed from age-matched normal eyes. Similar

control-eye effects have been found in previous studies of mRNA and protein expression in tree shrew sclera in which the control and treated eyes both differ from normal in the same direction (Gao et al., 2011; Guo et al., 2013; Frost & Norton, 2012). Among the possible causes of these binocular changes, one that seems likely is binocular regulation of blood flow in the choroid, which could produce similar effects in both choroid and sclera in both eyes. Choroidal blood flow during the development of monocular induced myopia has not been measured in mammals; in chicks there is a reduction in choroidal blood flow in form-deprived eyes. However, several studies have found evidence for a binocular reduction in choroidal blood flow during monocular treatment (Shih et al., 1993; Jin & Stjernschantz, 2000). According to Jin and Stjernschantz (Jin & Stjernschantz, 2000), during form deprivation the choroidal blood flow was markedly reduced (significantly) in the form-deprived eye, and tended to be reduced (non-significantly) in the contralateral eye, compared with the normal group; while during recovery, choroidal blood flow in both recovering and contralateral eyes were significantly increased compared with the normal group. Indeed, some of the sampled genes whose expression in the control eyes differed from normal eyes included ones related to vascular regulation and/or angiogenesis. To the extent that regulation of choroidal blood flow in mammals is binocular, alterations in choroidal blood flow in the control eye choroid may help to explain the effects that we observed in the control eyes in GO, STAY, and STOP conditions. Another potential reason for binocular effects on choroidal mRNA expression in the control eye is that the open goggle frame around the control eye may affect the peripheral visual field (Amedo & Norton, 2012). Although this is not sufficient to produce refractive ef-

fects, measured on the pupillary axis, it may produce small changes in peripheral areas of the choroid that were included in our choroidal samples.

In the Results, we noted several genes that were significantly different in the treated eyes vs. control eyes because mRNA levels in the control eye choroid differed from normal but the levels in the treated eye choroids did not. Comparing treated eye vs. normal eye and control eye vs. normal eye mRNA differences (for example, Figs. 6B and 6C), many of the fold-differences (both significant and non-significant) moved in the same direction of up- or down-regulation. The cases where the treated eyes did not change but the control eyes did differ from normal were included as differentially expressed genes under the assumption that the control eye change reflected binocular effects in the choroid. That the mRNA levels for these genes in treated eye choroid did not change in concert with the control eye choroid implied active regulation that prevented a binocular shift. We examined treated vs. normal eye mRNA differences comparing LIM-2 (GO) vs. LIM-11 (STAY), LIM-2 vs. REC-2 (STOP), and LIM-11 vs. REC-2, analogous to the treated vs. control eye differences shown in Figures 7, 9, and 10. GO and STAY produced a similar treated vs. normal gene expression pattern, that was very weakly correlated. GO vs. STOP and STAY vs. STOP, as in Figs 9 and 10, showed expression patterns that differed, but are not opposite, so there was not a significant correlation between GO vs. STOP or STAY vs. STOP.

Summary

This study examined differential mRNA expression by cells in mammalian choroid during normal development; early in minus-lens wear, a GO condition; after full

compensation for a minus lens, a STAY condition; and early in refractive recovery from lens-induced myopia, a STOP condition. We found that cells in the choroid respond with different mRNA expression signatures to the different emmetropization conditions. The STAY signature resembled, but differed from, the GO signature and both were very distinct from the STOP signature. Expression signatures were different from those seen in the RPE (Frost, He, & Norton, 2013) and in the sclera (Guo et al., 2013). Thus, the choroid is a unique way-station that receives emmetropization-related signals from the RPE and generates signals that produce changes in the sclera. Because a limited number of genes were examined, these signatures are incomplete; however, the large number of genes that are altered suggest that the emmetropization-related signals in choroid are complex and unlikely to depend on the regulation of a single gene, or even a small number of genes.

ACKNOWLEDGEMENTS

This study was supported by NIH grants EY005922 and EY003039 (P30). Li He was supported in part by a supplement to EY005922 and by funds from the Department of Vision Sciences. This work was performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Alabama at Birmingham (Li He). Preliminary results were presented in abstract form. We thank Lin Guo for technical contributions including demonstrating a way to dissect choroid away from both retina/RPE and from sclera.

REFERENCES

Amann, P. M., Luo, C., Owen, R. W., Hofmann, C., Freudenberger, M., Schadendorf, D., Eichmuller, S. B. & Bazhin, A. V. (2012). Vitamin A metabolism in benign and malignant melanocytic skin cells: importance of lecithin/retinol acyltransferase and RPE65. *J Cell Physiol,* 227, 718-728.

Amedo, A. O. & Norton, T. T. (2012). Visual guidance of recovery from lens-induced myopia in tree shrews (*Tupaia glis belangeri*). *Ophthalmic Physiol Opt,* 32, 89-99.

Birol, G., Wang, S., Budzynski, E., Wangsa-Wirawan, N. D. & Linsenmeier, R. A. (2007). Oxygen distribution and consumption in the macaque retina. *Am J Physiol Heart Circ Physiol,* 293, H1696-H1704.

Cui, D., Trier, K., Zeng, J., Wu, K., Yu, M. & Ge, J. (2010). Adenosine receptor protein changes in guinea pigs with form deprivation myopia. *Acta Ophthalmol,* 88, 759-765.

Diether, S. & Schaeffel, F. (1997). Local changes in eye growth induced by imposed local refractive error despite active accommodation. *Vision Res,* 37, 659-668.

Frost, M. R., He, L. & Norton, T. T. (2013). Differential gene expression in tree shrew retinal pigment epithelium (RPE) in response to six hours of minus-lens wear. *Ophthalmic Physiol Opt,* 33, 667.

Frost, M. R. & Norton, T. T. (2012). Alterations in protein expression in tree shrew sclera during development of lens-induced myopia and recovery. *Invest Ophthalmol Vis Sci,* 53, 322-336.

Gao, H., Frost, M. R., Siegwart, J. T., Jr. & Norton, T. T. (2011). Patterns of mRNA and protein expression during minus-lens compensation and recovery in tree shrew sclera. *Mol Vis,* 17, 903-919.

Gentle, A. & McBrien, N. A. (1999). Modulation of scleral DNA synthesis in development of and recovery from induced axial myopia in the tree shrew. *Exp Eye Res,* 68, 155-163.

Glickstein, M. & Millodot, M. (1970). Retinoscopy and eye size. *Science,* 168, 605-606.

Guo, L., Frost, M. R., He, L., Siegwart, J. T., Jr. & Norton, T. T. (2013). Gene expression signatures in tree shrew sclera in response to three myopiagenic conditions. *Invest Ophthalmol Vis Sci,* 54, 6806-6819.

Guo, L., Frost, M. R., Siegwart, J. T., Jr. & Norton, T. T. (2012). Gene expression signatures in tree shrew sclera in response to two STOP visual conditions: recovery from minus-lens wear, and plus-lens wear. *Invest Ophthalmol Vis Sci,* 53, ARVO E-Abstract 3455.

He, L., Frost, M. R., Siegwart, Jr. J. T., Filios, S. & Norton, T. T. (2011). Retinal gene expression signatures in tree shrew in response to three myopiagenic visual conditions: minus lens, form deprivation, and darkness. *Invest Ophthalmol Vis Sci,* 52, ARVO E-Abstract 6301.

Helfer, G., Ross, A. W., Russell, L., Thomson, L. M., Shearer, K. D., Goodman, T. H., McCaffery, P. J. & Morgan, P. J. (2012). Photoperiod regulates vitamin A and Wnt/beta-catenin signaling in F344 rats. *Endocrinology,* 153, 815-824.

Hinterhuber, G., Cauza, K., Brugger, K., Dingelmaier-Hovorka, R., Horvat, R., Wolff, K. & Foedinger, D. (2004). RPE65 of retinal pigment epithelium, a putative receptor molecule for plasma retinol-binding protein, is expressed in human keratinocytes. *J Invest Dermatol,* 122, 406-413.

Howlett, M. H. & McFadden, S. A. (2009). Spectacle lens compensation in the pigmented guinea pig. *Vision Res,* 49, 219-227.

Hung, L. F., Wallman, J. & Smith, E. L., III. (2000). Vision-dependent changes in the choroidal thickness of macaque monkeys. *Invest Ophthalmol Vis Sci,* 41, 1259-1269.

Irving, E. L., Callender, M. G. & Sivak, J. G. (1991). Inducing myopia, hyperopia, and astigmatism in chicks. *Optom Vis Sci,* 68, 364-368.

Irving, E. L., Callender, M. G. & Sivak, J. G. (1995). Inducing ametropias in hatchling chicks by defocus--aperture effects and cylindrical lenses. *Vision Res,* 35, 1165-1174.

Jin, N. & Stjernschantz, J. (2000). Regional blood flow in the myopic chick eye during and after form deprivation: a study with radioactively-labelled microspheres. *Exp Eye Res,* 71, 233-238.

Jobling, A. I., Wan, R., Gentle, A., Bui, B. V. & McBrien, N. A. (2009). Retinal and choroidal TGFb in the tree shrew model of myopia: isoform expression, activation and effects on function. *Exp Eye Res,* 88, 458-466.

Liu, Q., Wu, J., Wang, X. & Zeng, J. (2007). Changes in muscarinic acetylcholine receptor expression in form deprivation myopia in guinea pigs. *Mol Vis,* 13, 1234-1244.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDCt} method. *Methods,* 25, 402-408.

