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Gene Expression Signatures In Tree Shrew Sclera In Different Visual Conditions

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GENE EXPRESSION SIGNATURES IN TREE SHREW SCLERA IN DIFFERENT VISUAL CONDITIONS

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ABSTRACT

The sclera is a target tissue that receives signals that are initiated in the retina, cascade through retinal pigment epithelium (RPE) and choroid, and cause scleral extra cellular matrix remodeling. Biomechanical alterations of the sclera are produced by these biochemical changes, and in turn control the axial length of the eye. This dissertation project examined scleral gene expression changes in mRNA level of juvenile tree shrews.

Three specific aims were investigated: **specific aim one** tested the hypothesis that three different GO visual conditions that all produce axial elongation and myopia: minus-lens wear, form deprivation, and continuous darkness, will produce similar gene expression signatures in sclera.

Recovery (STOP) occurs in response to the myopia that is produced by discontinuing minus-lens wear. The axial elongation rate slows and the induced myopia dissipates. Scleral responses in STOP were examined in **specific aim two** to compare with GO conditions.

A very similar refractive myopia can also be produced by having normal juvenile eyes wear convex lenses. This treatment produces very little refractive change (IGNORE). In **specific aim three**, we compared the scleral response to IGNORE conditions with the STOP response.

In all three specific aims, scleral mRNA levels for 55 candidate genes were meas-

ured in treated and control eyes and compared between groups of tree shrew (n = 7 per group) exposed to GO, STOP, and IGNORE conditions. Normal interocular differences were also measured. In **specific aim one**, the gene expression in tree shrew sclera was very similar in the three different GO conditions, a GO signature. **Specific aim two** found a STOP response signature that included some bi-directionally-regulated genes, but the expression of other genes differed only in GO or STOP. Although plus-lens wear did not produce significant refractive changes in **specific aim three**, the IGNORE gene signature showed that signals arrive at the sclera produce a pattern that is similar to STOP signature with distinguishable differences in normal sclera.

Keywords: myopia, animal models, emmetropization, gene expression, sclera

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INTRODUCTION

REFRACTIVE ERROR AND AXIAL LENGTH

The eye is one of the most delicate organs of the human body and a large amount of information about the environment is obtained from vision. In order to see clearly, light must be focused on the retina.

Light rays from distant objects pass through cornea, anterior chamber, crystalline lens, vitreous chamber, and, in an emmetropic eye, are finally focused on the photoreceptors of the retina, where the visual information is transduced into neural signals, analyzed, and then sent to the visual cortex through the optic nerve, optic tract and lateral geniculate nucleus.

Refractive error is the result of a mismatch between the location of focal plane at which light is focused and the axial length of the eye. Genetics control the refractive power distribution of the cornea, the lens, and anterior chamber depth (McBrien & Norton, 1992; Siegwart, Jr. & Norton, 2011). If the axial length is too short relative to the focal plane, the eye is hyperopic. If the axial length is too long relative to the focal plane, the eye is myopic. Most human and animal eyes have substantial refractive error at birth, but this decreases during the postnatal period. The question of how emmetropia is achieved and maintained has long been a hot topic in studies of both human and animal eyes.

In humans, the axial length of the eye grows from about 17 mm at birth to 20 mm at 9 months of age (Mutti et al., 2005), and arrives about 24 mm for emmetropic adults.

Evidence from animal studies has shown that there is an active emmetropization mechanism that uses refractive error to guide the axial elongation of the developing eye so that the photoreceptors achieve a match to the focal plane, generally avoiding the mismatch that leads to myopia or hyperopia. Both human and animal studies suggest that the visual environment affects the axial length of the eye but not the focal plane.(Qiao-Grider, Hung, Kee, Ramamirtham, & Smith, III, 2010; Siegwart, Jr. & Norton, 1999)

EPIDEMIOLOGY

In the human population, myopia is one of the most prevalent ocular conditions. It affects about 25-40% of the population in North American, European and Australian populations (Attebo, Ivers, & Mitchell, 1999; Fledelius, 1988; Sperduto, Seigel, Roberts, & Rowland, 1983; Vitale, Ellwein, Cotch, Ferris, III, & Sperduto, 2008; Wang, Klein, Klein, & Moss, 1994; Wensor, McCarty, & Taylor, 1999), and the prevalence reaches as high as 85-96.5% of urban Asian populations in South-East Asia (Goh & Lam, 1994; He et al., 2004; Jung, Lee, Kakizaki, & Jee, 2012; Lin et al., 1999; Lu et al., 2009; Quek et al., 2004).

Elongated axial length, more specifically enlargement of the vitreous chamber of the eye, is the most common structural cause associated with juvenile-onset myopia (Curtin, 1985). Learning how refractive error can develop, despite the existence of an emmetropization mechanism, is an important goal. Achieving that goal depends upon understanding the normal function of the emmetropization mechanism and ways it changes with age.

THE EMMETROPIZATION MECHANISM

The emmetropization mechanism is a biological feedback mechanism in the juvenile eye, which uses the refractive state to bring the retina to the focal plane (Lauber, McGinnis, & Boyd, 1965; Mutti et al., 2005; Sherman, Norton, & Casagrande, 1977; Troilo & Judge, 1993; Wallman, Turkel, & Trachtman, 1978; Wiesel & Raviola, 1977). This coordinates the eye's postnatal axial elongation with the maturation of its refractive components (Bradley, Fernandes, Lynn, Tigges, & Boothe, 1999). The first clear indication that this emmetropization mechanism exists was that monkey eyes deprived of clear images (form deprivation) became too long for their own optics and were, therefore, myopic (Wiesel & Raviola, 1977). A similar discovery was made the same year in tree shrews (Sherman et al., 1977) and later in chick (Schaeffel & Howland, 1988; Wallman & Adams, 1987; Wallman et al., 1978; Wildsoet, Howland, Falconer, & Dick, 1993). Evidence that the emmetropization mechanism is regulated by visual feedback was obtained in many animal models by using lenses to stimulate the emmetropization mechanism and alter the axial elongation rate (Graham & Judge, 1999; Howlett & McFadden, 2009; Hung, Crawford, & Smith, 1995; Irving, Sivak, & Callender, 1992; Schaeffel, Hagel, Kohler, & Zrenner, 1992; Shen & Sivak, 2007; Siegwart, Jr. & Norton, 1999).

OUR ANIMAL MODEL

The tree shrew, a small mammal that is closely related to primates with a good response to both form deprivation and lens treatment, has been chosen to be our animal model in this study. They are diurnal animals with eye structures and a fibrous sclera that is similar to monkey and human. Their infants grow fast enough to enable this study to be

finished within a reasonable period of time, and their genome sequence is mostly known. Tree shrews are born with eyes closed, and the eyes open around 21days after birth (Marsh-Tootle & Norton, 1989; Norton & McBrien, 1992; Shaikh, Siegwart, & Norton, 1999). The first day when their eyes are both open is defined as first day of visual experience (DVE). At eye opening, the eyes are very hyperopic (about +25D) (McBrien & Norton, 1992) with axial length approximately 85% of the adult value at this time (Norton & McBrien, 1992).

As in human infants (Sorsby, Benjamin, Sheridan, Stone, & Leary, 1961), there are two relatively distinct growth phases of tree shrew refractive development starting from day one of visual experience (VE). During the first 15 DVE their hyperopia drops dramatically (over 20 D) (Norton & McBrien, 1992); this is produced by changes in the ocular component dimensions. As the refractive error becomes closer to emmetropia, the hyperopia decrease rate becomes slower within the second, juvenile emmetropization phase (Figure 1 purple line). Their refractive error declines from about 7 D at 11 DVE rapidly to about 1.5 D at 24 DVE, and then to less than 1 D at 35 DVE (Norton, Amedo, & Siegwart, Jr., 2006; Siegwart, Jr. & Norton, 2010). By 60 days of VE, eyes are gradually becoming close to emmetropia (Norton & McBrien, 1992; Norton, Siegwart, Jr., & Amedo, 2006). During the infantile and juvenile periods, the focal plane is continually moved away from the cornea by the maturation of the cornea and the lens. To achieve continuing emmetropia, the axial length must continually, but slowly, increase. This process also is visually guided (Norton, Amedo, & Siegwart, Jr., 2010).

During development, the refractive state and elongation rate of normal eyes serve as the baseline; in studies in which one eye is treated and the other is an untreated fellow

control eye, the refractions and elongation rate for control eyes are typically equal to normal eyes. Although minus lens wear is effective in producing axial elongation and lens compensation well into adulthood, there is a peak sensitive period starting around 10-15 days of VE and lasting for at least a month (Norton, 1990; Norton et al., 2010; Siegwart, 1997).

MANIPULATING THE EMMETROPIZATION MECHANISM

Minus Lens Induced Myopia (GO #1)

In minus lens "compensation", a concave (minus-power) lens is held in place in front of an eye by means of a goggle frame attached to the skull; this shifts the focal plane away from the cornea, making the eye hyperopic in comparison to a control eye or to normal eyes (Figure 1, left). In response, the juvenile eye increases its axial elongation rate, enlarging the vitreous chamber until the retina comes to reside at the location of the shifted focal plane, eliminating the refractive error.

Manipulating visual environment by applying minus lenses in front of animals' eyes to induce axial elongation has been well established on many animal models (Schaeffel, Glasser, & Howland, 1988; Siegwart & Norton, 1993; Troilo & Wallman, 1991). In this case, the treated eye elongates its axial length to match the new focal plane becoming emmetropic while wearing the lens (Figure 2, red symbols). With the lens removed (Figure 1) the eye is myopic, so this also is called lens-induced myopia (LIM).

An increase in vitreous chamber depth is the cause of axial elongation; there is no significant change to the corneal power, anterior chamber depth and lens thickness during lens-induced myopia formation (Hung et al., 1995; Irving, Callender, & Sivak, 1991;

Irving, Callender, & Sivak, 1995; Schaeffel et al., 1992; Shaikh et al., 1999). Based on the observation on many tree shrews in our lab, a 100 µm change in vitreous chamber depth produces a 4 D to 5 D refractive change in refraction (Siegwart, Jr. & Norton, 2010). As will be described later, in this condition the retina detects the hyperopia and generates signals that cause the sclera to remodel so that the eye becomes longer, a GO condition.

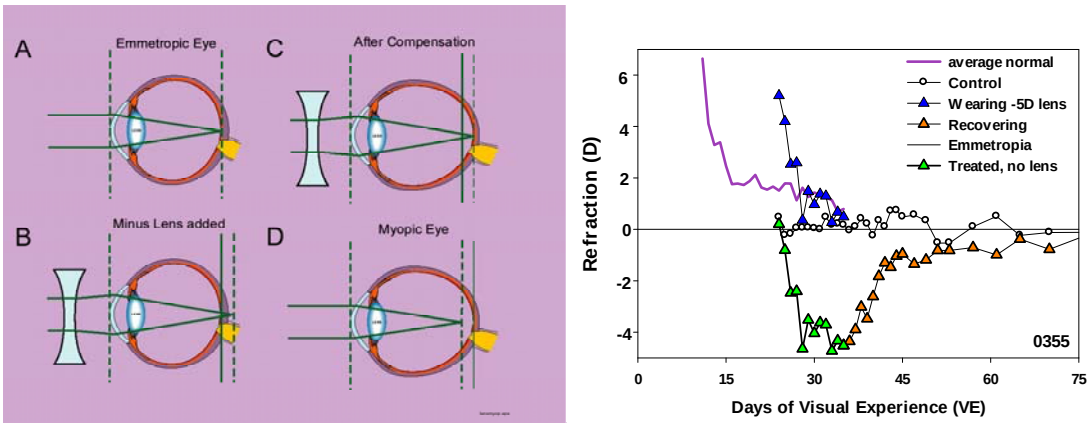

Figure 1. Stimulate the emmetropization mechanism.

Left figure: stimulating the emmetropization mechanism by placing a minus lens in front of normal eye. **A**. In an emmetropic eye, parallel light rays pass through the cornea and the crystalline lens and then focus on the retina; **B**. A minus (concave) lens moves the focal plane behind retina, which makes the eye hyperopic; **C**. After a few days, the eye elongates to move the retina to the new focal plane; **D**. If the minus lens is removed after axial length elongation, the eye is myopic. This process is referred to as “minus lens compensation” and lens-induced myopia (LIM). **Right figure**: The purple line shows the average refractive development of normal animals. The blue triangles show the effect of minus lens wear in a juvenile tree shrew measured while the lens is in place. The green triangles show the effect of minus lens wear measured without the lens. The eye becomes myopic; open circles denote the untreated control eye. The orange triangles show the effect of removing the minus lens. Source: Norton lab library

Form Depravation Induced Myopia (GO #2)

Wiesel and Raviola reported that form deprivation by eyelid closure in rhesus monkeys causes axial (vitreous chamber) elongation and myopia in the deprived eyes (Wiesel & Raviola, 1977). This myopiagenic method later has been confirmed on cats (Wilson & Sherman, 1977), tree shrews (Sherman et al., 1977), chicks (Wallman et al., 1978), rabbits (Mohan, Rao, & Dada, 1977) and other species. More recent studies have produced form deprivation by use of a translucent diffuser held in front of the eye. This eliminates high spatial-frequency images and dramatically reduces the contrast of all retinal images. The retina cannot detect clear visual stimulus under this situation, which creates an "open loop" situation where the eye never receives feedback that emmetropia has been achieved, so the eye keeps elongating until it becomes more than 10 D myopic (McBrien & Norton, 1992; Siegwart, Jr. & Norton, 1999).

The elongation gradually slows after 30 days or so; the eventual limit, however, has not been systematically explored. Tree shrews under approximately 15 days of VE, however, are not sensitive to form deprivation, which may be caused by the immaturity of the retinal signaling mechanism, but also might occur because the eye is already elongating as rapidly as is possible at that age (Siegwart, Jr. & Norton, 1998). Shorter treatment periods or older treatment ages result in less induced myopia. Yoking effects in which the control eye develops a transient myopic shift also have been reported (Gao, Frost, Siegwart, Jr., & Norton, 2011; McBrien & Norton, 1992).

Continuous Darkness Induced Myopia (GO #3)

Continuous darkness in tree shrews that achieved emmetropia in normal lighting is a third, recently discovered, way to increase the axial elongation rate and produce refractive myopia (Norton et al., 2006). Like form deprivation, this is also an open-loop visual environment where there is no end point image on the retina that could be used to slow the axial elongation rate (Hess, Schmid, Dumoulin, Field, & Brinkworth, 2006; Schmid & Wildsoet, 1997). The retinal mechanisms by which continuous darkness produces axial elongation and myopia is still undiscovered, however, evidence from retinal mRNA studies in tree shrews and other species suggest that minus lens wear, form deprivation and continuous darkness involve different retinal mechanisms (He, Frost, Siegwart, Filios, & Norton, 2011; Kee, Marzani, & Wallman, 2001; Schaeffel, Hagel, Bartmann, Kohler, & Zrenner, 1994).

In tree shrews, the corneal curvature does not differ between dark treated eyes and age-matched normal or control eyes (Norton et al., 2006). In contrast, the cornea of dark-treated chicks becomes flatter, causing the eyes to become hyperopic despite their increased axial elongation. (Gottlieb, Fugate-Wentzek, & Wallman, 1987; Lauber, 1991; Li & Howland, 2003; Oishi, Lauber, & Vriend, 1987; Troilo & Wallman, 1991). The finding that continuous darkness is myopiagenic reinforces the concept that emmetropization is an active process that requires a continuous visual guidance to maintain the match between the axial length and the focal plane of the eye.

The fact that all three myopiagenic conditions produce an increase in the axial elongation rate suggests that the scleral fibroblasts should respond similarly in all three conditions. This hypothesis examined in Specific Aim 1.

Recovery from Induced Myopia (STOP)

After myopiagenic treatment, if the myopiagenic conditions (minus lens, form deprivation, or dark treatment) are removed, the eye is optically myopic because the retina is behind the focal plane; the axial length is longer relative to contralateral control eyes or age-matched normal eyes. The retina detects the myopic refractive state, and produces a STOP signal that produces a slowed axial elongation rate that is below normal at the beginning of recovery(Siegwart, Jr. & Norton, 1999). Over time, the focal plane moves away from the cornea because the cornea flattens and lens-power is reduced, so the refractive state of the eye in juvenile tree shrews recovers back to match the control or age-matched normal eyes (Figure 1 Right, green symbols) (McBrien, Gentle, & Cottriall, 1999; Norton, 1990; Shaikh et al., 1999; Siegwart, Jr. & Norton, 1998; Siegwart, Jr. & Norton, 1999).

The slowed elongation rate is caused by what can be described as retinal STOP signals altering the scleral biochemistry and biomechanical properties. As a result of slowing down the axial elongation rate, in contrast to the normal elongation rate, the refractive state returns to normal (Moring, Baker, & Norton, 2007; Norton et al., 2010; Siegwart, Jr. & Norton, 2005). The sclera mRNA response to the STOP condition was examined in Specific Aim 2.

Plus Lens Wear in Juvenile Animals (IGNORE)

Placing a plus lens in front of an emmetropic eye, the light rays from distant objects focusing in front of the retina, makes the eye myopic. Plus lens wear thus produces a similar refractive situation to that experienced by eyes that have compensated for a minus

lens when lens wear is discontinued: both experience refractive myopia. As discussed above (Figure 1, Right, green symbols) elongated eyes recover from the induced myopia. However, the response of normal eyes differs from that of elongated eyes. Age-matched juvenile normal eyes have a greatly reduced ability to use myopia to slow their axial elongation rate below normal (Figure 2, blue symbols) Juvenile tree shrews, between 24 and 35 days of VE, generally do not respond to +5 D lens wear (Figure 2); most remain myopic with the lens on (Siegwart, Jr. & Norton, 2010). The juvenile normal eyes, while wearing plus lenses, ignore the STOP visual stimuli in contrast to the age-matched myopic eyes that recover form myopia (Figure 2, green symbols).

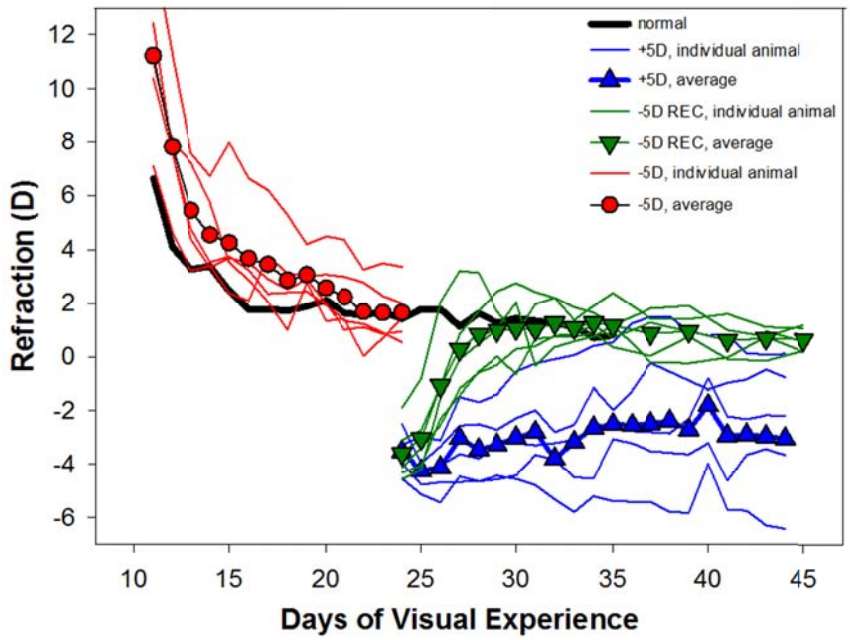

Figure 2. Refractive measures.

Refractive measures with the lens in place, of three groups of animals that underwent different lens treatments: (1) the black solid line represents the average refractive development of normal eyes. (2) The red symbols and lines show compensation to a -5D lens (GO). (3) Green symbols and lines show refractive recovery after the -5D lens wear was discontinued (STOP). (4) The blue symbols and lines show age-matched normal juvenile animals wore a +5D lens, creating the same refractive myopia (IGNORE). Source: Norton lab library

Because it is the sclera that controls the axial length of the eye, it would appear that the sclera responds differently in the two STOP conditions. The purpose of Specific Aim 3 is to examine how these two conditions affect the responses of scleral fibroblasts, as assessed by examining the pattern (signature) of mRNA expression levels.

COMMUNICATION WITH SCLERA IS LOCAL AND DIRECT

Although this dissertation project will deal only with the sclera, it is important to discuss how the signals generated in the retina arrive at sclera. Surprisingly, there is direct, spatially local communication from the retina, through the retinal pigment epithelium (RPE) and choroid to the sclera. The first indication for the existence of a direct pathway, not involving communication with central visual structures, was that cutting the optic nerve in rhesus monkeys did not prevent the development of lid-suture myopia (Raviola & Wiesel, 1985). Later, researchers found that the sectioning the optic nerve in chicks and guinea pigs also did not stop myopia development (Troilo, Gottlieb, & Wallman, 1987; Wildsoet, 2003; Wildsoet & McFadden, 2010; Wildsoet & Pettigrew, 1988). In agreement with this finding, intravitreous injection in chicks and tree shrews of tetrodotoxin (TTX), which blocks the communication between retinal and brain by inactivating voltage-dependent sodium channels in axons, blocking action potentials, (Hwang & Noguchi, 2007) did not block the axial elongation length of the eyes under myopiagenic conditions (Norton, Essinger, & McBrien, 1994; Wildsoet & Wallman, 1995).

Further evidence that the signal pathway is spatially local has been shown in tree shrew, chicks, guinea pigs, and rhesus monkeys by employing diffusers or minus lenses that only cover half of the visual field, which produces myopia development and axial

elongation only at the affected half field (Diether & Schaeffel, 1997; Gottlieb et al., 1987; Hodos & Kuenzel, 1984; McFadden, 2002; Norton & Siegwart, 1991; Siegwart & Norton, 1993; Smith et al., 2010; Smith, III et al., 2009; Troilo et al., 1987; Wallman, Gottlieb, Rajaram, & Fugate-Wentzek, 1987; Zeng & McFadden, 2010).

All aforementioned evidences corroborate that the effects of visual stimuli on refractive development and ocular growth that are shown on animal models are manipulated by emmetropization mechanism. Signals produced by the retina that cascade through RPE and choroid, communicate with sclera directly and locally (Figure 3). The nature of the signal molecules that pass information from retina to RPE, RPE to choroid and choroid to sclera is poorly understood.

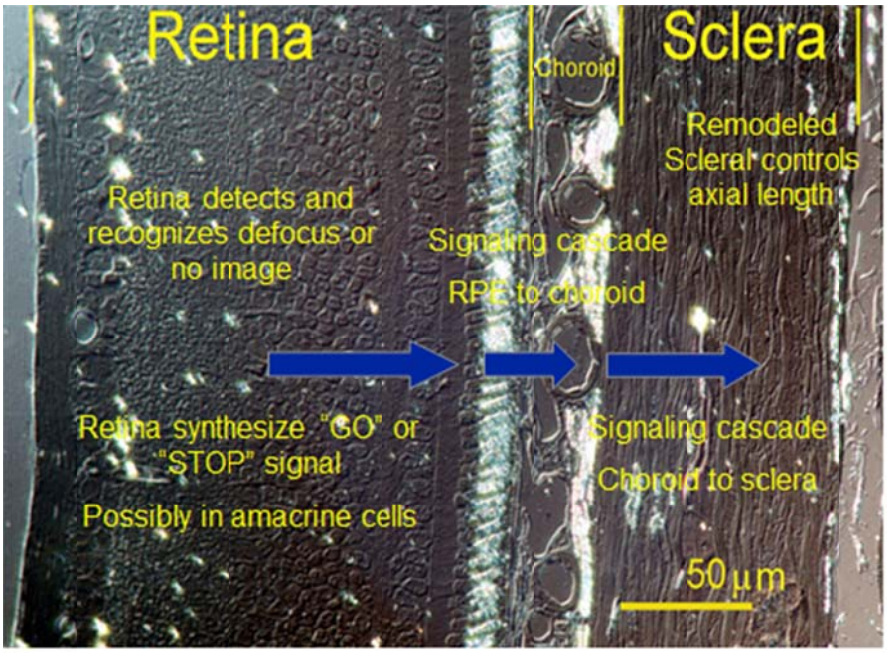

Figure 3. Local signaling cascade underlying emmetropization mechanism.
Retina detects and recognizes visual that the eye is hyperopic or myopic, then produces GO or STOP signals according to the refractive error. Signals cascade in stages through the RPE and then the choroid where signal modification or amplification may occur. Finally, signals arrive at the sclera modifying its biomechanical properties to control axial elongation rate. Source: Norton lab library

BIOMECHANICAL CHANGES IN THE SCLERA

The change of the axial elongation rate during normal maturation and in response to visual stimuli is caused by modulation of the scleral biochemistry that affects the viscoelasticity of the sclera and is measured by the creep rate (Siegwart, Jr. & Norton, 1999). The creep rate is the rate of increase in the length of a tissue when subjected to a constant tension over a certain period of time. Only the scleral strip where the posterior pole is located has been used to measure the creep rate. The previous studies in our lab have demonstrated that the changes in creep rate are closely related to alternations in the axial elongation rate under different visual stimuli.

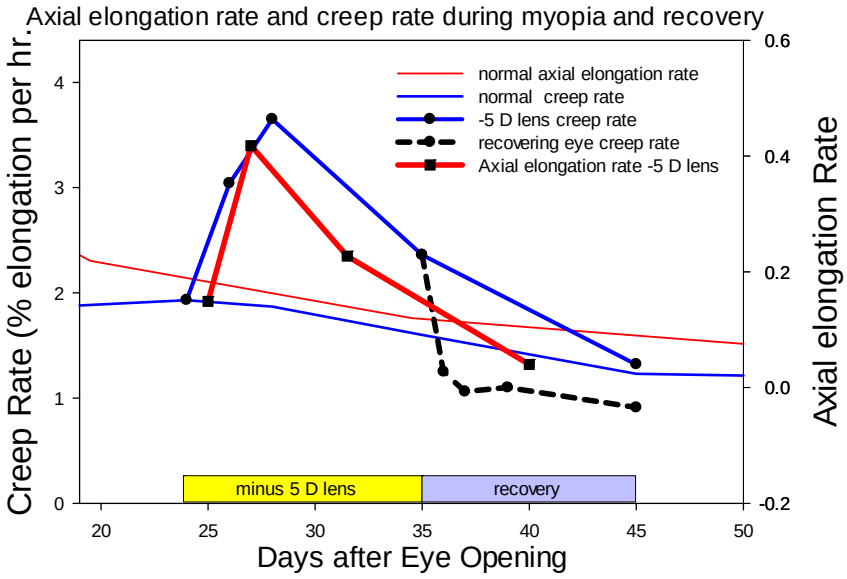

Figure 4. Axial elongation rate and creep rate during myopia and recovery.
Creep rate increases rapidly at the start of minus lens wear, in parallel with the increasing of the axial elongation rate, reaching its maximum at 4 days of lens wear and then declining with the declination decrease in axial elongation rate. Recovery from minus lens wear makes the creep rate decline lower than normal. Source: Norton lab library

Increased axial elongation rate and corresponding increased creep rate (Figure 4) are found in the treated eyes both on deprivation and minus lens wear animals as compared to control eyes. Maximum scleral creep rate reaches maximum at 4 days of -5 D lens treatment, then declines toward normal after 11 days when the axial length of the eye again matches the new focal plane (Figure 4). The increased creep rate may be caused by sclera lamella layers slipping across each other more easily, which gives rise to the increased axial elongation under normal intraocular pressure. Recovery from myopia by removing the minus lens results in a decline in both axial elongation rate and creep rate; the creep rate drops even lower than the normal level (Siegwart, Jr. & Norton, 1999).

SCLERAL MORPHOLOGY

Before describing the biochemical changes that have been found to occur in the sclera during myopia development and recovery, it is important to review the morphological characteristics of this tissue. As in other eutherian mammals, the tree shrew sclera is an extracellular matrix (ECM) arranged in layers (lamellae) of fibrillar collagen (Figure 5). The ECM is produced by the fibroblasts. In tree shrews, many of the fibroblasts contain smooth-muscle actin and, thus, are myofibroblasts (Phillips & McBrien, 2004). The scleral fibroblasts (Figure 5B) are located between the lamella.

SCLERAL COMPONENTS

Remodeling of the ECM is the underlying reason for the alterations of the scleral creep rate. The ECM provides support for cells and framework for tissue, which is a dynamic entity being remodeled constantly. The next sections will focus on several types of

proteins that are of structural importance (collagens and proteoglycans), and proteins that are involved in scleral remodeling: metalloproteinases and their inhibitors.

Figure 5. Tree shrew sclera. **A.** Light micrograph showing the outer segments of the photoreceptors, the retinal pigment epithelium (RPE), the choroid and the sclera lamellae layers which are composed largely of type I collagen. **B.** Low power electron micrograph showing fibroblasts (black) that are located in between the scleral lamellae. **C.** Higher power electron micrograph showing the edges of two lamellae with collagen fibrils of differing orientation. Collagen fibrils are oriented in the same direction within each lamella. Scale bar in C = 1 µm. Source: Norton lab library

Collagens

Collagen is the most abundant protein in animal bodies. In tree shrew, about 90% of sclera dry weight is from collagens, and 95% of these collagens are type I collagen (Norton & Miller, 1995), which is the primary structural element of the sclera. Collagen types III, V, VI, VIII, XIII (Gentle, Liu, Martin, Conti, & McBrien, 2003; Kittelberger, Davis, Flynn, & Greenhill, 1990; Marshall, Konstas, & Lee, 1993; Sandberg-Lall, Hagg, Wahlstrom, & Pihlajaniemi, 2000) and collagen subtypes VII, IX, XV, XVI, XVII (Gentle et al., 2003) also have been found in tree shrew sclera. Type V collagen plays an important role in mediating lateral accretion of collagen fibrils. It interacts with type I collagen during fibrillogenesis to mediate fibril diameter (Birk, Fitch, Babiarz, Doane, & Linsenmayer, 1990), and a higher type V/type I collagen ratio produces thinner fibrils (Paul, Birk, Oldberg, & Chakravarti, 2001). Collagen III is also associated with fibril diameter reduction (Birk & Trelstad, 1997). As with the type V/type I collagen ratio, an increase of type III/type I collagen ratio produces smaller diameter type I collagen fibrils (Gentle et al., 2003). Collagen fibrils in sclera are arranged irregularly and vary highly in diameters. The typical collagen fibril diameters in posterior pole of sclera in tree shrews are divided into the inner, middle, and outer third regions, and the average diameters is 76 nm (50-125 nm) (Kang & Norton, 1996; McBrien, Cornell, & Gentle, 2001). As shown in Figure 5, the orientation of the collagen fibrils within a lamella is the same, but typically differs from one lamella to the next. The scleral lamellae are variable in thickness and orientation, forming an interwoven pattern (Summers Rada, Shelton, & Norton, 2006).

Hydroxyproline is a major component of type I collagen and plays a role in collagen stability (Berg & Prockop, 1973). During monocular form deprivation, hydroxypro-

line levels become significantly lower in the treated eye, more specifically at the posterior pole of sclera (about -11.8% less), indicating that the reduced collagen accumulation happens mainly in this area (Norton & Rada, 1995). During myopia development, induced by either form deprivation or minus lens wear, expression of mRNA for type I collagen is reduced up to 35%, relative to normal eyes and to untreated fellow control eyes, which suggests the reduced collagen accumulation in myopic eye is at least partly due to a decrease in collagen synthesis (Gentle, Martin, & McBrien, 2002; Siegwart, Jr. & Norton, 2002). The expression levels of type III and V collagens for treated eyes during myopiagenic visual conditions remains the same as in control eyes (Gentle et al., 2002), which leads about a 20% increase in the collagen type V/I and type III /I ratio. Since type V collagen plays a critical role in controlling the diameter of the collagen fibers, the increased type V/I collagen ratio may result in a reduction of collagen fibril diameter (Birk et al., 1990). However, this can only occur at the edges of the lamellae, as Kang (Kang & Norton, 1996) found that fibril diameters and spacing were unaffected in the mid-regions of the lamellae after form deprivation. The loss of collagen is greatest during the first few days of myopia development (McBrien et al., 2001; McBrien, Lawlor, & Gentle, 2000; Norton & Miller, 1995). During recovery from induced myopia, there is an increase in mRNA for type I collagen (Gao et al., 2011) but little recovery of type I collagen accumulation (Norton & Miller, 1995).

Proteoglycans

Along with collagen and elastic fibers (Moses, Grodzki, Starcher, & Galione, 1978), proteoglycans (PG: aggrecan, biglycan, and decorin) are present in the scleral

ECM, and occupy approximately 0.7-0.9% of sclera dry weight (Rada, Achen, Perry, & Fox, 1997). Several biologic functions are served by proteoglycans in sclera, embracing regulation of hydration, structural integrity maintenance, growth regulation, matrix organization, cell adhesion, and the ability to bind to certain growth factors (Hassell, Blochberger, Rada, Chakravarti, & Noonan, 1993). Proteoglycans are comprised of a core protein and at least one attached glycosaminoglycan (GAG) side chain that made up of repeating disaccharide units (Summers Rada et al., 2006). GAGs can be mainly categorized into four groups: hyaluronan, chondroitin sulfate and dermatan sulfate, heparan sulfate and heparin (Brown, Vural, Johnson, & Trinkaus-Randall, 1994; Trier, Olsen, & Ammitzboll, 1990), and keratin sulfate (Borcherding et al., 1975). Hyaluronan or hyaluronic acid (HA) is an exception that does not have a core protein to attach to. It is a non-sulfated glycosaminoglycan (Brown et al., 1994; Trier et al., 1990) with the ability to bind with aggrecan monomers in the presence of link protein, which can imbibe water and produce resiliency in cartilage (Muir, 1983).

Scleral glycosaminoglycans (GAGs) content is probably a major factor involved in the viscoelastic properties of the sclera underlying the early changes during myopia development. Decreased GAG synthesis in the sclera has been found both in human and many mammalian myopia models (McBrien et al., 1999; Rada, Nickla, & Troilo, 2000). It occurs in the earliest stages of myopia development and continues in the medium term (McBrien, Gentle, & Anastasopoulos, 2001; McBrien et al., 2000), resulting in the overall GAGs content reduction shows a rapid differential decrease during myopia development and returns quickly to normal during recovery in tree shrews (Moring et al., 2007). GAGs synthesis rate increases rapidly during recovery, and reaches the rate of control

eye within 24 hours (McBrien et al., 2000). After the refractive errors of the recovery eye diminished, the GAGs synthesis level returns to normal as well (McBrien et al., 2000). Hyaluronan (HA) shows a rapid differential decrease during myopia development and returns to normal rapidly during recovery in tree shrews (Moring et al., 2007). Although HA has not been definitively localized within tree shrew sclera, it likely occurs in the regions between the lamellae, in association with aggrecan (Norton & Rada, 1995)..

During experimental induced myopia development, selective proteoglycans synthesis is down-regulated and up-regulated during recovery in sclera, which has been indicated in chicks (Marzani & Wallman, 1997; Rada, McFarland, Cornuet, & Hassell, 1992), tree shrews (McBrien et al., 2000; Norton & Rada, 1995; Norton, Siegwart, & German-Moring, 2002; Siegwart, Jr. & strang, 2007; Summers Rada et al., 2006) and monkeys (Troilo, Nickla, Mertz, & Summers Rada, 2006). Aggrecan, a big proteoglycan, is located primarily around the fibroblasts and between the lamellae in tree shrew sclera (Siegwart, Jr. & strang, 2007), where may provide a “resilient medium” between collagen lamellae. The level of mRNA for aggrecan shows down-regulation during myopiagenic treatment and up-regulation during recovery in tree shrews sclera (Siegwart, Jr. & strang, 2007; Summers Rada et al., 2006).