Luckett, W.P. (1980). Comparative Biology and Evolutionary Relationships of Tree Shrews. New York: Plenum Press.

Lutty, G. A., Hasegawa, T., Baba, T., Grebe, R., Bhutto, I. & McLeod, D. S. (2010). Development of the human choriocapillaris. *Eye (Lond),* 24, 408-415.

Malik, K. J., Chen, C. D. & Olsen, T. W. (2003). Stability of RNA from the retina and retinal pigment epithelium in a porcine model simulating human eye bank conditions. *Invest Ophthalmol Vis Sci,* 44, 2730-2735.

McBrien, N. A., Jobling, A. I., Truong, H. T., Cottriall, C. L. & Gentle, A. (2009). Expression of muscarinic receptor subtypes in tree shrew ocular tissues and their regulation during the development of myopia. *Mol Vis,* 2009, 464-475.

McFadden, S. A., Howlett, M. H. & Mertz, J. R. (2004). Retinoic acid signals the direction of ocular elongation in the guinea pig eye. *Vision Res,* 44, 643-653.

McKanna, J. A. & Casagrande, V. A. (1981). Atropine affects lid-suture myopia development. *Doc Ophthalmol Proc Ser,* 28, 187-192.

Mertz, J. R. & Wallman, J. (2000). Choroidal retinoic acid synthesis: a possible mediator between refractive error and compensatory eye growth. *Exp Eye Res,* 70, 519-527.

Mutti, D. O., Mitchell, G. L., Jones, L. A., Friedman, N. E., Frane, S. L., Lin, W. K., Moeschberger, M. L. & Zadnik, K. (2005). Axial growth and changes in lenticular and corneal power during emmetropization in infants. *Invest Ophthalmol Vis Sci,* 46, 3074-3080.

Nickla, D. L., Damyanova, P. & Lytle, G. (2009). Inhibiting the neuronal isoform of nitric oxide synthase has similar effects on the compensatory choroidal and axial responses to myopic defocus in chicks as does the non-specific inhibitor L-NAME. *Exp Eye Res,* 88, 1092-1099.

Nickla, D. L., Sharda, V. & Troilo, D. (2005). Temporal integration characteristics of the axial and choroidal responses to myopic defocus induced by prior form deprivation versus positive spectacle lens wear in chickens. *Optom Vis Sci,* 82, 318-327.

Nickla, D. L. & Wallman, J. (2010). The multifunctional choroid. *Prog Retin Eye Res,* 29, 144-168.

Nickla, D. L. & Wildsoet, C. F. (2004). The effect of the nonspecific nitric oxide synthase inhibitor NG-nitro-L-arginine methyl ester on the choroidal compensatory response to myopic defocus in chickens. *Optom Vis Sci,* 81, 111-118.

Nickla, D. L., Wilken, E., Lytle, G., Yom, S. & Mertz, J. (2006). Inhibiting the transient choroidal thickening response using the nitric oxide synthase inhibitor l-NAME prevents the ameliorative effects of visual experience on ocular growth in two different visual paradigms. *Exp Eye Res,* 83, 456-464.

Norton, T. T. (1999). Animal models of myopia: Learning how vision controls the size of the eye. *ILAR J,* 40, 59-77.

Norton, T. T., Amedo, A. O. & Siegwart, J. T., Jr. (2006a). Darkness causes myopia in visually experienced tree shrews. *Invest Ophthalmol Vis Sci,* 47, 4700-4707.

Norton, T. T., Amedo, A. O. & Siegwart, J. T., Jr. (2010). The effect of age on compensation for a negative lens and recovery from lens-induced myopia in tree shrews (*Tupaia glis belangeri*). *Vision Res,* 50, 564-576.

Norton, T. T., Essinger, J. A. & McBrien, N. A. (1994). Lid-suture myopia in tree shrews with retinal ganglion cell blockade. *Vis Neurosci,* 11, 143-153.

Norton, T. T. & McBrien, N. A. (1992). Normal development of refractive state and ocular component dimensions in the tree shrew (*Tupaia belangeri*). *Vision Res,* 32, 833-842.

Norton, T. T., Metlapally, R. & Young, T. L. (2008). Myopia. In Garner A. & Klintworth G.K. (Eds), *The Pathobiology of Ocular Disease.* New York: Taylor & Francis.

Norton, T. T. & Siegwart, J. T. (1991). Local myopia produced by partial visual-field deprivation in tree shrew. *Soc Neurosci Abstr,* 17, 558.

Norton, T. T., Siegwart, J. T., Jr. & Amedo, A. O. (2006b). Effectiveness of hyperopic defocus, minimal defocus, or myopic defocus in competition with a myopiagenic stimulus in tree shrew eyes. *Invest Ophthalmol Vis Sci,* 47, 4687-4699.

Norton, T. T., Wu, W. W. & Siegwart, J. T., Jr. (2003). Refractive state of tree shrew eyes measured with cortical visual evoked potentials. *Optom Vis Sci,* 80, 623-631.

Oyster, C.W. (1999). The Human Eye. Sunderland, MA: Sinauer Associated, Inc.

Phillips, J. R., Khalaj, M. & McBrien, N. A. (2000). Induced myopia associated with increased scleral creep in chick and tree shrew eyes. *Invest Ophthalmol Vis Sci,* 41, 2028-2034.

Rada, J. A., Hollaway, L. R., Lam, W., Li, N. & Napoli, J. L. (2012). Identification of RALDH2 as a visually regulated retinoic acid synthesizing enzyme in the chick choroid. *Invest Ophthalmol Vis Sci,* 53, 1649-1662.

Rada, J. A., Huang, Y. & Rada, K. G. (2001). Identification of choroidal ovotransferrin as a potential ocular growth regulator. *Curr Eye Res,* 22, 121-132.

Rada, J. A. & Wiechmann, A. F. (2009). Ocular expression of avian thymic hormone: changes during the recovery from induced myopia. *Mol Vis,* 15, 778-792.

Rada, J. A. S., Wiechmann, A. F., Hollaway, L. R., Baggenstoss, B. A. & Weigel, P. H. (2010). Increased hyaluronan synthase-2 mRNA expression and hyaluronan accumulation with choroidal thickening: Response during recovery from induced myopia. *Invest Ophthalmol Vis Sci,* 51, 6172-6179.

Rohrer, B. & Stell, W. K. (1994). Basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-b) act as stop and go signals to modulate postnatal ocular growth in the chick. *Exp Eye Res,* 58, 553-561.

Salvador-Silva, M., Ghosh, S., Bertazolli-Filho, R., Boatright, J. H., Nickerson, J. M., Garwin, G. G., Saari, J. C. & Coca-Prados, M. (2005). Retinoid processing proteins in the ocular ciliary epithelium. *Mol Vis,* 11, 356-365.

Schaeffel, F. & Howland, H. C. (1991). Properties of the feedback loops controlling eye growth and refractive state in the chicken. *Vision Res,* 31, 717-734.

Schippert, R., Brand, C., Schaeffel, F. & Feldkaemper, M. P. (2006). Changes in scleral MMP-2, TIMP-2 and TGFb-2 mRNA expression after imposed myopic and hyperopic defocus in chickens. *Exp Eye Res,* 82, 710-719.

Shelton, L., Troilo, D., Lerner, M. R., Gusev, Y., Brackett, D. J. & Rada, J. S. (2008). Microarray analysis of choroid/RPE gene expression in marmoset eyes undergoing changes in ocular growth and refraction. *Mol Vis,* 14, 1465-1479.

Shih, Y.-F., Fitzgerald, M. E. C., Norton, T. T., Gamlin, P. D. R., Hodos, W. & Reiner, A. (1993). Reduction in choroidal blood flow occurs in chicks wearing goggles that induce eye growth toward myopia. *Curr Eye Res,* 12, 219-227.

Siegwart, J. T. & Norton, T. T. (1994). Goggles for controlling the visual environment of small animals. *Lab Animal Sci,* 44, 292-294.

Siegwart, J. T., Jr. & Norton, T. T. (1998). The susceptible period for deprivation-induced myopia in tree shrew. *Vision Res,* 38, 3505-3515.

Siegwart, J. T., Jr. & Norton, T. T. (1999). Regulation of the mechanical properties of tree shrew sclera by the visual environment. *Vision Res,* 39, 387-407.

Siegwart, J. T., Jr. & Norton, T. T. (2005). Selective regulation of MMP and TIMP mRNA levels in tree shrew sclera during minus lens compensation and recovery. *Invest Ophthalmol Vis Sci,* 46, 3484-3492.

Siegwart, J. T., Jr. & Norton, T. T. (2013). Response to interrupted hyperopia after restraint of axial elongation in tree shrews. *Optom Vis Sci,* 90, 131-139.

Simon, P., Feldkaemper, M., Bitzer, M., Ohngemach, S. & Schaeffel, F. (2004). Early transcriptional changes of retinal and choroidal TGFb-2, RALDH-2, and ZENK following imposed positive and negative defocus in chickens. *Mol Vis,* 10, 588-597.

Smith, E. L., III, Hung, L. F., Huang, J., Blasdel, T. L., Humbird, T. L. & Bockhorst, K. H. (2010). Effects of optical defocus on refractive development in monkeys: evidence for local, regionally selective mechanisms. *Invest Ophthalmol Vis Sci,* 51, 3864-3873.

Stone, R. A., McGlinn, A. M., Baldwin, D. A., Tobias, J. W., Iuvone, P. M. & Khurana, T. S. (2011). Image defocus and altered retinal gene expression in chick: Clues to the pathogenesis of ametropia. *Invest Ophthalmol Vis Sci,* 52, 5765-5777.