Small, leucine-rich proteoglycans [SLRPs: decorin (DCN), fibromodulin (FMOD), keratocan (KERA), Nyctalopin (NYX), mimecan (OGN), prolargin (PRELP)] are present in tree shrew sclera and may play an important role in axial elongation regulation through a variety of mechanisms (Majava et al., 2007). Decorin and biglycan are located throughout the lamellae and closely related to the surface of collagen fibrils. Specifically, decorin is one of the most important proteoglycans of the mammalian sclera (Siegwart, Jr. &

Norton, 2001). People believe that although the change of decorin core protein mRNA is small their glycosaminoglycan side chains may be shorter (Pan, Colmers, & Williams, 1989) than normal, or the occupancy of their sulfate sites reduces during myopia development (McBrien & Gentle, 2003). There is no strong differential regulation between treated and control eyes for decorin, biglycan and lumican (Siegwart, Jr. & strang, 2007) during myopia development in tree shrew sclera (Rada, Achen, Penugonda, Schmidt, & Mount, 2000; Rada et al., 1997). Nevertheless, for biglycan, lumican, and aggrecan, the mRNA expression levels for both the treated and control eyes decreases during myopiagenic conditions and returns back to normal during recovery, the mechanism is unknown (Siegwart, Jr. & Norton, 2005; Siegwart, Jr. & strang, 2007).

Remodeling: Metalloproteinases (MPs) and Tissue Inhibitors of Metalloproteinases (TIMPs)

Matrix metalloproteinases (MMPs) are a family of neutral proteinases that can initiate the degradation of native fibrillar collagens, their breakdown products and other crucial ECM structural components (Aimes & Quigley, 1995; Woessner, Jr., 1994). At present, 25 vertebrate MMPs and 22 human homologues have been identified (Lohi, Wilson, Roby, & Parks, 2001; Nagase & Woessner, Jr., 1999). All mammalian MMPs share a conserved domain structure that contains a catalytic domain and an auto-inhibitory pro-domain. MMPs can be activated at neutral pH in the presence of active-site zinc and calcium ions, and are inhibited by tissue inhibitors of metalloproteinases (TIMPs). The functions of MMPs are summarized as: 1) serving as enzyme degrading structural components of the ECM; 2) producing specific substrate-cleavage fragments with independent biological activity; 3) regulating tissue architecture through effects on

the ECM and intercellular junctions; 4) activating, deactivating or modifying the activity of signaling molecules (Sternlicht & Werb, 2001); 5) affecting cellular functions by regulating the ECM proteins with which the cells interact (Page-McCaw, Ewald, & Werb, 2007; Streuli, 1999). The MMPs have a complex regulatory system in which the MMPs are tightly regulated at the transcriptional levels, post-transcriptional levels, and the protein levels via their activators, inhibitors, and their cell surface localization (Page-McCaw et al., 2007; Sternlicht & Werb, 2001).

Adamalysins (ADAMs) that share the metalloproteinase domain with MMPs are considered as subfamilies of zinc-dependent metalloproteinase. Two groups are distinguished in the adamlysin family: one is membrane-anchored ADAMs (a disintegrin and metalloproteinase) and the other is secreted-type ADAM-TSs (ADAMs with thrombospondin repeats). Both ADAMs and ADAMTSs play essential roles in biological processes such as shedding plasma-membrane-bound proteins, intracellular signaling, cellular adhesions, and cell fusion (Rocks et al., 2008). Under certain situations, adamalysins work more effectively than MMPs. During aggrecan degradation, for example, ADAMTS5 is about 100 times and 10 times more efficient at cleaving within the aggrecan interglobular domain (IGD) and the chondroitin sulfate-2 (CS-2) regions, respectively, compare to MMP3 (Durigova, Nagase, Mort, & Roughley, 2011). In previous study in tree shrew, ADAMTS5 showed significant mRNA regulation neither in four days of form deprivation nor four days of minus-lens wear (treated vs. control eyes), but showed down regulation after 1 day of recovery (-2.0 fold) {Gao, 2011 8800 /id;Guo, 2013 9694 /id}.

Another reason for the reduction in scleral collagen during myopia development is increased collagen degradation through the collagenolytic mechanism of the MMPs

(McBrien & Gentle, 2001; Siegwart, Jr. & Norton, 2005). The mRNA expression levels for MMP2, and the amount of latent and active MMP2 increase during myopia development and reverse during recovery in tree shrew sclera (both form deprivation and lens induced myopia) (Guggenheim & McBrien, 1996; Siegwart, Jr. & Norton, 2001; Siegwart, Jr. & Norton, 2002). The mRNA expression level for matrix metallopeptidase 14 (MT1-MMP or MMP14) also increases during myopia development and declines in recovery (Summers Rada et al., 2006). The mRNA level for MMP3, the other important MMPs family enzyme, however, do not show differential changes between the treated and control eyes during myopia development or recovery (Gao et al., 2011; Siegwart, Jr. & Norton, 2001).

Tissue inhibitors of metalloproteinase (TIMPs) represent a family of four secreted proteins (TIMPs 1-4) that are major cellular inhibitors of the MMPs (Maskos, 2005; Murphy et al., 1994) and other members of the zinc-dependent proteinase ADAMs and ADAMTSs families (Hashimoto, Aoki, Nakamura, Tanzawa, & Okada, 2001; Loechel, Fox, Murphy, Albrechtsen, & Wewer, 2000). In previous studies, mRNA for TIMP3 is the only TIMP mRNA that showed significant differential change between treated and control eyes: decreased during myopia development and reversed back to control eye level during recovery (Gao et al., 2011; Siegwart, Jr. & Norton, 2005). TIMP2 and MT1-MMP (MMP14) can form a complex that activates MMP2, a process is inhibited by TIMP3. Thus, during myopia development, with the increase of MT1-MMP, the TIMP2 and MT1-MMP complex increase as well, coupled with a decrease in TIMP3 level, which together can result in the activation of MMP2 in sclera (Siegwart, Jr. & Norton, 2005; Summers Rada et al., 2006).

SUMMARY

During the development of induced myopia, the retina generates GO signals that pass in a signaling cascade through the RPE and choroid to the sclera fibroblasts that produce biochemical changes in scleral extra cellular matrix. Due to biochemical scleral remodeling, the scleral lamella of myopic eyes may slide across each other more easily under normal intraocular pressure, which results in the increase of the scleral creep rate and the axial elongation rate of the eye. Refractive recovery (STOP) occurs in response to the myopia that exists after minus lens wear is discontinued. However, a very similar refractive myopia, produced by having a normal juvenile eye wear a plus-power lens, has very little effect (an IGNORE condition). It is unknown how the signals pass through so many tissues, and what signal pathways are used; moreover we are not sure which molecules are the messengers that cause the scleral fibroblasts to remodel the scleral ECM. The present project will deal solely with the responses of the sclera, measured by changes in mRNA levels.

There were three specific aims in this study: **Specific aim 1** tested the hypothesis that the three known GO visual conditions: minus lens wear, form deprivation, and continuous darkness, would produce very similar sclera responses, although the three myopiagenic conditions are very different. **Specific aim 2** tested the hypothesis that STOP scleral responses are very different from the GO responses in sclera. **Specific aim 3** tested the hypothesis that although similar myopic refractive conditions occur in recovery and plus lens wear, the sclera response with an IGNORE response pattern that differs from the STOP pattern. The results of the three specific aims are presented in the form of three papers. The paper reporting **Specific aim 1** has been published in *Investigative*

Ophthalmology and Visual Science (2013). The manuscript reporting **Specific aim 2** will be submitted to *Experimental Eye Research.* Submission is planned before January 31, 2014. A manuscript reporting **Specific Aim 3** is in preparation for submission to *Molecular Vision.*

Quantitative real time PCR was used to examine gene expression patterns of the sclera under different visual manipulation situations. The selected genes are ones that either have been shown to change in sclera in lens-induced myopia and/or recovery, either in studies of mRNA expression, or protein expression, or are representative of a class of genes that seem likely to change in these conditions. Although it is hoped that examining the changes of individual genes may help in understanding the biochemical changes that produce altered creep rate and, thus, changes in the axial elongation rate, the primary interest is to examine the pattern, the “signature” of mRNA changes in sclera GO, STOP and IGNORE conditions.

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

by

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ABSTRACT

Purpose: We compare gene expression signatures in tree shrew sclera produced by three different visual conditions that all produce ocular elongation and myopia: minus-lens wear, form deprivation, and dark treatment.

Methods: Six groups of tree shrews (n = 7 per group) were used. Starting 24 days after normal eye-opening (days of visual experience [DVE]), two minus-lens groups wore a monocular –5 D lens for two days (ML-2) or four days (ML-4); two form-deprivation groups wore a monocular translucent diffuser for two days (FD-2) or four days (FD-4). A dark-treatment (DK) group was placed in continuous darkness for 11 days after experiencing a light/dark environment until 17 DVE. A normal colony-reared group was examined at 28 DVE. Quantitative PCR was used to measure the relative differences in mRNA levels for 55 candidate genes in the sclera that were selected either because they showed differential expression changes in previous ML studies or because a whole-transcriptome analysis suggested they would change during myopia development.

Results: The treated eyes in all groups responded with a significant myopic shift indicating that the myopia was actively progressing. In the ML-2 group 27 genes were significantly down-regulated in the treated eyes, relative to control eyes. In the treated eyes of the FD-2 group, 16 of the same genes were also significantly downregulated and one was upregulated. The two gene expression patterns were significantly correlated ($r^2 = 0.90$, $p < 0.001$). After 4 days of treatment, 31 genes were significantly downregulated in the treated eyes of the ML-4 group and three were upregulated. Twenty-nine of the same genes (26 down- and 3 upregulated) and six additional genes (all downregulated) were

significantly affected in the FD-4 group. The response patterns were highly correlated ($r^2 = 0.95$, $p < 0.001$). When the DK group (mean of right & left eyes) was compared to the control eyes of the ML-4 group, the direction and magnitude of the gene expression patterns were similar to those of the ML-4 ($r^2 = 0.82$, $p < 0.001$, excluding *PENK*). Similar patterns were also found when the treated eyes of the ML-4, FD-4, and DK groups were compared with the age-matched normal eyes.

Conclusions: The very similar gene expression signatures produced in the sclera by the three different myopiagenic visual conditions at different time points suggests that there is a "scleral remodeling signature" in this mammal, closely related to primates. The scleral genes examined did not distinguish between the specific visual stimuli that initiate the signaling cascade that results in axial elongation and myopia.

INTRODUCTION

Emmetropia is a refractive condition that occurs when the images of distant objects are focused on the retina in an eye without accommodation. Refractive error occurs when there is a mismatch between the location of the focal plane and the axial length of the eye. If the axial length is short, the retina is in front of the focal plane and the eye is hyperopic. If the axial length is long, the retina is behind the focal plane and the eye is myopic. Myopia, the most prevalent type of refractive error, affects approximately 25 to 40% of adults in North America, Europe, and Australia.[1-5] In urban populations in East Asia, myopia prevalence can reach as high as 80 to 96.5%.[6-10] In juvenile-onset myopia the vitreous chamber is elongated, relative to emmetropic eyes;[11] myopia progression in

children is accompanied by an increase in vitreous chamber depth,[12] suggesting that re-modeling of the sclera, the outer shell of the globe, may be involved.

Studies in animal models (fish, chicks, monkeys, guinea pigs, tree shrews, and other species) have shown that there is a visually-guided emmetropization feedback mechanism that uses refractive error to guide axial elongation and achieve a match of the retinal location to the focal plane.[13-17] In these species, as in most humans, eyes initially are hyperopic and achieve emmetropia by increasing the axial length until only a small hyperopia remains.[18-21] The power distribution of the cornea, crystalline lens, and anterior chamber depth appear to be determined primarily by genetics and are not adjusted by visual guidance.[22-25] It is primarily vitreous chamber depth, which is determined by the size and shape of the scleral shell, that is modulated by the emmetropization mechanism.[26,27]

In animal models, two visual conditions, minus-lens wear and form deprivation, have been used frequently to manipulate the emmetropization mechanism. A minus (negative-power) lens, held in place in front of an eye with a goggle frame, produces a hyperopic shift in the eye's refractive state, moving the focal plane behind the retinal photoreceptors. This creates a refractive target and triggers axial (vitreous chamber) elongation, which continues until the induced refractive error is eliminated. The eye elongates only until the increase in axial length reestablishes age-appropriate emmetropia with the lens in place. This process is described commonly as minus-lens compensation, and the myopia that is present when the lens is removed is referred to as lens-induced myopia (LIM). Form deprivation, produced with a translucent diffuser that eliminates focused images, creates an open-loop situation that causes the eye to maintain an increased elongation rate

as long as the diffuser is in place. Measured with the diffuser removed, the eye has a form-deprivation myopia (FDM).

In minus lens (ML) and form deprivation (FD) treatments, the retina detects the hyperopic refractive state or the absence of focused visual images. Which retinal neurons are involved and how they encode the visual cues is not well understood. The net result, however, is that one or more neural signals are generated by the retinal neurons. Studies have shown that central communication via ganglion cell axons is not required.[28-30] Rather, as shown schematically in Figure 1, the information can pass via a signaling cascade through the RPE and choroid to the sclera, where it produces biochemical remodeling of the scleral extracellular matrix.[31,32] This, in tree shrew, increases the viscoelasticity of the sclera[22] allowing normal IOP to elongate the eye.

In addition to the two primary methods of inducing axial elongation and myopia in experimental animals, there is a third method – a period of continuous darkness. In juvenile tree shrews (diurnal, cone-dominated mammals closely related to primates) that have undergone emmetropization in standard animal colony conditions (100 - 300 lux on a light/dark cycle that establishes circadian rhythms),[33] an 11 day period of continuous darkness induces an increase in axial elongation rate, an increase in scleral viscoelasticity, and a myopic shift in refraction. No significant changes are produced in corneal power, anterior segment depth, or lens thickness;[19] the myopia, as in LIM and FDM, is due to vitreous chamber elongation.

Although minus-lens wear, form deprivation, and dark treatment produce similar increases in scleral viscoelasticity and axial elongation, studies have found that the way eyes respond to minus-lens wear and to form deprivation are not identical (He L, et al.

IOVS 2011;52:ARVO E-Abstract 6301).[34-36] Dark treatment has been studied in less detail, but appears to act more slowly than minus-lens wear or form deprivation such that 11 days of dark treatment produces similar amounts of elongation and myopia as does 4 days of minus-lens wear or form deprivation.[19] It is unknown if, or at what point in the signaling cascade, the signals produced by these different visual stimuli might merge to produce a similar scleral biomechanical response (Norton TT, et al. *IOVS* 2007;48:ARVO E-Abstract 1531)[22] and an increase in axial elongation.

In this study we used these three different myopiagenic conditions to induce axial elongation and myopia, and examined the changes in scleral mRNA expression associated with each condition to ask whether the different visual conditions produced distinct scleral gene expression patterns. Dissimilar patterns would suggest that the differing retinal responses are preserved and passed through the signaling cascade to produce differing scleral responses. Similar scleral response patterns would suggest that the differing retinal responses to the visual conditions are, at some point in the signaling cascade, combined into a common "remodeling signal/response" before, or as, they reach the sclera.

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 hour light/10-hour dark cycle. Tree shrew pups open their eyes at approximately 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 males and females, and avoided pups from the same parents wherever possible.

Six groups of animals (n = 7 per group) were used in this study (Fig. 2). Two minus-lens wear groups (ML-2 and ML-4) wore a monocular –5 D (spherical power) lens for either 2 or 4 days, starting at 24 ± 1 DVE. Two form deprivation groups (FD-2 and FD-4) wore a monocular translucent diffuser for either 2 or 4 days, also starting at 24 ± 1 DVE. Six of the seven animals in the ML-4 group also provided RNA for the "4-day –5 D lens wear group" reported by Gao et al.[37] In all of these groups the visual treatment induced myopia; the 4-day treatment duration was chosen to ensure that the sclera would be undergoing maximal remodeling when examined. The 2-day treatment duration was selected to examine earlier changes when the refractive changes had begun, but the axial length had changed only a little.[22] In all ML and FD groups, the untreated fellow eye served as a control. The darkness (DK) group was kept in continuous darkness for 11 days, from 17 DVE until 28 DVE, because dark-induced elongation and myopia develop more slowly than ML or FD myopias.[19] The treatment duration was intended to produce elongation and myopia that was nearly equivalent to the ML and FD groups. An age-matched (28 DVE) normal group also was studied.

Goggle Installation

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 following procedures described by Siegwart and Norton.[38] In the ML, FD, and normal

groups the pedestal was installed at 21 ± 1 DVE (Fig. 2); in the DK group it was installed at 14 ± 1 DVE. 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 ML and FD groups, a goggle frame holding either 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/diffuser in front of the randomly selected treated eye. The 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 minutes) removed to clean the lens/diffuser under dim illumination During goggle cleaning, animals were kept in a darkened nest box to minimize exposure to visual stimuli. Animals in the DK group began dark treatment 3 days after pedestal installation. The normal group received a pedestal at 21 DVE but did not wear a goggle.

Refractive and Axial Measures

Noncycloplegic 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).[39] DK animals remained in darkness during the terminal refractive measures. Normal animals were measured just before euthanasia. Cycloplegic refractive measures were omitted to prevent any interference by atropine on retinoscleral signaling.[40] However, previous studies have shown that noncycloplegic measures provide a valid estimate of the refractive state and of induced myopia in tree shrews. When compared, cycloplegic refractions are approximately 0.8 D hyperopic compared with noncycloplegic refractions in myopic, control, and normal eyes.[39,41] Further, treated-eye versus

control-eye differences are essentially identical between noncycloplegic and cycloplegic measures.[41] All refractive values were corrected for the small eye artifact,[42] previously shown to be approximately +4 D in tree shrews.[39]

At the time the pedestal was attached, ocular component dimensions were measured whilst under anesthesia with A-scan ultrasound, as described by Norton and McBrien,[43] to ensure that the treated, control, and normal 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 anesthesia required for the A-scan procedure might alter gene expression. In the ML-2 and FD-2 groups, posttreatment axial component measures were made with a Lenstar LS-900 optical biometer (Haag-Streit USA, Mason, OH). This instrument was placed into service after the ML-4, FD-4, and DK groups were completed and allowed measures to be made quickly, 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).

Gene Expression Analysis

On completion of the final refractive measures, approximately 2 to 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 along the corneoscleral junction. After removing the lens, vitreous humor, and optic nerve head, both surfaces of the sclera were scraped gently to remove the retina, RPE, choroid, and any residual extraocular tissue, before freezing the tissue in liquid nitrogen. Animals in the DK group were euthanized in the dark and the scleral tissue collected rapidly under minimal illumination.

Frozen sclera was pulverized to a fine powder in a chilled Teflon freezer mill (Sartorius Stedim, Bohemia, NY) 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) and the quality 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 55 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. Primer sequences, amplicon size, and efficiencies are listed in Supplementary Table S1. The selected candidate genes included representatives of three major groupings: signaling, metallopeptidases and TIMPs, and extracellular matrix (ECM) proteins. They were selected from genes that were found to change in previous studies of tree shrew sclera during LIM (He L, et al. *IOVS* 2010; 51:ARVO E-Abstract 3681),[37,44] along with additional genes that were suggested by stud-

ies in other species and by a whole-transcriptome analysis of three of the ML-4 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 ΔΔCt method[45] 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

Paired *t*-tests were used to assess treated versus control eye differences; unpaired *t*-tests were used to test for differences between all independent groups; $p < 0.05$ was considered significant and no adjustment for possible false discovery rate was applied (see Discussion). One-way Analysis of Variance (ANOVA; Statistica, Statsoft, Tulsa,

OK) was used to compare refractive data across groups of animals. Linear regressions between expression differences were made in SigmaPlot (Systat Software, San Jose, CA).

RESULTS

Refraction

The refractive changes produced by the three visual treatments are shown in Figure 3. As expected in tree shrews of this age, both eyes of the normal group were slightly hyperopic (right eyes, 1.4 ± 0.3 D; left eyes, 1.3 ± 0.3 D; mean ± SEM). After two days of treatment, the treated eyes of the ML-2 and the FD-2 showed small, statistically significant myopic shifts (treated eyes – control eyes); the ML-2 treated eyes were −1.0 ± 0.2 D myopic in comparison to the control eyes and the FD-2 treated eyes were −1.9 ± 0.2 D myopic. After 4 days of treatment, the myopic shift in the ML-4 group was −2.8 ± 0.3 D, while in the FD-4 group it was −3.2 ± 0.3 D; both were statistically significant. After 11 days of dark treatment the refractions in the DK group were: right eyes, −2.6 ± 0.4 D; left eyes, −2.5 ± 0.7 D. The difference (–3.9 ± 0.6 D) between the normal eyes (right and left eyes averaged, 1.4 ± 0.3 D) and the DK eyes (right and left eyes averaged, –2.6 ± 0.5 D) was statistically significant. The control eyes in the ML and FD groups did not differ significantly from the normal eyes (one-way ANOVA, $p = 0.76$). Note that the ML-4 treated eyes had not fully compensated for the –5 D lens and thus were still actively elongating, as were the treated eyes in the FD-4 group, and both eyes in the darkness group. Ocular component dimensions, measured with the Lenstar in the ML-2 and FD-2 groups confirmed that the vitreous chamber of the treated eyes had elongated slightly, relative to the control eyes, by 0.016 ± 0.004 mm (ML-2) and by 0.038 ± 0.011 mm (FD-2). Although

the axial changes were not measured in the other animals, the results of previous studies in tree shrews make it reasonable to assume that the myopic shifts after 4 days of ML or FD and 11 days of DK were due to an increase in vitreous chamber depth of approximately 0.060 to 0.075 mm.[46-48]

Gene Expression

Normal Left versus Right Eye Differences

Figure 4A compares the gene expression in the right and left eyes of the normal animals, measured at 28 DVE. The fold differences (without regard to sign) were very small (1.11 ± 0.08 fold; mean ± SEM) and only one of the genes in our sample, the alpha chain of type 1 collagen (*COL1A1*), was significantly different between left and right eyes (1.17 ± 0.05, $p = 0.0208$).

Differential Effects – Treated versus Control Eyes

2-day treatments: The fold differences in gene expression between the treated and control eyes in the ML and FD groups after 2 days of monocular treatment are shown in Figures 4B and 4C; expression values are also listed in Table 2. In both treatments, a pattern of gene expression differences had developed. As in previous studies of scleral gene expression in the eyes of trees shrews that were developing induced myopia (He L, et al. *IOVS* 2010;51:ARVO E-Abstract 3681),[37,44] most of the sampled genes were downregulated in the treated eyes, relative to the control eyes. In the ML-2 group, all 27 of the genes that were significantly different between the two eyes were downregulated in the treated eyes. In the FD-2 group, 16 of the same genes were downregulated; only one gene was upregu-

lated: the membrane-bound degradative enzyme, MMP14. The variability in expression across animals within each group was low, as evidenced by the small SEM values.

It is evident from examination of Figures 4B and 4C that the pattern of differential gene expression is very similar in the ML-2 and FD-2 groups across these 55 genes. The two patterns (Figs. 4B versus Fig. 4C) are compared quantitatively in Figure 5, which plots the fold differences in the ML-2 group (Fig. 4B) against those in the FD-2 group (Fig. 4C). The correlation was very high ($r^2 = 0.90$, $p < 0.001$) with no outliers, suggesting that the examined genes responded in a similar way to these two different myopiagenic visual conditions, not only in terms of which genes showed significant differential expression, but also the magnitude of the fold-changes.

4-day treatments: Figure 6 shows the fold-differences in gene expression between the treated and control eyes in the ML (Fig. 6A) and FD (Fig. 6B) groups after 4 days of monocular treatment, and the DK group (Fig. 6C) after 11 days of dark treatment. Because both eyes in the DK group were in darkness there was not an untreated control eye. Therefore, the mean gene expression of the right and left DK eyes were therefore compared to the control eyes of the ML-4 group. Expression values are also listed in Table 2. The pattern in all three treatments is very similar; 31 genes were significantly downregulated in the ML-4 treated eyes and three were up-regulated. Twenty-nine of the same genes (26 down- and 3 upregulated) and 6 additional genes (all downregulated) were significantly affected in the FD-4 group.

The patterns seen in Figure 6 are compared in Figure 7. Figure 7A compares the pattern in Figure 6A (ML-4) and 6B (FD-4); Figure 7B compares the pattern in Figure

6A (ML-4) with that in Figure 6C (DK). As was the case for the 2-day ML and FD treatments, the response patterns after 4 days in the ML and FD groups (Fig. 7A) were highly correlated (Fig. 7A, $r^2 = 0.95$, $p < 0.001$). Downregulated genes included ones for signaling molecules (cell surface receptors, cytoskeletal related proteins, transcription regulators, secreted signal proteins, matricellular proteins), for TIMPs, collagens, and proteoglycans. The upregulated genes included two in the signaling group (*FBLN1, TGFBI*) and one in the metallopeptidase group (*MMP14*).

When the DK group (mean of right and left eyes) was compared to the control eyes of the ML-4 group (Fig. 6C) the direction and magnitude of the gene expression patterns were similar to those of the ML-4 and FD-4 groups, but differed in two ways: (1) fewer of the differences were statistically significant, which may reflect the fact that independent groups were compared, rather than treated and control eyes within animals, and (2) one gene in the DK group, *PENK*, showed a very large downregulation (-29.27 fold). In other respects, there was a similar pattern of gene expression to the ML-4 group ($r^2 = 0.82$, $p < 0.001$, excluding *PENK*). When the pattern in Figure 6B (FD-4) was compared with that in Figure 6C (DK), the result was nearly identical to that shown in Figure 7B ($r^2 = 0.80$, $p < 0.001$, excluding *PENK*).

Comparison of 2-Day versus. 4-Day Treatments

The degree to which the pattern of gene expression differences that had emerged after 2 days of treatment were similar to those after four days of treatment is examined in Figure 8 for the ML (Fig. 8A) and FD groups (Fig. 8B). In general, the patterns after 2 and 4 days of treatment are similar for both types of treatment in that the differential ex-

pression of all genes moved in the same direction after 4 days as after 2 days. Several genes, however, showed significant expression differences after two days of treatment that were not statistically different after 4 days (Table 2). For ML and FD treatments, genes that followed this pattern included a secreted protein (*TGFBI*), matricellular proteins (*FBLN1*, *SPARC*, and *TNC*), and proteoglycan core proteins (*FMOD* and *NYX*). For the ML treatment, five additional genes not significantly different at 2 days were significantly different at 4 days (*DCN, KERA, MMP14, RARB*, and *SDC2*). For the FD treatment, 12 additional genes not significantly different after 2 days of treatment were significantly different after 4 days (*ANXA1, CAPN2, COL1A1, COL12A1, FGFR2, NOV, OGN, PRELP, SERPINH1, TGFB1, THBS1,* and *VDR*).

Treated Eyes versus Normal Eyes

Previous studies in tree shrews and other species have found refractive or mRNA changes in the control eyes that might affect treated vs. control eye comparisons (Rucker FJ, et al. *IOVS* 2009;50:ARVO E-Abstract 3931).[37,49,50] To examine the effect of treatments specifically on the treated eyes, we compared gene expression in the treated eyes of ML-4, FD-4, and DK groups with expression in the age-matched normal eyes (28 DVE). This comparison examined the effect on gene expression in the treated eye alone. It also allowed the treated eyes in all three treatment conditions to be compared to a common reference. Figure 9 shows the fold difference in mRNA expression levels in the treated eyes versus normal eyes for the ML-4 (Fig. 9A), the FD-4 (Fig. 9B), and the DK groups (Fig. 9C); the expression difference values are presented in Table 2. Overall, the pattern in this figure is similar to that in Figure 6, suggesting that the treated versus con-

trol-eye differences were primarily due to changes in the treated eyes. Indeed, when control eye gene expression was compared with normal eyes, only two control-eye genes in the ML-4 group were significantly different from normal (*NPR3* and *RARB*), five control-eye genes in the FD-4 group were significantly different from normal (*ADAMTS5, IGF2, RARB, RXRB,* and *WISP1*).

The treated-eyes versus normal eyes patterns seen in Figure 9 are compared in Figure 10. Figure 10A compares the pattern in Figures 9A (ML-4) and 9B (FD-4); Figure 10B compares the pattern in Figure 9A (ML-4) with that in Figure 9C (DK). The response patterns after 4 days in the ML, FD, and DK groups were highly correlated. The correlations between the ML and FD groups ($r^2 = 0.67$, $p < 0.001$) and the ML and DK groups ($r^2 = 0.76$, $p < 0.001$, excluding *PENK*) were similar to each other and to the correlation comparing treated with control eyes (Fig. 7). When the pattern in Figure 9B (FD-4) was compared with that in Figure 9C (DK), the result was nearly identical to that shown in Figure 10B ($r^2 = 0.78$, $p < 0.001$, excluding *PENK*).

DISCUSSION

The scleral gene expression patterns produced by the three myopiagenic visual conditions were very similar in terms of which genes were affected as well as the direction and relative magnitude of each gene's response. Thus, it appears that scleral fibroblast expression of specific genes is controlled tightly when the emmetropization mechanism calls for an increase in axial elongation even if the visual stimuli that initiate the signaling cascade are very different. In particular, the gene expression changes in the ML-4 and FD-4 groups (Fig. 7) were as close to identical ($r^2 = 0.95$, slope = 0.86) as

could be expected comparing the patterns in two groups, each containing seven animals that responded similarly, but not identically, to the visual conditions. The slightly lower correlation between the DK group and ML-4 group ($r^2 = 0.82$, slope = 0.91, excluding *PENK*) may occur because the comparisons are made between eyes in different groups of animals, rather than between treated and control eyes in the same animal that normally have very similar gene expression (Fig. 4A).

When the treated eye mRNA levels in the ML-4, FD-4, and DK groups were compared with the expression in the age-matched normal group, the scleral remodeling signatures (Figs. 9, 10) were similar to the treated eye vs. control eye patterns, confirming that most of the altered mRNA expression occurred in the treated eyes. Taken together, the similarity of the gene expression differences in our sample of 55 genes, produced by the three different myopiagenic conditions, lead us to conclude that these are part of a "scleral remodeling signature" that results in increased viscoelasticity and the axial (vitreous chamber) elongation that produces the myopia.

A single remodeling signature that does not differentiate between the visual conditions that produce it is consistent with the fact that all three visual conditions produce a similar increase in scleral creep rate that is closely related to the increase in vitreous chamber elongation and the myopic shift in refractive state (Norton TT, et al. *IOVS* 2007;48:ARVO E-Abstract 1531).[22] Although there are undoubtedly differences in the patterns of retinal neural activity generated by these very different visual conditions, and there may be differences in the signaling pathways at the level of the RPE and choroid (He L, et al. *IOVS* 2013;54:ARVO E-Abstract 3675), these data show that by the time the

signaling cascade has reached the scleral fibroblasts, the signals have converged to produce a common response by the scleral fibroblasts.

Development of the Gene Expression Signature

The availability of mRNA data from animals after 2 days of ML and FD treatment, when there has been little refractive or axial change, allowed us to examine the development of the gene expression signatures by comparing the patterns with those that were found after 4 days of treatment, when larger refractive (and, presumably axial) changes had occurred. This allowed us to learn whether the signatures developed at the same pace in both visual conditions and also the extent to which the signature was related to the amount of elongation and myopia.

A previous study that examined a subset of these genes after 1 day of ML treatment found very few differences between the treated and control eyes.[37] In the present study, the 2-day (ML-2 and FD-2) patterns were generally similar to the 4-day patterns (Fig. 8). The lower slopes (ML, 0.88; FD, 0.70) suggest that the magnitude of the mRNA differences after 2 days was less than after 4 days. This is consistent with the fold-differences increasing in magnitude as a function of time, and is in agreement with measures of scleral viscoelasticity (creep rate) which is elevated after 2 days of ML or FD treatment, and is more strongly elevated after 4 days.[22] Paradoxically, the amount of myopia that developed in the FD-2 group was larger than the amount in the ML-2 group, yet there were fewer large fold differences in the FD-2 group than in the ML-2 group (Fig. 5, slope = 0.65).

Comparison of the 2-day and 4-day patterns (Fig. 8A & 8B) may also help to distinguish gene expression changes that are due primarily to signals from the choroid versus changes that may occur in response to the gradual elongation of the globe. It is well known that fibroblasts can respond to mechanical deformation with changes in gene expression.[51-53] The altered mechanical tension on the fibroblasts after 4 days of axial elongation may have provided mechanical stimulation. Thus, some of the gene expression differences in the present study that occurred after 4 days, but were absent at 2 days, may have been related to mechanical effects of axial elongation rather than to signals arriving from choroid; changes that occurred after 2 days were less likely to result from intrascleral changes. However, the gene expression differences after 2 days generally involved the same genes and the expression changes were in the same direction, but of smaller magnitude, as found after 4 days (Table 2). Four genes also were involved with cell-surface receptors (*ACVRL1*, *TRPV4*, and *UNC5B*) and cytoskeleton (*CAPN2*) that showed significant downregulation after 2 days of ML treatment, but non-significant downregulation after 4 days of ML treatment (Table 2). Changes in these genes may comprise early signaling into the sclera that initiate remodeling, but are not involved in the sustained remodeling process.

We asked if the same scleral gene expression signature has been found in cell culture, particularly in studies that subjected fibroblasts in cell culture to mechanical deformation and found changes due to the mechanical deformation itself, in the absence of potential signals arriving from another structure.[54-59] However, the overall patterns in these studies do not duplicate, or even closely resemble, the pattern we have found, suggesting that the scleral response signature we found may be uniquely produced by (unknown)

molecules arriving from the choroid with, perhaps, some additional changes due to the elongated axial length.

Gene Expression Differences

The focus of this study was to learn whether or not the sclera responded with the same remodeling response in reaction to different myopiagenic stimuli. The genes that were selected encode proteins that represent a wide range of functions, with a focus on ones involved in cell signaling. The 22 genes that had been examined in previous studies of tree shrew sclera generally responded as previously found (He L, et al. *IOVS* 2010;51:ARVO E-Abstract 3681).[37,60] Of the 33 newly-examined genes, most showed down-regulation. As we found previously, the differences in gene expression produced in the sclera were generally small, mostly less than 4-fold, with the exception of *PENK*, which showed a huge (–29.27-fold) unexplained binocular downregulation in the dark-treated group, compared with ML-4 control eyes.

The general pattern of downregulation is consistent with prior reports that during myopia development there is a small loss of scleral ECM including a reduction in dry weight (about 3 - 5 %), a small loss of collagen, hyaluronan, and glycosaminoglycans.[32,61,62] It is of interest that there appeared to be selective regulation of gene expression within each of the functional groupings. Some genes within a group showed (relatively) strong differences in expression (such as *NPR3*, Table 2), while others (such as *TGFBR3*) appeared unaffected. Amongst the genes coding for matricellular proteins, most were downregulated whereas one (*FBLN1*) showed upregulation. Whatever molecules, presumably arriving from choroid,[63,64] produce the scleral remodeling sig-

nature, they produce effects on the expression of some mRNAs, but not others within the same class.

The differences in scleral mRNA expression, while consistent, do not directly reveal the identity of the molecule or molecules that initiate the remodeling process. However, the scleral remodeling signature found in this study could allow application of various candidate choroidal signaling molecules to scleral organ culture to learn if, either singly or in combination, they reproduce this remodeling signature.