Summers, J. A. (2013). The choroid as a sclera growth regulator. *Exp Eye Res,* 114, 120-127.

Tang, P. H., Buhusi, M. C., Ma, J. X. & Crouch, R. K. (2011). RPE65 is present in human green/red cones and promotes photopigment regeneration in an *in vitro* cone cell model. *J Neurosci,* 31, 18618-18626.

Troilo, D. (1990). Experimental studies of emmetropization in the chick. In *Myopia and the Control of Eye Growth.* New York: Wiley & Sons.

Troilo, D., Nickla, D. L., Mertz, J. R. & Summers Rada, J. A. (2006). Change in the synthesis rates of ocular retinoic acid and scleral glycosaminoglycan during experimentally altered eye growth in marmosets. *Invest Ophthalmol Vis Sci,* 47, 1768-1777.

Troilo, D., Nickla, D. L. & Wildsoet, C. F. (2000). Choroidal thickness changes during altered eye growth and refractive state in a primate. *Invest Ophthalmol Vis Sci,* 41, 1249-1258.

Troilo, D. & Wallman, J. (1991). The regulation of eye growth and refractive state: an experimental study of emmetropization. *Vision Res,* 31, 1237-1250.

Wallman, J., Wildsoet, C., Xu, A., Gottlieb, M. D., Nickla, D. L., Marran, L., Krebs, W. & Christensen, A. M. (1995). Moving the retina: choroidal modulation of refractive state. *Vision Res,* 35, 37-50.

Wallman, J. & Winawer, J. (2004). Homeostasis of eye growth and the question of myopia. *Neuron,* 43, 447-468.

Wang, C. X.-Z., Zhang, K., Aredo, B., Lu, H. & Ufret-Vincenty, R. L. (2012). Novel method for the rapid isolation of RPE cells specifically for RNA extraction and analysis. *Exp Eye Res,* 102, 1-9.

Wildsoet, C. F. & McFadden, S. A. (2010). Optic nerve section does not prevent form deprivation-induced myopia or recovery from it in the mammalian eye. *Invest Ophthalmol Vis Sci,* 51, ARVO E-Abstract 1737.

Zhang, Y., Liu, Y. & Wildsoet, C. F. (2012). Bidirectional, optical sign-dependent regulation of BMP2 gene expression in chick retina pigment epithelium. *Invest Opth almol Vis Sci,* 53, 6072-6080

SUMMARY AND DISCUSSION

The overall purpose of this dissertation was to determine mRNA gene expression signatures in the retina, RPE, and choroid in response to different visual stimuli. Here, we examined 44 genes in the retina, RPE, and combined retina+RPE after 6 and 24 hours of minus lens wear, and compared the gene expression signatures to help us to better understand how the GO signals are transmitted from retina to RPE. Also, we examined 77 genes in the choroid under different visual conditions – GO, STAY, and STOP, and learned how the emmetropization related signals were transmitted and transformed in the choroid.

In **specific aim 1**, we found gene expression signatures in the retina and combined retina+RPE are similar, while the signature in the RPE is hidden in the combined retina+RPE. Only a few genes were found to be significantly regulated in the retina and in the combined retina+RPE. The relative lack of mRNA regulation in the retina may be due to several reasons: the primary function of most retinal neurons is visual perception, rather than emmetropization, so the emmetropization related signals are minor part of retinal activity, making them difficult to detect; the emmetropization related signals are produced by a small portion of cells, for example, a subset of amacrine cells, so the signal is not very intense. The notable exception is VIP, which is produced by a subset of amacrine cells, and was detectable in the retina and combined retina+RPE. A larger number of genes with significant regulation were found in the RPE, suggesting the signals get

transformed, and possibly amplified. However, when the combined retina+RPE was examined, the response only represented the retina, the effect in the RPE was hidden.

The genes with significant regulation in retina and RPE when we stimulated the emmetropization mechanism may play a role in the emmetropization. In the retina, EGR1 was down-regulated after 6 hours of minus lens wear, which was consistent with the finding in chick and mouse (Ashby et al., 2010; Stone et al., 2011; Brand et al., 2007; Fischer et al., 1999). The protein product of EGR1 functions as a transcription factor, and may be involved in the initiation of emmetropization signaling. Interestingly, for the other retinal genes showing significant regulation (BMP2, CTGF, SST, IGF2, and VIP), their protein product are all secreted protein, which may serve as the signals by diffusing or being transported from retina to the next compartment – RPE. BMP2 has been studied in the chick retina and RPE, and may play a "STOP" role in the ocular growth regulation (Stone et al., 2011; McGlinn et al., 2007; Zhang et al., 2012). IGF2 has a similar growth-promoting effect as insulin does, the up-regulation of IGF2 may lead to an increased ocular growth, and play a "GO" role. VIP is a secreted peptide with multiple functions, and has been found to be up-regulated in the myopia development in monkey, and now in tree shrew, suggesting a "GO" role (Tkatchenko et al., 2006; Stone et al., 1988).

In the RPE, more genes with significant regulation were found, but the gene expression signature was very different from that in the retina, suggesting a transformation of the signals. The regulated genes may indicate a complex signaling network and multiple pathways existing in the RPE, including dopamine signaling, NO signaling, and neuropeptides signaling (PENK, VIP). The transformed signals in the RPE are then relayed to the next compartment – choroid.

In **specific aim 2a**, we compared gene expression signatures under three myopiagenic GO conditions – minus lens, form deprivation, and continuous darkness. In the sclera, it has been reported that different visual conditions caused similar gene expression responses and similar effect on axial elongation (Guo et al., 2013). In the retina, these different visual stimuli presumably induce mostly different responses. So how the different responses in the retina converge to a similar effect in the sclera is an important question if we are to decode emmetropization related signaling. Based on our data, the gene expression signatures in the choroid were similar between these treatments, suggesting the emmetropization related signals induced by different visual stimuli have converged before or at the level of choroid. A common set of genes with significant regulation indicates they may be part of the core of GO signals. In **specific aim 2b**, we defined three gene expression signatures – GO, STAY, and STOP – in the tree shrew choroid in response to different visual stimuli. After 2 days of minus lens wear, the myopia was actively progressing in the treatment group, characterized with a GO pattern in the choroid. After 11 days of lens wear, the treated eyes have compensated completely for the minus lens, and exhibited a STAY pattern, which was similar to GO pattern. After 2 days of recovery from complete minus lens compensation, the treated eyes showed a STOP pattern, which was different from either GO or STAY.

Emmetropization Related Signaling

Although the signaling underlying emmetropization mechanism is not well elucidated, our studies, which found many potential genes involved, can enhance the knowledge of how emmetropization works and changes that produce myopia. The gene

products of the majority of the candidate genes we examined in retina, RPE, and choroid participate in cell signaling, and they may play direct or indirect roles in the initiation, transmission and transformation of emmetropization signaling cascade from retina to choroid. The genes with significant regulation under different vision conditions in retina, RPE, or choroid were discussed below in detail.

In most of previous studies on emmetropization related signaling, the experimental animals were chick. Although chick was established as a myopia animal model, it has a separate cartilaginous layer outside the choroid, and evolutionally different from mammals. So our data on tree shrew could not only confirm the previous chick data, but also be more relevant and relatable to the human condition.

Retina – Origin of Signaling

EGR1 (protein name: Early growth response protein 1) is a transcriptional regulator, that activates the transcription of target genes whose product are required for mitogenesis and differentiation. It is also known as ZENK in chick. The induction of ZENK expression by visual stimuli is mainly restricted to glucagon amacrine cells in chick retina, and dependent on sign of defocus (Fischer et al., 1999). ZENK was considered as a myopia activity marker in the chick retina, and its expression level could be regulated by different pharmacological interventions related to choline, dopamine, and insulin (Bitzer, Kovacs, Feldkaemper, & Schaeffel, 2006; Ashby, McCarthy, Maleszka, Megaw, & Morgan, 2007; Feldkaemper et al., 2009). ZENK mRNA expression in chick retina was down-regulated by both minus lens and form deprivation (Ashby et al., 2010; Stone et al., 2011). In our study, EGR1, the mammal ortholog of ZENK, was also down-regulated in

retina after 6 hours of minus lens treatment. The down-regulation of EGR1, may play as an early GO signal in the myopia development initiating the increased axial elongation, and may interact with other signal pathways including dopamine, cholinergic, and insulin/glucagon signaling.

BMP2 (protein name: Bone morphogenetic protein 2) belongs to TGFβ superfamily, and was originally found to be involved in embryogenesis and osteogenesis. In recent years, BMP2 has been found to exist in the retina and/or RPE, and may play a regulatory role in retinal differentiation and ocular growth (Belecky-Adams & Adler, 2001; Sakuta et al., 2006; Stone et al., 2011; McGlinn et al., 2007; Zhang et al., 2012). The BMP2 protein expression was found to exist in all layers of retina and RPE, and mRNA expression of three BMP2 receptors – BMPR1A, BMPR2B, BMPR2 were found to exist in retina, RPE, and choroid (Zhang et al., 2012). BMP2 mRNA expression was down-regulated in chick combined retina+RPE after minus lens wear, also down-regulated in chick RPE treated by minus lens. Our data confirmed the down-regulation of BMP2 in retina or combined retina+RPE, further indicating a negative regulatory role for BMP2 during ocular growth.