Statistical Significance

As noted in the Methods, we assessed whether expression differences were statistically significant by applying either a paired (dependent) *t*-test for treated and control eyes in the same animal, or an independent t-test for eyes that were in different animals. We did not apply a correction for false discovery rate for two primary reasons. When the mRNA levels were compared between the left and right eyes of the normal animals (Fig. 4A), the fold differences and the standard errors of the mean were very small. One gene, *COL1A1*, had mRNA levels 1.17 fold higher in the right eyes and, because the variability in the group was small (1.11 ± 0.08), the difference was statistically significant. Thus, normally, the mRNA levels in the right and left eyes are very close to identical in the 55 genes that were examined and provide a baseline against which the substantial alterations in mRNA levels could be assessed.

In addition, 2 and 4 days of ML or FD produced nearly universal downregulation of the sampled genes in the treated eyes, including ones that were significant by our *t*-tests and ones that were not (unfilled bars in Fig. 4, 6, & 9). These “non-significant”

mRNA levels clearly differed from the normally very similar levels in right and left eyes. Further, the pattern of differential expression was very similar across visual conditions and time-points, suggesting that the expression differences are repeatable and meaningful, whether or not the p-value was less than the alpha of 0.05. When we examined the correlation between the visual conditions (Fig. 8A & 8B) using only the genes whose expression differences did not reach our criterion for statistical significance, the correlation amongst these "non-significant" expression differences was also statistically significant, with slopes that were very similar to the overall pattern. This suggests that there were differences in gene expression that, even when they did not meet our pre-selected alpha, were consistent across visual conditions, both after 2 days and 4 days of treatment. Thus, it appears in this study, that an alpha of 0.05 may actually have been more conservative than needed.

Limitations

It is important to note that the remodeling signature reported here is the pattern of mRNA changes for 55 genes for which we developed primers for quantitative PCR. The expression levels of many other genes that we did not measure are likely involved in altering the biomechanical properties of the sclera. RNA-Seq (whole-transcriptome) analysis of treated and control eyes from three of the ML-4 animals suggests that perhaps 400 to 500 genes (of the over 20,000 transcripts found to be expressed in sclera; Frost MR, et al. *IOVS* 2012;53:ARVO E-Abstract 3452) may be up- or downregulated by at least 1.20 fold, with approximately equal numbers changing in each direction (M. R. Frost, personal communication, 2013). The results of the present study, along with the RNA-Seq analysis,

serve as a reminder that gene expression is a complex and interactive process. Although these data show highly specific control of gene expression, a change in one or two genes in isolation may not provide a good picture of the overall process, and may reflect indirect downstream influences as well as direct responses to the signaling cascade.

We recognize that changes in mRNA levels may, or may not, produce changes in protein levels and that proteins are the "effectors" that actually produce the scleral remodeling. We have previously examined changes in protein expression after 4 days of minus lens wear and after 4 days of recovery from minus lens wear to directly address protein levels.[49] There were 14 genes whose protein abundance levels were found to differ significantly in the sclera after 4 days of minus-lens wear [37,49] whose mRNA expression was also analyzed in the present study. The correlation between the mRNA and protein differential expression was not statistically significant ($r^2 = 0.16$, $p > 0.05$). This was not surprising; the correlation between mRNA and protein abundances in complex biological samples is typically poor.[65-68] This is because mRNA levels reflect the activity of cells at the time the sample is collected whereas protein levels reflect the cumulative activity of synthetic and degradative processes over time, influenced by a large repertoire of systems that enhance or repress the synthesis of proteins from a specific copy number of mRNA transcripts. In the present study, our focus was to learn if the scleral fibroblasts respond differently to the three different myopiagenic visual conditions. The changes in mRNA levels, as found in this study and others,[37,60,69] provide a useful way to examine, and compare, the way cells respond to signals. Because the mRNA responses to the three different visual conditions are so similar, it is likely that any changes in protein levels would also be similar.

In summary, when retinally-generated signals coding an increase in axial elongation reach the sclera, the scleral fibroblasts respond with a "scleral remodeling gene expression signature" that does not distinguish between the type of visual stimulus that initiates the signaling cascade. At what point in the signaling cascade the initially very different retinal activity is integrated into a common pathway is unclear. This may have occurred by the level of the choroid, based on the fact that similar thinning of the choroid occurs in response to both form deprivation and minus-lens wear.[70] However, this integration also could occur as the signals pass through the RPE. Although these scleral fibroblast responses do not specifically identify the signaling molecules that initiate the changes in gene expression, they do suggest that the sclera might become a therapeutic target for controlling axial elongation without affecting vision. Agents that selectively suppress the changes in gene expression might be applied sub-conjunctivally to suppress the remodeling, prevent the increase in viscoelasticity, and thus control axial elongation and the development of myopia.

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FIGURE LEGENDS

Figure 1. Information flow produced by three myopiagenic conditions (minus-lens wear, form deprivation, and dark treatment). Retinal neurons detect these stimuli and generate signals that cascade through the retinal pigment epithelium (RPE) and choroid to produce remodeling in the sclera. The remodeled sclera has increased viscoelasticity that produces an increase in the axial elongation rate. The focus of this paper (central box) is the gene expression changes that occur in the sclera in response to these three visual conditions, including altered gene expression related to signaling, degradative enzymes and inhibitors, and extracellular matrix.

Figure 2. 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.

Figure 3. End-of-treatment refractive measures for the normal, minus lens (ML), form deprived (FD), and dark treatment (DK) groups. Values are the mean refraction ± SEM for the right (R) and left (L) eyes of the normal and DK groups, and for the treated (T) and control (C) eyes of the ML and FD groups. Treated eyes in all groups were significantly myopic relative to control (or normal) eyes.

Figure 4. Gene expression fold differences. (**A**) Normal eyes (right eyes vs. left eyes). (**B**) 2 days of minus-lens wear (treated eyes vs. control eyes). (**C**) 2 days of form deprivation

(treated eyes vs. control eyes). 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. In B, the off-scale fold difference for NPR3 (-7.66) is indicated next to the bar.

Figure 5. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4B (ML-2) with the differences in Fig. 4C (FD-2). The amount of differential expression in both conditions was very similar. Stars = significant fold differences for both ML and FD; triangles = significant fold differences only for ML; squares = significant fold differences only for FD; circles = fold differences not significant in either treatment.

Figure 6. Gene expression fold differences. (**A**) 4 days of minus-lens wear (treated eyes vs. control eyes). (**B**) 4 days of form deprivation (treated eyes vs. control eyes). (**C**) 11 days of dark treatment (mean R & L vs. ML-4 control eyes).

Figure 7. Comparison of the treated vs. control gene expression differences in Fig. 6. (**A**) ML-4 (Fig. 6A) vs. FD-4 (Fig. 6B) (**B**) ML-4 (Fig. 6A) vs. 11 days of continuous darkness (Fig. 6C). Stars = significant fold differences for both treatments; triangles = significant fold differences only for ML-4; squares = significant fold differences only for FD-4 (A) or DK (B); circles = fold differences not significant for either treatment.

Figure 8. Comparison of the 4 day (Fig. 6) vs. 2 day (Fig. 4) treated vs. control gene expression differences produced by (**A**) minus-lens wear (Fig 6A vs. Fig. 4B) and (**B**) form

deprivation (Fig. 6B vs. Fig. 4C). Stars = significant fold differences for both 2- and 4-day treatments; triangles = significant fold differences only for 4-day; squares = significant fold differences only for 2-day; circles = fold differences not significant at either treatment duration.

Figure 9. Gene expression fold differences. (**A**) 4 days of minus-lens wear (treated eyes vs. normal eyes). (**B**) 4 days of form deprivation (treated eyes vs. normal eyes). (**C**) 11 days of dark treatment (mean R & L vs. normal eyes).

Figure 10. Comparison of gene expression patterns in the treated-eyes of ML, FD, and DK with normal eyes as a common reference (Fig. 9). (**A**) minus-lens wear compared with form deprivation (Fig. 9A vs. Fig. 9B). (**B**) minus-lens wear compared with continuous darkness (Fig. 9A vs. Fig. 9C). Because the DK group treatment was binocular, the fold difference for DK eyes is the mean of values of the R & L eyes.

Table 1. Genes examined, divided into functional categories, with cellular location of the protein encoded by the gene

Gene symbol	Protein name	Location
Signaling − Cell surface receptors		
ACVRL1	Activin A receptor 2-like 1	Cell surface
FGFR2	FGF receptor 2	Cell surface
NPR3	Atrial natriuretic peptide receptor 3	Cell surface
SDC2	Syndecan 2	Cell surface
TGFBR3	TGFβ receptor III	Cell surface
TRPV4	Transient receptor potential cation channel V4	Cell surface
UNC5B	Netrin receptor UNC5B	Cell surface
EFNA1	Ephrin A1	Cell surface
Signaling − Cytoskeleton related		
ANXA1	Annexin A1	Cell surface
ANXA2	Annexin A2	Cell surface
CAPN2	Calpain 2	Cell surface
CAPNS1	Calpain small subunit 1	Cell surface
GJA1	Connexin 43	Cell surface
ACTA2	Smooth muscle actin	Intracellular
NGEF	Ephexin 1	Intracellular
Signaling − Transcription regulators		
HIF1A	Hypoxia-inducible factor 1α	Intracellular
RARB	Retinoic acid receptor β	Intracellular
RXRB	Retinoid X receptor β	Intracellular
VDR	Vitamin D receptor	Intracellular
Signaling − Secreted		
ANGPTL7	Angiopoietin-related protein 7	Extracellular
IGF1	Insulin-like growth factor 1	Extracellular
IGF2	Insulin-like growth factor 2	Extracellular
IL18	Interleukin 18	Extracellular
PENK	Proenkephalin A	Extracellular
TGFB1	Transforming growth factor β1	Extracellular
TGFB2	Transforming growth factor β2	Extracellular
TGFBI	TGFβ-induced protein	Extracellular
Signaling − Matricellular		
CTGF	Connective tissue growth factor	Extracellular
CYR61	Protein CYR61	Extracellular
FBLN1	Fibulin 1	Extracellular
NOV	Nephroblastoma overexpressed gene	Extracellular
SPARC	Secreted protein acidic and rich in cysteine	Extracellular
SPP1	Osteopontin	Extracellular
THBS1	Thrombospondin 1	Extracellular
THBS2	Thrombospondin 2	Extracellular
TNC	Tenascin C	Extracellular
WISP1	WNT1 inducible signaling pathway protein 1	Extracellular
MPs / TIMPs		
ADAMTS5	ADAM metallopeptidase with thrombospondin motif, 5	Extracellular
MMP2	Matrix metallopeptidase 2	Extracellular
MMP14	Matrix metallopeptidase 14	Cell surface
TIMP1	TIMP metallopeptidase inhibitor 1	Extracellular
TIMP2	TIMP metallopeptidase inhibitor 2	Extracellular
TIMP3	TIMP metallopeptidase inhibitor	Extracellular
Extracellular matrix − Collagens		
COL1A1	Collagen type I, α1	Extracellular
COL12A1	Collagen type XII, α1	Extracellular
COL14A1	Collagen type XIV, α1	Extracellular
Extracellular matrix − Proteoglycans		
ACAN	Aggrecan	Extracellular
DCN	Decorin	Extracellular
FMOD	Fibromodulin	Extracellular
KERA	Keratocan	Extracellular
NYX	Nyctalopin	Extracellular
OGN	Mimecan	Extracellular
PRELP	Prolargin	Extracellular
Extracellular matrix − Other		
HS6ST1	Heparan-sulfate 6-O-sulfotransferase 1	Cell surface
SERPINH1	Serpin H1	Intracellular

Table 2. Gene expression differences comparing treated vs. control and treated vs. normal eyes. Red text = significant down-regulation, blue = significant up-regulation, grey = expression difference not statistically significant

	RE vs. LE	Treated vs. control		Treated vs. normal		Treated vs. control			Treated vs. normal		
	Normal	*ML-2*	*FD-2*	*ML-2*	*FD-2*	*ML-4*	*FD-4*	*DK*	*ML-4*	*FD-4*	*DK*
Signaling − Cell surface receptors											
ACVRL1	1.05	**-1.96**	**-1.52**	-1.27	1.12	-1.77	**-1.42**	**-1.68**	-1.70	-1.19	**-1.61**
FGFR2	1.15	-1.59	-1.15	-1.84	1.11	-1.61	**-1.46**	-1.63	-1.30	-1.34	-1.31
NPR3	1.15	**-7.66**	**-4.57**	**-2.58**	**-1.72**	**-5.71**	**-4.52**	**-5.36**	**-1.90**	**-3.25**	**-1.78**
SDC2	1.11	-1.42	-1.12	-1.45	1.22	**-1.26**	-1.16	-1.84	-1.36	-1.41	**-2.00**
TGFBR3	1.12	-1.16	1.46	-1.34	1.95	1.40	1.34	1.44	1.20	1.59	1.23
TRPV4	-1.05	**-1.44**	-1.28	-1.17	-1.24	-1.35	-1.18	**-1.37**	-1.14	-1.03	-1.17
UNC5B	-1.02	**-1.79**	**-1.56**	**-1.53**	-1.28	-1.84	**-1.74**	**-1.51**	-1.59	-1.45	-1.30
EFNA1	-1.00	-1.05	1.01	1.02	1.23	-1.21	-1.10	1.22	-1.23	1.05	1.20
Signaling − Cytoskeleton related											
ANXA1	1.14	**-1.53**	-1.29	-1.60	-1.24	**-1.70**	**-1.40**	-1.30	-1.72	**-1.64**	-1.31
ANXA2	1.13	**-1.94**	**-1.62**	**-1.76**	-1.19	**-2.09**	**-1.92**	**-1.97**	**-1.89**	**-2.68**	**-1.78**
CAPN2	-1.02	**-1.36**	-1.18	-1.34	-1.05	-1.26	**-1.22**	-1.23	-1.01	1.11	1.01
CAPNS1	-1.04	**-1.26**	**-1.24**	**-1.54**	**-1.45**	**-1.46**	**-1.30**	**-1.48**	-1.22	-1.13	-1.23
GJA1	1.15	-1.43	1.11	-1.23	1.74	-1.38	-1.55	-1.71	-1.05	-1.43	-1.30
ACTA2	-1.15	1.11	1.08	-1.01	-1.38	1.22	1.12	1.36	1.04	1.14	1.16
NGEF	-1.00	**-4.71**	**-3.95**	**-3.27**	**-2.10**	**-3.95**	**-3.68**	**-4.71**	**-2.42**	**-4.32**	**-2.88**
Signaling − Transcription regulators											
HIF1A	-1.08	**-1.56**	-1.21	-1.05	1.51	**-1.43**	-1.11	-1.34	1.06	1.06	1.14
RARB	1.07	-1.36	-1.26	-1.33	1.12	**-1.49**	-1.22	**-1.88**	1.12	1.24	-1.13
RXRB	-1.01	-1.21	-1.03	**-2.17**	**-1.84**	-1.21	-1.06	-1.05	1.07	**1.43**	1.23
VDR	1.17	-1.43	-1.34	-1.80	-1.03	-1.29	**-1.53**	1.27	-1.10	-1.28	1.49
Signaling − Secreted											
ANGPTL7	1.19	-1.13	1.33	-1.38	1.75	-1.06	-1.02	-1.18	-1.00	-1.03	-1.12
IGF1	-1.03	-1.10	1.12	1.08	1.21	1.06	1.07	1.22	1.30	**1.59**	1.50
IGF2	-1.06	1.00	-1.01	-1.37	**-1.48**	-1.08	1.04	-1.02	1.22	**1.54**	1.29
IL18	1.12	**-2.59**	**-2.25**	**-1.98**	**-2.23**	**-3.06**	**-2.73**	**-3.13**	**-2.43**	**-3.28**	**-2.49**
PENK	1.12	**-1.74**	**-1.36**	-2.34	-1.30	**-3.67**	**-3.88**	**-29.27**	-1.58	**-3.82**	**-12.58**
TGFB1	-1.01	**-1.18**	-1.13	-1.31	**-1.46**	**-1.39**	**-1.21**	-1.39	-1.40	-1.29	**-1.40**
TGFB2	1.11	**-2.41**	**-2.06**	-1.86	-1.44	**-1.87**	**-1.69**	-1.74	-1.44	-1.08	-1.34
TGFBI	1.01	-1.14	1.44	-1.41	1.62	**1.59**	**1.68**	1.11	**2.74**	**2.02**	1.91
Signaling − Matricellular											
CTGF	1.15	**-3.54**	**-3.19**	**-3.23**	**-2.43**	**-3.02**	**-2.64**	**-1.91**	**-2.71**	**-2.48**	-1.71
CYR61	1.18	**-2.96**	**-2.25**	-1.69	-1.22	**-3.34**	**-2.63**	**-2.39**	**-2.84**	**-2.59**	**-2.03**
FBLN1	1.15	-1.08	1.32	-1.24	1.68	**1.69**	**1.83**	1.72	1.52	1.72	1.54
NOV	1.07	**-1.81**	-1.36	-2.34	-1.75	**-1.93**	**-1.67**	-1.00	-2.06	-2.27	-1.07
SPARC	1.16	-1.42	-1.14	-1.77	1.01	**-1.43**	**-1.42**	-1.12	-1.43	-1.26	-1.11
SPP1	1.19	**-1.52**	**-1.42**	**-2.12**	-1.25	**-1.58**	**-1.41**	1.03	-1.80	**-1.68**	-1.10
THBS1	1.18	**-2.90**	-1.76	-2.05	1.07	**-2.67**	**-2.67**	-1.69	-2.26	**-2.68**	-1.43
THBS2	1.20	-1.42	-1.06	-1.30	1.25	-1.26	-1.14	-1.09	-1.13	-1.02	1.02
TNC	1.24	-1.77	-1.30	-2.42	-1.48	**-2.13**	**-1.94**	-1.99	-2.54	**-3.61**	-2.37
WISP1	1.02	-1.12	1.06	**1.51**	**1.62**	1.17	1.15	**1.45**	**1.49**	**1.60**	**1.85**
MPs / TIMPs											
ADAMTS5	1.03	-1.19	1.02	-1.33	-1.15	-1.10	-1.11	1.27	-1.07	**1.67**	1.30
MMP2	1.18	-1.26	1.15	-1.11	1.90	1.16	1.15	1.06	-1.02	-1.09	-1.12
MMP14	1.01	1.07	**1.45**	1.21	**2.03**	**2.11**	**1.86**	1.99	**3.87**	**3.00**	**3.65**
TIMP1	1.02	**-1.26**	**-1.21**	**-1.56**	**-1.87**	**-1.54**	**-1.37**	**-1.79**	-1.24	**-1.24**	**-1.43**
TIMP2	1.03	-1.31	1.00	-1.13	1.29	-1.22	-1.15	-1.08	-1.07	1.12	1.06
TIMP3	1.26	**-2.63**	**-2.05**	**-2.07**	-1.70	**-3.33**	**-3.21**	**-2.86**	**-2.32**	**-2.80**	**-2.00**
Extracellular matrix − Collagens											
COL1A1	**1.17**	**-1.15**	-1.24	-1.16	-1.08	**-1.40**	**-1.56**	1.07	-1.40	-1.19	1.07
COL12A1	1.21	**-2.26**	-1.66	-1.53	1.06	**-1.98**	**-2.09**	**-2.30**	-1.38	**-2.30**	-1.60
COL14A1	1.04	-1.47	-1.19	1.20	**2.15**	-1.05	-1.07	1.10	1.50	1.42	**1.73**
Extracellular matrix − Proteoglycans											
ACAN	1.13	**-2.08**	**-1.82**	-1.70	-1.15	**-2.53**	**-2.21**	-1.71	-2.06	-1.69	-1.39
DCN	1.13	-1.33	-1.01	-1.35	1.18	**-1.53**	-1.38	-1.67	-1.22	-1.61	-1.33
FMOD	1.17	-1.45	-1.14	-1.62	-1.15	**-1.73**	**-1.72**	-1.19	-1.54	-1.77	-1.06
KERA	1.21	-1.49	-1.16	1.02	1.69	**-1.59**	-1.34	**-2.27**	1.09	-1.43	-1.31
NYX	1.06	-1.00	1.02	-1.60	**-1.78**	**-1.36**	**-1.67**	-1.56	-1.09	-1.80	-1.25
OGN	1.37	**-2.00**	-1.58	**-2.12**	-1.42	**-3.05**	**-3.01**	**-3.80**	**-1.93**	**-3.33**	**-2.40**
PRELP	1.06	-1.17	1.01	-1.10	1.44	-1.18	**-1.28**	-1.25	1.03	-1.38	-1.03
Extracellular matrix − Other											
HS6ST1	1.16	**-1.72**	**-1.55**	-1.13	-1.15	**-1.77**	**-1.61**	-1.29	-1.43	-1.29	-1.04
SERPINH1	1.18	**-1.75**	-1.40	-1.32	1.17	**-1.69**	**-1.45**	-1.51	-1.38	-1.42	-1.24

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

Gene	*Forward sequence*	*Reverse sequence*	*Amplicon (bp)*	*Efficiency (%)*
ACAN	CTGTGACATAGACAAGGAGAT	GACGATGCTGCTCAGG	142	92
ACTA2	CGTGATCTCACCGACTACCT	GCGGAAGCGTTCGTTCCCG	222	98
ACVRL1	CGTACTGGTCAAGAGCAA	ATGTCAGTCCACTTGTAGG	189	94
ADAMTS5	TCTTCCATCCTAACCAGCATTG	GGTGGCATCATAAGTCTGTCC	165	98
ANGPTL7	GCTGTCACTTGGCTCTG	AGTCCTTCTCCTGCTTCTTG	193	94
ANXA1	ACAAAGTTCTGGACCTGGAGTTGA	AGAGCGGGAAACCATAATCCTGAT	167	95
ANXA2	CTTCCGCAAGCTGATG	CATGATGCTGATCCACTT	154	99
CAPN2	GGCTGACCAGACGGCATGAAG	CTCCATCTTGGTGAGTTTCCATTTCTTG	146	88
CAPNS1	TGACCGATCAGGGACCATCTG	GGATTTGTCCACTGCCATCTTTG	215	92
COL12A1	CAGGTGTCCCGATCAAAGAG	GCCTGGTGGAATGGTGG	95	97
COL14A1	TCACCTCCTACTCAACCACCAACT	GGCTCCAACAGTGCTATACAGAAAGT	165	94
COL1A1	GCCCTTCTGGAAATGC	GACCAACTTCACCAGGA	111	95
CTGF	CCAACTATGATTCGAGCCAACTG	TTCTCTTCCAGGTCTGCTTCA	173	96
CYR61	TGGAACCTCGCATTCTGTATAACC	GCCACAAGGTCGCACTTCAC	176	97
DCN	CTGCGTGCCCATGAGAACGAGATC	GGTATCAGCAATGCGGATGTACGAAAGC	168	96
EFNA1	CCACCATCAAGAAGACCAGT	CCAGGCAAGAGGGAAGAG	178	97
FBLN1	CGAGGAGGAGCAAGAG	AGCACGAGCAGACTAC	104	94
FGFR2	TGAAGGAAGGACACAGGATGGATAAG	GAGGCTGACTGAGGTCCAAGTATTC	180	93
FMOD	TCCTGCTGGACCTGAG	TTGTTGGTGAGGCTGTTAT	172	96
GJA1	CCTAGCCATTGTGGACCA	ATCTCCAGGTCATCAGGC	81	99
HIF1A	ACTGGTTGCATCTCCGTCTCCTACTCA	TCCTGCTCTGTTTGGTGAGGCTGTC	109	94
HS6ST1	CGTTCAACCTCAAGTTCATCC	GGTCCTTGGCGTAGTCAT	141	98
IGF1	GTCCTCCTCACATCTCTTCTACC	CAGCACCGCAGAGTGTCTC	89	99
IGF2	TGGCATTGTTGAAGAGTGTTG	GAAGCACGGTCGGAGAG	113	100
IL18	AACTCTTTCCTTTAAGAATATCAGTCGTCCT	GTTGGCTTCCACAGTAAACATTACAGATT	226	94
KERA	TCTCACAATCAGCTCACCAAGGTT	CCAGACGGAGGTAGCGAAGATG	175	101
MMP14	CCCTGGAACCTGGCTACCC	ATAGGTCTTTCCATTGGGCATCC	104	96
MMP2	TGACCTTGACCAGAACACCATCG	GAGCGAAGGCATCATCCACTGT	176	95
NGEF	ACCACTTCTCCGTGTAC	GACTAGGAGCTTGAGGC	194	96
NOV	CGAACAGACTACAGAGTGGAGTG	GTGATTTCTTGGTGCGGAGACA	191	97
NPR3	GGACTACGCCTTCTTCAACATCG	AGAGTGACTGTTTGGAGGGATGA	126	96
NYX	GTGAGCAGGTTCAGCAG	GACAGCGAGGTGTTAGC	101	96
OGN	CTCTCCTTCCTCTACCTGGACCACAAC	CTGAAGATGGATCACGCGCAGACTC	84	96
PENK	TCCTTGCCAAGCGATACG	TCTTGCTCATTTCTTCGTCGTT	162	98
PRELP	TTCGGCTGAACTACAACAAGC	GATCTGGGTCCCGTTGATTTTCT	182	99
RARB	CGGCTTGACCATCGCAGAC	GCTGGTTGGCAAAGGTGAACA	197	97
RXRB	CCTGACCTACTCGTGCC	TACCGCCTCCCTCTTCA	118	91
SDC2	TGATGACGACTACGCTTCTGC	CAGGCATCTTGTTCTGTGTCTTC	155	95
SERPINH1	ACAAGAACAAGGCAGAC	GCACCAGGAAGATGAAG	186	103
SPARC	GCGAGTTTGAGAAGGTGTGC	GCCCGATGTAGTCCAGGTG	126	102
SPP1	CCGACGACACCGACCATCC	GGCTTTGACCTCACTCTGTAAACC	190	92
TGFB1	ACCAGAAATACAGCAACAATTCC	AACCCGTTGATGTCCACTTG	205	91
TGFB2	GCAGAGTTTAGGGTCTTTCGTTTG	CTCGTGAACAGCATCAGTTACATC	189	93
TGFBI	CCTCGGCACTCATCTCTCC	GCAAATTCTTCATCTTGGCATCG	107	94
TGFBR3	CCCTGGTCTGGCGTCTGAAG	GTAACTGCTCCATACTCGTTTCGG	190	93
THBS1	CTGTCAGAACTCAGTCACCATC	CCACGGAGACCAGCCATC	136	91
THBS2	GAGACCGACTTCAGGAACTTC	CGAAACCCACTGCGATGC	142	100
TIMP1	CACTTGCACATCACCACCT	CAAGGGATGGATAAACAGGGAAA	134	99
TIMP2	AGATGGGCTGTGAATGC	CCGTCGCTTCTCTTGAT	151	96
TIMP3	CCGTGTCTATGATGGCAAGATG	ACAAAGCAAGGCAGGTAATAGC	153	99
TNC	AGACGCCAAGACTCGCTACAG	CAGGTTGACACGGTGACAGTTC	184	94
TRPV4	AGAGCAAGCACATCTGGAA	CCACGGTCACCATCTCC	111	94
UNC5B	CACCTGCTGGCTCCCTG	ATGGACAGCGGGATCTTGAA	94	96
VDR	AACTTGCACGAGGAGGAAC	CTTCTGGATCATCTTGGCATAG	189	93
WISP1	AGGCACCCATGAACTT	GACACTGGAAGGAGACA	135	96
POLR2A	CTACCAGCCCCAAGTATTC	GGTGAGTAAGTAGGAGACG	106	98

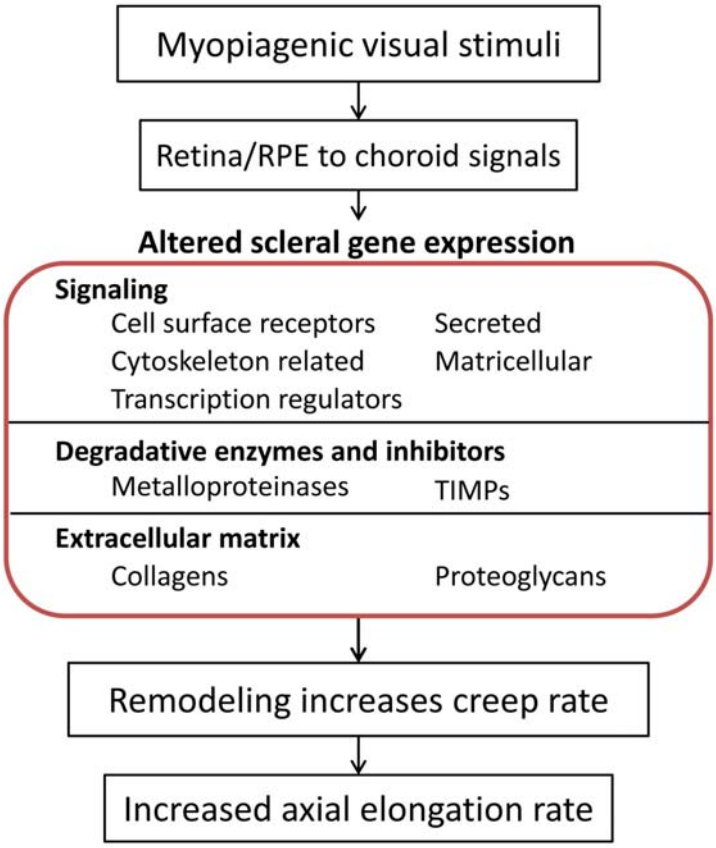

FIGURE 1. Information flow produced by three myopiagenic conditions (minus-lens wear, form deprivation, and dark treatment). Retinal neurons detect these stimuli, and generate signals that cascade through the RPE and choroid to produce remodeling in the sclera. The remodeled sclera has increased viscoelasticity that produces an increase in the axial elongation rate. The focus of this paper (central box) is the gene expression changes that occur in the sclera in response to these three visual conditions, including altered gene expression related to signaling, degradative enzymes and inhibitors, and extracellular matrix.

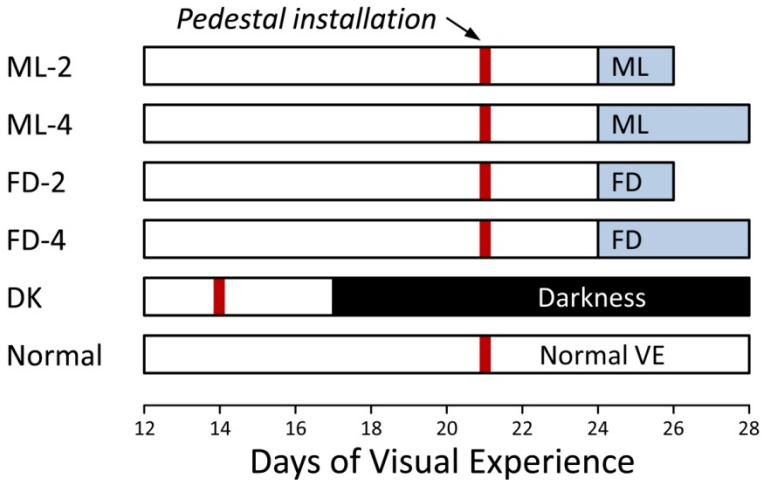

Figure 2. Experimental groups and duration of treatments. The red vertical bar indicates the point when a dental acrylic pedestal was installed under anesthesia. Filled regions indicate the type and duration of visual treatment. The right end of each bar indicates the time point when mRNA levels were measured.

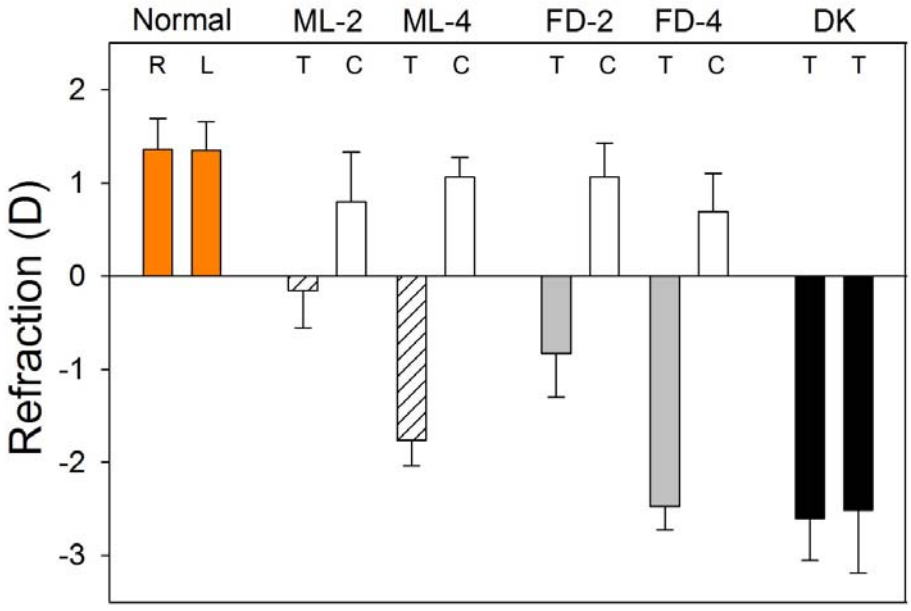

Figure 3. End-of-treatment refractive measures for the normal, minus lens (ML), form deprived (FD), and dark treatment (DK) groups. Values are the mean refraction ± SEM for the right (R) and left (L) eyes of the normal and DK groups, and for the treated (T) and control (C) eyes of the ML and FD groups. Treated eyes in all groups were significantly myopic relative to control (or normal) eyes.