VIP (protein name: Vasoactive intestinal peptide) was originally isolated from porcine intestine, whose function was relaxant of smooth muscle in the digestive system (Said & Mutt, 1972). In the retina, VIP can stimulate the adenylate cyclase and induce the formation of cyclic AMP (Longshore & Makman, 1981; Schorderet, Sovilla, & Magistretti, 1981). VIP in the retina is localized to a subset of amacrine cells (Terubayashi et al., 1983; Tornqvist, Uddman, Sundler, & Ehinger, 1982). Based on two studies on monkey (Stone et al., 1988; Tkatchenko et al., 2006), the mRNA and protein

levels of VIP were increased in form deprived or lid sutured eyes compared to open eyes, suggesting the possible link between VIP expression and ocular growth regulation. Surprisingly, in chick retina, VIP mRNA expression was down-regulated in response to minus lens or form deprivation treatment (Stone et al., 2011; McGlinn et al., 2007). Our finding of up-regulation of VIP in retina and combined tissue was consistent with previous results on mammals, and supported the possible GO role of VIP in the ocular growth.

SST (protein name: Somatostatin) is a neuropeptide that has a wide range of physiological effect on neurotransmission, secretion and proliferation. In mammalian retina, SST immunoreactivity is predominantly localized to a sparse population of amacrine cells (Johnson, Rickman, & Brecha, 2000). The detailed role of SST in the ocular growth and myopia development has not yet been studied. However, SST mediates nitric oxide (NO) production by activating SSTR2 in rat retina (Vasilaki, Mouratidou, Schulz, & Thermos, 2002), and nitric oxide is considered to be a potential regulator on choroid thickness and ocular growth (Fujikado et al., 1997; Nickla, Damyanova, & Lytle, 2009). Further study is encouraged to investigate the signaling cascade of SST – SSTR2 – NOS – NO, which may span from the retina to the choroid.

RPE – a Signaling Compartment

In the RPE exposed to short period of lens wear, we found several genes with significant regulation, most of them were from two functional categories: signaling receptors, and secreted molecules. mRNA levels for several signal receptors – DRD1, GRM5, P2RY1, SSTR2, and VIPR1 – were significantly down-regulated in RPE in response under early GO conditions. The regulation of message for these receptors indicates a possi-

ble involvement of signaling of dopamine, glutamate, purine, somatostatin (SST), and VIP, which could be secreted by retina and diffused to RPE (paracrine), or made by RPE itself (autocrine). As discussed in the SST section earlier, the NOS1 may be an important signaling intermediate regulated by SST and produce NO which could diffuse into choroid. RPE is a polarized tissue, so secreted molecules can be released through either the apical (TGF-β, APOE) or basal (APOE) side of RPE cells (Kay, Yang, & Paraoan, 2013; Ishida et al., 2004; Li, Wen, Banzon, Maminishkis, & Miller, 2011). The secreted molecules produced by RPE – APOE, FGF1, NRG1, PENK, TGFB1, and VIP (of which genes were significantly regulated) – may function as a paracrine or autocrine signal. How these secreted signals are released and diffuse to the target tissue requires further examination.

Several of these genes are of interest because their altered mRNA levels are consistent with previous findings or theories: DRD1 (receptor of dopamine signaling), LRP2 (lrp2 mutant zebrafish become myopic) (Veth et al., 2011), NOS1 (involved in nitric oxide production), SSTR2 (receptor of somatostatin), and VIP and VIPR1 (both down-regulated in the RPE, but VIP up-regulated in the retina). So our findings in the RPE improved the knowledge of emmetropization signaling, added evidence to the possible pathways communicating between retina, RPE and choroid.

Choroid – Another Signaling Compartment

TGFβ/BMP signaling: A few genes directly related to TGFβ/BMP signaling pathway were found to be significantly regulated in the choroid under different visual conditions. The regulation of these genes - SCUBE3 (protein binds to TGFBR2 and acti-

vates TGFβ signaling), SOSTDC1 (directly antagonize activity of BMP2, BMP4), CILP (interacts with TGFβ), BMP2, BMP4, TGFβ2, TGFβ3, and TGFBI – strongly suggest the active participation of TGFβ/BMP signaling in the choroid when emmetropization related signals were transmitted. In addition, the secreted molecules produced by these genes (BMP2, BMP4, TGFβ2, TGFβ3) are able to diffuse and enter the sclera to modulate tissue remodeling.

Retinoid/Retinoic acid signaling: The synthesis of atRA (All-trans-retinoic acid) has been found to be regulated in the choroid during myopia development: down-regulated in chick and up-regulated in the mammalian choroid (Mertz & Wallman, 2000; McFadden et al., 2004; Troilo et al., 2006). The enzyme RALDH2 (retinal dehydrogenase 2), involved in atRA synthesis, was up-regulated in chick choroid during recovery (Rada et al., 2012). Although the previous reports on atRA are not consistent between chick and mammal, Retinoid/Retinoic acid signaling is still considered to be an important ocular size modulator. Tissue concentrations of atRA are tightly controlled by activities of several synthesize enzymes and metabolizing enzymes (Napoli, 2012). In our regulated gene list, CYP26B1 protein is involved in metabolism of atRA, while BCO2 produces apocarotenals which may be the precursors for the biosynthesis of retinoic acid. RLBP1 and RPE65 are related to retinoid signaling. These data suggest the potential involvement of Retinoid/Retinoic acid signaling in the choroid, more details left to be clarified.

Limitations of This Study

Although we studied mRNA changes in a list of genes with potential involvement in emmetropization mechanism in different tissues, only the changes in mRNA transcrip-

tion levels were examined. The changes in the transcription level may not necessarily cause corresponding changes in the translation level. Meanwhile, a system can be regulated at even more levels: pre-transcription (microRNA, DNA methylation, etc) and post-translation level (different types of protein modification). Also, the emmetropization related signals may be contained in some inorganic or organic molecules which are not directly encoded by DNA, like dopamine, atRA, NO. So the significantly regulated genes, and the pattern composed of those genes, may not represent the whole picture of what is happening. Nonetheless, the focus and aim of this study was primarily to learn the overall patterns in each tissue under different conditions, and we indeed discovered the distinguishable patterns in each tissue under different visual conditions. Also, since in the myopia research field, most of the hypotheses we were testing have not been fully examined and understood (especially in mammal), our results on emmetropization mechanism in transcription level were original and meaningful.

When studying different tissues, we tested 44 genes in the retina and RPE and 77 genes in the choroid. Although the genes we selected were targeted specifically on tissues and based on preliminary data, it is still possible or likely that some genes are involved in emmetropization related signaling in important ways were not included in our study. However, it appears that the amount genes we selected were sufficient enough to distinguish each pattern, and reveal potential signaling pathways.

Although we found many genes with significant regulation in RPE and choroid, only a few genes were found in the retina. As discussed before, the emmetropization related signals in the retina may be submerged by the active visual processing. Considering the amacrine cells are making different important signals (EGR1, SST, VIP, and dopa-

mine), collecting and analyzing amacrine cells alone by laser microdissection technique might avoid the noise of visual processing and provide more useful information.

Future Directions

In this study, we revealed different gene expression signatures in retina, RPE, and choroid under different visual conditions, and found many genes with significant regulation which may play important roles in emmetropization related signaling. Since myopia and emmetropization are not controlled by a single gene or a small number of genes, a comprehensive study of RNA or proteins, like microarray, RNAseq, two-dimensional electrophoresis might be useful in these ocular tissues. To fully understand emmetropization mechanism, deeper investigation on each gene or each signaling pathway is also needed. Different molecular techniques, including immunohistochemistry, radiolabeling, *in situ* hybridization, transgenic animal models, etc, should be applied to track the localization, movement and cascading of signals. Since the direct emmetropization signaling is composed of several compartment (Retina – RPE – Choroid – Sclera), signals appear to be transformed when transmitted to the next compartment. So it is necessary to analyze each compartment alone. For retina, different cell types should be analyzed separated to obtain high signal resolution and prevent noise from visual processing.

LIST OF GENERAL REFERENCES

Algvere, P. V., Jahnberg, P., & Textorius, O. (1999). The Swedish Retinal Detachment Register. I. A database for epidemiological and clinical studies. *Graefes Arch.Clin.Exp.Ophthalmol., 237,* 137-144.

Amann, P. M., Luo, C., Owen, R. W., Hofmann, C., Freudenberger, M., Schadendorf, D. et al. (2012). Vitamin A metabolism in benign and malignant melanocytic skin cells: importance of lecithin/retinol acyltransferase and RPE65. *J.Cell Physiol, 227,* 718-728.

Amedo, A. O. & Norton, T. T. (2003). Comparison of Infrared photoretinoscope and autorefractor in tree shrews with and without induced myopia. Optometry and Vision Science 80 (suppl.), 120.
Ref Type: Abstract

Amedo, A. O. & Norton, T. T. (2012). Visual guidance of recovery from lens-induced myopia in tree shrews (*Tupaia glis belangeri*). *Ophthalmic and Physiological Optics, 32,* 89-99.

Ashby, R., Kozulin, P., Megaw, P. L., & Morgan, I. G. (2010). Alterations in ZENK and glucagon RNA transcript expression during increased ocular growth in chickens. *Mol.Vis., 16,* 639-649.

Ashby, R., McCarthy, C. S., Maleszka, R., Megaw, P., & Morgan, I. G. (2007). A muscarinic cholinergic antagonist and a dopamine agonist rapidly increase ZENK mRNA expression in the form-deprived chicken retina. *Experimental Eye Research, 85,* 15-22.