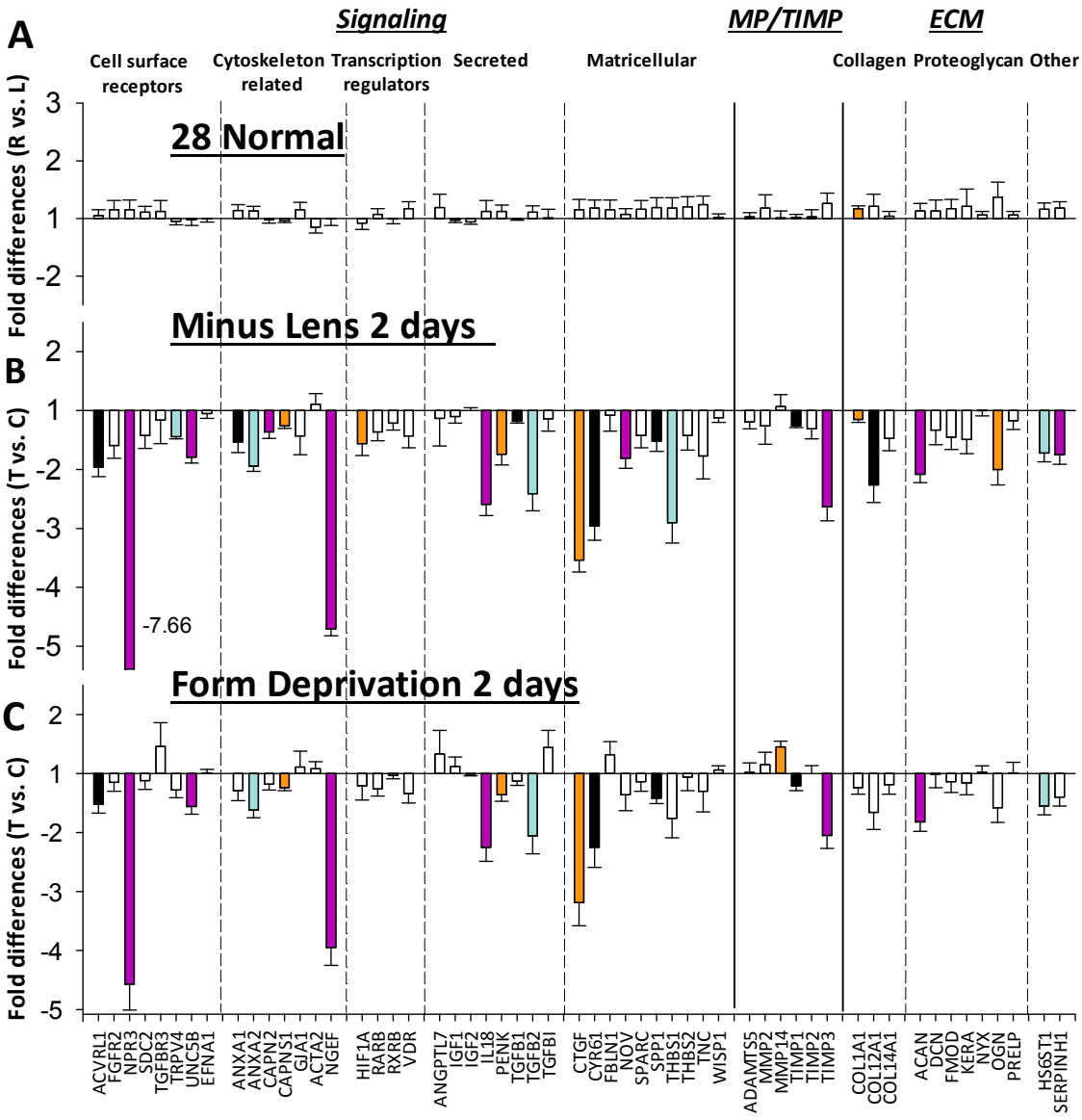

Figure 4. Gene expression fold differences. (**A**) Normal eyes (right eyes vs. left eyes). (**B**) 2 days of minus-lens wear (treated eyes vs. control eyes). (**C**) 2 days of form deprivation (treated eyes vs. control eyes). Filled bars represent statistically significant differences between the treated 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. In B, the off-scale fold difference for NPR3 (-7.66) is indicated next to the bar.

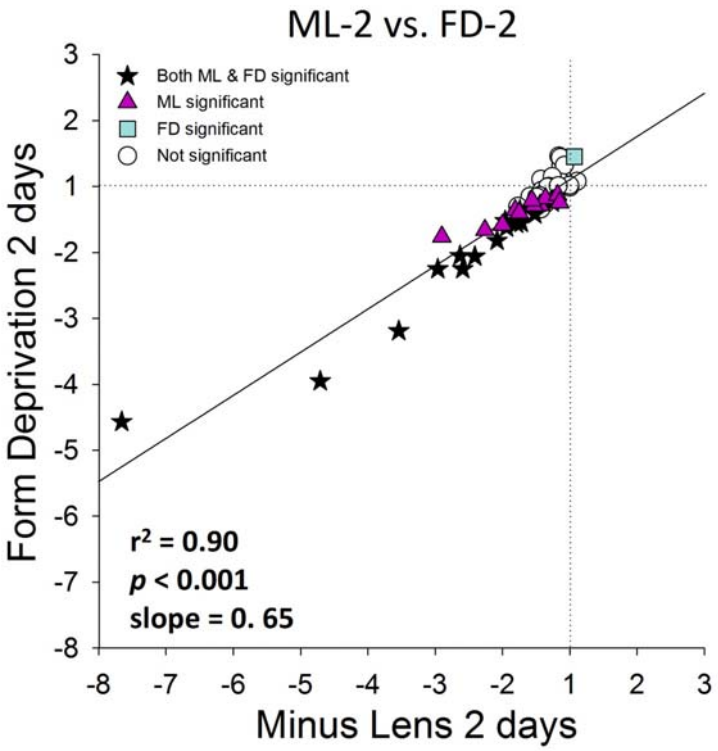

Figure 5. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4B (ML-2) with the differences in Fig. 4C (FD-2). The amount of differential expression in both conditions was very similar. Stars = significant fold differences for both ML and FD; triangles = significant fold differences only for ML; squares = significant fold differences only for FD; circles = fold differences not significant in either treatment.

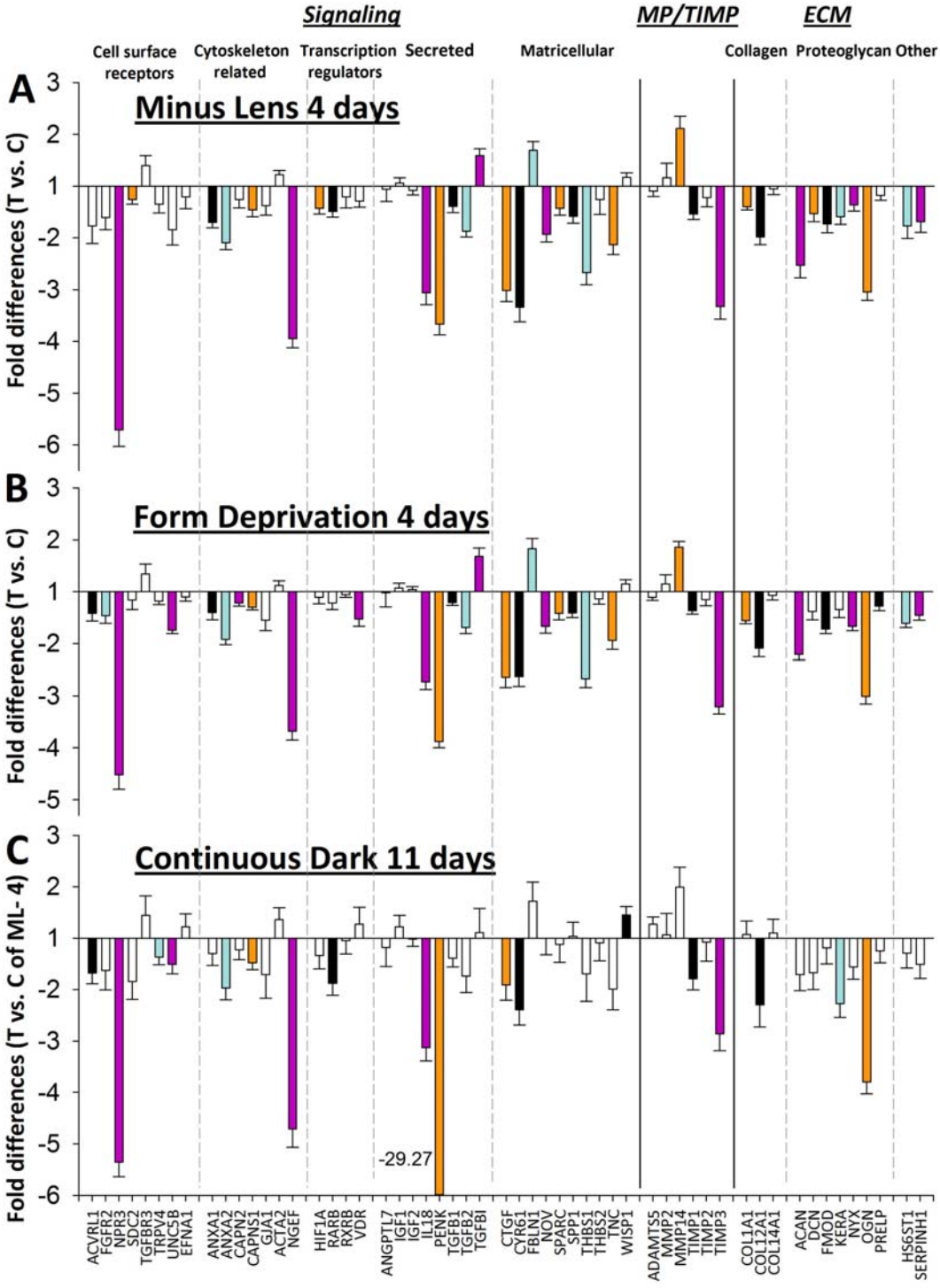

Figure 6. Gene expression fold differences. (**A**) 4 days of minus-lens wear (treated eyes vs. control eyes). (**B**) 4 days of form deprivation (treated eyes vs. control eyes). (**C**) 11 days of dark treatment (mean R & L vs. ML-4 control eyes).

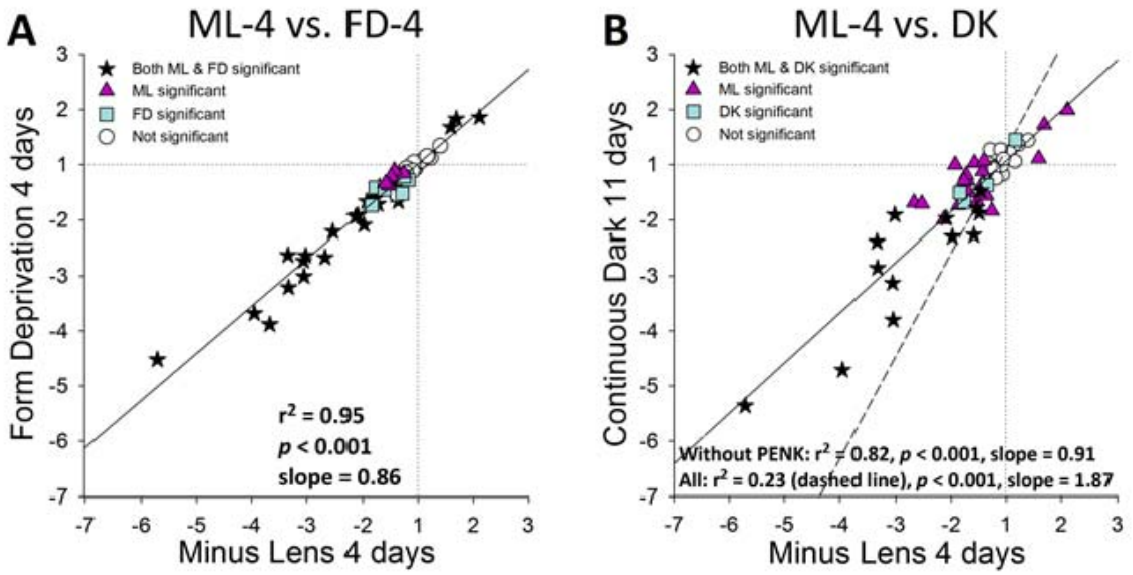

Figure 7. Comparison of the treated vs. control gene expression differences in Fig. 6. (**A**) ML-4 (Fig. 6A) vs. FD-4 (Fig. 6B) (**B**) ML-4 (Fig. 6A) vs. 11 days of continuous darkness (Fig. 6C). Stars = significant fold differences for both treatments; triangles = significant fold differences only for ML-4; squares = significant fold differences only for FD-4 (A) or DK (B); circles = fold differences not significant for either treatment.

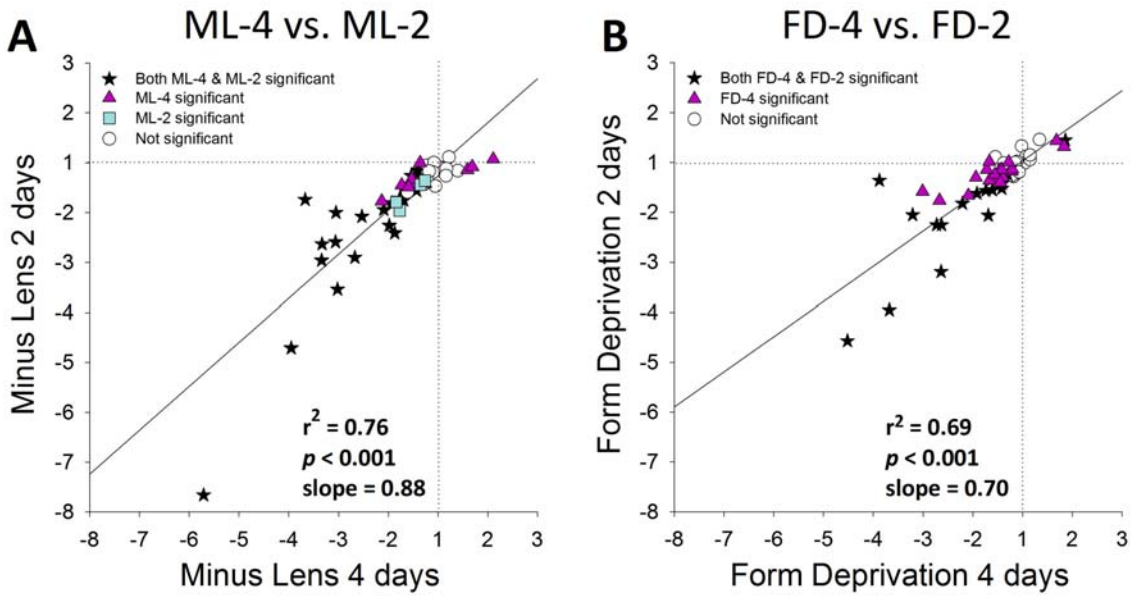

Figure 8. Comparison of the 4 day (Fig. 6) vs. 2 day (Fig. 4) treated vs. control gene expression differences produced by (**A**) minus-lens wear (Fig 6A vs. Fig. 4B) and (**B**) form deprivation (Fig. 6B vs. Fig. 4C). Stars = significant fold differences for both 2- and 4-day treatments; triangles = significant fold differences only for 4-day; squares = significant fold differences only for 2-day; circles = fold differences not significant at either treatment duration.

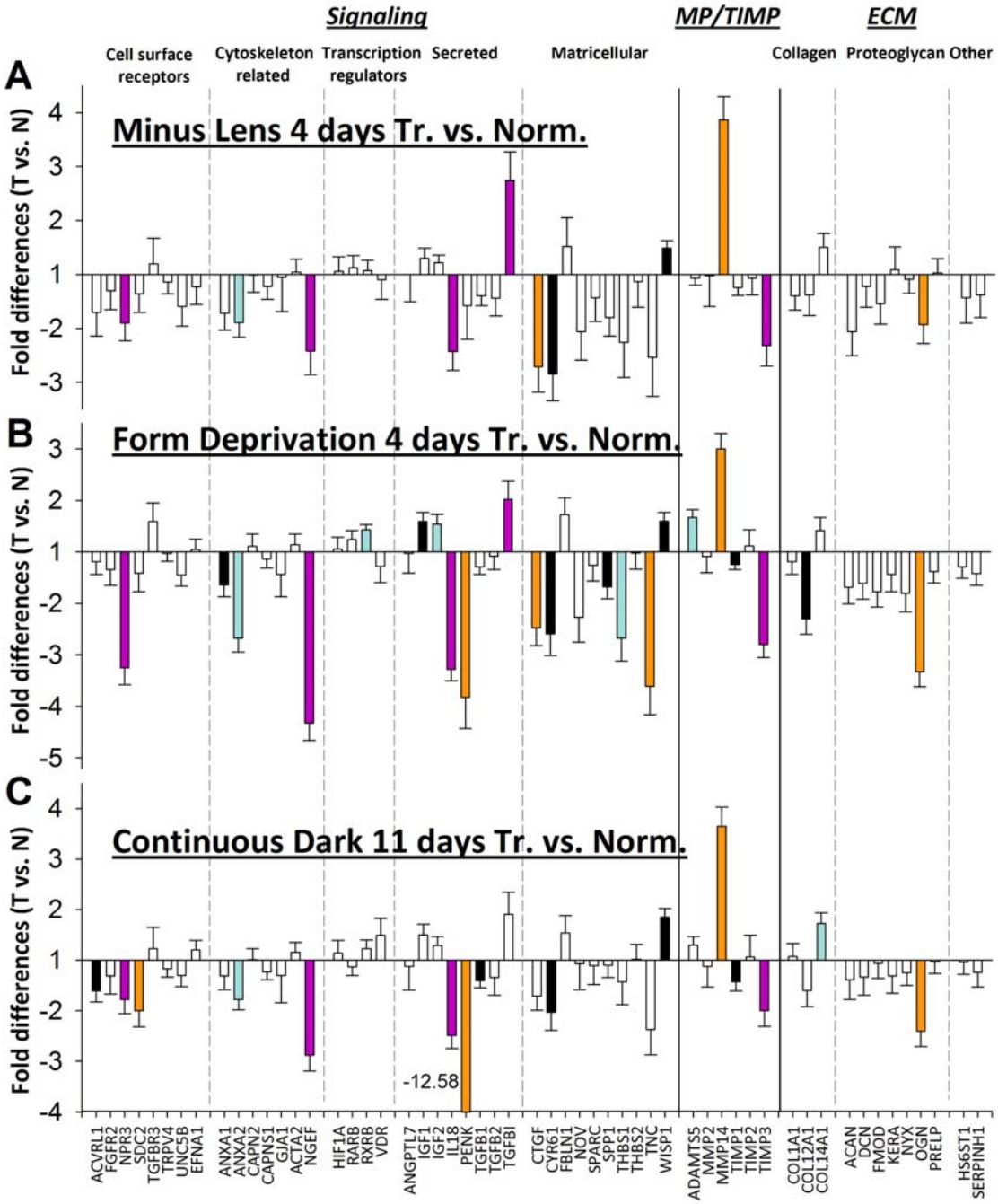

Figure 9. Gene expression fold differences. (**A**) 4 days of minus-lens wear (treated eyes vs. normal eyes). (**B**) 4 days of form deprivation (treated eyes vs. normal eyes). (**C**) 11 days of dark treatment (mean R & L vs. normal eyes).

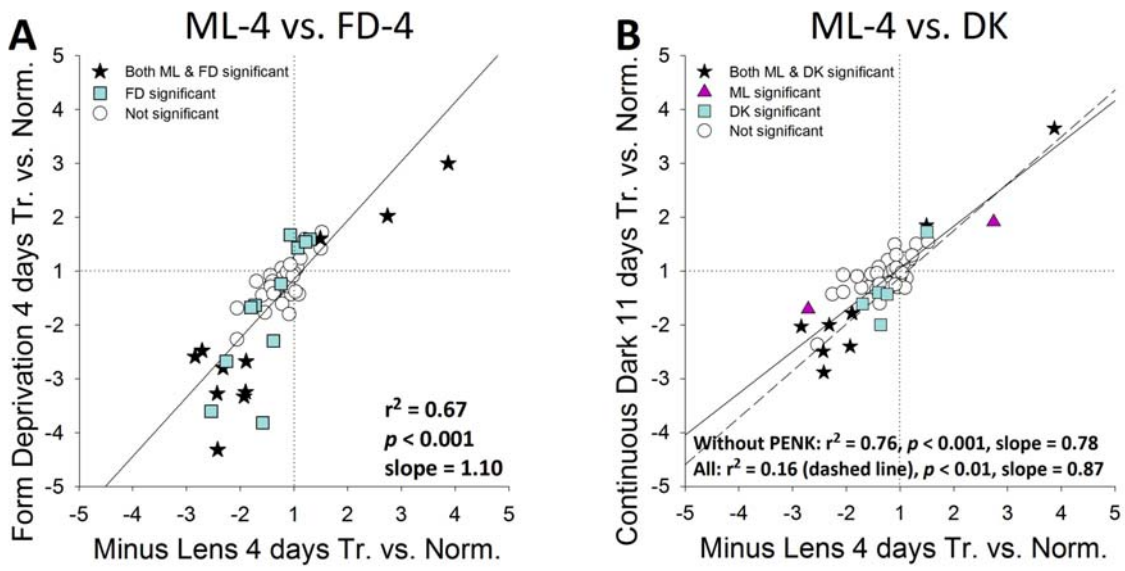

Figure 10. Comparison of gene expression patterns in the treated-eyes of ML, FD, and DK with normal eyes as a common reference (Fig. 9). (**A**) minus-lens wear compared with form deprivation (Fig. 9A vs. Fig. 9B). (**B**) minus-lens wear compared with continuous darkness (Fig. 9A vs. Fig. 9C). Because the DK group treatment was binocular, the fold difference for DK eyes is the mean of values of the R & L eyes.

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

by

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ABSTRACT

During postnatal refractive development, the sclera receives retinally-generated signals that regulate its biochemical properties. This regulation produces scleral remodeling that modulates the biomechanical properties of the sclera and the axial elongation rate of the eye. To learn more about the underlying molecular mechanisms controlling scleral remodeling during the development of lens-induced myopia and recovery from induced myopia, we examined scleral gene expression in juvenile tree shrews under three conditions. GO: monocular –5 D lens wear for 2 days (ML-2) or 4 days (ML-4); STAY: –5 D lens wear for 11 days (ML-11); STOP: recovery (without the lens), after 11 days of −5D lens wear, for 2 days (REC-2) or 4 days (REC-4). The untreated contralateral eyes served as a control in all groups. Two age-matched normal groups provided a comparison with the treated groups. Quantitative real-time PCR (qPCR) was used to measure mRNA levels for 55 candidate genes. The two GO groups developed small amounts of lens-induced myopia in the treated eyes relative to the control eyes (ML-2, −1.0 ± 0.2 D; ML-4, –2.8 ± 0.3 D; mean ± SEM). The STAY group compensated fully for the lens (ML-11, –5.1 ± 0.2 D). Wearing the lens, the hyperopic signal for elongation had dissipated (ML-11 with lens, –0.3 ± 0.3 D). In the STOP groups, the refraction in the recovering eyes became less myopic relative to their control eyes (REC-2, +1.3 ± 0.3 D; REC-4, +2.6 ± 0.4 D). Gene expression differences in the two GO conditions involved mostly down-regulation with similar fold differences at both time points. In the STAY group, only 3 genes showed significant down-regulation. However, many genes that were significantly altered in GO showed smaller, non-significant, expression differences in the same direction in STAY, suggesting the gene expression signature in STAY is a greatly weakened form of the GO

signature. In the STOP groups, a very different gene expression pattern was observed, characterized by mostly up-regulation with larger fold differences after 4 days than after 2 days of recovery. Eleven of the 55 genes examined showed significant bi-directional regulation in the ML-2 and REC-2 groups, and 13 genes showed bi-directional regulation in the ML-4 and REC-4 groups. Eight of these genes (NPR3, CAPNS1, NGEF, TGFB1, CTGF, NOV, TIMP1, and HS6ST1) were bi-directionally regulated at both time points in the GO and STOP conditions. An additional 15 genes showed significant regulation in either GO or STOP conditions, but not in both. These data show that the differing gene expression signatures during the development of induced myopia and recovery involve the selective regulation of many genes in distinct patterns.

Key Words: myopia; sclera; emmetropization; animal models; gene expression; refractive error; axial elongation

1. INTRODUCTION

Refractive error occurs when there is a mismatch between the focal plane, where images are in focus, and the location of the retina, which is controlled by the axial length of the eye. When distant objects are in focus on the retina without accommodation, the eye is emmetropic. If the axial length is shorter than the focal plane, the eye is hyperopic. If the axial elongation of the globe moves the retina behind the focal plane, the eye is myopic. Myopia is the most prevalent type of refractive error worldwide, affecting 25 -

42% of the population in the US and in European countries (Attebo et al., 1999; Fledelius, 1988; Sperduto et al., 1983; Vitale et al., 2009; Wang et al., 1994; Wensor et al., 1999). In Asia, the prevalence reaches as high as 85-96.5% (Goh and Lam, 1994; He et al., 2004; Jung et al., 2012; Lin et al., 1999; Lu et al., 2009; Quek et al., 2004). Myopia is not only a refractive problem, but also an important risk factor for blinding conditions such as glaucoma, cataract, retinal detachment, choroidal degeneration, and other conditions (Avila et al., 1984; Burton, 1989; Lim et al., 1999; Saw et al., 2005). Thus, it is important to try to determine the causes of human myopia and, in particular, the mechanisms that regulate the axial length of the eye.

Studies of postnatal refractive development in both children and animal models (fish, chicks, monkeys, guinea pigs, tree shrews, and other species) have found that there is a visually-guided emmetropization mechanism that uses visual cues to modulate the elongation of the globe so that the retina comes to be located at the focal plane (Mutti et al., 2005; Norton, 1999; Norton et al., 2010; Schaeffel et al., 1988; Schaeffel and Howland, 1988; Shen and Sivak, 2007; Smith, III et al., 1999; Troilo et al., 2000; Wallman and Winawer, 2004; Wildsoet, 1997). As demonstrated in animal studies by severing or blocking the retinal output to central visual structures, this mechanism includes a direct, spatially local, vision-dependent pathway from the retina, through the retinal pigment epithelium (RPE) and choroid to the sclera, that modulates the normal elongation of the eye by remodeling the sclera (McBrien et al., 1995; Norton et al., 1994; Smith, III et al., 2013; Troilo et al., 1987; Wildsoet and McFadden, 2010; Wildsoet and Wallman, 1995).

The sclera is an extracellular matrix (ECM) that, in mammals, is comprised primarily of layers (lamellae) of fibrillar type I collagen (Moses et al., 1978), along with many other components (proteoglycans, elastin, matricellular proteins, etc.) that are typically associated with fibrous connective tissue (Rada et al., 1997). This laboratory investigates the changes that are produced in the sclera by visual conditions that increase, maintain, and slow, the axial elongation rate in the eyes of juvenile tree shrews (mammals closely related to primates).

The emmetropization mechanism can be stimulated to produce increased axial elongation and myopia in animal models by placing a concave (minus-power) lens in front of an emmetropizing eye. This moves the focal plane away from the cornea, producing a hyperopic mismatch between the focal plane and the retina. In response, neurons in the retina generate what has been characterized as GO signals (Rohrer and Stell, 1994; Schaeffel and Howland, 1991). These pass in a signaling cascade through the RPE and choroid, and produce biochemical and biomechanical changes in the sclera that increase the axial elongation rate. Over a period of a few days, the retina is moved to the shifted focal plane, restoring age-normal emmetropia (Irving et al., 1991; Irving et al., 1995; Norton et al., 2010). Increased axial elongation can be detected in tree shrews after as little as two days of wearing a –5 diopter (D) lens (Norton et al., 2010). After 11 days of continuous –5 D lens wear, the axial length of the eye matches the new focal plane and the eye's refraction, while wearing the lens, matches that of the untreated fellow control eye.

The underlying mechanism of the increased axial elongation rate is the scleral remodeling that involves an overall reduction in scleral ECM. There is a reduction in scle-

ral dry weight (McBrien et al., 2001; McBrien and Gentle, 2003; Moring et al., 2007), a slight decrease in the amount of type I collagen, generally reduced levels of numerous ECM proteins (Frost and Norton, 2012), and increased viscoelasticity of the sclera (measured by creep rate) (Phillips et al., 2000; Siegwart, Jr. and Norton, 1999). The remodeling appears to cause the eyeball to expand under normal intraocular pressure. The creep rate reaches a peak after four days of –5 D lens wear, and then gradually decreases, suggesting that the GO signal is strongest at this time point. When minus-lens compensation is complete, the eyes remain elongated as long as the lens is left in place (Norton et al., 2010).

When minus-lens wear is discontinued after compensation has occurred, the eye experiences lens-induced myopia (LIM) due to the elongated globe. The retina then produces STOP signals that pass through RPE and choroid, altering gene expression, and producing a rapid (2-day) reduction of scleral creep rate below normal (Siegwart, Jr. and Norton, 1999). This slows the axial elongation rate below normal while the eye's optic power continues to mature, producing refractive recovery. Recovery continues until the axial length and the refractive power of the recovering eye matches those of the control eye and of age-matched normal eyes (Amedo and Norton, 2012; Siegwart, Jr. and Norton, 1998).

Previous studies from this laboratory and others have found changes in mRNA expression, and protein and glycosaminoglycan levels, in tree shrew sclera during minus-lens wear and recovery, and have examined the time-course of these changes (Arumugam and McBrien, 2012; Frost and Norton, 2012; Gao et al., 2011; Guo et al., 2013; McBrien et al., 2012; Moring et al., 2007; Norton and Rada, 1995; Siegwart, Jr. and Norton, 2005).

Although the number of genes in tree shrews whose expression has been examined has been relatively small, it is clear that the remodeling of the sclera involves the selective regulation of a substantial number of genes. After one day of minus-lens wear or recovery, few differences in gene expression were detected by Gao et al. (2011) suggesting that more time is required for the signaling cascade from retina, through RPE and choroid, to initiate scleral remodeling. However, after four days of lens-wear or recovery, changes in mRNA and protein expression were well established. Moreover, we recently found (Guo et al., 2013) that each of the three methods that produce axial elongation and myopia (minus-lens wear, form deprivation, or several days of total darkness in animals that emmetropized in the light) produce a very similar pattern of gene expression differences in the sclera that we characterized as a GO signature.

In the present study, we compare the GO signature produced by minus-lens wear with the STOP response that occurs during recovery from minus-lens induced myopia to learn if STOP involves bi-directional expression changes in the same genes as are affected during GO (whether the STOP signature is the reverse of the GO signature). In order to compare the early alterations in gene expression with those occurring later, we examined groups after two and four days of lens wear along with two and four days of recovery. Further, because a recent study in tree shrew choroid (He et al., 2013b) found evidence for a STAY gene expression signature in animals that had fully compensated for a minus lens but were still wearing the lens, we examined scleral gene expression in a group that also had compensated fully for the lens after 11 days of treatment. To more fully examine the scleral STOP response pattern in this study, we increased the number

of genes examined to include the same 55 that were examined in GO conditions in a previous study (Guo et al., 2013).

2. MATERIALS AND METHODS

2.1. Experimental Groups

The methods used in this study are very similar to those employed in previous studies from this laboratory (Guo et al., 2013; He et al., 2013b). The juvenile tree shrews (*Tupaia glis belangeri*) used in this study were raised in breeding colony by their mothers on a 14 hr light/10 hr dark cycle. All procedures complied with the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. The first day both eyes are open, which occurs about three weeks after birth, is considered to be the first day of visual experience (DVE). Experimental groups were balanced to include both males and females, and avoided pups from the same parents wherever possible. Right and left eyes were balanced as treated and control eyes in each group.

Seven groups of animals (n = 7 per group) were used in this study (Fig. 1). Two minus-lens wear groups (ML-2 and ML-4; GO) wore a monocular –5 D (spherical power) lens for either 2 or 4 days, starting at 24 ± 1 DVE. These animals were also included in our previous report of the GO signature (Guo et al., 2013) and are used here to compare the GO signature with the STAY and STOP responses. A third minus-lens wear group (ML-11; STAY) wore a monocular −5 D lens for 11 days and fully compensated for the lens. At this point the refractive hyperopia that produced the retinal GO signal had dissipated, yet eyes maintain with-the-lens emmetropia until lens wear is discontinued

(Norton et al., 2010).The animals in this group provided mRNA that was also used in a previous study (Gao et al., 2011).Two STOP groups began recovery at 35 ± 1 DVE, after 11 days of monocular minus-lens wear. They experienced unrestricted vision for 2 (REC-2) or 4 (REC-4) days. In all ML and REC groups, the untreated fellow eye served as a control. Two age-matched normal groups were used, one at 28 DVE (28N) for comparison with the ML-2 and ML-4 groups, the other at 38 DVE (38N) for comparison with the ML-11, REC-2, and REC-4 groups.

In both the GO and STOP conditions, the 2-day treatment duration was selected to examine gene expression soon after the start of treatment or recovery. The 4-day duration was chosen to examine gene expression when the sclera would be undergoing maximal remodeling in both conditions.

2.2. Goggle Installation

At 21 ± 1 DVE, animals in all groups were anesthetized (17.5 mg ketamine, 1.2 mg xylazine; supplemented with 0.5 - 2.0% isoflurane as needed) in order for a dental acrylic pedestal to be attached to the skull (Siegwart and Norton, 1994) (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 ML 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 treated eye. The untreated contralateral 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 for lens cleaning under dim illumina-

tion, while the animals were kept in a darkened nest box to minimize exposure to visual stimuli. The normal groups did not wear a goggle.

2.3. Refractive and Axial Measures

At the start and end of the treatments, non-cycloplegic refractive measures were taken in awake animals with a Nidek ARK-700A infrared autorefractor (Marco Ophthalmic, Jacksonville, FL) (Norton et al., 2003). Measures were made at intermediate time points in some animals. Normal animals were measured just before euthanasia. Since atropine may interfere with retino-scleral signaling, cycloplegic refractive measures were omitted (McKanna and 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).

Ocular component dimensions were measured by A-scan ultrasound under anesthesia at the time the pedestal was installed, as previously described (Norton and McBrien, 1992), to ensure that experimental eyes did not differ significantly in axial length before treatment began. Post-treatment A-scan measures were avoided to eliminate any possibility that the anesthesia required for the A-scan procedure might alter gene expression. A Lenstar LS-900 optical biometer (Haag-Streit USA, Mason, OH) was used to make post-treatment axial component measures in the ML-2 group only. This instrument was placed into service after the other groups were completed, and allowed measures to be quickly made, in awake animals, before euthanasia. Comparison of A-scan and

Lenstar measures of the vitreous chamber in animals in this laboratory showed that the axial differences measured with the Lenstar were very similar to those measured with A-scan ultrasound (Gann, personal communication, 2013).

2.4. Tissue Preparation

After 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. After removing the lens, vitreous humor, and optic nerve head, both surfaces of the sclera were gently 'scraped' to remove the retina, RPE, choroid, and any extraocular tissue, before freezing the tissue in liquid nitrogen.

2.5. Gene Expression Analysis

Individual frozen scleras were pulverized to a fine powder in a chilled Teflon freezer mill (Sartorius Stedim, Bohemia, NY) 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 sclera of 6.8 ± 1.6 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 modi-

fications (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 55 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. 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 previous studies of tree shrew sclera during minus-lens wear (Gao et al., 2011; He et al., 2010; Siegwart, Jr. and Strang, 2007) along with additional genes that were suggested by studies in other species and by a preliminary whole-transcriptome analysis of three of the ML-4 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 and 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 (mean of right and left eyes), and right vs. left eyes of normal animals. 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.

2.6. 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).

3. RESULTS

3.1. Refraction

As shown in Fig. 2A, the refractive differences between right and left eyes of the two normal groups were negligible (28N, 0.01 ± 0.2 D; 38N, −0.02 ± 0.2 D; mean, right eyes – left eyes ± SEM). The ML-2 treated eyes (Fig. 2B) showed a small, statistically significant, myopic shift (−1.0 ± 0.2 D) relative to the control eyes. The myopic shift in

the ML-4 group was −2.8 ± 0.3 D. The myopia in the ML-11 group was −5.1 ± 0.2 D. Measured with the lens in place, the treated eyes were very slightly myopic (−0.3 ± 0.3 D) compared with their fellow control eyes, and all treated eyes were within 1 D of the control eyes. The lens-induced hyperopia present at the start of lens wear had dissipated. After fully compensating for the minus lens, the REC-2 treated eyes became less myopic by 1.3 ± 0.3 D (Fig 2C). The REC-4 group recovered by 2.6 ± 0.4 D. The control eyes in the ML and REC groups did not differ significantly from the normal eyes in the 28N and 38N groups (one-way ANOVA, p = 0.17).

Post-treatment ocular component dimensions of the eyes in the ML-2 group confirmed that the vitreous chamber of the treated eyes had elongated slightly, relative to the control eyes, by 0.016 ± 0.004 mm. Although the axial changes were not measured in the other groups, the results of previous studies in tree shrew (McBrien and Norton, 1992; Moring et al., 2007; Norton et al., 2006; Norton and Rada, 1995; Shaikh et al., 1999; Siegwart, Jr. and Norton, 1999; Siegwart, Jr. and Norton, 2005) make it reasonable to assume that the myopic shifts after 4 days and 11 days of ML were due to an increase in vitreous chamber depth of approximately 0.060 to 0.075 mm, and that the differences in the recovery groups were smaller than they had been after compensation to the minus lens.