Ashby, R. S. & Feldkaemper, M. P. (2010). Gene expression within the amacrine cell layer of chicks after myopic and hyperopic defocus. *Invest Ophthalmol.Vis.Sci., 51,* 3726-3735.

Attebo, K., Ivers, R. Q., & Mitchell, P. (1999). Refractive errors in an older population: The Blue Mountains Eye Study. *Ophthalmology, 106,* 1066-1072.

Bartmann, M., Schaeffel, F., Hagel, G., & Zrenner, E. (1994). Constant light affects retinal dopamine levels and blocks deprivation myopia but not lens-induced refractive errors in chickens. *Visual Neuroscience, 11,* 199-208.

Belecky-Adams, T. & Adler, R. (2001). Developmental expression patterns of bone morphogenetic proteins, receptors, and binding proteins in the chick retina. *J.Comp Neurol., 430,* 562-572.

Bitzer, M., Feldkaemper, M., & Schaeffel, F. (2000). Visually induced changes in components of the retinoic acid system in fundal layers of the chick. *Experimental Eye Research, 70,* 97-106.

Bitzer, M., Kovacs, B., Feldkaemper, M., & Schaeffel, F. (2006). Effects of muscarinic antagonists on ZENK expression in the chicken retina. *Experimental Eye Research, 82,* 379-388.

Bitzer, M. & Schaeffel, F. (2002). Defocus-induced changes in ZENK expression in the chicken retina. *Invest Ophthalmol.Vis.Sci., 43,* 246-252.

Bradley, D. V., Fernandes, A., Lynn, M., Tigges, M., & Boothe, R. G. (1999). Emmetropization in the rhesus monkey (*Macaca mulatta*): birth to young adulthood. *Invest Ophthalmol.Vis.Sci., 40,* 214-229.

Brand, C., Schaeffel, F., & Feldkaemper, M. P. (2007). A microarray analysis of retinal transcripts that are controlled by image contrast in mice. *Mol.Vis., 13,* 920-932.

Buckerfield, M., Oliver, J., Chubb, I. W., & Morgan, I. G. (1981). Somatostatin-like immunoreactivity in amacrine cells of the chicken retina. *Neuroscience, 6,* 689-695.

Cervia, D., Casini, G., & Bagnoli, P. (2008). Physiology and pathology of somatostatin in the mammalian retina: a current view. *Mol Cell Endocrinol., 286,* 112-122.

Chao, W. & D'Amore, P. A. (2008). IGF2: epigenetic regulation and role in development and disease. *Cytokine Growth Factor Rev, 19,* 111-120.

Cui, D., Trier, K., Zeng, J., Wu, K., Yu, M., & Ge, J. (2010). Adenosine receptor protein changes in guinea pigs with form deprivation myopia. *Acta Ophthalmologica, 88,* 759-765.

Dillingham, C. M., Guggenheim, J. A., & Erichsen, J. T. (2013). Disruption of the centrifugal visual system inhibits early eye growth in chicks. *Invest Ophthalmol Vis Sci, 54,* 3632-3643.

Dillingham, C. M., Guggenheim, J. A., & Erichsen, J. T. (2013). Disruption of the Centrifugal Visual System Inhibits Early Eye Growth in Chicks. *Investigative Ophthalmology & Visual Science*.

Feldkaemper, M. & Schaeffel, F. (2013). An updated view on the role of dopamine in myopia. *Experimental Eye Research, 114,* 106-119.

Feldkaemper, M. P., Neacsu, I., & Schaeffel, F. (2009). Insulin acts as a powerful stimulator of axial myopia in chicks. *Invest Ophthalmol Vis Sci, 50,* 13-23.

Fischer, A. J., McGuire, J. J., Schaeffel, F., & Stell, W. K. (1999). Light- and focus-dependent expression of the transcription factor ZENK in the chick retina. *Nat.Neurosci., 2,* 706-712.

Fischer, A. J., Seltner, R. P., & Stell, W. K. (1997). N-methyl-d-aspartate-induced excitotoxicity causes myopia in hatched chicks. *Canadian Journal of Ophthalmology - Journal Canadien d Ophtalmologie, 32,* 373-377.

Fledelius, H. C. (1988). Myopia prevalence in Scandinavia. A survey, with emphasis on factors of relevance for epidemiological refraction studies in general. *Acta Ophthalmol.Suppl, 185,* 44-50.

Frost, M. R., He, L., & Norton, T. T. (2013). Differential gene expression in tree shrew retinal pigment epithelium (RPE) in response to six hours of minus-lens wear. *Ophthalmic and Physiological Optics, 33,* 667.

Frost, M. R. & Norton, T. T. (2007). Differential protein expression in tree shrew sclera during development of lens-induced myopia and recovery. *Molecular Vision, 13,* 1580-1588.

Frost, M. R. & Norton, T. T. (2012). Alterations in protein expression in tree shrew sclera during development of lens-induced myopia and recovery. *Invest Ophthalmol Vis Sci, 53,* 322-336.

Fujikado, T., Kawasaki, Y., Fujii, J., Taniguchi, N., Okada, M., Suzuki, A. et al. (1997). The effect of nitric oxide synthase inhibitor on form-deprivation myopia. *Current Eye Research, 16,* 992-996.

Fujikado, T., Kawasaki, Y., Suzuki, A., Ohmi, G., & Tano, Y. (1997). Retinal function with lens-induced myopia compared with form-deprivation myopia in chicks. *Graefes Arch Clin.Exp.Ophthalmol, 235,* 320-324.

Gamlin, P. D. (1999). Subcortical neural circuits for ocular accommodation and vergence in primates. *Ophthalmic and Physiological Optics, 19,* 81-89.

Gamlin, P. D. R., Zhang H., Harlow A., & Barbur, J. L. (1998). Pupil responses to stimulus color, structure and light flux increments in the rhesus monkey. *Vision Research, 38,* 3353-3358.

Gao, H., Frost, M. R., Siegwart, J. T., Jr., & Norton, T. T. (2011). Patterns of mRNA and protein expression during minus-lens compensation and recovery in tree shrew sclera. *Mol.Vis., 17,* 903-919.

Glickstein, M. & Millodot, M. (1970). Retinoscopy and eye size. *Science, 168,* 605-606.

Goh, W. S. H. & Lam, C. S. Y. (1994). Changes in refractive trends and optical components of Hong Kong Chinese aged 19-39 years. *Ophthalmic and Physiological Optics, 14,* 378-382.

Graham, B. & Judge, S. J. (1999). The effects of spectacle wear in infancy on eye growth and refractive error in the marmoset (*Callithrix jacchus*). *Vision Research, 39,* 189-206.

Guo, L., Frost, M. R., He, L., Siegwart, J. T., Jr., & Norton, T. T. (2013). Gene expression signatures in tree shrew sclera in response to three myopiagenic conditions. *Invest Ophthalmol Vis Sci, 54,* 6806-6819.

Guo, L., Frost, M. R., Siegwart, Jr. J. T., & Norton, T. T. (2014). Scleral gene expression signatures in tree shrew sclera during lens-induced myopia and recovery. *Experimental Eye Research, submitted for publication*.

He, L., Frost, M. R., Siegwart, J. T., & Norton, T. T. (2014). Gene expression sigatures in tree shrew choroid in response to three myopiagenic conditions. *Vision Research, submitted for publication*.

He, L., Frost, M. R., & Norton, T. T. (2014). Differential gene expression in tree shrew retina compared with retinal pigment epithelium (RPE) in response to six hours of minus-lens wear. Invest Ophthalmol Vis Sci 55, ARVO E-Abstract 3037.
Ref Type: Abstract

He, L., Frost, M. R., Siegwart, J. T., Jr., & Norton, T. T. (2010). Adhesion-related protein and vitamin D receptor mRNA levels in tree shrew sclera during minus lens treatment and during recovery. *Invest Ophthalmol Vis Sci, 51,* ARVO E-Abstract 3681.

He, L., Frost, M. R., Siegwart, J. T., Jr., & Norton, T. T. (2014). Gene expression signatures in tree shrew choroid during lens-induced myopia and recovery. *Exp Eye Res, 123,* 56-71.

He, L., Frost, M. R., Siegwart, Jr. J. T., Filios, S., & Norton, T. T. (2011). Retinal gene expression signatures in tree shrew in response to three myopiagenic visual conditions: minus lens, form deprivation, and darkness. Invest Ophthalmol Vis Sci 52, ARVO E-Abstract 6301.
Ref Type: Abstract

He, M., Zeng, J., Liu, Y., Xu, J., Pokharel, G. P., & Ellwein, L. B. (2004). Refractive error and visual impairment in urban children in southern china. *Invest Ophthalmol Vis Sci, 45,* 793-799.

Helfer, G., Ross, A. W., Russell, L., Thomson, L. M., Shearer, K. D., Goodman, T. H. et al. (2012). Photoperiod regulates vitamin A and Wnt/beta-catenin signaling in F344 rats. *Endocrinology, 153,* 815-824.

Hinterhuber, G., Cauza, K., Brugger, K., Dingelmaier-Hovorka, R., Horvat, R., Wolff, K. et al. (2004). RPE65 of retinal pigment epithelium, a putative receptor molecule for plasma retinol-binding protein, is expressed in human keratinocytes. *J.Invest Dermatol., 122,* 406-413.

Howlett, M. H. & McFadden, S. A. (2006). Form-deprivation myopia in the guinea pig (Cavia porcellus). *Vision Research, 46,* 267-283.

Howlett, M. H. & McFadden, S. A. (2009). Spectacle lens compensation in the pigmented guinea pig. *Vision Research, 49,* 219-227.

Hu, W., Criswell, M. H., Fong, S. L., Temm, C. J., Rajashekhar, G., Cornell, T. L. et al. (2009). Differences in the temporal expression of regulatory growth factors during choroidal neovascular development. *Experimental Eye Research, 88,* 79-91.

Hung, L. F., Crawford, M. L., & Smith, E. L. (1995). Spectacle lenses alter eye growth and the refractive status of young monkeys. *Nature Medicine, 1,* 761-765.

Irving, E. L., Callender, M. G., & Sivak, J. G. (1991). Inducing myopia, hyperopia, and astigmatism in chicks. *Optometry and Vision Science, 68,* 364-368.

Irving, E. L., Callender, M. G., & Sivak, J. G. (1995). Inducing ametropias in hatchling chicks by defocus--aperture effects and cylindrical lenses. *Vision Research, 35,* 1165-1174.

Irving, E. L., Sivak, J. G., & Callender, M. G. (1992). Refractive plasticity of the developing chick eye. *Ophthalmic and Physiological Optics, 12,* 448-456.

Ishida, B. Y., Bailey, K. R., Duncan, K. G., Chalkley, R. J., Burlingame, A. L., Kane, J. P. et al. (2004). Regulated expression of apolipoprotein E by human retinal pigment epithelial cells. *J.Lipid Res., 45,* 263-271.

Iuvone, P. M., Tigges, M., Stone, R. A., Lambert, S., & Laties, A. M. (1991). Effects of apomorphine, a dopamine receptor agonist, on ocular refraction and axial elongation in a primate model of myopia. *Invest Ophthalmol Vis Sci, 32,* 1674-1677.

Janssen, A., Hoellenriegel, J., Fogarasi, M., Schrewe, H., Seeliger, M., Tamm, E. et al. (2008). Abnormal vessel formation in the choroid of mice lacking tissue inhibitor of metalloprotease-3. *Invest Ophthalmol.Vis.Sci., 49,* 2812-2822.

Jin, N. & Stjernschantz, J. (2000). Regional blood flow in the myopic chick eye during and after form deprivation: a study with radioactively-labelled microspheres. *Exp Eye Res, 71,* 233-238.

Jobling, A. I., Wan, R., Gentle, A., Bui, B. V., & McBrien, N. A. (2009). Retinal and choroidal TGFβ in the tree shrew model of myopia: isoform expression, activation and effects on function. *Exp Eye Res, 88,* 458-466.

Johnson, J., Rickman, D. W., & Brecha, N. C. (2000). Somatostatin and somatostatin subtype 2A expression in the mammalian retina. *Microsc.Res.Tech., 50,* 103-111.

Jung, S. K., Lee, J. H., Kakizaki, H., & Jee, D. (2012). Prevalence of myopia and its association with body stature and educational level in 19-year-old male conscripts in Seoul, South Korea. *Invest Ophthalmol Vis Sci, 53,* 5579-5583.

Kang, R. N. & Norton, T. T. (1996). Electronmicroscopic examination of tree shrew sclera during normal development, induced myopia, and recovery [ARVO Abstract]. Investigative Ophthalmology and Visual Science 37, S324.
Ref Type: Abstract

Kay, P., Yang, Y. C., & Paraoan, L. (2013). Directional protein secretion by the retinal pigment epithelium: roles in retinal health and the development of age-related macular degeneration. *J.Cell Mol.Med., 17,* 833-843.

Kee, C. S., Marzani, D., & Wallman, J. (2001). Differences in time course and visual requirements of ocular responses to lenses and diffusers. *Invest Ophthalmol.Vis.Sci., 42,* 575-583.

Lauber, J. K. (1991). Three avian eye enlargement protcols as myopia models: effects of pharmacological intervention. *J Ocular Pharmacol, 7,* 65-75.

Lauber, J. K., McGinnis, J., & Boyd, J. (1965). Influence of mitotics, diamox and vision occluders on light- induced buphthalmos in domestic fowl. *Proceedings of the Society for Experimental Biology and Medicine, 120,* 572-575.

Leske, M. C., Chylack, L. T., Jr., & Wu, S. Y. (1991). The Lens Opacities Case-Control Study. Risk factors for cataract. *Arch.Ophthalmol., 109,* 244-251.

Li, R., Wen, R., Banzon, T., Maminishkis, A., & Miller, S. S. (2011). CNTF mediates neurotrophic factor secretion and fluid absorption in human retinal pigment epithelium. *PLoS ONE, 6,* e23148.

Lin, L. L., Shih, Y. F., Tsai, C. B., Chen, C. J., Lee, L. A., Hung, P. T. et al. (1999). Epidemiologic study of ocular refraction among schoolchildren in Taiwan in 1995. *Optom.Vis.Sci., 76,* 275-281.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods, 25,* 402-408.

Longshore, M. A. & Makman, M. H. (1981). Stimulation of retinal adenylate cyclase by vasoactive intestinal peptide (VIP). *Eur.J.Pharmacol., 70,* 237-240.

Lu, B., Congdon, N., Liu, X., Choi, K., Lam, D. S., Zhang, M. et al. (2009). Associations between near work, outdoor activity, and myopia among adolescent students in rural China: the Xichang Pediatric Refractive Error Study report no. 2. *Archives of Ophthalmology, 127,* 769-775.

Malik, K. J., Chen, C. D., & Olsen, T. W. (2003). Stability of RNA from the retina and retinal pigment epithelium in a porcine model simulating human eye bank conditions. *Invest Ophthalmol.Vis.Sci., 44,* 2730-2735.

Marcus, M. W., de Vries, M. M., Junoy Montolio, F. G., & Jansonius, N. M. (2011). Myopia as a risk factor for open-angle glaucoma: a systematic review and meta-analysis. *Ophthalmology, 118,* 1989-1994.

Marsh-Tootle, W. L. & Norton, T. T. (1989). Refractive and structural measures of lid-suture myopia in tree shrew. *Investigative Ophthalmology and Visual Science, 30,* 2245-2257.

McBrien, N. A. & Norton, T. T. (1987). Experimentally induced myopia in tree shrew. *American Journal of Optometry and Physiological Optics, 64,* 10.

McBrien, N. A. & Norton, T. T. (1992). The development of experimental myopia and ocular component dimensions in monocularly lid-sutured tree shrews (*Tupaia belangeri*). *Vision Research, 32,* 843-852.

McFadden, S. A., Howlett, M. H., & Mertz, J. R. (2004). Retinoic acid signals the direction of ocular elongation in the guinea pig eye. *Vision Research, 44,* 643-653.

McFadden, S. A. & Wildsoet, C. (2009). Mammalian eyes need an intact optic nerve to detect the sign of defocus during emmetropisation. *Invest Ophthalmol Vis Sci, 50,* ARVO E-Abstract 1620.

McGlinn, A. M., Baldwin, D. A., Tobias, J. W., Budak, M. T., Khurana, T. S., & Stone, R. A. (2007). Form-deprivation myopia in chick induces limited changes in retinal gene expression. *Invest Ophthalmol.Vis.Sci., 48,* 3430-3436.

McKanna, J. A. & Casagrande, V. A. (1981). Atropine affects lid-suture myopia development. *Doc Ophthalmol Proc Ser, 28,* 187-192.

McKanna, J. A., Casagrande, V. A., Norton, T. T., & Marsh, W. L. (1983). Dark-reared tree shrews do not develop lid-suture myopia. *Investigative Ophthalmology and Visual Science, 24 Suppl,* 226.

Mertz, J. R. & Wallman, J. (2000). Choroidal retinoic acid synthesis: a possible mediator between refractive error and compensatory eye growth. *Experimental Eye Research, 70,* 519-527.

Morgan, I. G., Ashby, R. S., & Nickla, D. L. (2013). Form deprivation and lens-induced myopia: are they different? *Ophthalmic and Physiological Optics, 33,* 355-361.

Moring, A. G., Baker, J. R., & Norton, T. T. (2007). Modulation of glycosaminoglycan levels in tree shrew sclera during lens-induced myopia development and recovery. *Invest Ophthalmol.Vis.Sci., 48,* 2947-2956.

Mutti, D. O., Mitchell, G. L., Jones, L. A., Friedman, N. E., Frane, S. L., Lin, W. K. et al. (2005). Axial growth and changes in lenticular and corneal power during emmetropization in infants. *Invest Ophthalmol.Vis.Sci., 46,* 3074-3080.

Napoli, J. L. (2012). Physiological insights into all-trans-retinoic acid biosynthesis. *Biochim.Biophys.Acta, 1821,* 152-167.

Nickla, D. L., Damyanova, P., & Lytle, G. (2009). Inhibiting the neuronal isoform of nitric oxide synthase has similar effects on the compensatory choroidal and axial responses to myopic defocus in chicks as does the non-specific inhibitor L-NAME. *Exp Eye Res, 88,* 1092-1099.

Nickla, D. L., Sharda, V., & Troilo, D. (2005). Temporal integration characteristics of the axial and choroidal responses to myopic defocus induced by prior form deprivation versus positive spectacle lens wear in chickens. *Optom.Vis.Sci., 82,* 318-327.

Nickla, D. L. & Wallman, J. (2010). The multifunctional choroid. *Prog.Retin.Eye Res, 29,* 144-168.

Nickla, D. L., Wildsoet, C., & Wallman, J. (1998). The circadian rhythm in intraocular pressure and its relation to diurnal ocular growth changes in chicks. *Experimental Eye Research, 66,* 183-193.