3.2. Gene Expression

3.2.1. Normal Animals

Fig. 3 compares gene expression in the right and left eyes of the two groups of normal animals, measured at 28 DVE and 38 DVE. Fold differences (listed in Table 2)

were very small – without regard to sign, 1.11 ± 0.08 fold in the 28N group and 1.12 ± 0.15 in the 38N group (mean ± SD). Only one of the genes in our sample at 28N, the alpha chain of type 1 collagen (COL1A1), was slightly, but significantly, higher in the right eyes (1.17 ± 0.05, mean ± SEM, $p = 0.0208$), as reported previously (Guo et al., 2013). None of the genes in the 38N group differed significantly.

3.2.2. GO: Two-Day and Four-Day Minus Lens Treatment

The fold differences in gene expression between the treated and control eyes in the GO groups (ML-2 and ML-4) are shown in Fig. 4A and 4B; expression values are also listed in Table 2. The variability in expression across animals within each group was low, as evidenced by the small SEM values. The GO patterns were reported previously (Guo et al., 2013) and are presented here to allow comparison with the STAY and STOP expression patterns. Most, but not all, of the sampled genes were down-regulated in the treated eyes, relative to the control eyes.

It is evident in Fig. 4A and 4B that the gene expression pattern of the ML-2 group was very similar to the pattern of the ML-4 group. The two patterns were compared quantitatively in our previous study (Guo et al., 2013). The correlation between the fold differences in the ML-2 group (Fig. 4A) vs. those in the ML-4 group (Fig. 4B) was very high ($r^2 = 0.76$, $p < 0.001$) with no outliers, suggesting that there is a consistent GO signature. The slope of the correlation (0.88) indicated that the signature was stronger after 4 days.

3.2.3. STAY: Eleven-Day Minus Lens Treatment

Fig. 4C shows the fold differences in gene expression between the treated and control eyes in the ML-11 group; expression values are also listed in Table 2. Three genes showed significant down-regulation (PENK, TGFB1, and ACAN). Comparing the patterns in Fig. 4B and 4C, the (non-significant) fold differences for most of the genes in the ML-11 group were similar to those of the ML-4 group, except that they were smaller. This similarity is shown in Fig. 5 which compares the ML-4 group pattern (Fig. 4B) with that of the ML-11 group (Fig. 4C). The correlation between the two ($r^2 = 0.46$) was statistically significant ($p < 0.001$). The low slope (0.27) reflected the finding that the magnitude of the fold differences was smaller in the ML-11 group.

3.2.4. STOP: Two-Day and Four-Day Recovery

As shown in Fig. 4, the gene expression pattern of both the REC-2 and REC-4 groups were similar to each other and very different from those of the GO and STAY groups. Few genes were down-regulated (two in the REC-2 group, three in the REC-4 group) and many were up-regulated (REC-2, 12; REC-4, 17). The down-regulated genes included members of the signaling group (cell surface receptors and secreted signal proteins) and the extracellular matrix group (proteoglycans).The up-regulated genes included ones for signaling molecules (cell surface receptors, cytoskeletal related proteins, secreted signal proteins, and matricellular proteins), for TIMPs, collagens, and proteoglycans.

The fold differences seen in Fig. 4D (REC-2) and Fig. 4E (REC-4) are compared in Fig. 6. The gene expression patterns were highly correlated ($r^2 = 0.76$, $p < 0.001$). The fold differences for all genes in both groups were in the same direction, but differed in

that fewer of the differences in the REC-2 group were statistically significant. mRNA levels of 13 genes were significantly different in both the REC-2 and REC-4 groups (Table 3), and an additional 7 genes were significant only in REC-4. One gene (PENK) was significantly affected in the REC-2 group but not the REC-4 group. The slope of the correlation (0.80) suggested that the overall magnitude of the fold differences in the REC-4 group was somewhat greater than in the REC-2 group.

3.2.5. Comparison of the GO and STOP Patterns

An aim of this study was to learn how the STOP gene expression pattern differed from the GO pattern. The two GO groups and the two STOP groups are compared in Fig. 7: Fig. 7A compares the REC-2 pattern (Fig. 4D) with the ML-2 pattern (Fig. 4A); Fig. 7B compares the REC-4 pattern (Fig. 4E) with the ML-4 pattern (Fig. 4B). As expected from Fig. 4, the GO and STOP patterns are very different from each other and differ in a similar way at both time points. The correlation between GO and STOP in each case is highly significant (2-day, $p < 0.001$, $r^2 = 0.62$; 4-day, $p < 0.001$, $r^2 = 0.57$) and has a negative slope (–0.40 and –0.46 respectively), indicating that the STOP pattern is, in general, opposite to the GO pattern and that the overall fold-difference magnitude was lower in STOP than in GO.

At both time points, there were genes (indicated by stars) that showed significant expression differences in both GO and STOP. All were down-regulated in GO and all but two (PENK and NYX) were significantly up-regulated in STOP, indicating that they were bi-directionally regulated. Eleven genes were bi-directionally regulated after 2 days, and 13 genes were bi-directionally regulated after 4 days; eight of these (NPR3, CAPNS1,

NGEF, TGFB1, CTGF, NOV, TIMP1, and HS6ST1) showed bi-directional regulation at both time points (Table 3). Additional genes at both time-points (triangles) showed significant expression differences in GO, but not in STOP. All were down-regulated after 2 days and all but three were down-regulated after 4 days. Expression of other genes (squares) was not significantly altered during GO, but showed significant expression differences in STOP. There were two such genes in the 2-day GO vs. STOP comparison and six in the 4-day comparison. TGFBI, FBLN1, and MMP14 showed significant up-regulation in the ML-4 group but not in the ML-2 group. PENK was down-regulated significantly in both GO groups, in the STAY group, and in the REC-2 group, but was not differentially regulated in the REC-4 group. Significant down-regulation of TGFBR3 and IGF1 occurred in the REC-4 group but not in any other group. NYX was significantly down-regulated after 4 days of GO and remained down-regulated both STOP groups.

3.2.6. Comparison with Normal Eyes

As in previous studies of gene-expression differences during minus-lens wear and recovery, genes in the control eyes of the treated groups showed significant expression differences from the normal eyes even though, refractively, they did not differ from normal eyes. This raised the question of how the treated eye gene expression values compare with normal eye values – is the differential gene expression due mostly to change in the treated eyes? This comparison in the two GO groups (ML-2 and ML-4) with the 28N group was reported in our previous paper (Guo et al., 2013), where it was found that the treated vs. normal pattern in GO was very similar to the treated eye vs. control eye pattern. When the GO vs. STOP patterns at 2 days and 4 days were re-plotted using treated

eye expression values compared with normal eye values (data not shown), a pattern similar to that shown in Fig. 7 was found. Most of the genes whose expression was significantly down-regulated vs. the control eyes in the GO condition at 2 and 4 days were also down-regulated when compared with the normal eyes; during both STOP conditions, most were up-regulated.

4. DISCUSSION

4.1. The STOP Signature

Differential patterns, in which the treated eyes differ from the control eyes, are of interest because it is the treated eyes that increase their creep rate and axial elongation rate during lens compensation and decrease them during recovery, whereas the control eyes remain relatively normal (Norton et al., 2010; Siegwart, Jr. and Norton, 1999). In a previous paper (Guo et al., 2013) we found that very similar differential mRNA expression patterns for 55 genes were produced in scleral fibroblasts by three different GO conditions: minus-lens wear, form deprivation, and an 11-day period of darkness in animals that had emmetropized in normal light/dark conditions. We suggested that the consistent pattern during GO is a component of a GO signature.

The purpose of the present study was to learn if there is a scleral fibroblast STOP mRNA expression signature for the same 55 genes and, if so, to compare it with the GO signature. The sampled genes clearly show that after two days of recovery from lens-induced myopia, a time-point when refractive recovery has begun, a STOP response pattern has developed that is very similar to the response pattern found after 4 days, when refractive recovery is well underway. The regulation of mRNA expression was selective;

there was a consistent group of genes whose expression was not significantly affected at either time point. The 22 genes that had been examined in previous studies of tree shrew sclera generally responded in this study as previously reported (Gao et al., 2011; He et al., 2010; Siegwart, Jr. and Norton, 2005).

As shown in Figs. 4 and 6, the gene expression patterns in the REC-2 and REC-4 groups were very similar in terms of which genes were affected as well as the direction and relative magnitude of each gene's response. The gene expression pattern appeared to become stronger over time. After two days, 14 genes were differentially expressed, of which 12 were up-regulated. After 4 days, all but one of the same genes were differentially expressed in the same direction and 7 additional genes were differentially regulated, five of them up-regulated. The expression differences for these additional genes were in the same direction after four as after two days, but did not reach statistical significant at the earlier time point. This pattern of mRNA expression differences, and absence of expression differences, between the treated and control eyes may be considered to be a STOP signature for the 55 genes that we examined. It appears that scleral fibroblast gene expression is controlled with some precision when the emmetropization mechanism calls for a slowing of the axial elongation rate.

The general pattern of mRNA up-regulation is consistent with prior reports that during recovery from induced myopia the viscoelasticity of the sclera, measured as the creep rate, rapidly decreases. There is a small gain in scleral ECM and many protein levels return to normal, as does hyaluronan (Frost and Norton, 2012; Gentle et al., 2003; McBrien et al., 2009; Moring et al., 2007).

4.2. STOP vs. GO Gene Expression Signatures

The presence of both GO and STOP gene expression signatures for the 55 candidate genes used in this study allows us to compare the two to learn if gene expression in STOP is the opposite (inverse) of that in GO. Although the general pattern in GO was for down-regulation of most of the genes examined and the general pattern in STOP was for up-regulation, the STOP signature was not an exact inverse of the GO signature. This is reasonable; the scleral remodeling needed to reduce the creep rate and slow axial elongation during recovery need not be the opposite of that required to increase the creep rate during myopia development. As summarized in Table 3, there was a subset of sampled genes whose expression in STOP (after both two and four days) was the opposite of their expression in GO – they were bi-directionally regulated. However, there were additional genes whose mRNA expression was affected only in GO and still others whose expression was affected only in STOP. Thus, there is a "core" of bi-directionally-regulated genes along with many additional genes whose expression is altered in only GO or STOP but not both and others in our sample not significantly altered in either condition.

It is clear from examination of Table 2, Table 3, and Fig. 4, that genes belonging to many functional categories are included in the GO and/or STOP signatures. The three main categories in our sample were genes whose protein products are involved in signaling, metallopeptidases (MP) and tissue inhibitors of metallopeptidases (TIMPS), and the ECM. The signaling category was subdivided into genes whose protein products serve as cell surface receptors, are related to cytoskeleton and cell-cell contacts, nuclear transcription regulators, secreted signaling proteins, and matricellular proteins. The ECM category was subdivided into collagens, proteoglycans, and other proteins. As shown in Table 3,

genes whose mRNA levels were differentially affected during GO or STOP included representatives from all of these nine categories. Examples from all categories, except for transcriptional regulation, showed differential expression in both STOP and GO. The eight bi-directionally regulated genes at both time points in GO and STOP were distributed across six of the categories. It is not known if the bi-directionally regulated genes are more important in producing the scleral remodeling that controls axial elongation than are genes that were affected only in GO or STOP, but they are naturally of interest because the effect on axial elongation, and on scleral viscoelasticity, is opposite.

The 55 genes examined in this study presumably are a subset of a much larger group of genes that show differential expression in GO and STOP conditions. A preliminary whole-transcriptome (RNA-Seq) analysis of treated and control eyes from three of the ML-4 animals and three of the REC-4 animals suggested that perhaps almost 500 genes in ML and 400 in REC (from the nearly 15,000 genes found to be expressed in tree shrew sclera) may be up- or down-regulated by at least 1.20-fold (Frost, personal communication, 2013). Thus, our sampled genes do not represent the whole expression signature in GO and STAY conditions. However, expression differences in this sample of 55 genes is sufficient to distinguish GO and STOP signatures and to provide strong evidence that alterations in the expression of many genes with a wide range of functions are involved. The sclera clearly is a tissue in which complex biological processes interact; examining these changes in intact eyes in their "native" state allows us to learn more about these interactions. The GO and STAY signatures may resemble an orchestra playing a concerto. At different points in the score, some instruments play loudly, others play softly, and still others remaining silent. The interaction of the notes produced by the individual

instruments produces the unique orchestral sound. Although this study did not examine the protein products of these genes, or whether their protein levels are altered, these genes reflect altered fibroblast responses to the emmetropization-related signals from the choroid that are involved in regulating axial elongation of the sclera.

4.3. A STAY Signature

As noted in Fig. 2, after 11 days of minus-lens wear, the refractive hyperopia initially produced by the lens at the start of lens wear had dissipated. The refractions of the treated eyes, while wearing the −5 D lens, were very similar to the refractions of the control eyes. Yet, from studies in other groups of tree shrews, we assume that the axial length of the treated eyes remained elongated, keeping the retina located at the shifted focal plane (Moring et al., 2007; Norton et al., 2010; Siegwart, Jr. and Norton, 1999). That something (the STAY signal) actively maintained the with-the-lens emmetropia is demonstrated by the rapid recovery that developed soon after minus lens-wear was stopped. If the GO condition is one of an accelerated axial elongation rate, and STOP one where the elongation rate decelerates, then STAY is a condition of maintained axial elongation rate. A STAY signal would be analogous to the pressure on the accelerator pedal of a car needed to maintain highway speed. Evidence for a STAY signature in the choroid was found in a previous study of mRNA expression from this lab (He et al., 2013a). This led us to examine the gene expression pattern in the ML-11 group to learn if there was evidence for a STAY signature in the responses of the scleral fibroblasts. In our sample of genes, only three (IL18, TGFB1, and ACAN) showed statistically significant differential expression in the ML-11 group. These three also showed significant differential regu-

lation in the same direction (down-regulation) in both the ML-2 and ML-4 groups. Significant expression of these few genes would not seem to constitute a "signature". However, when comparing the differential expression of all 55 genes at ML-11 and ML-4 (Fig. 5), we noted that there also were small differences in the expression of numerous other genes that were significantly affected in ML-4 but whose expression did not reach statistical significance at ML-11. These non-significant fold differences included 29 genes regulated in the same direction (26 down-regulated) as they were at ML-4 when the differences were statistically significant. Only two genes did not follow this pattern. Whether or not to give weight to these consistent, but non-significant differences is an issue of interest considering the relatively low statistical power that can be achieved with groups of seven animals. We suggest here that there may be evidence of a STAY signature in the sclera. If so, it appears to be weaker than the choroidal STAY signature.

4.4. Summary

This study examined differential mRNA expression by mammalian scleral fibroblasts during normal development; in minus-lens wear, a GO condition; and during refractive recovery from lens-induced myopia, a STOP condition. Based on a sample of 55 genes, we found that the scleral fibroblasts respond with distinctly different mRNA expression signatures to the different emmetropization conditions. The signature for this sample of genes is mostly, but not entirely, down-regulation in the GO conditions and up-regulation in the STOP conditions. In both, the four day time-points showed stronger alterations in gene expression level and a greater number of significantly affected genes than the two day treatment. We also found evidence in sclera for the presence of a STAY

response in eyes that had completed compensation for a minus lens. In the STAY condition, only 3 genes showed significant differences, but other genes may also have been slightly affected. The STAY signature appeared as a weakened form of the GO signature and both were very distinct from the STOP signature. Because a limited number of genes were examined, these signatures are incomplete; however, the many sampled genes that are altered suggest that the emmetropization-related responses in sclera are complex and unlikely to depend on the regulation of a single gene, or even a small number of genes.

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FIGURE LEGENDS

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.

Figure 2. Refractive differences of the (**A**) normal and (**B**) minus lens groups. (**C**) Refractive recovery from full compensation. Values are the mean refractive differences ± SEM for the right – left eyes of the normal groups, and treated – control eyes for the ML and REC groups. The with-lens values in (**B**) show the treated eye – control eye difference while the –5 D lens was in place. Treated eyes in all groups were significantly myopic relative to their fellow control eyes. The upward bars in **(C)** indicate the amount of recovery (decrease in myopia) between the start and end of recovery.

Figure 3. Comparison of gene expression fold differences in normal eyes (right vs. left) in the (**A**) 28 DVE normal and (**B**) 38 DVE normal groups. Filled bars represent statistically significant differences between the right and left eyes ($p < 0.05$). A positive bar indicates that expression was higher in the right eyes. Error bars = SEM.

Figure 4. Comparison of gene expression differences (treated eye vs. control eye) produced by (**A**) 2 days of minus-lens wear, (**B**) 4 days of minus-lens wear, (**C**) 11 days of minus-lens wear, (**D**) 2 days of recovery from 11 days of minus-lens wear, and (**E**) 4 days

of recovery from 11 days of minus-lens wear. Bar color is arbitrary and intended to help in comparing the same gene in the five different conditions. Error bars = SEM.

Figure 5. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4C (ML-11) with the differences in Fig. 4B (ML-4). The patterns of differential expression in both conditions were similar. Stars = significant fold differences for both ML-11 and ML-4; triangles = significant fold differences only for ML-4; circles = fold differences not significant in either treatment.

Figure 6. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4E (REC-4) with the differences in Fig. 4D (REC-2). The amount of differential expression in both conditions was very similar. Stars = significant fold differences for both REC-4 and REC-2; triangles = significant fold differences only for REC-4; squares = significant fold differences only for REC-2; circles = fold differences not significant in either treatment.

Figure 7. Comparison of treated vs. control eye gene expression differences in Fig. 4. (**A**) ML-2 (Fig. 4A) vs. REC-2 (Fig. 4D), (**B**) ML-4 (Fig. 4B) vs. REC-4 (Fig. 4E). Stars = significant fold differences for both treatments; triangles = significant fold differences only for ML; squares = significant fold differences only for REC; circles = fold differences not significant for either treatment.

Table 1. Genes examined, divided into functional categories, with cellular location of the protein encoded by the gene

Gene symbol	Protein name	Location
Signaling – Cell surface receptors		
ACVRL1	Activin A receptor 2-like 1	Cell surface
FGFR2	FGF receptor 2	Cell surface
NPR3	Atrial natriuretic peptide receptor 3	Cell surface
SDC2	Syndecan 2	Cell surface
TGFBR3	TGFβ receptor III	Cell surface
TRPV4	Transient receptor potential cation channel V4	Cell surface
UNC5B	Netrin receptor UNC5B	Cell surface
EFNA1	Ephrin A1	Cell surface
Signaling – Cytoskeleton related		
ANXA1	Annexin A1	Cell surface
ANXA2	Annexin A2	Cell surface
CAPN2	Calpain 2	Cell surface
CAPNS1	Calpain small subunit 1	Cell surface
GJA1	Connexin 43	Cell surface
ACTA2	Smooth muscle actin	Intracellular
NGEF	Neuronal guanine nucleotide exchange factor	Intracellular
Signaling – Transcription regulators		
HIF1A	Hypoxia-inducible factor 1α	Intracellular
RARB	Retinoic acid receptor β	Intracellular
RXRB	Retinoid X receptor β	Intracellular
VDR	Vitamin D receptor	Intracellular
Signaling – Secreted		
ANGPTL7	Angiopoietin-related protein 7	Extracellular
IGF1	Insulin-like growth factor 1	Extracellular
IGF2	Insulin-like growth factor 2	Extracellular
IL18	Interleukin 18	Extracellular
PENK	Proenkephalin A	Extracellular
TGFB1	Transforming growth factor β1	Extracellular
TGFB2	Transforming growth factor β2	Extracellular
TGFBI	TGFβ-induced protein	Extracellular
Signaling – Matricellular		
CTGF	Connective tissue growth factor	Extracellular
CYR61	Protein CYR61	Extracellular
FBLN1	Fibulin 1	Extracellular
NOV	Nephroblastoma overexpressed gene	Extracellular
SPARC	Secreted protein acidic and rich in cysteine	Extracellular
SPP1	Osteopontin	Extracellular
THBS1	Thrombospondin 1	Extracellular
THBS2	Thrombospondin 2	Extracellular
TNC	Tenascin C	Extracellular
WISP1	WNT1 inducible signaling pathway protein 1	Extracellular
MPs / TIMPs		
ADAMTS5	ADAM metallopeptidase with thrombospondin motif, 5	Extracellular
MMP2	Matrix metallopeptidase 2	Extracellular
MMP14	Matrix metallopeptidase 14	Cell surface
TIMP1	TIMP metallopeptidase inhibitor 1	Extracellular
TIMP2	TIMP metallopeptidase inhibitor 2	Extracellular
TIMP3	TIMP metallopeptidase inhibitor 3	Extracellular
Extracellular matrix – Collagens		
COL1A1	Collagen type I, α1	Extracellular
COL12A1	Collagen type XII, α1	Extracellular
COL14A1	Collagen type XIV, α1	Extracellular
Extracellular matrix – Proteoglycans		
ACAN	Aggrecan	Extracellular
DCN	Decorin	Extracellular
FMOD	Fibromodulin	Extracellular
KERA	Keratocan	Extracellular
NYX	Nyctalopin	Extracellular
OGN	Mimecan	Extracellular
PRELP	Prolargin	Extracellular
Extracellular matrix – Other		
HS6ST1	Heparan-sulfate 6-O-sulfotransferase 1	Cell surface
SERPINH1	Serpin H1	Intracellular

Table 2. Gene expression differences comparing right vs. left eyes (*Normals*) or treated vs. control eyes (*ML* and *REC*). Red text = significant down-regulation, blue = significant up-regulation, grey = expression difference not statistically significant.

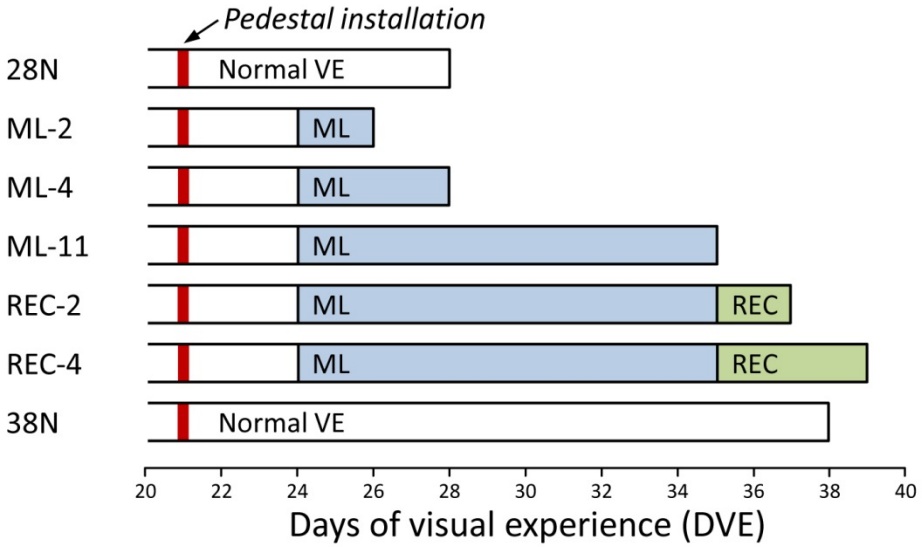
	Normals		GO		STAY	STOP	
	28N	*38N*	*ML-2*	*ML-4*	*ML-11*	*REC-2*	*REC-4*
Signaling – Cell surface receptors							
ACVRL1	1.05	-1.50	**-1.96**	-1.77	-1.27	**1.81**	**1.83**
FGFR2	1.15	-1.02	-1.59	-1.61	-1.19	1.21	1.07
NPR3	1.15	-1.28	**-7.66**	**-5.71**	-1.80	**3.42**	**4.50**
SDC2	1.11	1.01	-1.42	**-1.26**	-1.05	1.28	1.20
TGFBR3	1.12	-1.01	-1.16	1.40	-1.08	-1.79	**-1.79**
TRPV4	-1.05	-1.11	**-1.44**	-1.35	-1.13	**1.47**	**1.37**
UNC5B	-1.02	-1.01	**-1.79**	-1.84	-1.32	**1.58**	**1.51**
EFNA1	-1.00	-1.05	-1.05	-1.21	-1.09	-1.02	1.03
Signaling – Cytoskeleton related							
ANXA1	1.14	-1.07	**-1.53**	**-1.70**	-1.11	1.35	1.15
ANXA2	1.13	-1.12	**-1.94**	**-2.09**	-1.32	1.31	**1.20**
CAPN2	-1.02	-1.03	**-1.36**	-1.26	-1.11	1.18	1.16
CAPNS1	-1.04	-1.08	**-1.26**	**-1.46**	-1.02	**1.28**	**1.18**
GJA1	1.15	-1.08	-1.43	-1.38	-1.27	-1.11	-1.23
ACTA2	-1.15	-1.15	1.11	1.22	1.21	-1.18	1.02
NGEF	-1.00	-1.57	**-4.71**	**-3.95**	-1.80	**2.64**	**1.91**
Signaling – Transcription regulators							
HIF1A	-1.08	-1.07	**-1.56**	**-1.43**	-1.07	1.15	1.05
RARB	1.07	-1.03	-1.36	**-1.49**	-1.16	1.14	1.06
RXRB	-1.01	1.08	-1.21	-1.21	-1.39	1.18	-1.09
VDR	1.17	1.12	-1.43	-1.29	-1.51	1.33	1.09
Signaling – Secreted							
ANGPTL7	1.19	-1.01	-1.13	-1.06	-1.05	-1.16	-1.33
IGF1	-1.03	1.02	-1.10	1.06	1.01	-1.26	**-1.29**
IGF2	-1.06	-1.07	1.00	-1.08	1.04	-1.06	-1.01
IL18	1.12	-1.13	**-2.59**	**-3.06**	-1.50	2.09	**2.03**
PENK	1.12	-1.82	**-1.74**	**-3.67**	**-3.24**	**-2.05**	-1.01
TGFB1	-1.01	-1.08	**-1.18**	**-1.39**	**-1.16**	**1.22**	**1.23**
TGFB2	1.11	-1.07	**-2.41**	**-1.87**	-1.25	1.39	1.25
TGFBI	1.01	1.18	-1.14	**1.59**	1.06	1.03	-1.30
Signaling – Matricellular							
CTGF	1.15	-1.08	**-3.54**	**-3.02**	-1.39	**2.76**	**2.45**
CYR61	1.18	-1.15	**-2.96**	**-3.34**	-1.55	1.74	1.73
FBLN1	1.15	1.10	-1.08	**1.69**	1.32	1.64	1.16
NOV	1.07	-1.08	**-1.81**	**-1.93**	1.01	**1.58**	**1.48**
SPARC	1.16	-1.06	-1.42	**-1.43**	-1.23	1.11	1.19
SPP1	1.19	-1.19	**-1.52**	**-1.58**	1.01	1.25	1.27
THBS1	1.18	-1.23	**-2.90**	**-2.67**	-2.23	1.19	1.40
THBS2	1.20	1.08	-1.42	-1.26	-1.64	1.49	1.28
TNC	1.24	-1.23	-1.77	**-2.13**	-1.31	1.51	1.16
WISP1	1.02	1.06	-1.12	1.17	-1.04	**1.25**	**1.40**
MPs / TIMPs							
ADAMTS5	1.03	-1.04	-1.19	-1.10	-1.03	-1.26	-1.21
MMP2	1.18	-1.01	-1.26	1.16	-1.08	1.02	-1.22
MMP14	1.01	1.18	1.07	**2.11**	1.14	1.24	1.05
TIMP1	1.02	-1.12	**-1.26**	**-1.54**	-1.04	**1.20**	**1.23**
TIMP2	1.03	1.03	-1.31	-1.22	-1.54	1.07	-1.07
TIMP3	1.26	-1.13	**-2.63**	**-3.33**	-1.59	1.38	**1.75**
Extracellular matrix - Collagens							
COL1A1	**1.17**	1.04	**-1.15**	**-1.40**	-1.34	1.09	**1.48**
COL12A1	1.21	-1.06	**-2.26**	**-1.98**	-1.71	1.30	1.58
COL14A1	1.04	-1.02	-1.47	-1.05	1.02	1.10	1.28
Extracellular matrix - Proteoglycans							
ACAN	1.13	-1.13	**-2.08**	**-2.53**	**-1.42**	1.54	**1.70**
DCN	1.13	-1.10	-1.33	**-1.53**	-1.17	-1.09	-1.09
FMOD	1.17	-1.05	-1.45	**-1.73**	-1.47	1.14	1.06
KERA	1.21	-1.17	-1.49	**-1.59**	-1.03	-1.06	1.20
NYX	1.06	-1.04	-1.00	**-1.36**	-1.32	**-1.36**	**-1.29**
OGN	1.37	-1.18	**-2.00**	**-3.05**	-1.53	1.14	1.39
PRELP	1.06	-1.09	-1.17	-1.18	-1.37	-1.11	1.02
Extracellular matrix - Other							
HS6ST1	1.16	-1.40	**-1.72**	**-1.77**	-1.13	**1.96**	**2.36**
SERPINH1	1.18	-1.05	**-1.75**	**-1.69**	-1.23	1.43	1.38

Table 3. Genes showing significant regulation at both time-points in G0 and STOP. Red text = significant down-regulation, blue = significant up-regulation, bold italics = bi-directional regulation.

	Signaling					MP/TIMP	ECM		
	Cell surface receptors	*Cytoskeleton related*	*Transcription regulators*	*Secreted*	*Matricelular*		*Collagens*	*Proteoglycans*	*Other*
GO (ML)	***NPR3***	ANXA1 ANXA2 ***CAPNS1*** ***NGEF***	HIF1A	IL18 PENK ***TGFB1*** TGFB2	***CTGF*** CYR61 ***NOV*** SPP1 THBS1	***TIMP1*** TIMP3	COL1A1 COL12A1	ACAN OGN	***HS6ST1*** SERPINH1
STOP (REC)	ACVRL1 ***NPR3*** TRPV4 UNC5B	***CAPNS1*** ***NGEF***		***TGFB1***	***CTGF*** ***NOV*** WISP1	***TIMP1***		NYX	***HS6ST1***

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

Gene	*Forward sequence*	*Reverse sequence*	*Amplicon (bp)*	*Efficiency (%)*
ACAN	CTGTGACATAGACAAGGAGAT	GACGATGCTGCTCAGG	142	92
ACTA2	CGTGATCTCACCGACTACCT	GCGGAAGCGTTCGTTCCCG	222	98
ACVRL1	CGTACTGGTCAAGAGCAA	ATGTCAGTCCACTTGTAGG	189	94
ADAMTS5	TCTTCCATCCTAACCAGCATTG	GGTGGCATCATAAGTCTGTCC	165	98
ANGPTL7	GCTGTCACTTGGCTCTG	AGTCCTTCTCCTGCTTCTTG	193	94
ANXA1	ACAAAGTTCTGGACCTGGAGTTGA	AGAGCGGGAAACCATAATCCTGAT	167	95
ANXA2	CTTCCGCAAGCTGATG	CATGATGCTGATCCACTT	154	99
CAPN2	GGCTGACCAGACGGCATGAAG	CTCCATCTTGGTGAGTTTCCATTTCTTG	146	88
CAPNS1	TGACCGATCAGGGACCATCTG	GGATTTGTCCACTGCCATCTTTG	215	92
COL12A1	CAGGTGTCCCGATCAAAGAG	GCCTGGTGGAATGGTGG	95	97
COL14A1	TCACCTCCTACTCAACCACCAACT	GGCTCCAACAGTGCTATACAGAAAGT	165	94
COL1A1	GCCCTTCTGGAAATGC	GACCAACTTCACCAGGA	111	95
CTGF	CCAACTATGATTCGAGCCAACTG	TTCTCTTCCAGGTCTGCTTCA	173	96
CYR61	TGGAACCTCGCATTCTGTATAACC	GCCACAAGGTCGCACTTCAC	176	97
DCN	CTGCGTGCCCATGAGAACGAGATC	GGTATCAGCAATGCGGATGTACGAAAGC	168	96
EFNA1	CCACCATCAAGAAGACCAGT	CCAGGCAAGAGGGAAGAG	178	97
FBLN1	CGAGGAGGAGCAAGAG	AGCACGAGCAGACTAC	104	94
FGFR2	TGAAGGAAGGACACAGGATGGATAAG	GAGGCTGACTGAGGTCCAAGTATTC	180	93
FMOD	TCCTGCTGGACCTGAG	TTGTTGGTGAGGCTGTTAT	172	96
GJA1	CCTAGCCATTGTGGACCA	ATCTCCAGGTCATCAGGC	81	99
HIF1A	ACTGGTTGCATCTCCGTCTCCTACTCA	TCCTGCTCTGTTTGGTGAGGCTGTC	109	94
HS6ST1	CGTTCAACCTCAAGTTCATCC	GGTCCTTGGCGTAGTCAT	141	98
IGF1	GTCCTCCTCACATCTCTTCTACC	CAGCACCGCAGAGTGTCTC	89	99
IGF2	TGGCATTGTTGAAGAGTGTTG	GAAGCACGGTCGGAGAG	113	100
IL18	AACTCTTTCCTTTAAGAATATCAGTCGTCCT	GTTGGCTTCCACAGTAAACATTACAGATT	226	94
KERA	TCTCACAATCAGCTCACCAAGGTT	CCAGACGGAGGTAGCGAAGATG	175	101
MMP14	CCCTGGAACCTGGCTACCC	ATAGGTCTTTCCATTGGGCATCC	104	96
MMP2	TGACCTTGACCAGAACACCATCG	GAGCGAAGGCATCATCCACTGT	176	95
NGEF	ACCACTTCTCCGTGTAC	GACTAGGAGCTTGAGGC	194	96
NOV	CGAACAGACTACAGAGTGGAGTG	GTGATTTCTTGGTGCGGAGACA	191	97
NPR3	GGACTACGCCTTCTTCAACATCG	AGAGTGACTGTTTGGAGGGATGA	126	96
NYX	GTGAGCAGGTTCAGCAG	GACAGCGAGGTGTTAGC	101	96
OGN	CTCTCCTTCCTCTACCTGGACCACAAC	CTGAAGATGGATCACGCGCAGACTC	84	96
PENK	TCCTTGCCAAGCGATACG	TCTTGCTCATTTCTTCGTCGTT	162	98
PRELP	TTCGGCTGAACTACAACAAGC	GATCTGGGTCCCGTTGATTTTCT	182	99
RARB	CGGCTTGACCATCGCAGAC	GCTGGTTGGCAAAGGTGAACA	197	97
RXRB	CCTGACCTACTCGTGCC	TACCGCCTCCCTCTTCA	118	91
SDC2	TGATGACGACTACGCTTCTGC	CAGGCATCTTGTTCTGTGTCTTC	155	95
SERPINH1	ACAAGAACAAGGCAGAC	GCACCAGGAAGATGAAG	186	103
SPARC	GCGAGTTTGAGAAGGTGTGC	GCCCGATGTAGTCCAGGTG	126	102
SPP1	CCGACGACACCGACCATCC	GGCTTTGACCTCACTCTGTAAACC	190	92
TGFB1	ACCAGAAATACAGCAACAATTCC	AACCCGTTGATGTCCACTTG	205	91
TGFB2	GCAGAGTTTAGGGTCTTTCGTTTG	CTCGTGAACAGCATCAGTTACATC	189	93
TGFBI	CCTCGGCACTCATCTCTCC	GCAAATTCTTCATCTTGGCATCG	107	94
TGFBR3	CCCTGGTCTGGCGTCTGAAG	GTAACTGCTCCATACTCGTTTCGG	190	93
THBS1	CTGTCAGAACTCAGTCACCATC	CCACGGAGACCAGCCATC	136	91
THBS2	GAGACCGACTTCAGGAACTTC	CGAAACCCACTGCGATGC	142	100
TIMP1	CACTTGCACATCACCACCT	CAAGGGATGGATAAACAGGGAAA	134	99
TIMP2	AGATGGGCTGTGAATGC	CCGTCGCTTCTCTTGAT	151	96
TIMP3	CCGTGTCTATGATGGCAAGATG	ACAAAGCAAGGCAGGTAATAGC	153	99
TNC	AGACGCCAAGACTCGCTACAG	CAGGTTGACACGGTGACAGTTC	184	94
TRPV4	AGAGCAAGCACATCTGGAA	CCACGGTCACCATCTCC	111	94
UNC5B	CACCTGCTGGCTCCCTG	ATGGACAGCGGGATCTTGAA	94	96
VDR	AACTTGCACGAGGAGGAAC	CTTCTGGATCATCTTGGCATAG	189	93
WISP1	AGGCACCCATGAACTT	GACACTGGAAGGAGACA	135	96
POLR2A	CTACCAGCCCCAAGTATTC	GGTGAGTAAGTAGGAGACG	106	98