Norton, T. T. (1999). Animal models of myopia: Learning how vision controls the size of the eye. *Institute for Laboratory Animal Research Journal, 40,* 59-77.

Norton, T. T., Amedo, A. O., & Siegwart, J. T., Jr. (2006). Darkness causes myopia in visually experienced tree shrews. *Invest Ophthalmol Vis Sci, 47,* 4700-4707.

Norton, T. T., Amedo, A. O., & Siegwart, J. T., Jr. (2010). The effect of age on compensation for a negative lens and recovery from lens-induced myopia in tree shrews (*Tupaia glis belangeri*). *Vision Research, 50,* 564-576.

Norton, T. T., Essinger, J. A., & McBrien, N. A. (1994). Lid-suture myopia in tree shrews with retinal ganglion cell blockade. *Visual Neuroscience, 11,* 143-153.

Norton, T. T. & McBrien, N. A. (1992). Normal development of refractive state and ocular component dimensions in the tree shrew (*Tupaia belangeri*). *Vision Research, 32,* 833-842.

Norton, T. T., Metlapally, R., & Young, T. L. (2008). Myopia. In A.Garner & G. K. Klintworth (Eds.), *The Pathobiology of Ocular Disease* (pp. 537-556). New York: Taylor & Francis.

Norton, T. T. & Siegwart, J. T., Jr. (1995). Animal models of emmetropization: matching axial length to the focal plane. *J.Am.Optom.Assoc., 66,* 405-414.

Norton, T. T., Siegwart, J. T., Jr., & Amedo, A. O. (2006). Effectiveness of hyperopic defocus, minimal defocus, or myopic defocus in competition with a myopiagenic stimulus in tree shrew eyes. *Invest Ophthalmol Vis Sci, 47,* 4687-4699.

Norton, T. T., Siegwart, J. T., German, A. J., Robertson, J., & Wu, W. W. (2000). Comparison of cycloplegic streak retinoscopy with autorefractor measures in tree shrew eyes with, and without, induced myopia. *Invest Ophthalmol Vis Sci, 41,* ARVO Abstract 563.

Norton, T. T., Wu, W. W., & Siegwart, J. T., Jr. (2003). Refractive state of tree shrew eyes measured with cortical visual evoked potentials. *Optom.Vis.Sci., 80,* 623-631.

Penha, A. M., Schaeffel, F., & Feldkaemper, M. (2011). Insulin, insulin-like growth factor-1, insulin receptor, and insulin-like growth factor-1 receptor expression in the chick eye and their regulation with imposed myopic or hyperopic defocus. *Mol.Vis., 17,* 1436-1448.

Phillips, J. R., Khalaj, M., & McBrien, N. A. (2000). Induced myopia associated with increased scleral creep in chick and tree shrew eyes. *Invest Ophthalmol.Vis.Sci., 41,* 2028-2034.

Quek, T. P., Chua, C. G., Chong, C. S., Chong, J. H., Hey, H. W., Lee, J. et al. (2004). Prevalence of refractive errors in teenage high school students in Singapore. *Ophthalmic and Physiological Optics, 24,* 47-55.

Rada, J. A., Hollaway, L. R., Lam, W., Li, N., & Napoli, J. L. (2012). Identification of RALDH2 as a visually regulated retinoic acid synthesizing enzyme in the chick choroid. *Invest Ophthalmol Vis Sci, 53,* 1649-1662.

Rada, J. A., Huang, Y., & Rada, K. G. (2001). Identification of choroidal ovotransferrin as a potential ocular growth regulator. *Curr.Eye Res., 22,* 121-132.

Rada, J. A. & Wiechmann, A. F. (2009). Ocular expression of avian thymic hormone: changes during the recovery from induced myopia. *Mol.Vis, 15,* 778-792.

Rada, J. A. S., Wiechmann, A. F., Hollaway, L. R., Baggenstoss, B. A., & Weigel, P. H. (2010). Increased hyaluronan synthase-2 mRNA expression and hyaluronan accumulation with choroidal thickening: Response during recovery from induced myopia. *Invest Ophthalmol Vis Sci, 51,* 6172-6179.

Raviola, E. & Wiesel, T. N. (1985). An animal model of myopia. *N Engl J Med, 312,* 1609-1615.

Rohrer, B., Negishi, K., Tao, J., & Stell, W. K. (1993). A role for basic fibroblast growth factor (bFGF) in the visually guided regulation of eye growth in the chick. *Invest Ophthalmol Vis Sci, 34,* ARVO Abstract 2489.

Rohrer, B. & Stell, W. K. (1994). Basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF-β) act as stop and go signals to modulate postnatal ocular growth in the chick. *Experimental Eye Research, 58,* 553-561.

Said, S. I. & Mutt, V. (1972). Isolation from porcine-intestinal wall of a vasoactive octacosapeptide related to secretin and to glucagon. *Eur.J.Biochem., 28,* 199-204.

Sakuta, H., Takahashi, H., Shintani, T., Etani, K., Aoshima, A., & Noda, M. (2006). Role of bone morphogenic protein 2 in retinal patterning and retinotectal projection. *J.Neurosci., 26,* 10868-10878.

Salvador-Silva, M., Ghosh, S., Bertazolli-Filho, R., Boatright, J. H., Nickerson, J. M., Garwin, G. G. et al. (2005). Retinoid processing proteins in the ocular ciliary epithelium. *Mol.Vis., 11,* 356-365.

Schaeffel, F., Burkhardt, E., Howland, H. C., & Williams, R. W. (2004). Measurement of refractive state and deprivation myopia in two strains of mice. *Optom.Vis Sci, 81,* 99-110.

Schaeffel, F., Glasser, A., & Howland, H. C. (1988). Accommodation, refractive error and eye growth in chickens. *Vision Research, 28,* 639-657.

Schaeffel, F., Hagel, G., Bartmann, M., Kohler, K., & Zrenner, E. (1994). 6-Hydroxy dopamine does not affect lens-induced refractive errors but suppresses deprivation myopia. *Vision Research, 34,* 143-149.

Schaeffel, F., Hagel, G., Kohler, K., & Zrenner, E. (1992). Deprivation myopia and ametropia induced by spectacle lenses result from two different mechanisms in chicks. *Investigative Ophthalmology and Visual Science, 33 Suppl,* 1052.

Schaeffel, F. & Howland, H. C. (1988). Mathematical model of emmetropization in the chicken. *Journal of the Optical Society of America, 5,* 2080-2086.

Schaeffel, F. & Howland, H. C. (1991). Properties of the feedback loops controlling eye growth and refractive state in the chicken. *Vision Research, 31,* 717-734.

Schaeffel, F., Troilo, D., Wallman, J., & Howland, H. C. (1990). Developing eyes that lack accommodation grow to compensate for imposed defocus. *Vis Neurosci, 4,* 177-183.

Schippert, R., Brand, C., Schaeffel, F., & Feldkaemper, M. P. (2006). Changes in scleral MMP-2, TIMP-2 and TGFβ-2 mRNA expression after imposed myopic and hyperopic defocus in chickens. *Experimental Eye Research, 82,* 710-719.

Schorderet, M., Sovilla, J. Y., & Magistretti, P. J. (1981). VIP-and glucagon-induced formation of cyclic AMP in intact retinae in vitro. *Eur.J.Pharmacol., 71,* 131-133.

Shaikh, A. W., Siegwart, J. T., & Norton, T. T. (1999). Effect of interrupted lens wear on compensation for a minus lens in tree shrews. *Optom.Vis.Sci., 76,* 308-315.

Shelton, L., Troilo, D., Lerner, M. R., Gusev, Y., Brackett, D. J., & Rada, J. S. (2008). Microarray analysis of choroid/RPE gene expression in marmoset eyes undergoing changes in ocular growth and refraction. *Mol.Vis, 14,* 1465-1479.

Shen, W. & Sivak, J. G. (2007). Eyes of a lower vertebrate are susceptible to the visual environment. *Invest Ophthalmol.Vis.Sci., 48,* 4829-4837.

Sherman, S. M., Norton, T. T., & Casagrande, V. A. (1977). Myopia in the lid-sutured tree shrew (*Tupaia glis*). *Brain Research, 124,* 154-157.

Shih, Y.-F., Fitzgerald, M. E. C., Norton, T. T., Gamlin, P. D. R., Hodos, W., & Reiner, A. (1993). Reduction in choroidal blood flow occurs in chicks wearing goggles that induce eye growth toward myopia. *Current Eye Research, 12,* 219-227.

Siegwart, J. T. & Norton, T. T. (1993). Refractive and ocular changes in tree shrews raised with plus or minus lenses. Investigative Ophthalmology and Visual Science 34, ARVO Abstract S1208.
Ref Type: Abstract

Siegwart, J. T. & Norton, T. T. (1994). Goggles for controlling the visual environment of small animals. *Laboratory Animal Science, 44,* 292-294.

Siegwart, J. T., Jr. & Norton, T. T. (1998). The susceptible period for deprivation-induced myopia in tree shrew. *Vision Research, 38,* 3505-3515.

Siegwart, J. T., Jr. & Norton, T. T. (1999). Regulation of the mechanical properties of tree shrew sclera by the visual environment. *Vision Research, 39,* 387-407.

Siegwart, J. T., Jr. & Norton, T. T. (2005). Selective regulation of MMP and TIMP mRNA levels in tree shrew sclera during minus lens compensation and recovery. *Invest Ophthalmol.Vis.Sci., 46,* 3484-3492.

Siegwart, J. T., Jr. & Norton, T. T. (2013). Response to interrupted hyperopia after restraint of axial elongation in tree shrews. *Optometry and Vision Science, 90,* 131-139.

Simon, P., Feldkaemper, M., Bitzer, M., Ohngemach, S., & Schaeffel, F. (2004). Early transcriptional changes of retinal and choroidal TGFβ-2, RALDH-2, and ZENK following imposed positive and negative defocus in chickens. *Mol.Vis., 10,* 588-597.

Smith, E. L., III, Hung, L. F., & Harwerth, R. S. (1999). Developmental visual system anomalies and the limits of emmetropization. *Ophthalmic Physiol Opt., 19,* 90-102.

Smith, E. L., III, Huang, J., Hung, L. F., Blasdel, T. L., Humbird, T. L., & Bockhorst, K. H. (2009). Hemiretinal Form Deprivation: Evidence for Local Control of Eye Growth and Refractive Development in Infant Monkeys. *Investigative Ophthalmology Visual Science, 50,* 5057-5069.

Sperduto, R. D., Seigel, D., Roberts, J., & Rowland, M. (1983). Prevalence of myopia in the United States. *Arch.Ophthalmol., 101,* 405-407.

Stell, W. K., Tao, J., Karkhanis, A., Siegwart, J. T., Jr., & Norton, T. T. (2004). Amacrine Cells responsive to optical conditions in the tree shrew, *Tupaia glis belangeri*. Invest Ophthalmol Vis Sci 45, E-abstract 1159.
Ref Type: Abstract

Stone, R. A., Laties, A. M., Raviola, E., & Wiesel, T. N. (1988). Increase in retinal vasoactive intestinal polypeptide after eyelid fusion in primates. *Proc.Natl.Acad.Sci U.S.A, 85,* 257-260.

Stone, R. A., Lin, T., Iuvone, P. M., & Laties, A. M. (1990). Postnatal control of ocular growth: dopaminergic mechanisms. In *Myopia and the Control of Eye Growth* (155 ed., pp. 45-57). New York: Wiley & Sons.

Stone, R. A., McGlinn, A. M., Baldwin, D. A., Tobias, J. W., Iuvone, P. M., & Khurana, T. S. (2011). Image defocus and altered retinal gene expression in chick: Clues to the pathogenesis of ametropia. *Invest Ophthalmol Vis Sci, 52,* 5765-5777.

Strauss, O. (2005). The retinal pigment epithelium in visual function. *Physiol Rev., 85,* 845-881.

Summers Rada, J. A., Shelton, S., & Norton, T. T. (2006). The sclera and myopia. *Experimental Eye Research, 82,* 185-200.

Summers, J. A. (2013). The choroid as a sclera growth regulator. *Exp Eye Res, 114,* 120-127.

Tang, P. H., Buhusi, M. C., Ma, J. X., & Crouch, R. K. (2011). RPE65 is present in human green/red cones and promotes photopigment regeneration in an *in vitro* cone cell model. *J.Neurosci., 31,* 18618-18626.

Terubayashi, H., Tsuto, T., Fukui, K., Obata, H. L., Okamura, H., Fujisawa, H. et al. (1983). VIP (vasoactive intestinal polypeptide)-like immunoreactive amacrine cells in the retina of the rat. *Experimental Eye Research, 36,* 743-749.

Tkatchenko, A. V., Walsh, P. A., Tkatchenko, T. V., Gustincich, S., & Raviola, E. (2006). Form deprivation modulates retinal neurogenesis in primate experimental myopia. *Proc.Natl.Acad.Sci.U.S.A, 103,* 4681-4686.

Tornqvist, K., Uddman, R., Sundler, F., & Ehinger, B. (1982). Somatostatin and VIP neurons in the retina of different species. *Histochemistry, 76,* 137-152.

Troilo, D. (1990). Experimental studies of emmetropization in the chick. In *Myopia and the Control of Eye Growth* (pp. 89-114). New York: Wiley & Sons.

Troilo, D., Gottlieb, M. D., & Wallman, J. (1987). Visual deprivation causes myopia in chicks with optic nerve section. *Curr.Eye Res., 6,* 993-999.

Troilo, D. & Judge, S. J. (1993). Ocular development and visual deprivation myopia in the common marmoset (*Callithrix jacchus*). *Vision Research, 33,* 1311-1324.

Troilo, D., Nickla, D. L., Mertz, J. R., & Summers Rada, J. A. (2006). Change in the synthesis rates of ocular retinoic acid and scleral glycosaminoglycan during experimentally altered eye growth in marmosets. *Invest Ophthalmol Vis Sci, 47,* 1768-1777.

Troilo, D. & Wallman, J. (1991). The regulation of eye growth and refractive state: an experimental study of emmetropization. *Vision Research, 31,* 1237-1250.

Vasilaki, A., Mouratidou, M., Schulz, S., & Thermos, K. (2002). Somatostatin mediates nitric oxide production by activating sst(2) receptors in the rat retina. *Neuropharmacology, 43,* 899-909.

Vessey, K. A., Rushforth, D. A., & Stell, W. K. (2005). Glucagon- and secretin-related peptides differentially alter ocular growth and the development of form-deprivation myopia in chicks. *Invest Ophthalmol Vis Sci, 46,* 3932-3942.

Veth, K. N., Willer, J. R., Collery, R. F., Gray, M. P., Willer, G. B., Wagner, D. S. et al. (2011). Mutations in zebrafish lrp2 result in adult-onset ocular pathogenesis that models myopia and other risk factors for glaucoma. *PLoS.Genet., 7,* e1001310.

Vitale, S., Ellwein, L., Cotch, M. F., Ferris, F. L., III, & Sperduto, R. (2008). Prevalence of refractive error in the United States, 1999-2004. *Arch.Ophthalmol., 126,* 1111-1119.

Wallman, J., Gottlieb, M. D., Rajaram, V., & Fugate-Wentzek, L. A. (1987). Local retinal regions control local eye growth and myopia. *Science, 237,* 73-77.

Wallman, J., Turkel, J., & Trachtman, J. (1978). Extreme myopia produced by modest change in early visual experience. *Science, 201,* 1249-1251.

Wallman, J., Wildsoet, C., Xu, A., Gottlieb, M. D., Nickla, D. L., Marran, L. et al. (1995). Moving the retina: choroidal modulation of refractive state. *Vision Research, 35,* 37-50.

Wallman, J. & Winawer, J. (2004). Homeostasis of eye growth and the question of myopia. *Neuron, 43,* 447-468.

Wang, C. X.-Z., Zhang, K., Aredo, B., Lu, H., & Ufret-Vincenty, R. L. (2012). Novel method for the rapid isolation of RPE cells specifically for RNA extraction and analysis. *Experimental Eye Research, 102,* 1-9.

Wang, Q., Klein, B. E., Klein, R., & Moss, S. E. (1994). Refractive status in the Beaver Dam Eye Study. *Invest Ophthalmol Vis Sci, 35,* 4344-4347.

Wensor, M., McCarty, C. A., & Taylor, H. R. (1999). Prevalence and risk factors of myopia in Victoria, Australia. *Arch.Ophthalmol., 117,* 658-663.

Wiesel, T. N. & Raviola, E. (1977). Myopia and eye enlargement after neonatal lid fusion in monkeys. *Nature, 266,* 66-68.

Wildsoet, C. (2003). Neural pathways subserving negative lens-induced emmetropization in chicks--insights from selective lesions of the optic nerve and ciliary nerve. *Current Eye Research, 27,* 371-385.

Wildsoet, C. F. & Pettigrew, J. D. (1988). Experimental myopia and anomalous eye growth patterns unaffected by optic nerve section in chickens: Evidence for local control of eye growth. *Clinical Vision Science, 3,* 99-107.

Wildsoet, C. F. & Wallman, J. (1995). Choroidal and scleral mechanisms of compensation for spectacle lenses in chicks. *Vision Research, 35,* 1175-1194.

Yew, K. & Wildsoet, C. F. (2003). The usual effects of high-power negative lens and diffusers show differential susceptibility to disruption to the diurnal light cycle. *Invest Ophthalmol Vis Sci, 44,* ARVO E-Abstract 1979.

Young, T. L., Raviola, E., Russell, M. E., & Wiesel, T. N. (1994). Upregulation of vasoactive intestinal polypeptide (VIP) gene expression in the retina of myopic eyes following lid fusion in monkeys [ARVO Abstract]. Investigative Ophthalmology and Visual Science 35[suppl.], S2069.
Ref Type: Abstract

Zhang, Y., Liu, Y., Ho, C., & Wildsoet, C. F. (2013). Effects of imposed defocus of opposite sign on temporal gene expression patterns of BMP4 and BMP7 in chick RPE. *Exp Eye Res, 109,* 98-106.

Zhang, Y., Liu, Y., & Wildsoet, C. F. (2012). Bidirectional, optical sign-dependent regulation of BMP2 gene expression in chick retinal pigment epithelium. *Invest Ophthalmol Vis Sci, 53,* 6072-6080.

APPENDIX A

IACUC Approval Form

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: February 23, 2012

TO: THOMAS T NORTON, PhD
WORB-606 4390
FAX: (205) 934-5725

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: Mechanisms of Ocular Development
Sponsor: NIH
Animal Project Number: 120208727

As of February 23, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Tree Shrews	A	50
Tree Shrews	B	100

Animal use must be renewed by February 22, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120208727 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
933 19th Street South
205.934.7692
FAX 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE S
BIRMINGHAM AL 35294-0019