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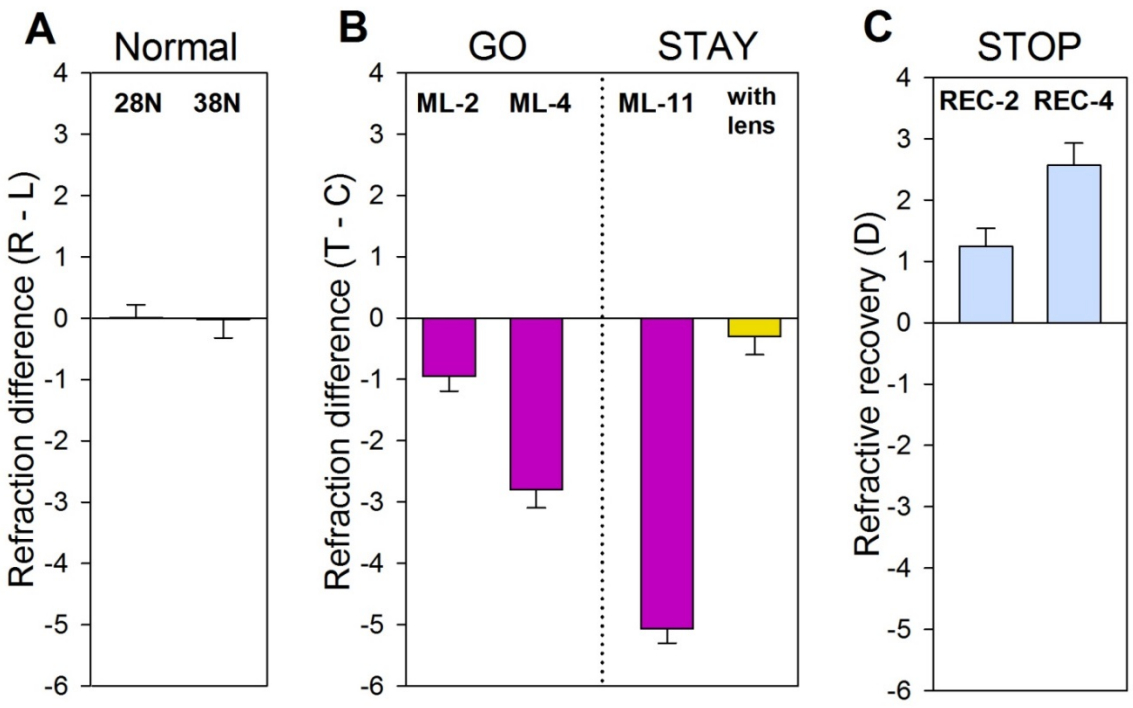

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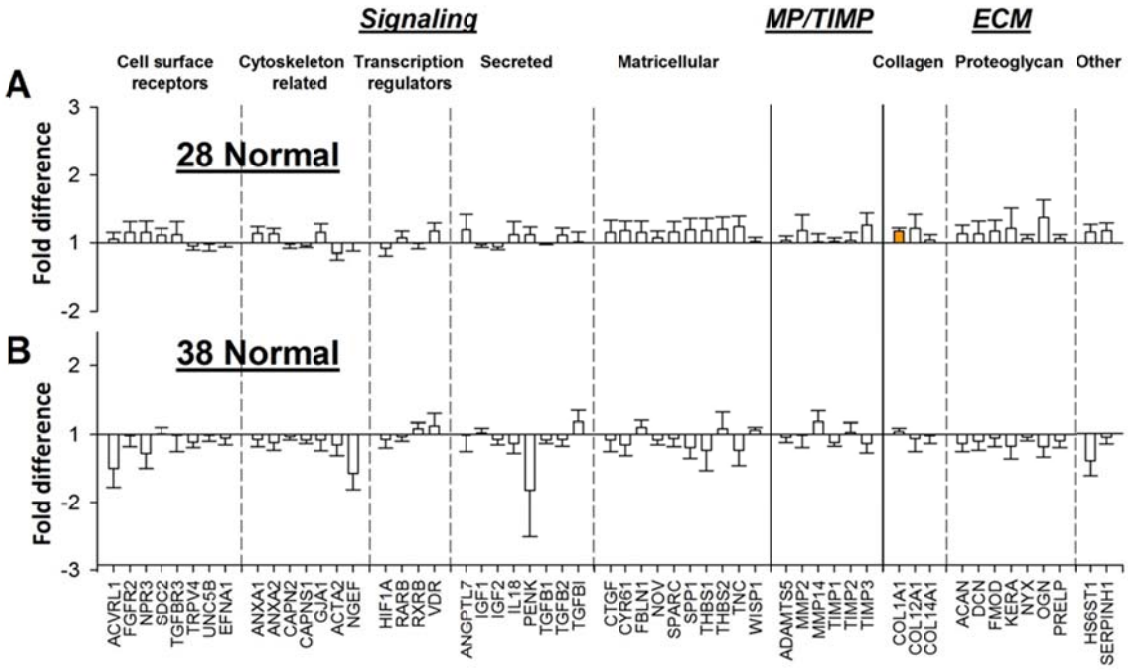

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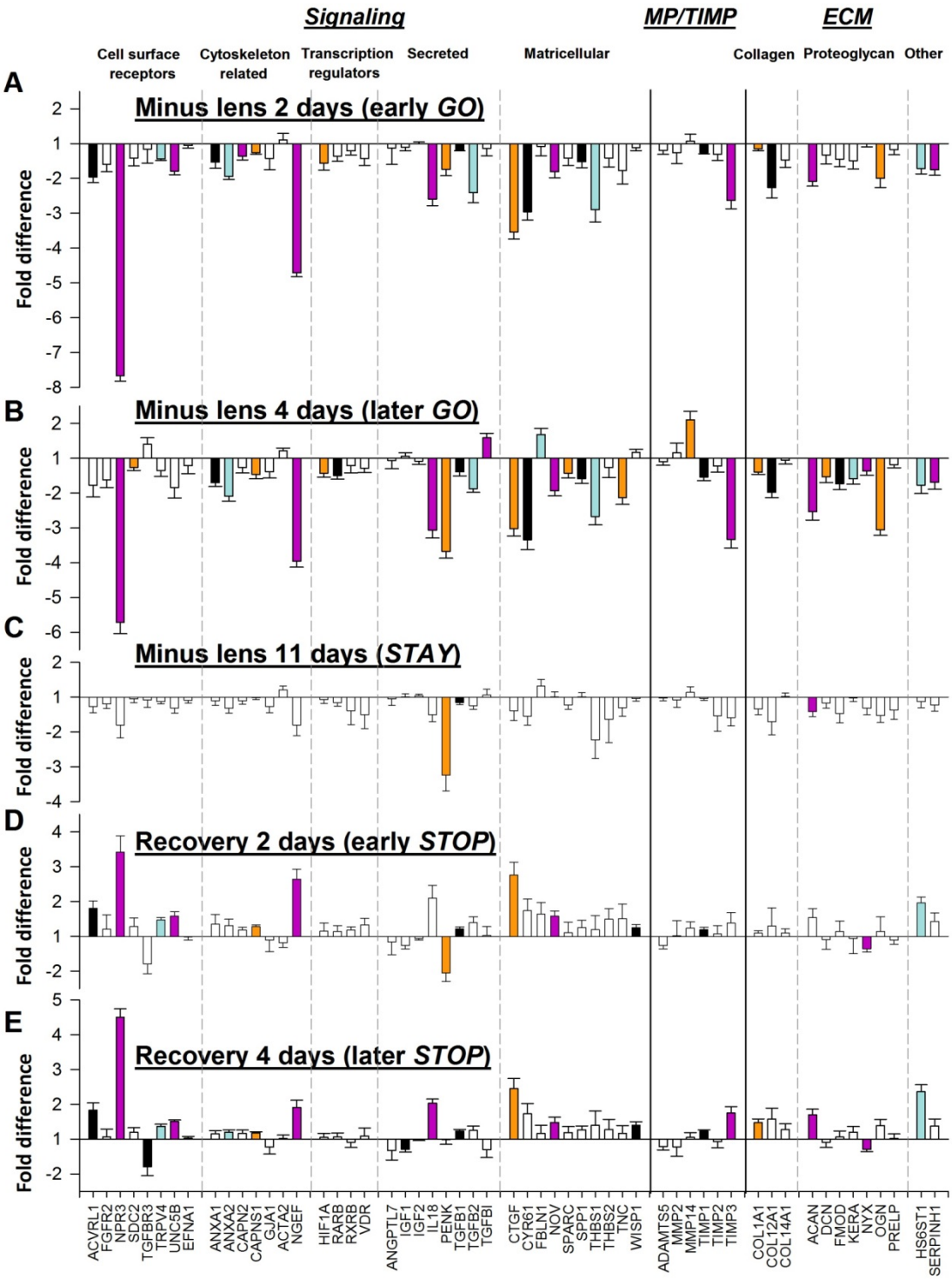

Figure 4. Comparison of gene expression differences (treated eye vs. control eye).They are produced by (**A**) 2 days of minus-lens wear, (**B**) 4 days of minus-lens wear, (**C**) 11 days of minus-lens wear, (**D**) 2 days of recovery from 11 days of minus-lens wear, and (**E**) 4 days of recovery from 11 days of minus-lens wear. Bar color is arbitrary and intended to help in comparing the same gene in the five different conditions. Error bars = SEM.

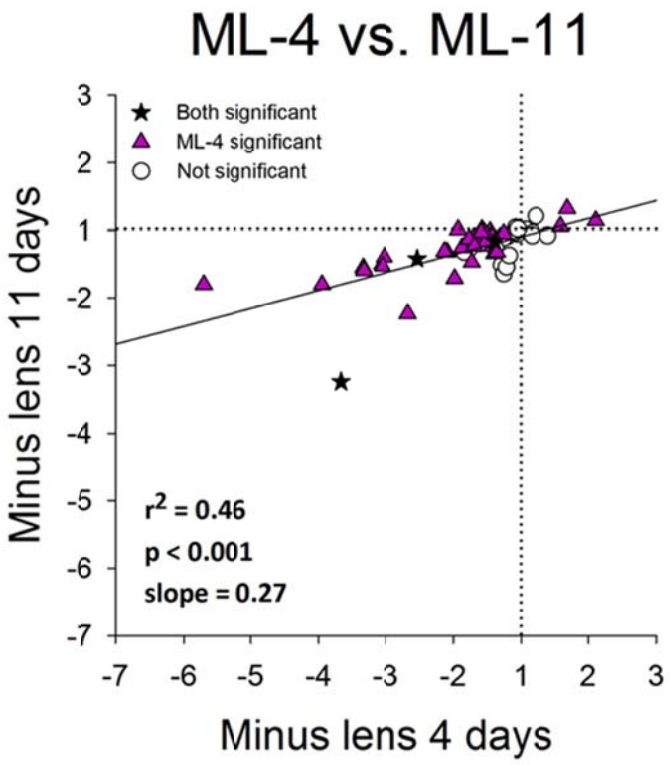

Figure 5. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4C (ML-11) with the differences in Fig. 4B **(**ML-4). The patterns of differential expression in both conditions were similar. Stars = significant fold differences for both ML-11 and ML-4; triangles = significant fold differences only for ML-4; circles = fold differences not significant in either treatment.

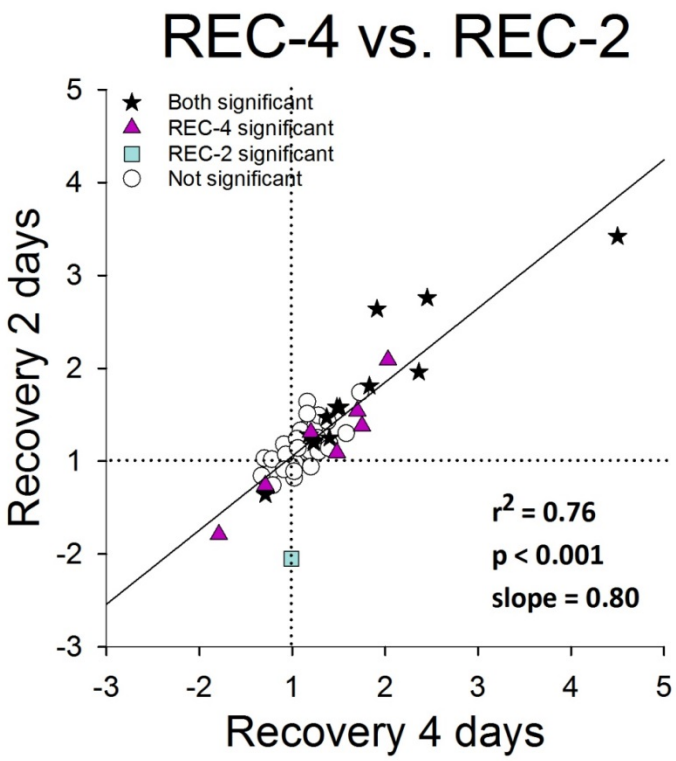

Figure 6. Comparison of the gene expression differences (treated eye vs. control eye) in Fig. 4E (REC-4) with the differences in Fig. 4D (REC-2). The amount of differential expression in both conditions was very similar. Stars = significant fold differences for both REC-4 and REC-2; triangles = significant fold differences only for REC-4; squares = significant fold differences only for REC-2; circles = fold differences not significant in either treatment.

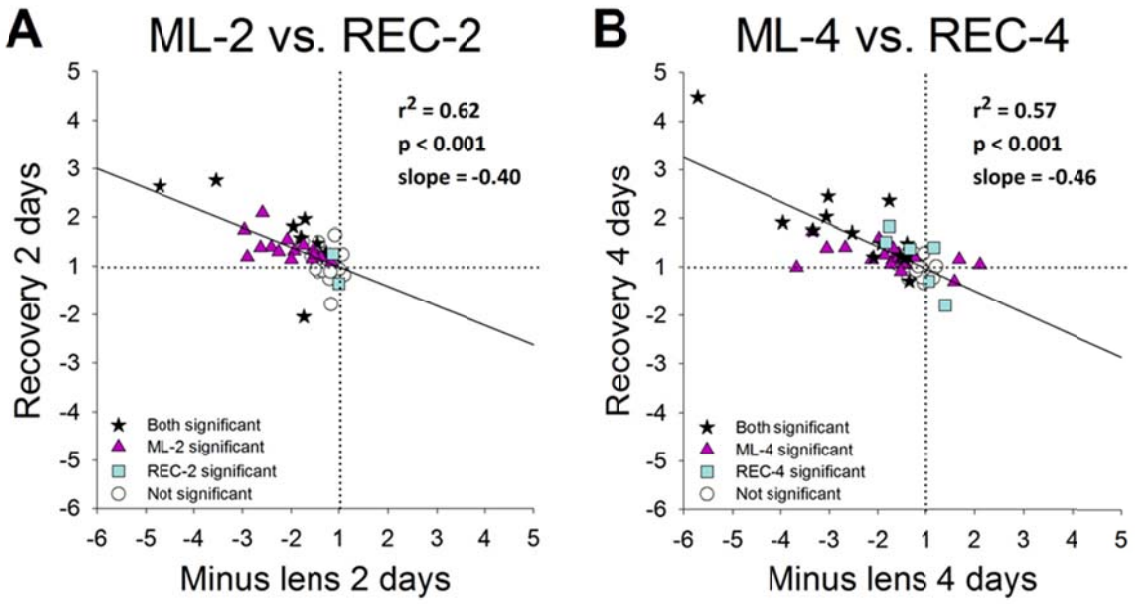

Figure 7. Comparison of treated vs. control eye gene expression differences in Fig. 4. (**A**) ML-2 (Fig. 4A) vs. REC-2 (Fig. 4D), (**B**) ML-4 (Fig. 4B) vs. REC-4 (Fig. 4E). Stars = significant fold differences for both treatments; triangles = significant fold differences only for ML; squares = significant fold differences only for REC; circles = fold differences not significant for either treatment.

GENE EXPRESSION SIGNATURES IN TREE SHREW SCLERA DURING RECOVERY FROM MINUS-LENS WEAR AND DURING PLUS-LENS WEAR

by

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ABSTRACT

Purpose: After developing minus lens-induced myopia, if lens treatment is discontinued, refractive recovery (REC) occurs in juvenile tree shrews. In age-matched animals, plus-lens wear (PLW) produces little refractive change even though the visual stimulus (myopia) is similar. Because the sclera controls axial elongation and refractive error, we compared gene expression signatures in the sclera, produced by REC and PLW, to learn if these similar refractive conditions produce differing scleral responses.

Methods: Four groups of tree shrews (n=7 per group) wore a monocular −5 D lens for 11 days from 24 to 35 days of visual experience (DVE). Lens-wear was then discontinued and the animals recovered for 0 hr (REC-0), 2 hr (REC-2hr), 1 day (REC-1d), or 4 days (REC-4d). Three additional age-matched groups (n=7 per group) wore a +5 D lens for 2 hr (PLW-2hr), 1 day (PLW-1d), or 4 days (PLW-4d). A normal group was examined at 38 DVE to provide baseline measures (PLW-0). Using quantitative real-time PCR (qPCR), we examined mRNA levels for 55 candidate genes including signaling molecules, metalloproteinases and their inhibitors (MPs/TIMPs), extracellular matrix proteins, and other functional molecules.

Results: The scleral mRNA expression pattern in the REC-2hr group was similar to the REC-0 group. The myopia in the REC-1d group changed little, but the mRNA expression pattern did differ somewhat from the REC-2hr expression pattern. The REC-4d group recovered refractively by 2.6 ± 0.4 D (mean ± SEM) and displayed a STOP gene expression signature, of mostly up-regulated mRNA expression in the recovering eyes. The PLW-2hr group showed no significant differential gene expression. The PLW-1d group showed a small refractive change (0.1 ± 0.2 D). and the PLW-4d group became slightly

hyperopic (0.67 ± 0.3 D), confirming that the plus-lens-induced myopia was refractively ignored. In the PLW-1d group there were two differentially expressed genes (NPR3, up-regulated, IGF1, down-regulated). In the PLW-4d group there was a pattern of general differential up-regulation (22 up-regulated, 1 down-regulated) in the treated eyes. Ten genes were up-regulated in both REC-4d and PLW-4d but 10 other genes were differentially expressed in STOP but not in IGNORE, while 12 additional genes were differentially-expressed in IGNORE but not in STOP. The magnitude of the fold-differences was greater in the REC-4d group.

Conclusions: One day is not long enough for the emmetropization mechanism to produce significant refractive or gene expression changes in the sclera. After 4 days of treatment, REC (STOP) and PLW (IGNORE) produced scleral gene expression signatures that included some shared, up-regulated genes. The expression of other genes was altered in response to one treatment, but not the other. Thus, plus-lens wear produces responses in normal sclera. However, the sclera in the elongated myopic eye responds differently than does a normal sclera, apparently because the recovering sclera has remodeled during minus lens wear.

INTRODUCTION

Refractive error occurs when the location of the focal plane does not match the axial length of the eye. When distant objects are in focus on the retina without accommodation, the eye is emmetropic. If the axial length is shorter than the focal plane, the eye is hyperopic. If the axial length is longer than the focal plane, images are in focus in front of the retina and the eye is myopic. Myopia is the most prevalent type of refractive error

worldwide, affecting 25 – 40% of the population in the US and in European countries (Attebo et al., 1999; Fledelius, 1988; Sperduto et al., 1983; Wang et al., 1994; Wensor et al., 1999). In Asia, the prevalence is higher: 85% – 96.5% (Goh and Lam, 1994; He et al., 2004; Jung et al., 2012; Lin et al., 1999; Lu et al., 2009; Quek et al., 2004). Myopia is a risk factor for blinding conditions, including retinal detachment, glaucoma, choroidal degeneration, and cataract (Avila et al., 1984; Burton, 1989; Lim et al., 1999; Saw et al., 2005). Thus, it is important to understand the mechanisms that underlie the development of myopia.

A visually-guided emmetropization mechanism has been found to operate in children and in animal models (fish, chicks, monkeys, guinea pigs, tree shrews, and other species). It uses refractive error cues to modulate the elongation of the eye so that the location of the retina comes to be located at the focal plane (Mutti et al., 2005; Norton, 1999; Norton et al., 2010; Schaeffel et al., 1988; Schaeffel and Howland, 1988; Shen and Sivak, 2007; Smith, III et al., 1999b; Troilo et al., 2000; Wallman and Winawer, 2004). The retina senses the refractive error and produces a signal that then passes in a signaling cascade through the retinal pigment epithelium (RPE) and choroid to alter gene expression in fibroblasts in the sclera. This produces remodeling of the scleral extracellular matrix (ECM) that alters the axial elongation rate of the developing juvenile eye. This mechanism is local and direct, as has been demonstrated in animal studies by severing or blocking the retinal output to the brain (McBrien et al., 1995; Norton et al., 1994; Troilo et al., 1987; Wildsoet and McFadden, 2010; Wildsoet and Wallman, 1995).

The emmetropization mechanism can be manipulated to increase or decrease the axial elongation rate of the growing eye. Placing a minus lens held in a goggle frame in

front of an emmetropic eye moves the focal plane away from the cornea, producing refractive hyperopia while the lens is worn. This produces retinal responses that have been described as a GO signal (Rohrer and Stell, 1994). Through the signaling cascade, this produces an increase in the axial elongation rate, moving the retina toward the shifted focal plane. As the eye elongates, the refractive error is reduced. When the retina has reached the shifted focal plane, its refraction matches that of untreated eyes while the lens is worn. The eye has compensated for the minus lens.

After compensation, if minus-lens wear is discontinued, the eye is optically myopic because the retina in the elongated eye is now behind the focal plane. Refractive and axial recovery occurs. The retina detects the myopic refractive state and produces STOP signals that, through the signaling cascade, produce scleral remodeling that slows the axial elongation rate. Over time, the focal plane moves away from the cornea because the cornea flattens and lens-power decreases; the refractive myopia decreases until it once again matches that of untreated normal eyes (McBrien et al., 1999; Moring et al., 2007; Norton, 1990; Norton et al., 2010; Shaikh et al., 1999; Siegwart, Jr. and Norton, 1998; Siegwart, Jr. and Norton, 1999; Siegwart, Jr. and Norton, 2005).

Scleral remodeling is the mechanism that underlies both the increase and decrease in the axial elongation rate of the eye. During the development of induced myopia, there is a general loss of scleral ECM that includes a reduction in the amount of type I collagen and other ECM proteins (Frost and Norton, 2012) and increases in matrix metalloproteinase levels (Guggenheim and McBrien, 1995). The remodeling increases the viscoelasticity of the sclera (measured by creep rate) (Phillips et al., 2000; Siegwart, Jr. and Norton, 1999) and appears to allow the globe to expand under normal intraocular pressure. In a

previous study that examined mRNA levels for 55 candidate genes during the development of lens-induced myopia, we found a gene expression "scleral GO signature" that characterizes the expression pattern as the scleral fibroblasts respond to the incoming signals.

During recovery, scleral remodeling occurs that is not a mirror image of the pattern during myopia development. At both the mRNA and protein levels, the expression levels of many genes that were decreased during myopia development returned to normal, or slightly above normal (Frost and Norton, 2012). We previously characterized a "scleral STOP signature" of mRNA expression for the same 55 genes examined during GO (Guo et al., 2014).

A myopic shift in the eye's refractive state can also be produced by placing a plus-power (convex) lens in front of an emmetropic eye. Light rays from distant objects then focus in front of the retina, making the eye myopic. Plus-lens wear thus produces a similar refractive situation to that experienced by eyes that are recovering from lens-induced myopia: both experience refractive myopia. An important difference is that the recovering eye is elongated but the plus-lens wearing eye is not. In infantile tree shrews, plus-lens wear slows the axial elongation rate until the eyes become emmetropic while wearing the lens. With the lens removed, the eyes are hyperopic. In older, juvenile tree shrews, plus-lens wear has little effect on the axial elongation rate or on refraction (Siegwart, Jr. and Norton, 2010). In most cases, the eyes appear to ignore the myopic refractive error and remain myopic while wearing the lens.

The different responses to similar refractive conditions – a STOP response during recovery and an IGNORE response during plus-lens wear – led to the hypothesis that the

gene expression pattern in the sclera must differ in these two conditions. Possibly, a normal sclera might not respond at all to the incoming STOP signals. Therefore, the purpose of this study was to examine gene expression in the sclera in juvenile tree shrews that experienced myopia produced by plus-lens wear and to compare it to the STOP pattern found during recovery.

METHODS

Experimental Groups

All of the juvenile tree shrews (*Tupaia glis belangeri*) used in this study were produced in our breeding colony and raised by their mothers under a 14 hr light/10 hr dark cycle. Tree shrew pups are born with their eyes closed and open them about three weeks after birth, which we describe as 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, avoided pups from the same parents wherever possible, and picked the treated eye randomly.

Recovery Groups

Four groups of animals (n=7 per group) were used to examine the effect of recovery from lens- induced myopia on mRNA expression patterns. As shown in Figure 1, all animals in the recovery groups received 11 days of –5 D lens treatment starting at 24 DVE and showed full compensation for the lens, becoming approximately 5 D myopic

when the lens was removed. The untreated fellow eyes served as a within-animal control. Recovery began by discontinuing lens wear at 35 DVE. The REC-0 group experienced no recovery. The REC-2hr group recovered for 2 hours, the REC-1d group recovered for one day and the REC-4d group recovered for 4 days. A group with no recovery (REC-0) was needed because, even though full compensation occurred, it has been found in previous studies (Gao et al., 2011; Guo et al., 2014) that the scleral mRNA levels were not completely normal at this time-point; the REC-0 group served as a baseline for the other recovery groups and also provided mRNA for a previous study (Gao et al., 2011). mRNA results from two of the REC groups, (REC-0 and REC-4d) were reported previously (Guo et al., 2014) as the ML-11 and REC-4 groups. The shorter recovery groups (REC-2h and REC-1d) were added to provide earlier time-points on the development of the scleral STOP signature.

Plus Lens Groups

An additional four groups of animals (n=7 per group) were used to examine the effect of plus-lens wear on the refractive state of the eyes and on mRNA expression (Figure 1). Starting at 35 DVE, the three plus-lens wear groups, which had normal visual experience until this point, began to wear a monocular +5 D lens. The PLW-2hr group wore the lens for 2 hours, the PLW-1d group wore it for one day, and the PLW-4d group wore the lens for four days. These animals experienced approximately the same amount of refractive myopia as was initially experienced by the animals in the recovery groups and for similar periods of time. The untreated fellow eye served as a within-animal control. A normal group of animals (n=7) at 38 DVE, in between the shortest (PLW-2hr) and long-

est (PLW-4d) groups, provided baseline data and is described as the plus-lens wear for 0 hours (PLW-0) group. Data from this group was reported previously (Guo et al., 2014) as the 38N group.

Goggle Installation

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 following procedures described by Siegwart and Norton (Siegwart and Norton, 1994). In all eight groups, the pedestal was installed at 21 ± 1 DVE. 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 four 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 –5 D lens in front of the randomly selected treated eye. The PLW groups also received a pedestal at 21 DVE but did not begin to wear a goggle with a monocular +5 D lens until 35 DVE. The normal group (PLW-0) received a pedestal at 21 DVE but did not wear a goggle. In all lens-wear groups, the 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.

Refractive and Axial Measures

Non-cycloplegic refractive measures were made, in awake animals, with a Nidek ARK-700A infrared autorefractor (Marco Ophthalmic, Jacksonville, FL) (Norton et al., 2003). Recovery animals were measured without the lens to show the amount of myopia that existed in the treated eyes relative to their control eyes. The REC-0 group was measured after 11 days of –5 D lens wear, with no recovery period. The REC-2hr group was also measured at the end of 11 days of –5 D lens wear and was not measured a second time after two hours of recovery because no significant refractive change was expected. The REC-1d and REC-2d groups were measured at the end of lens wear and again at the end of their recovery period.

The PLW-0 (normal group) was measured just before euthanasia. The refractive measures in the treated and control eyes of the other PLW groups were made while the animals were wearing the +5 D lens. This provided a measure of the amount of refractive myopia experienced by the treated eyes relative to their fellow control eyes. The PLW-2hr group was measured only at the start of lens wear. The PLW-1d and PLW-4d groups were measured at the start, and again at the end of the plus-lens wear period.

Cycloplegic refractive measures were omitted to prevent any interference by atropine on retino-scleral signaling (McKanna and 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. When compared, cycloplegic refractions are approximately 0.8 D hyperopic compared with non-cycloplegic refractions in myopic, control, and normal eyes (Norton et al., 2003; Norton et al., 2006). Further, treated-eye vs. control-eye differences are essentially identical between non-cycloplegic and cycloplegic

measures (Norton et al., 2006). All refractive values were corrected for the small eye artifact (Glickstein and 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, as described by Norton and McBrien (1992), to ensure that the treated, control, and normal 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 anesthesia required for the A-scan procedure might alter gene expression.

Gene Expression Analysis

On completion of the final refractive measures, animals were terminally anesthetized and the scleral tissue collected in RNAlater according to published procedures (Guo et al., 2013) before freezing the tissue in liquid nitrogen. Frozen sclera was pulverized to a fine powder in a chilled Teflon freezer mill (Sartorius Stedim, Bohemia, NY) from which total RNA was isolated using a RiboPure kit (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, with the addition of an on-filter DNase treatment. The purified RNA was quantified (NanoDrop Technologies, Wilmington, DE) and the quality 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 55 genes of interest (Table 1 and Table S1) 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. Primer sequences are listed in Supplementary Table S1. The selected candidate genes included representatives of three major groupings: signaling, MPs & TIMPs, and extracellular matrix (ECM) proteins. They were the same 55 genes studied previously to characterize the scleral GO and STOP signatures (Guo et al., 2014). 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 and 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 and treated 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

At the start of recovery, the treated eyes in all REC groups had fully compensated to the minus lens. Measured with the lens in place, the refractive difference between the treated eyes and the control eyes across groups was 0.1 ± 0.2 D (mean ± SEM). Thus, the refractive hyperopia present at the start of lens wear had dissipated. With the –5 D lens removed, the treated eyes were all myopic compared with the fellow untreated control eyes (Figure 2). The relative myopia in the REC-0 treated eyes was –5.1 ± 0.2 D and the REC-2hr treated eyes were –4.6 ± 0.3 D myopic. The REC-1d treated eyes were –4.4 ± 0.3 D myopic, and the REC-4d treated eyes were –5.2 ± 0.5 D myopic. A very small refractive recovery occurred after one day in the REC-1d group. The myopia was reduced

by 0.1 ± 0.1 D. Substantial refractive recovery occurred after four days in the REC-4d group; the treated eye vs. control eye myopia decreased by 2.6 ± 0.4 D.

The PLW-0 (38 DVE normal) group did not experience plus-lens wear and the difference between right eyes and left eyes was negligible (–0.02 ± 0.2 D, right eyes – left eyes). Plus-lens wear produced a refractive myopia in these groups that was comparable to the myopia experienced by the treated eyes in the recovery groups. Measured with the +5 D lens in place, the PLW-2hr group had a relative myopia of –5.1 ± 0.3 D. In the PLW-1d group, the plus lens initially produced a myopia of –4.7 ± 0.4 D. After one day, the myopia was slightly less –4.6 ± 0.3 D, a hyperopic shift of 0.1 ± 0.2 D. In the PLW-4d group, the +5 D lens initially produced a myopia of –4.2 ± 0.5 D. After 4 days, the myopia decreased slightly to –3.9 ± 0.4 D, a hyperopic shift of 0.3 ± 0.4 D. Thus, as reported previously (Siegwart, Jr. and Norton, 2010), plus-lens wear in juvenile tree shrews had little refractive effect. Any STOP signal produced in the retina by the refractive myopia was essentially ignored by the plus-lens wearing eyes.

Differential Gene Expression

Recovery Groups

Figure 3 shows the gene expression differences between the treated eyes and control eyes in all recovery groups. The expression fold-differences are listed in Table 2. The data from the REC-0 and REC-4d groups were reported previously (Guo et al., 2014) and are shown here for comparison with the PLW groups. The overall pattern at REC-0 and REC-2hr was very similar. mRNA levels generally were lower in the recovering eyes than in the control eyes. The primary difference was that more of the mRNA differences

in the REC-2hr group were statistically significant. The similarity of the overall patterns is shown in Figure 4A which plots the correlation between the REC-0 pattern (Figure 3A) and the REC-2hr pattern (Figure 3B). Both appear to represent responses of the scleral fibroblasts at the end of minus-lens wear when the eye has elongated but the refractive hyperopia that produced the elongation has dissipated. This has been described as a scleral STAY response (Guo et al., 2014).

After one day of recovery, the gene expression pattern (Figure 3B) still resembled the pattern at the start of recovery; most mRNA levels were still lower in the recovering eye sclera. However, as seen in Figure 4B the very similar pattern seen at REC-0 and REC-2hr had begun to change. Fewer genes were significantly down-regulated and the size of the fold-differences was lower. By four days of recovery (Figure 4C), a STOP gene expression pattern, very different from that at the start of recovery, was well established (Guo et al., 2014).

Plus Lens Groups

The mRNA levels in the right and left eyes of the PLW-0 group (normal animals) are compared in Figure 5A. None of the candidate genes differed significantly between the two eyes. However, in the majority of genes the mRNA levels in the left eyes were slightly higher than in the right eyes, so that there were more negative fold differences than positive ones.

Figure 5 also shows the expression differences between the treated eyes and control eyes in the PLW-2hr, PLW-1d, and PLW-4d groups. The expression values are listed in Table 2. After 2 hours of plus-lens wear, the mRNA expression levels in the treated-

eye scleral fibroblasts did not differ significantly from those of the control eyes for any of the measured genes (Figure 5B). The similarity of the overall pattern to that of the PLW-0 group is shown in Figure 6A which plots the correlation between the PLW-0 pattern (Figure 5A) and the PLW-2hr pattern (Figure 5B). Most of the fold differences were clustered around 1.0, indicating no significant differential expression. After 1 day, the overall pattern showed similarity with PLW-0 and PLW-2hr groups, with NPR3 and IGF1 showing significant changes (Figure 4C). After four days of plus-lens wear, the gene expression pattern (Figure 5B) still showed little alteration from
the pattern after 2 hours of PLW; two genes showed significant differential expression, one (NPR3) was up-regulated and the other (IGF1) was down-regulated. However, as seen in Figure 6B, the pattern at PLW-1d had begun to change; there were more genes that were non-significantly up-regulated. By four days of PLW (Figure 6D), a gene expression pattern, very different from that at the start of PLW, was well established. Twenty-three genes were differentially expressed; all but one (ADAMTS5) were up-regulated.

Comparison of STOP and IGNORE Signatures

The overall pattern of differential mRNA expression in both the REC-4 and the PLW-4 groups was for up-regulation. Similar numbers of genes were significantly up-regulated in REC-4 (17) and in PLW-4 (22). However, the patterns in these two conditions differed in several ways. Although there were ten genes up-regulated in both, there were an additional seven genes significantly regulated in REC-4 that were not up-regulated in PLW-4. Twelve genes that were significantly up-regulated in PLW-4 were not significantly affected in the REC-4 group. Figure 7 compared the two patterns. In ad-

dition, the magnitude of the significant fold differences was larger in the REC-4 group than in the PLW-4 group as indicated by the low slope (0.33) of the regression line.

DISCUSION

As expected from previous studies, the myopia in the recovery groups was substantially reduced over time but the plus lens induced myopia produced little refractive change (Amedo and Norton, 2012; Moring et al., 2007; Siegwart, Jr. and Norton, 1998; Siegwart, Jr. and Norton, 2010). A primary aim of this study was to learn if scleral fibroblasts in normal eyes respond with altered mRNA expression to the myopia produced by plus-lens wear and, if so, whether the differential mRNA expression pattern is different from that which occurs in the sclera of elongated eyes exposed to a similar refractive myopia. As seen in the PLW-4d group, the emmetropization signaling cascade clearly causes the scleral fibroblasts to respond; some aspect of the retinal STOP signal reaches the sclera and produces altered mRNA levels. Because this response pattern is not the same as the scleral STOP response signature, we suggest that the PLW expression differences are part of a scleral fibroblast IGNORE signature. Comparing the mRNA expression in the REC-2hr and the PLW-2hr, and the REC-1d and the PLW-1d groups, it appears that there is a similar time-lag for the scleral fibroblast response patterns to develop. Two hours is too short a time for retinally-generated signals to pass through the emmetropization cascade and produce altered mRNA expression in the sclera. After 1 day, there are small changes from the 2 hour groups, but the four-day pattern has not yet emerged.

Signal or Response

Tree shrews at the juvenile stage used in this study are less susceptible to plus lens produced myopia than are younger, infantile tree shrews. In a previous study, most tree shrews that started to wear +4 D lenses from 11 DVE fully compensated to the lens (Siegwart, Jr. and Norton, 2010). This difference in response between younger and older animals suggests that the ability to use myopic refractive error to decrease the eye elongation rate is age-related (Metlapally and McBrien, 2008; Norton et al., 2010; Siegwart, Jr. and Norton, 2010). Similar early responses to imposed myopia have also been observed in other species: infant rhesus monkeys compensated to binocular plus lenses (Smith, III and Hung, 1999). Late infant-aged marmoset monkeys responded refractively to soft contact lenses with plus power (Troilo et al., 2009), and infant guinea pigs also showed reduced eye elongation to plus-lens wear (McFadden and Wildsoet, 2009). To our knowledge, the response of older animals of these species to plus-lens wear has not been explored.

Age factors affect not only plus-lens wear responses, but also minus lens wear and recovery, but to a far lesser degree. For negative-lens compensation, older tree shrews (24 DVE compared to 11 DVE) compensated more slowly to the lens (Norton et al., 2010) as did form-deprived animals (Siegwart, Jr. and Norton, 1998). Similarly, the decreased susceptibility to GO signals has also been proved on older chicks and monkeys (Smith, III et al., 1999a; Wallman and Adams, 1987). Note, however, that young adult animals remain susceptible to minus-lens wear (Norton et al., 2010). This is also true for recovery. Although juvenile and young adult tree shrews can use myopic visual cues to slow the axial elongation rate in order to recover refractively, compared with infantile animals,

their recovery rates were slower and showed greater variability (Norton et al., 2010). Similar results of reduced rates of recovery were also observed on chicks (Wallman and Adams, 1987; Wildsoet and Schmid, 2000), marmosets (Qiao-Grider et al., 2004), and macaque monkeys (Smith, III and Hung, 1999). These results reinforce the idea that age is a factor that affects the response of emmetropization mechanism. However, because the animals in the REC and the PLW groups were age-matched, age alone cannot explain why the eyes did not refractively respond to the STOP signals in the PLW groups.

An important distinction between the sclera of the REC groups and the PLW groups is that the sclera in the PLW groups was normal at the onset of exposure to myopia whereas the sclera in the REC groups had remodeled as a result of minus-lens wear and the treated eyes had become elongated. Thus, the retinally generated signals that arrived in the sclera from the choroid reached fibroblasts that were in a differing state. The lack of refractive response does not seem to stem from an inability of the scleral fibroblasts to respond; rather, it would appear that the (presumably similar) signals produce different response signatures primarily because the sclera in the PLW groups is normal whereas the sclera in the recovery groups has been remodeled (Moring et al., 2007; Siegwart, Jr. and Norton, 1999). This suggestion is consistent with previous finding that "eye-size" of "eye-shape" factors exist and have effects on emmetropization process (Nickla et al., 2005; Schaeffel and Howland, 1991; Troilo and Wallman, 1991). Potentially, elongated eyes may be more likely to respond to STOP signals even in an older age.

Summary

This study examined differential mRNA expression by cells in mammalian sclera during refractive recovery from minus-lens wear for 2 hours, 1 day and 4 days, STOP conditions, and in plus-lens wear for 2 hours, 1 day and 4 days, IGNORE conditions. We found that cells in the sclera responded with different mRNA expression signatures to the similar refractive stimuli. In both groups, small mRNA expression changes were detectable after 1 day, but it was after 4 days that STOP and IGNORE gene expression signatures became evident. We can deduce from these results that the lack of refractive responses in sclera during plus-lens wear is not caused by a lack of responses of sclera fibroblasts, but is more likely derived from the condition of the scleral fibroblasts, an eye size factor. Because a limited number of genes were examined, these signatures are incomplete; however, the large number of genes that are altered suggests that the emmetropization-related signals in sclera are complex and unlikely to depend on the regulation of a single gene, or even a small number of genes.

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FIGURE LEGENDS

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

Figure 2. Refractive difference between treated and control eyes for each group (mean ± SEM). For the REC groups, the green bars show the amount of myopia at the end of recovery. For the REC-1d and REC-4d groups, the amount of myopia at the start of recovery is shown by the gray bars and the orange bars show the amount of refractive recovery. For the PLW groups, the purple bars show the plus-lens myopia present at the end of PLW. For the PLW-1d and PLW-4d groups, the amount of myopia at the start of PLW is shown by the open bars. The yellow bars adjacent to the PLW-1d and PLW-4d groups show the refractive change (reduction in myopia while wearing the +5 D lens) between the start and end of PLW.

Figure 3. Gene expression fold differences (treated eyes vs. control eyes). (A) REC-0, (B) Recovery for 2 hours, (C) Recovery for 1 day, (D) Recovery for 4 days. 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 4. Change in gene expression patterns over time during recovery from minus-lens-induced myopia. (A) compares the pattern at the start of recovery (Figure 3A) with that observed after 2 hours of recovery (Figure 3B); (B) compares the differential expres-

sion after 2 hours of recovery with that after one day (Figure 3C); (C) compares the pattern after 2 hours with the pattern after 4 days (Figure 3D).

Figure 5. Gene expression fold differences: (A) 38 DVE (PLW-0; right eyes vs. left eyes). Treated eye vs. control eye differ-ences: (B) Plus-lens wear for 2 hours at 35 DVE. (C) Plus-lens wear for one day from 35 DVE. (D) Plus-lens wear for 4 days from 35 DVE. 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 6. Change in gene expression patterns over time during plus-lens wear. (A) compares the pattern before plus-lens wear (Figure 5A) with that observed after 2 hours of plus-lens wear (Figure 5B). (B) compares the change in the differential expression between the PLW-2hr group (Figure 5B) and the PLW-1d group (Figure 5C). (C) compares the change from the PLW-2hr group (Figure 5C) to the PLW-4d group (Figure 5D).

Figure 7. Comparison of the treated vs. control gene expression differences in REC and PLW: REC-4d (Figure 3D) vs. PLW-4d (Figure 4D). Stars = significant fold differences for both treatments; triangles = significant fold differences only for REC group; squares = significant fold differences only for PLW group; circles = fold differences not significant for either treatment.

Table 1. Genes examined, divided into functional categories, with cellular location of the protein encoded by the gene

Gene symbol	Protein name	Location
Signaling − Cell surface receptors		
ACVRL1	Activin A receptor 2-like 1	Cell surface
FGFR2	FGF receptor 2	Cell surface
NPR3	Atrial natriuretic peptide receptor 3	Cell surface
SDC2	Syndecan 2	Cell surface
TGFBR3	TGFβ receptor III	Cell surface
TRPV4	Transient receptor potential cation channel V4	Cell surface
UNC5B	Netrin receptor UNC5B	Cell surface
EFNA1	Ephrin A1	Cell surface
Signaling − Cytoskeleton related		
ANXA1	Annexin A1	Cell surface
ANXA2	Annexin A2	Cell surface
CAPN2	Calpain 2	Cell surface
CAPNS1	Calpain small subunit 1	Cell surface
GJA1	Connexin 43	Cell surface
ACTA2	Smooth muscle actin	Intracellular
NGEF	Neuronal guanine nucleotide exchange factor	Intracellular
Signaling − Transcription regulators		
HIF1A	Hypoxia-inducible factor 1α	Intracellular
RARB	Retinoic acid receptor β	Intracellular
RXRB	Retinoid X receptor β	Intracellular
VDR	Vitamin D receptor	Intracellular
Signaling − Secreted		
ANGPTL7	Angiopoietin-related protein 7	Extracellular
IGF1	Insulin-like growth factor 1	Extracellular
IGF2	Insulin-like growth factor 2	Extracellular
IL18	Interleukin 18	Extracellular
PENK	Proenkephalin A	Extracellular
TGFB1	Transforming growth factor β1	Extracellular
TGFB2	Transforming growth factor β2	Extracellular
TGFBI	TGFβ-induced protein	Extracellular
Signaling − Matricellular		
CTGF	Connective tissue growth factor	Extracellular
CYR61	Protein CYR61	Extracellular
FBLN1	Fibulin 1	Extracellular
NOV	Nephroblastoma overexpressed gene	Extracellular
SPARC	Secreted protein acidic and rich in cysteine	Extracellular
SPP1	Osteopontin	Extracellular
THBS1	Thrombospondin 1	Extracellular
THBS2	Thrombospondin 2	Extracellular
TNC	Tenascin C	Extracellular
WISP1	WNT1 inducible signaling pathway protein 1	Extracellular
MPs / TIMPs		
ADAMTS5	ADAM metallopeptidase with thrombospondin motif, 5	Extracellular
MMP2	Matrix metallopeptidase 2	Extracellular
MMP14	Matrix metallopeptidase 14	Cell surface
TIMP1	TIMP metallopeptidase inhibitor 1	Extracellular
TIMP2	TIMP metallopeptidase inhibitor 2	Extracellular
TIMP3	TIMP metallopeptidase inhibitor 3	Extracellular
Extracellular matrix − Collagens		
COL1A1	Collagen type I, α1	Extracellular
COL12A1	Collagen type XII, α1	Extracellular
COL14A1	Collagen type XIV, α1	Extracellular
Extracellular matrix − Proteoglycans		
ACAN	Aggrecan	Extracellular
DCN	Decorin	Extracellular
FMOD	Fibromodulin	Extracellular
KERA	Keratocan	Extracellular
NYX	Nyctalopin	Extracellular
OGN	Mimecan	Extracellular
PRELP	Prolargin	Extracellular
Extracellular matrix − Other		
HS6ST1	Heparan-sulfate 6-O-sulfotransferase 1	Cell surface
SERPINH1	Serpin H1	Intracellular

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

	STAY	STOP			38 Normal	IGNORE		
	REC-0	*REC-2hr*	*REC-1d*	*REC-4d*	*PLW-0*	*PLW-2hr*	*PLW-1d*	*PLW-4d*
Signaling − Cell surface receptors								
ACVRL1	-1.27	**-1.52**	-1.23	**1.83**	-1.50	*-1.06*	*1.12*	**1.60**
FGFR2	-1.19	-1.42	-1.84	1.07	-1.02	-1.10	1.04	**1.40**
NPR3	-1.80	**-2.29**	1.60	**4.50**	-1.28	1.10	**1.75**	**2.22**
SDC2	-1.05	-1.19	-1.63	1.20	1.01	-1.04	1.01	1.13
TGFBR3	-1.08	1.07	**-2.68**	**-1.79**	-1.01	1.01	-1.24	1.04
TRPV4	-1.13	**-1.19**	1.09	**1.37**	-1.11	-1.00	1.17	1.12
UNC5B	-1.32	**-1.48**	1.02	**1.51**	-1.01	1.02	1.20	1.27
EFNA1	-1.09	-1.04	-1.35	1.03	-1.05	-1.11	-1.05	-1.03
Signaling − Cytoskeleton related								
ANXA1	-1.11	-1.19	-1.34	1.15	-1.07	-1.04	-1.00	**1.27**
ANXA2	-1.32	**-1.41**	-1.34	**1.20**	-1.12	-1.03	1.04	**1.31**
CAPN2	-1.11	-1.15	-1.54	1.16	-1.03	-1.02	-1.05	1.15
CAPNS1	-1.02	-1.07	-1.01	**1.18**	-1.08	1.02	1.06	**1.16**
GJA1	-1.27	-1.18	**-2.16**	-1.23	-1.08	-1.03	-1.08	1.23
ACTA2	1.21	1.11	1.07	1.02	-1.15	-1.10	1.36	-1.13
NGEF	-1.80	**-2.07**	1.13	**1.91**	-1.57	1.01	1.32	**1.81**
Signaling − Transcription regulators								
HIF1A	-1.07	-1.26	-1.61	1.05	-1.07	-1.26	-1.09	1.24
RARB	-1.16	**-1.32**	-1.63	1.06	-1.03	-1.09	-1.02	1.22
RXRB	-1.39	-1.05	-1.28	-1.09	1.08	1.03	1.03	1.08
VDR	-1.51	-1.37	-1.33	1.09	1.12	1.03	1.13	1.12
Signaling − Secreted								
ANGPTL7	-1.05	-1.13	-1.94	-1.33	-1.01	-1.05	-1.14	1.17
IGF1	1.01	-1.03	-1.18	**-1.29**	1.02	-1.05	**-1.17**	-1.22
IGF2	1.04	-1.01	-1.03	-1.01	-1.07	-1.07	-1.02	-1.02
IL18	-1.50	**-1.78**	1.01	**2.03**	-1.13	1.00	1.31	**1.68**
PENK	**-3.24**	**-5.20**	**-3.47**	-1.01	-1.82	-1.22	1.05	**1.57**
TGFB1	**-1.16**	**-1.17**	-1.15	**1.23**	-1.08	1.04	-1.05	1.18
TGFB2	-1.25	**-1.71**	-1.44	1.25	-1.07	-1.03	1.06	**1.49**
TGFBI	1.06	1.06	-1.19	-1.30	1.18	-1.21	-1.17	1.21
Signaling − Matricellular								
CTGF	-1.38	-1.60	1.15	**2.45**	-1.08	1.08	1.30	**1.73**
CYR61	-1.55	-1.71	1.00	1.73	-1.15	1.06	1.26	**1.58**
FBLN1	1.32	**1.64**	-1.17	1.16	1.10	1.11	-1.10	1.05
NOV	1.01	-1.19	-1.12	**1.48**	-1.08	1.07	1.12	1.32
SPARC	-1.23	-1.39	-1.49	1.19	-1.06	-1.01	1.07	**1.36**
SPP1	1.01	**-1.45**	-1.19	1.27	-1.19	1.03	1.10	1.16
THBS1	-2.23	**-2.17**	-2.43	1.40	-1.23	1.06	1.08	1.58
THBS2	-1.64	-1.10	-1.11	1.28	1.08	1.09	1.23	1.5
TNC	-1.31	**-1.59**	-1.52	1.16	-1.23	-1.08	-1.09	1.31
WISP1	-1.04	1.00	-1.18	**1.40**	1.06	1.00	-1.06	1.14
MPs / TIMPs								
ADAMTS5	-1.03	**-1.18**	**-1.60**	-1.21	-1.04	-1.02	-1.14	**-1.19**
MMP2	-1.08	1.00	-1.82	-1.22	-1.01	-1.01	1.03	1.33
MMP14	1.14	**1.53**	-1.01	1.05	1.18	1.10	1.00	**1.28**
TIMP1	-1.04	**-1.23**	1.07	**1.23**	-1.12	-1.02	1.01	1.18
TIMP2	-1.54	-1.20	-1.48	-1.07	1.03	1.04	-1.03	**1.24**
TIMP3	-1.59	**-1.84**	-1.46	**1.75**	-1.13	1.03	1.14	**1.56**
Extracellular matrix − Collagens								
COL1A1	-1.34	-1.4	-1.25	**1.48**	1.04	1.03	1.03	1.22
COL12A1	-1.71	-1.87	-1.89	1.58	-1.06	1.07	1.12	**1.61**
COL14A1	1.02	-1.16	-1.61	1.28	-1.02	-1.00	-1.05	1.15
Extracellular matrix − Proteoglycans								
ACAN	**-1.42**	**-1.69**	-1.44	**1.70**	-1.13	1.09	1.16	**1.62**
DCN	-1.17	-1.36	-1.76	-1.09	-1.10	-1.05	-1.00	1.27
FMOD	-1.47	-1.44	-1.48	1.06	-1.05	1.02	1.05	**1.36**
KERA	-1.03	-1.24	-2.01	1.20	-1.17	-1.07	1.10	1.45
NYX	-1.32	-1.22	**-1.98**	**-1.29**	-1.04	-1.08	1.05	1.04
OGN	-1.53	**-1.91**	-2.03	1.39	-1.18	-1.03	1.07	**1.61**
PRELP	-1.37	-1.24	-1.37	1.02	-1.09	1.05	1.03	1.12
Extracellular matrix − Other								
HS6ST1	-1.13	-1.06	1.13	**2.36**	-1.40	1.06	1.23	**1.45**
SERPINH1	-1.23	-1.26	-1.34	1.38	-1.05	1.06	1.10	**1.41**

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

Gene	*Forward sequence*	*Reverse sequence*	*Amplicon (bp)*	*Efficiency (%)*
ACAN	CTGTGACATAGACAAGGAGAT	GACGATGCTGCTCAGG	142	92
ACTA2	CGTGATCTCACCGACTACCT	GCGGAAGCGTTCGTTCCCG	222	98
ACVRL1	CGTACTGGTCAAGAGCAA	ATGTCAGTCCACTTGTAGG	189	94
ADAMTS5	TCTTCCATCCTAACCAGCATTG	GGTGGCATCATAAGTCTGTCC	165	98
ANGPTL7	GCTGTCACTTGGCTCTG	AGTCCTTCTCCTGCTTCTTG	193	94
ANXA1	ACAAAGTTCTGGACCTGGAGTTGA	AGAGCGGGAAACCATAATCCTGAT	167	95
ANXA2	CTTCCGCAAGCTGATG	CATGATGCTGATCCACTT	154	99
CAPN2	GGCTGACCAGACGGCATGAAG	CTCCATCTTGGTGAGTTTCCATTTCTTG	146	88
CAPNS1	TGACCGATCAGGGACCATCTG	GGATTTGTCCACTGCCATCTTTG	215	92
COL12A1	CAGGTGTCCCGATCAAAGAG	GCCTGGTGGAATGGTGG	95	97
COL14A1	TCACCTCCTACTCAACCACCAACT	GGCTCCAACAGTGCTATACAGAAAGT	165	94
COL1A1	GCCCTTCTGGAAATGC	GACCAACTTCACCAGGA	111	95
CTGF	CCAACTATGATTCGAGCCAACTG	TTCTCTTCCAGGTCTGCTTCA	173	96
CYR61	TGGAACCTCGCATTCTGTATAACC	GCCACAAGGTCGCACTTCAC	176	97
DCN	CTGCGTGCCCATGAGAACGAGATC	GGTATCAGCAATGCGGATGTACGAAAGC	168	96
EFNA1	CCACCATCAAGAAGACCAGT	CCAGGCAAGAGGGAAGAG	178	97
FBLN1	CGAGGAGGAGCAAGAG	AGCACGAGCAGACTAC	104	94
FGFR2	TGAAGGAAGGACACAGGATGGATAAG	GAGGCTGACTGAGGTCCAAGTATTC	180	93
FMOD	TCCTGCTGGACCTGAG	TTGTTGGTGAGGCTGTTAT	172	96
GJA1	CCTAGCCATTGTGGACCA	ATCTCCAGGTCATCAGGC	81	99
HIF1A	ACTGGTTGCATCTCCGTCTCCTACTCA	TCCTGCTCTGTTTGGTGAGGCTGTC	109	94
HS6ST1	CGTTCAACCTCAAGTTCATCC	GGTCCTTGGCGTAGTCAT	141	98
IGF1	GTCCTCCTCACATCTCTTCTACC	CAGCACCGCAGAGTGTCTC	89	99
IGF2	TGGCATTGTTGAAGAGTGTTG	GAAGCACGGTCGGAGAG	113	100
IL18	AACTCTTTCCTTTAAGAATATCAGTCGTCCT	GTTGGCTTCCACAGTAAACATTACAGATT	226	94
KERA	TCTCACAATCAGCTCACCAAGGTT	CCAGACGGAGGTAGCGAAGATG	175	101
MMP14	CCCTGGAACCTGGCTACCC	ATAGGTCTTTCCATTGGGCATCC	104	96
MMP2	TGACCTTGACCAGAACACCATCG	GAGCGAAGGCATCATCCACTGT	176	95
NGEF	ACCACTTCTCCGTGTAC	GACTAGGAGCTTGAGGC	194	96
NOV	CGAACAGACTACAGAGTGGAGTG	GTGATTTCTTGGTGCGGAGACA	191	97
NPR3	GGACTACGCCTTCTTCAACATCG	AGAGTGACTGTTTGGAGGGATGA	126	96
NYX	GTGAGCAGGTTCAGCAG	GACAGCGAGGTGTTAGC	101	96
OGN	CTCTCCTTCCTCTACCTGGACCACAAC	CTGAAGATGGATCACGCGCAGACTC	84	96
PENK	TCCTTGCCAAGCGATACG	TCTTGCTCATTTCTTCGTCGTT	162	98
PRELP	TTCGGCTGAACTACAACAAGC	GATCTGGGTCCCGTTGATTTTCT	182	99
RARB	CGGCTTGACCATCGCAGAC	GCTGGTTGGCAAAGGTGAACA	197	97
RXRB	CCTGACCTACTCGTGCC	TACCGCCTCCCTCTTCA	118	91
SDC2	TGATGACGACTACGCTTCTGC	CAGGCATCTTGTTCTGTGTCTTC	155	95
SERPINH1	ACAAGAACAAGGCAGAC	GCACCAGGAAGATGAAG	186	103
SPARC	GCGAGTTTGAGAAGGTGTGC	GCCCGATGTAGTCCAGGTG	126	102
SPP1	CCGACGACACCGACCATCC	GGCTTTGACCTCACTCTGTAAACC	190	92
TGFB1	ACCAGAAATACAGCAACAATTCC	AACCCGTTGATGTCCACTTG	205	91
TGFB2	GCAGAGTTTAGGGTCTTTCGTTTG	CTCGTGAACAGCATCAGTTACATC	189	93
TGFBI	CCTCGGCACTCATCTCTCC	GCAAATTCTTCATCTTGGCATCG	107	94
TGFBR3	CCCTGGTCTGGCGTCTGAAG	GTAACTGCTCCATACTCGTTTCGG	190	93
THBS1	CTGTCAGAACTCAGTCACCATC	CCACGGAGACCAGCCATC	136	91
THBS2	GAGACCGACTTCAGGAACTTC	CGAAACCCACTGCGATGC	142	100
TIMP1	CACTTGCACATCACCACCT	CAAGGGATGGATAAACAGGGAAA	134	99
TIMP2	AGATGGGCTGTGAATGC	CCGTCGCTTCTCTTGAT	151	96
TIMP3	CCGTGTCTATGATGGCAAGATG	ACAAAGCAAGGCAGGTAATAGC	153	99
TNC	AGACGCCAAGACTCGCTACAG	CAGGTTGACACGGTGACAGTTC	184	94
TRPV4	AGAGCAAGCACATCTGGAA	CCACGGTCACCATCTCC	111	94
UNC5B	CACCTGCTGGCTCCCTG	ATGGACAGCGGGATCTTGAA	94	96
VDR	AACTTGCACGAGGAGGAAC	CTTCTGGATCATCTTGGCATAG	189	93
WISP1	AGGCACCCATGAACTT	GACACTGGAAGGAGACA	135	96
POLR2A	CTACCAGCCCCAAGTATTC	GGTGAGTAAGTAGGAGACG	106	98

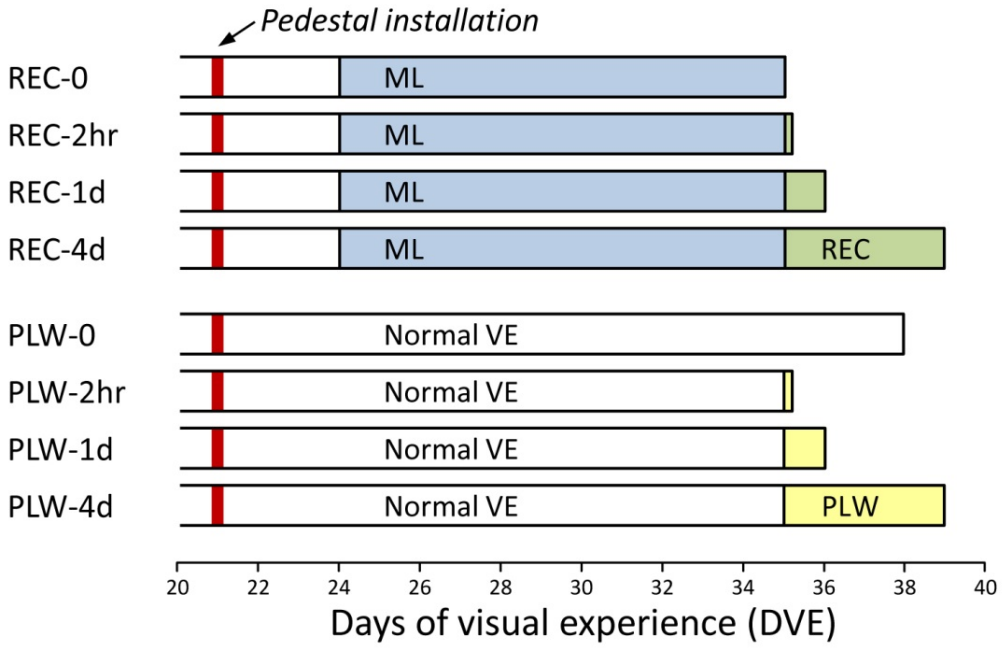

Figure 1. Experiment groups and duration of treatments. The red vertical bar indicates the point when a dental acrylic pedestal was attached to the skull under anesthesia. Filled regions indicate the type and duration of the visual treatment. The right end of each bar indicates the time point when mRNA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

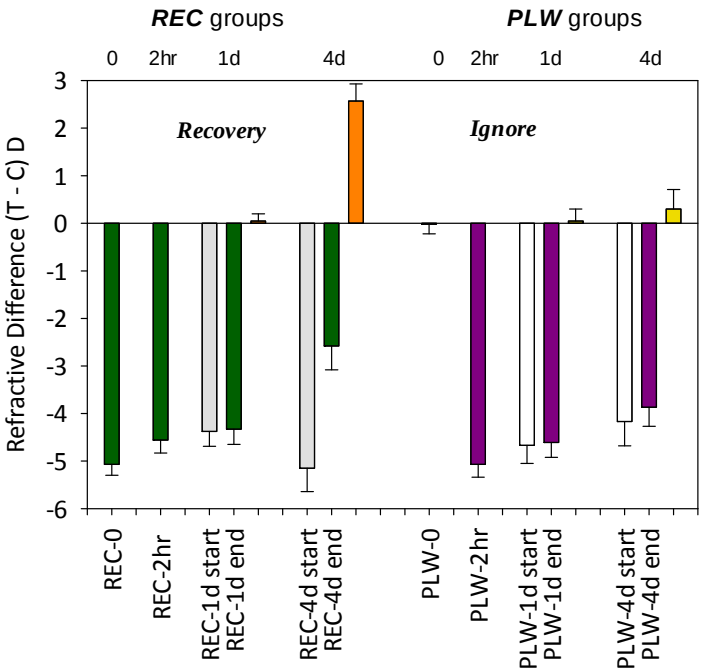

Figure 2. Refractive difference between treated and control eyes for each group (mean ± SEM). For the REC groups, the green bars show the amount of myopia at the end of recovery. For the REC-1d and REC-4d groups, the amount of myopia at the start of recovery is shown by the gray bars and the orange bars show the amount of refractive recovery. For the PLW groups, the purple bars show the plus-lens myopia present at the end of PLW. For the PLW-1d and PLW-4d groups, the amount of myopia at the start of PLW is shown by the open bars. The yellow bars adjacent to the PLW-1d and PLW-4d groups show the refractive change (reduction in myopia while wearing the +5 D lens) between the start and end of PLW.

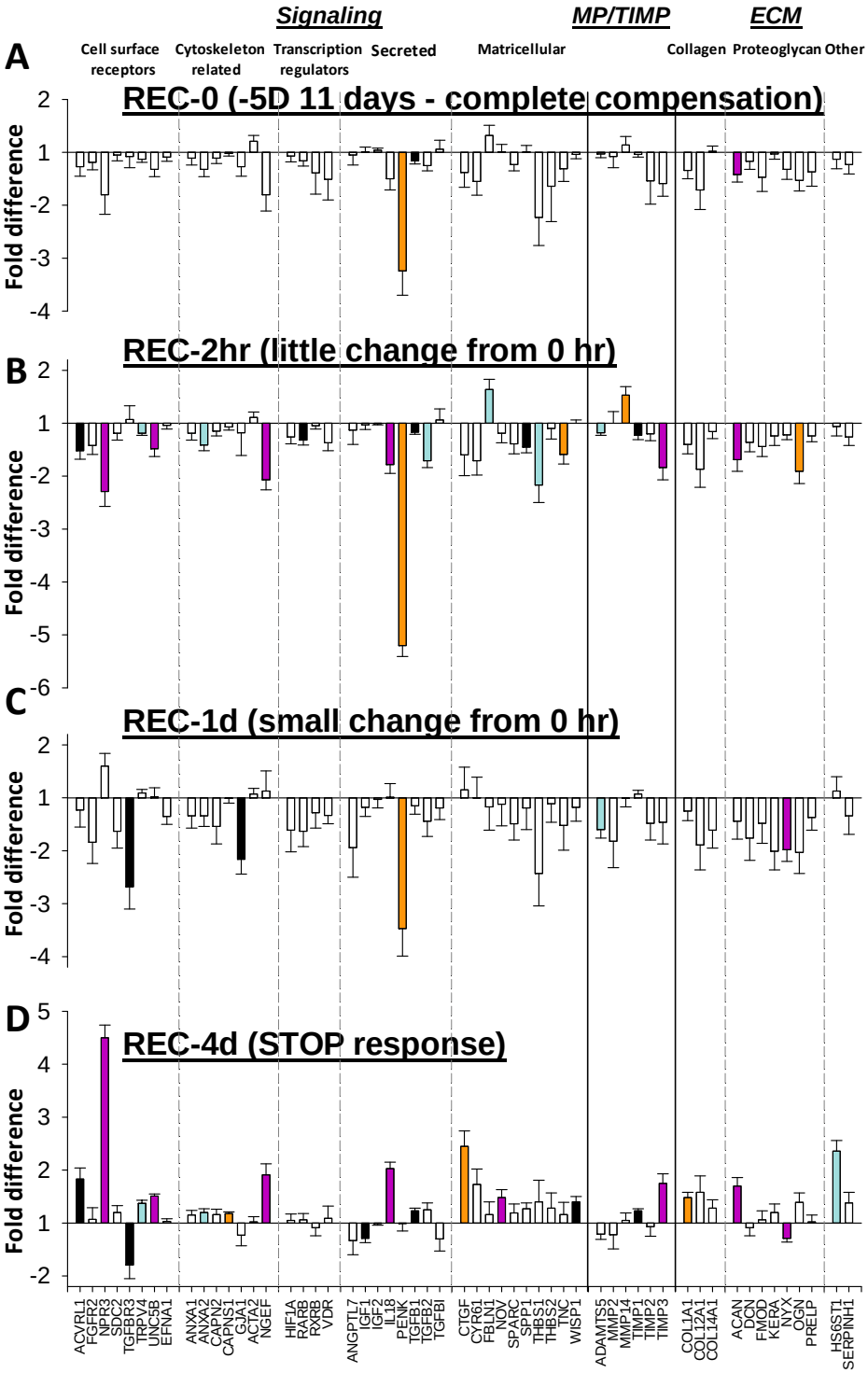

Figure 3. Gene expression fold differences (treated eyes vs. control eyes). (A) REC-0, (B) Recovery for 2 hours, (C) Recovery for 1 day, (D) Recovery for 4 days. Filled bars represent statistically significant differences between the treated and control eyes ($p < 0.05$). Bar color is arbitrary and intended to help in comparing the same gene in the three different conditions. Error bars = SEM.

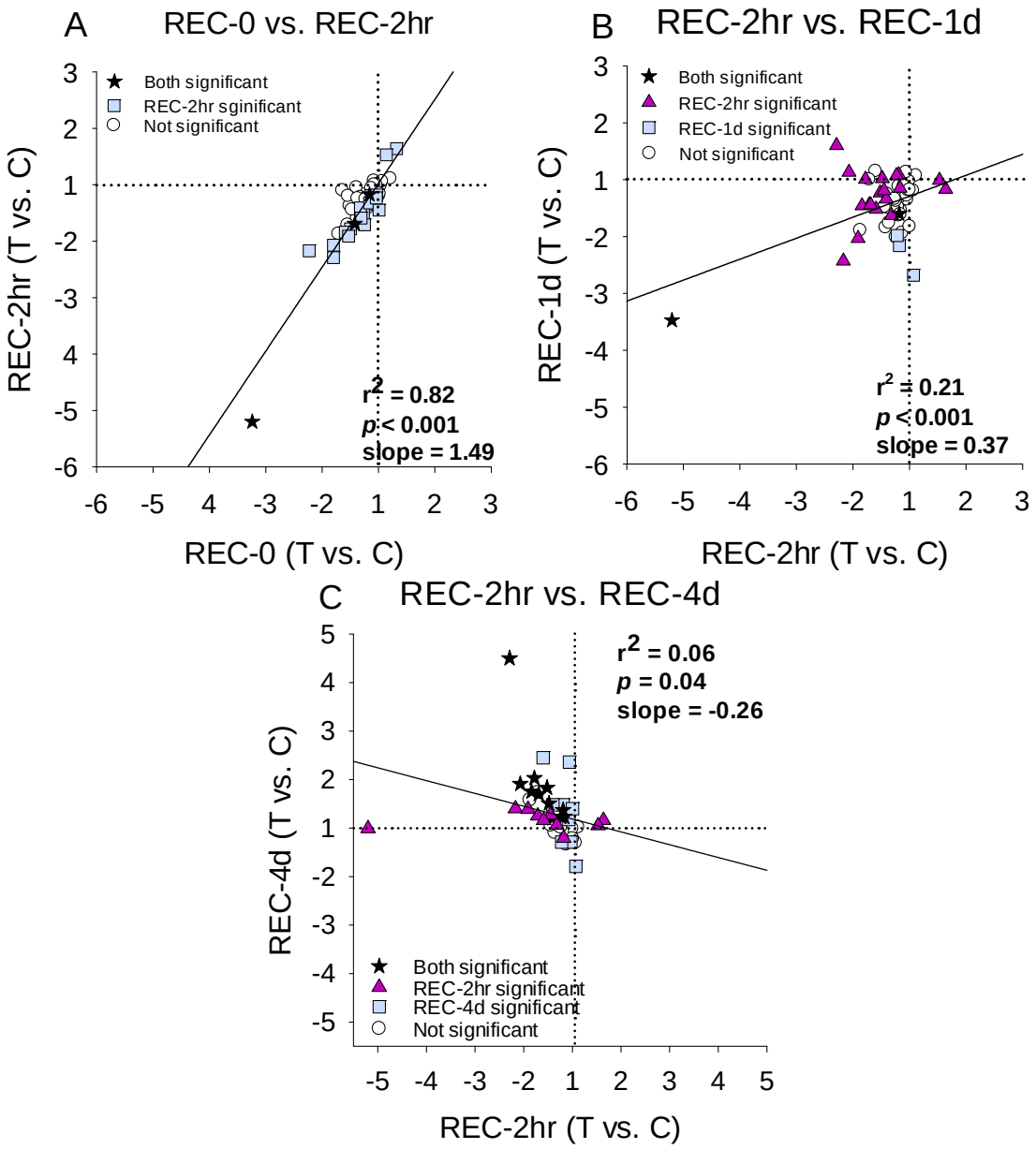

Figure 4. Change in gene expression patterns over time during recovery from minus-lens-induced myopia. (A) compares the pattern at the start of recovery (Figure 3A) with that observed after 2 hours of recovery (Figure 3B); (B) compares the differential expression after 2 hours of recovery with that after one day (Figure 3C); (C) compares the pattern after 2 hours with the pattern after 4 days (Figure 3D).

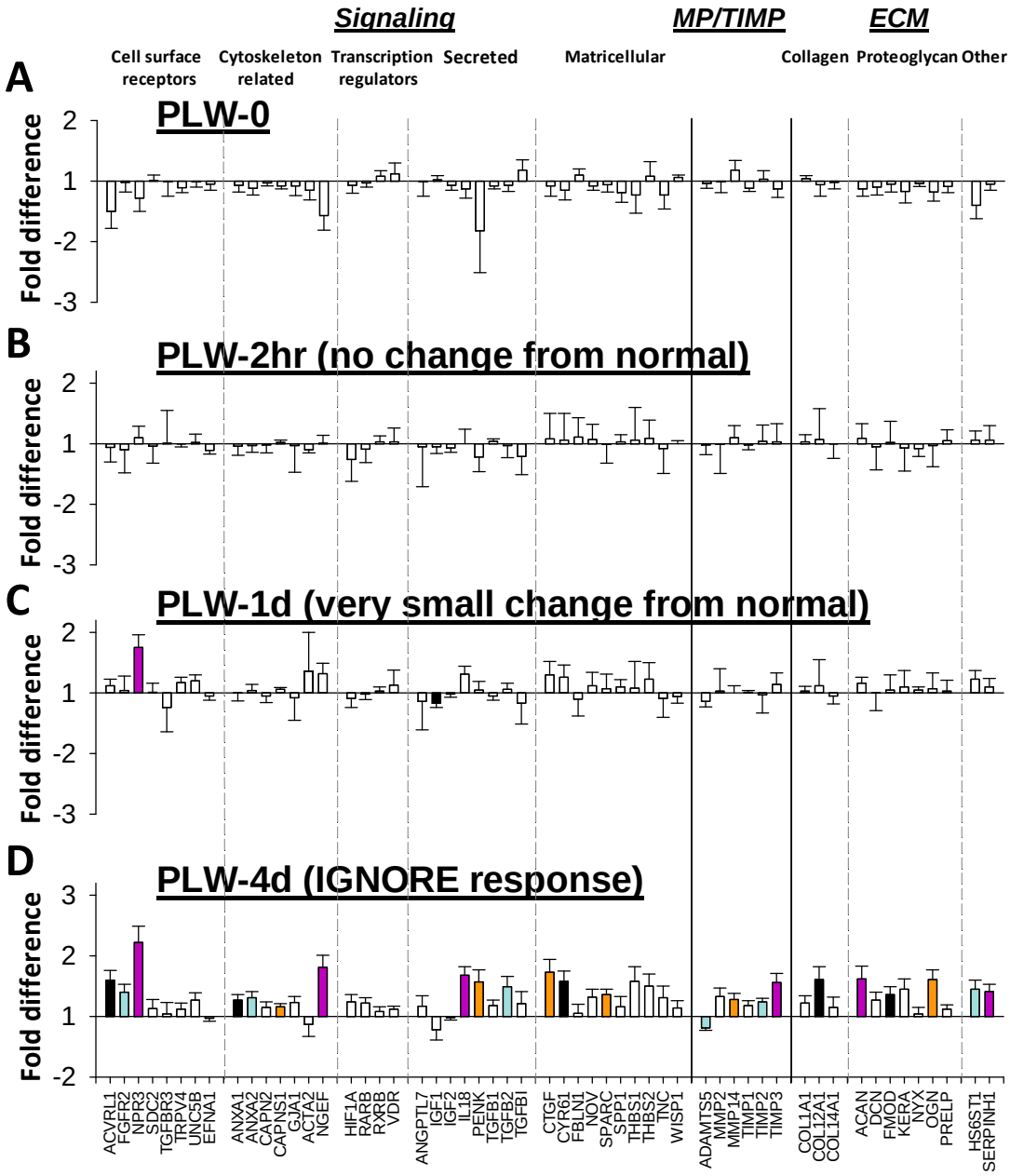

Figure 5. Gene expression fold differences: (A) 38 DVE (PLW-0; right eyes vs. left eyes). Treated eye vs. control eye differ-ences: (B) Plus-lens wear for 2 hours at 35 DVE. (C) Plus-lens wear for one day from 35 DVE. (D) Plus-lens wear for 4 days from 35 DVE. Filled bars represent statistically significant 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.

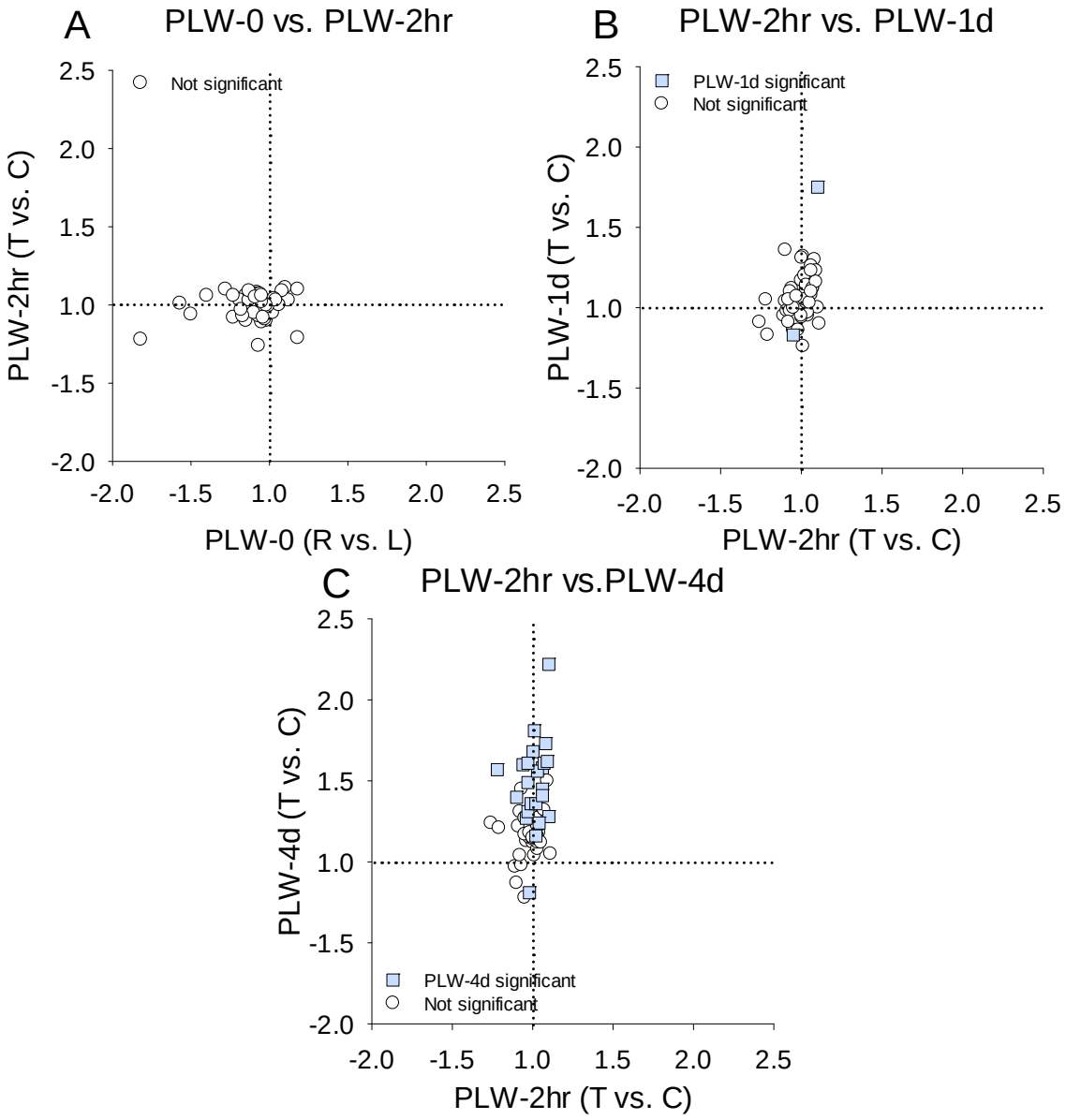

Figure 6. Change in gene expression patterns over time during plus-lens wear. (A) compares the pattern before plus-lens wear (Figure 5A) with that observed after 2 hours of plus-lens wear (Figure 5B). (B) compares the change in the differential expression between the PLW-2hr group (Figure 5B) and the PLW-1d group (Figure 5C). (C) compares the change from the PLW-2hr group (Figure 5C) to the PLW-4d group (Figure 5D).

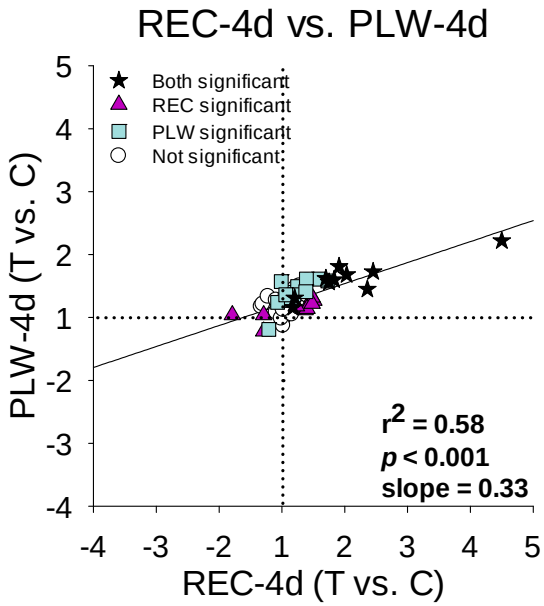

Figure 7. Comparison of the treated vs. control gene expression differences in REC and PLW: REC-4d (Figure 3D) vs. PLW-4d (Figure 4D). Stars = significant fold differences for both treatments; triangles = significant fold differences only for REC group; squares = significant fold differences only for PLW group; circles = fold differences not significant for either treatment.

SUMMARY AND DISCUSSION

The overall purpose of this dissertation was to determine mRNA gene expression signatures when the sclera responded to visual conditions (GO, STOP, and IGNORE). Here, we examined 55 genes in the sclera in response to different visual conditions and compared the mRNA expression signatures to help us to better understand the remodeling that occurs in the sclera that controls refractive error.

In **Specific Aim 1,** we found very similar gene expression signatures in tree shrew sclera in response to the three myopiagenic conditions (minus lens, form deprivation, and continuous darkness). Although there are undoubtedly many differences, at the retinal level, in the neural activity generated by these three very different visual conditions, by the time the signaling cascade has reached the scleral fibroblasts, the scleral remodeling response is nearly identical. The 2-day (ML-2 and FD-2) patterns generally were similar to the 4-day patterns. Four days of ML and FD treatment showed higher gene expression magnitude than the corresponding two days of treatment; this is consistent with the increased scleral viscoelasticity (creep rate) after two days of ML or FD treatment, the further increase after 4 days, and also is consistent with the decreased sclera dry weight (about 4% during lens compensation) found after 4 or more days of treatment (Moring et al., 2007; Norton & Miller, 1995; Norton & Rada, 1995).

During active myopia development, down-regulation was a prominent feature of the overall expression pattern. The mRNA levels for all sampled genes whose protein

products are structural proteins, were lower in the treated eyes (matricellular and extracellular matrix), mRNA levels for MMPs involved in tissue degradation were higher and levels for the selected TIMPs were lower. The changes presumably give rise to the scleral biomechanical changes, which may lead to the scleral lamella slipping across each other more easily under normal intraocular pressure.

Specific Aim 2 compared the gene expression signatures during lens induced myopia (GO) and recovery (STOP). The results showed that STOP responses occurred in the sclera when eyes were recovering from induced myopia. Comparing the STOP with the GO gene expression signatures, the general pattern in STOP was mRNA up-regulation, but the STOP signature was not an exact inverse of the GO signature. Eight genes (NPR3, CAPNS1, NGEF, TGFB1, CTGF, NOV, TIMP1, and HS6ST1) showed significant bidirectional regulation at both 2 days and 4 days in both GO and in STOP conditions, which may constitute a "core" of the two signatures. mRNA levels for other proteins changed only in GO or STOP. Their effect of the scleral remodeling on axial elongation and on scleral viscoelasticity was opposite (increased in GO, decreased in STOP) (Siegwart, Jr. & Norton, 1999). However, it is unknown that if those bidirectional regulated genes play more important roles in the extracellular matrix remodeling of the sclera that controls axial elongation than do ones that only were affected in GO or STOP.

Just as the GO signatures after 2 and 4 days of minus lens wear and form deprivation were similar, the STOP gene expression patterns in the REC-2 and REC-4 groups were also very similar in terms of which genes were affected, as well as the direction and relative magnitude of each gene's response. The gene expression signature after 4 days of recovery showed higher magnitude fold-differences with more genes differing signifi-

cantly than after 2 days of recovery, which suggests that the STOP gene expression pattern becomes stronger over time. This strengthening of the STOP pattern is consistent with prior studies showing that the scleral ECM gradually returned to normal, including a lessening of the reduction in dry weight that occurred during minus lens wear, a returned to normal level of hyaluronan, and gradual increase in glycosaminoglycans to normal level during recovery from induced myopia (Gentle et al., 2003; Moring et al., 2007; Norton & Rada, 1995).

After 11 days of minus-lens wear, the axial length of the treated eyes had increased to the point that the location of the retina was once again at the focal plane while the minus power lens was in place (Moring et al., 2007; Siegwart, Jr. & Norton, 1999). This suggests that the GO signals from the retina should have dissipated in ML-11 group, but STAY signals may exist in the sclera that cause the eyes to remain elongated and refractively emmetropic with the lens in place. The existence of STAY signals in choroid was found in a previous study of mRNA expression from this lab (He, Frost, Siegwart, & Norton, 2013). In sclera, only three genes (IL18, TGFB1, and ACAN) showed significant regulation in the STAY situation. However, when fold differences that were not significant were included, 29 of 55 non-significant genes in ML-11 showed the same regulation direction with ML-4. The gene expression pattern in ML-11 was also highly correlated with ML-4 with much lower magnitude. These data may suggest that a STAY signature in the sclera is weaker than the STAY signature in choroid.

Data from **Specific Aim 3** are summarized in a paper that is in preparation for publication. The results showed that in response to similar myopic refractive error, the sclera responded differently in plus-lens (IGNORE) condition groups than in the STOP

groups. Recovery produced a stronger response in terms of both refractive changes and mRNA expression differences than did plus-lens wear. The REC-1d group showed little refractive change, but the refraction recovered by 2.6 ± 0.4 D after 4 days of recovery. Compared to the REC groups, the PLW groups showed little refractive change at any time point; the refractive response was 0.7 ± 0.3 D after 4 days of +5 D lens wear.

For both the REC and the PLW groups, after 2 hr and 1 day treatment the scleral gene expression patterns were similar to those at their baseline (REC-0 for the REC groups, and PLW-0 for the PLW groups). After 4 days, both REC (STOP) and PLW (IGNORE) showed substantial fold differences, and began to show a positive correlation ($r^2 = 0.58$). However, the gene expression amplitude was greater in REC-4 than in PLW-4 (slope = 0.33), and they generally involved different genes, which suggests that STOP and IGNORE responses are similar but have distinguishable differences. The STOP signals may be similar as they arrive in the sclera from the choroid, but they produce stronger responses in the elongated, remodeled sclera.

The underlying reason for the lack of response of sclera in the PLW groups is unlikely to be retinal insensitivity to the myopia or the lack of response of scleral fibroblasts (mRNA changes do occur). It is more likely due to a difference between an elongated sclera and normal sclera. It appears that the same incoming signals cause similar but distinguishable differences in the fibroblasts because the fibroblasts are in a different "state" or condition. In a sense, it may be relatively easy to decrease the elongation rate of an elongated, myopic eye, but difficult to decrease the elongation rate of a normal eye to a lower-than-default value, which would be needed in order for the eye to respond refrac-

tively to plus lens wear. In addition, the RPE and choroid may respond differently under these conditions, and pass diverse signals to sclera.

SIGNALING

The majority of the signaling molecules that we examined in this study participate in cell signaling and play either direct or indirect roles in scleral extracellular matrix re-modeling. The genes that showed significant changes in expression in GO, STOP, or INGORE conditions are discussed below.

Extracellular Matrix Receptors

In connective tissues, cell-surface receptors such as integrins and syndecans play critical roles in cell-ECM adhesion. The major collagen-binding integrins α1β1 and α2β1, that are major receptors responsible for regulating ECM remodeling, have been found in tree shrew sclera, associated with type I, III, IV, and V collagen (McBrien, Metlapally, Jobling, & Gentle, 2006). The α1β1 integrin receptor preferentially binds to basement membrane collagen and modifies the activity of genes that lead to the down-regulation of collagen synthesis in human skin fibroblasts (Langholz et al., 1995). The α2β1 integrin receptor has the ability to adjust cell spread on type I-V collagen (Nykvist et al., 2000), and induce collagenase (MMP-1) (Langholz et al., 1995). The mRNA expression level (treated vs. normal eyes) of α1 and β1 subunits of integrin, the major collagen-binding receptor in mammalian sclera, is decreased early during the development of myopia, whereas the α2 subunit decrease occurs later (about 5 days of myopia development) in tree shrew sclera (McBrien et al., 2006). The decreased α1β1 level in the early myopia

development stage may result in slowing scleral fibroblast proliferation (Gentle & McBrien, 1999). Moreover, the decrease of the α2 subunit may depress the contractile ability of myofibroblasts or play a role in ocular growth (McBrien et al., 2006).

In connective tissues, cell adhesion proteins play critical roles in cell-ECM interaction. Syndecans represent a family of integral membrane proteoglycans, consisting of a protein core with covalently attached chondroitin sulfate and heparan sulfate glycosaminoglycan chains that are conserved in syndecans 1-4 (Bernfield & Sanderson, 1990). Syndecans behave as a matrix receptor, which binds both extracellular matrix components (fibronectin, collagen type I, III, and V, and thrombospondin) and growth factors (such as bFGF and VEGF) (Lopes, Dietrich, & Nader, 2006). They are involved in both modulation of growth factor signaling and cell adhesion modification. There has been little previous investigation on the possible involvement of syndecans in myopia development.

Activin A receptor type II-like 1 (ACVRL1) encodes a type I cell-surface receptor for the TGF- β superfamily of ligands (Ten et al., 1993), forming a receptor complex consisting of two type II and two type I transmembrane serine/threonine kinases that initiates TGF-β family signaling. Serine/threonine kinase receptor type II receptors phosphorylate and activate type I receptors, and then bind and activate (R)-SMAD transcriptional regulators (Itoh & Ten, 2007; Ten & Hill, 2004). In the eye, bone morphogenetic proteins (BMPs), members of the TGF-β superfamily, are essential for lens formation and development. Increased apoptosis of lens epithelial and fiber cells were observed in an AVCR1 knockout mouse (Rajagopal et al., 2008). AVCRL1 plays an important role in angiogenesis. Development of distinct arterial and venous vascular beds requires ACVRL1 (Urness,

Sorensen, & Li, 2000) and it is critical for postnatal retinal vascular remodeling and vascularization in mouse (Ricard et al., 2012). Since the choroid in eyes is also a vascular organ, the alteration of ACVRL1 expression level in sclera may be relevant to the signaling cascade from the choroid to the sclera during myopia development.

Natriuretic peptide receptor-3 (NPR3) is a cell surface receptor that is primarily used to remove natriuretic peptides by a process of internalization and degradation (Potter, 2011). It may also have a signaling function. The main physiological role of the natriuretic peptide receptors is in homeostasis of body fluid volume. The significant down-regulation in GO conditions and significant up-regulation in STOP and INGORE conditions in sclera may indicate a role in scleral structural modulation.

Transient receptor potential cation channel, subfamily V, member 4 (TRPV4) is a mechanosensitive member of the vanilloid receptor family (TRPV) of ion channels. The encoded protein is a non-selective cation channel that is thought to be involved in the regulation of systemic osmotic pressure (Liedtke et al., 2000; Strotmann, Harteneck, Nunnenmacher, Schultz, & Plant, 2000). A recent study found that TRPV4 plays an important role in regulating cardiac fibroblast differentiation to myofibroblasts by integrating signals from TGF-β1 and mechanical factors (Adapala et al., 2013); this may also be true in scleral fibroblasts.

Unc-5 homolog B (UNC5B) is a member of the netrin family of receptors that may play a role in in axonal growth and angiogenesis (Colamarino & Tessier-Lavigne, 1995). Studies found that vascular patterning during embryonic development requires UNC5B, which indicates a possible contribution to postnatal and pathological angiogene-

sis (Larrivee et al., 2007; Lu et al., 2004). This process may also include ocular development.

Cytoskeleton Related Signaling

AnnexinsA1 and A2 (ANXA1 and ANXA2) are Ca^2+-dependent phospholipid binding proteins, preferentially located on the cytosolic face of the plasma membrane. ANXA1 is known to be involved in important cellular regulatory pathways including cell proliferation, differentiation, and apoptosis (Lim & Pervaiz, 2007; Swa, Blackstock, Lim, & Gunaratne, 2012). ANXA2 plays a role in targeting Rho (a critical part in focal adhesion) to cellular membranes, thereby modulating Rho-related signaling events regulating cytoskeletal reorganization (Babbin et al., 2007).

Calpains are calcium-dependent cytosolic cysteine proteases, functioning as downstream targets of hormone receptors, membrane-type tyrosine kinases, and adhesion molecules and they proteolyze many signaling-related substrates (Perrin & Huttenlocher, 2002; Sato & Kawashima, 2001). Calpains 1 and 2 (CAPN1 and CAPN2) encode the large catalytic subunits, while calpain 4 (CAPNS1) encodes the small regulatory subunit of the heterodimer. CAPNS1 plays an important role in the production of mechanical forces and in mediating mechanosensing during fibroblast migration (Undyala et al., 2008).

Neuronal guanine nucleotide exchange factor (NGEF) differentially activates the GTPases RHOA, RAC1, and CDC42. It plays a role in axon guidance regulating ephrin-

induced growth cone collapse and dendritic spine morphogenesis. NGEF was found to be related to metabolic stress in human fibroblasts (Kalman et al., 2013).

Secreted Signaling

Many aspects of scleral extracellular matrix remodeling are under the control of specific growth factors or their downstream effectors. Angiopoietin-like 7 (ANGPTL7) is a secreted protein. The overexpression of this protein in human trabecular meshwork cells can alter the expression of fibronectin, collagens type I, IV & V, myocilin, versican, and MMP1, and also interferes with the fibrillar assembly of fibronectin (Comes, Buie, & Borras, 2011).

Insulin-like growth factor 1 (IGF-1) is effective in up-regulating proteoglycan synthesis (Summers Rada et al., 2006). Insulin is an antagonist of glucagon, and it acts in the opposite direction with glucagon (a STOP signal in chicken) as a strong stimulator of axial myopia. Previous studies showed that insulin injections into the vitreous chamber in chicken can accelerate the axial elongation rate and make the eye myopic (Feldkaemper, Neacsu, & Schaeffel, 2009; Zhu & Wallman, 2009). IGF1R and IR (insulin receptor) showed up-regulation in chicken fibrous sclera during minus lens-induced myopia (Penha, Schaeffel, & Feldkaemper, 2011). The significant mRNA down-regulation of IGF1 in both REC-4 and PLW-4 in our study during the slowing down of axial elongation in both STOP and IGNORE conditions is consistent with previous studies.

Interleukin 18 (IL18) is a pro-inflammatory cytokine that has been shown to induce profibrotic changes, apoptosis, and collagen production in tubular epithelial cells via signal transducer and activator of transcription 3 (STAT3) activation (Bani-Hani et al.,

2009; Matsui, Rhee, Hile, Zhang, & Meldrum, 2013; Zhang et al., 2011). In our study, IL18 mRNA levels were significantly down-regulated in GO conditions, and significantly up-regulated in both STOP and IGNORE conditions. This may indicate a positive role in collagen production that IL18 may play in tree shrew sclera.

Proenkephalin (PENK) play a role in several physiologic functions, including pain perception and responses to stress. Proteolytic cleavage of PENK produces two enkephalin peptides, both of which can increase glutamate release and decrease GABA concentration in the striatum (Comb, Seeburg, Adelman, Eiden, & Herbert, 1982; Noda et al., 1982). Glutamate is a positive autocrine signal for glucagon release by acting on iGluRs of the AMPA/kainate type (Cabrera et al., 2008). Glucagon is a known STOP signal in chicken (Bitzer & Schaeffel, 2004; Mathis & Schaeffel, 2006; Vessey, Lencses, Rushforth, Hruby, & Stell, 2005; Vessey, Rushforth, & Stell, 2005). *amma-amino butyric acid (GABA) is the major inhibitory neurotransmitter of the retina and the brain, is localized in diverse retinal cell populations (Nguyen-Legros, Versaux-Botteri, & Savy, 1997), and is involved in both amacrine and horizontal cells signaling (Barnstable, 1993; Kolb, 1997; Qian, 1995). GABA receptors are also expressed in both the fibrous and cartilaginous layers of chick sclera (Cheng, Chebib, & Schmid, 2011). Studies have shown that GABA antagonists can inhibit myopia development in the chick model (Chebib et al., 2009; Gibbs & Johnston, 2005; Stone et al., 2003). At the cellular level, studies of chicken scleral fibroblasts indicate that GABA antagonists increased scleral GAG content, acting via a scleral mechanism utilizing the RPE/choroid (Christian, Harkin, & Schmid, 2014). The significant mRNA down-regulation of PENK in all GO conditions in tree

shrew sclera in this study may be consistent with a reduced release of glutamate and increased release of GABA, which in turn may lead to myopia development.

Basic fibroblast growth factor (FGF2) mRNA is unchanged during myopia development (Gentle & McBrien, 2002; Seko, Shimokawa, & Tokoro, 1995). The FGF receptor-1 (FGFR1), however, shows up-regulation in myopia-developing sclera in tree shrew, which may suggest an indirect role of FGF in tissue remodeling (Gao et al., 2011; Gentle & McBrien, 2002).

Transforming growth factor β (TGFβ) is another controller of cell cycle activity and collagen, proteoglycan, and MMP2 production in ECM. In chick, it also inhibits the FGF effect on myopia development (Jobling, Nguyen, Gentle, & McBrien, 2004; Rohrer & Stell, 1994). Three isoforms (TGFB1, TGFB2, and TGFB3) are found to be significantly down-regulated (treated vs. control eyes) in tree shrew sclera after 1 day of form deprivation, and the down-regulation is even more obvious for TGFB2 after 5 days of myopia development (Gao et al., 2011; Jobling et al., 2004). TGFBR3 showed up-regulation after 4 days of myopia development and significant down-regulation after one day recovery (Gao et al., 2011; Guo, Frost, Siegwart, Jr., & Norton, 2012; Guo, Frost, Siegwart, & Norton, 2011). TGFβ plays an important role in ECM turnover, and the three mammalian isoforms of TGFβ have an effect on regulating collagen production (Jobling et al., 2004). However, TGFβ has a limited ability to bind ligands, and TGFBR3 can regulate access by presenting TGFβ to the signaling receptors via its core protein. TGFBR3, also known as betaglycan, is a membrane-anchored proteoglycan, and the non-signaling receptor of TGFβ (Lopez-Casillas, Wrana, & Massague, 1993). It is a potent inhibitor that sequesters TGFβ in the extracellular space preventing it from binding to the signaling

receptors (Lopez-Casillas, Payne, Andres, & Massague, 1994). Soluble TGFBR3 is produced from the membrane-anchored form by MMP14-mediated cleavage (Velasco-Loyden, Arribas, & Lopez-Casillas, 2004). The relative increase in MMP14 and TGFBR3 mRNA levels during three retina GO sclera GO conditions may indicate that there is an increased production of the inhibitory soluble TGFBR3 during this process. The relative decrease in TGFBR3 mRNA and the increase in TGFβ mRNA, along with the return to normal of mRNA for MMP14 during recovery, may suggest increased TGFβ pathway signaling.

TGF-β induced protein (TGFBI) is a secreted RGD-containing ECM protein that binds to type I, II and IV collagens and plays a role in cell-collagen interactions. In sclera, this protein plays an inhibitory role in human scleral fibroblasts attachment to collagen type I in vitro (Shelton & Rada, 2009). We found that TGFBI mRNA was up-regulated after 4 days of myopia development and returned to normal levels during STOP and IGNORE conditions.

Matricellular Proteins

Matricellular proteins are a group of proteins that do not contribute directly to the formation of structural elements but serve to modulate cell-matrix interactions and cell function through interactions with cell-surface receptors, structural components, proteases, and signaling molecules such as TGFβ (Bornstein & Sage, 2002; Sage, 2001). Thrombospondin 1 (THBS1) functions to activate TGFβ (Murphy-Ullrich & Poczatek, 2000) which, in turn, raises type I collagen production via the SMAD (Sma and Mad re-

lated family) signal transduction pathway (Miyazono, Ten, & Heldin, 2000; Overall, Wrana, & Sodek, 1989). Previous studies in our lab found down-regulation of THBS1 at both the mRNA (Gao et al., 2011) and protein levels (Frost & Norton, 2007; Frost & Norton, 2012) during lens-induced myopia development. This reduced THBS1 level may lead to a reduction in TGFβ activation, which, in turn, decreases the level of type I collagen in the sclera and decreases cell adhesion.

SPARC and SPP1 are secreted glycoproteins that modulate cell shape and cell-matrix interactions (Gilbert et al., 1999). TNC is another glycoprotein. It may regulate cellular behavior during tissue remodeling by enhancing migration and differentiation of myofibroblasts and by inducing matrix metalloproteinases (Imanaka-Yoshida, Hiroe, & Yoshida, 2004). The results in this study are consistent with our previous study; mRNA levels for SPARC, SPP1, and TNC, showed down-regulation in GO conditions, and then reversed during recovery (Gao et al., 2011). The CCN proteins (CYR61, CTGF, NOV, and WISP1) are an important family of matricellular regulatory factors involved in internal and external cell signaling. They are induced by growth factors and cytokines like TGFβ, or cellular stress such as hypoxia. They signal through integrins and proteoglycans, and modulate focal adhesions, cell attachment, and migration, along with the activity of a variety of different growth factors (Leask & Abraham, 2006; Perbal, 2004). The differential changes in mRNA levels of the matricellular candidate genes suggest their general involvement in scleral tissue remodeling.

Fibulin 1 (FBLN1) showed up-regulation while the other ECM genes were down-regulated during myopia development. Its levels returned to normal in tree shrew sclera during recovery. Studies in guinea pig have shown that the increased level of retinoic ac-

id could up-regulate the expression of FBLN1 and down-regulate the expression level of aggrecan (Li et al., 2010). The reason is unknown, but it might be because FBNL1 has the ability to enhance ADAMTS-mediated proteolysis of aggrecan, which might enhance the cleavage of aggrecan and tissue remodeling by form a ternary complex with ADAMTS1(de, Iwamoto, & Yamada, 2009).

Overall, results from all three specific aims in this dissertation have helped us to better understand how the sclera is remodeled during normal development, GO, STOP, IGNORE, and STAY conditions. We found that scleral fibroblasts respond with different mRNA expression signatures to the different emmetropization conditions. Genes that showed significant fold differences treated versus control eyes under those visual conditions are involved in different signaling pathway and may participate in scleral extracellular matrix remodeling.

Limitations of This Study

For all genes of interest in this study, only the changes in mRNA transcription levels were examined by quantitative PCR. We know that changes in transcription levels often do not correspond with the changes in protein levels in complex biological systems (Maier, Guell, & Serrano, 2009; Nie, Wu, & Zhang, 2006; Wang, 2008), and some signals may be contained pre- or post-transcriptional levels, like microRNA, protein translation and modification. Thus, the patterns that occur in the three specific aims give an incomplete picture of the remodeling events that occur in the sclera. Nonetheless, in our study, our focus was to learn if the scleral fibroblasts respond differently to the different

visual conditions. Also, because the genes that were selected among those that did respond during different visual conditions in previous studies, the changes in mRNA levels provided a useful way to examine and compare the way cells respond to the visual conditions and distinct patterns were obtained.

It is possible, even likely, that some genes that are involved in important ways in altering the sclera in the visual conditions we examined were not included in our study, since we did not examine all active genes. Another limitation is that, during the design of the tree shrew-specific primers, some gene expression levels in sclera were too low to be detected disqualifying some genes from inclusion in the study. However, it appears that a sufficient number of genes were included such that clear similarities and differences were found in specific aims 1, 2, and 3.

Another limitation is that the scleral mRNA changes do not clearly show what molecule(s) come into the sclera from the choroid to produce the GO, STAY, STOP, and IGNORE signatures. No single substance, or even collection of substances, is known to produce the pattern of altered gene expression in sclera or any other extracellular matrix. Additional experiments, perhaps applying candidate choroidal signaling molecules to the sclera will be needed to learn what produces these scleral gene-expression signatures.

Future Directions

Since this study has established the scleral gene expression signatures under different visual conditions, potentially these signatures could help to distinguish genes from choroid that initiate the scleral remodeling by applying genes that showed significant

changes in choroid to see if they can produce similar gene expression pattern in cultured sclera. Because the sclera is the “last stop” in the emmetropization cascade, it is possible that the sclera could be a potential therapeutic target for controlling axial elongation that would not interfere with vision. In order to control the sclera, perhaps with sub-conjunctivally administered drugs, it would be necessary to find out the up-stream signal molecules that can initiate or completely block the scleral extracellular matrix remodeling process. In addition, because we examined a limited (but large) number of genes, it would be useful to use RNA-Seq (a whole-transcriptome analysis method) to examine a much larger number of genes. Preliminary work has been done (Frost, Guo, & Norton, 2012), but this area has not yet been fully explored.

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APPENDIX

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.

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