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Gene Profiling in Experimental Models of Eye Growth: Clues to Myopia Pathogenesis

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Abstract

To understand the complex regulatory pathways that underlie the development of refractive errors, expression profiling has evaluated gene expression in ocular tissues of well-characterized experimental models that alter postnatal eye growth and induce refractive errors. Derived from a variety of platforms (e.g. differential display, spotted microarrays or Affymetrix GeneChips), gene expression patterns are now being identified in species that include chicken, mouse and primate. Reconciling available results is hindered by varied experimental designs and analytical/statistical features. Continued application of these methods offers promise to provide the much-needed mechanistic framework to develop therapies to normalize refractive development in children.

The basis of refractive errors

Ocular refraction depends primarily on the interactions of axial length, corneal curvature and lens power, with anterior chamber depth having a lesser effect (Curtin, 1985). With accommodation relaxed, distant images focus at the retinal photoreceptors in emmetropia, the condition with no evident refractive error. At birth, the eye generally is hyperopic (farsighted). During childhood, an active regulatory process termed emmetropization harmonizes the expanding eye length to match the powers of the cornea and lens to result in emmetropia. Emmetropization failure results in refractive errors. In myopia (nearsightedness), the eye is relatively long for the optical power of the cornea and lens, and distant images focus in front of the photoreceptors; in hyperopia, the eye is relatively short, and distant images focus behind the photoreceptors.

The public health impact of refractive errors

Besides requiring optical correction for daily activities, refractive errors predispose to numerous serious eye diseases. Myopia, the most common refractive error (Vitale, Ellwein, Cotch, Ferris & Sperduto, 2008), increases the risk for retinal detachment, certain macular/retinal degenerations, glaucoma and cataract (Curtin, 1985, Stone, 2008). Presumably because the retina thins as it stretches to line the enlarged vitreous chamber, retinal tears and

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other peripheral retinal pathologies develop with increased prevalence in myopia (Hyams & Neumann, 1969) and account for the increased incidence of retinal detachment, with the risk rising markedly as myopia worsens (Eye Disease Case-Control Study Group, 1993, Ogawa & Tanaka, 1988, Perkins, 1979). Myopic retinopathy comprises a variety of diseases affecting the posterior retina, with the prevalence also increasing with increasing degrees of myopia (Vongphanit, Mitchell & Wang, 2002). Across its entire spectrum, myopia predisposes to both open-angle glaucoma and normal tension glaucoma (Grødum, Heijl & Bengtsson, 2001, Leske, Nemesure, He, Wu, Hejtmancik, Hennis & Group, 2001, Mitchell, Hourihan, Sandbach & Wang, 1999, Wong, Klein, Klein, Knudtson & Lee, 2003), although the physiological basis for the glaucoma risk is unclear. Hyperopia also is a risk factor for glaucoma, in this case for angle-closure glaucoma because of the small eye and shallow anterior chamber (Ritch & Lowe, 1996). Higher and probably lower degrees of myopia are associated with cataract (Wong, Klein, Klein, Tomany & Lee, 2001), specifically nuclear and posterior subcapsular cataract (Harding, Harding & Egerton, 1989, Leske, Chylack, He, Wu, Schoenfeld, Friend, Wolfe & Group, 1998, Leske, Wu, Nemesure, Hennis & Group, 2002, McCarty, Mukesh, Fu & Taylor, 1999, Wong et al., 2001). The myopic refractive shift from nuclear sclerosis complicates interpreting the associations of myopia with nuclear cataract. Less ambiguous, earlier onset myopia predisposes to posterior subcapsular cataract in later life, a relation that increases with increasing myopia (Lim, Mitchell & Cumming, 1999, Younan, Mitchell, Cumming, Rochtchina & Wang, 2002).

Myopia, especially in its extreme degrees, ranks as a leading cause of visual impairment and blindness because of these associated diseases. Epidemiologic studies usually do not isolate a specific contribution of myopia to blindness from glaucoma, cataract or retinal detachment, but repeatedly these studies look for and identify a major impact of myopic retinopathy on visual impairment and blindness. Because myopic retinopathy frequently afflicts patients in mid-life, it has important economic, social, family and other quality of life consequences (Klaver, Wolfs, Vingerling, Hofman & de Jong, 1998, Soubrane, 2008). While prevalences vary between racial and ethnic groups, a major contribution of myopic retinopathy to visual impairment has been reported in many societies. Particularly severe in Asian societies, myopic retinopathy is the second leading cause of blindness (after cataract) in both Taiwan (Hsu, Cheng, Liu, Tsai & Chou, 2004) and mainland China (Xu, Wang, Li, Wang, Cui, Li & Jonas, 2006); in Japan, it represents the leading cause of unilateral blindness and third leading cause of bilateral blindness (Iwase, Araie, Tomidokoro, Yamamoto, Shimizu, Kitazawa & Tajimi Study Group, 2006). As some other examples, myopic retinopathy is the third leading cause of blindness in Israel (Avisar, Friling, Snir, Avisar & Weinberger, 2006), accounts for some 26% of blindness among subjects below 65 years-old in Denmark (Buch, Vinding, la Cour, Appleyard, Jensen & Nielsen, 2004), and is the leading cause of blindness among 40–50 year-olds in England (Vongphanit et al., 2002). In the US, myopic retinopathy accounts for some 4% of cases of visual impairment overall and has long ranked as the 7th leading cause of blindness (Hotchkiss & Fine, 1981, http://www.lighthouse.org/research/statistics-on-vision-impairment/causes/). The diseases associated with myopia are neither prevented nor lessened by any optical or surgical approaches to correct the image defocus.

Of added concern, the prevalence of myopia is increasing world-wide. In developed regions of Asia, it now affects some 80% of young adults (Lin, Shih, Tsai, Chen, Lee, Hung & Hou, 1999, Xu, Huang, Gao, Gao & Han, 2001). Whether or not myopia prevalence is increasing in Western societies has been more controversial (Fledelius, 2000, Mutti & Zadnik, 2000). A commonly cited myopia prevalence of 25% in the US derives from 1971–1972 data in the National Health and Nutrition Examination Survey (NHANES) (Sperduto, Siegel, Roberts & Rowland, 1983); but the most recent assessment of myopia prevalence in a comparable 12–54 year-old age group during 1999–2004 finds a significant increase to 41.6%

prevalence in the US (Vitale, Sperduto & Ferris, 2009). Besides myopia's increasing prevalence, the associated eye diseases and the resulting visual impairment and blindness, the costs of diagnosis and optical correction of refractive errors in the US alone are estimated at $3.8–$7.2 billion/year (Rein, Zhang, Wirth, Lee, Hoerger, McCall, Klein, Tielsch, Vijan & Saaddine, 2006, Vitale, Cotch, Sperduto & Ellwein, 2006, Vitale et al., 2008).

Why refractive errors?

The underlying mechanisms responsible for refractive errors and for the apparent increase in myopia prevalence are unknown. Most research on the pathogenesis of refractive errors has addressed myopia because of its high public health impact. Epidemiologic studies typically survey conventional parameters long hypothesized to account for myopia, such as family history, ocular accommodation, visual activities at near distances, socioeconomic status, education, intelligence, etc. (Angle & Wissmann, 1980, Curtin, 1985, Saw, Katz, Schein, Chew & Chan, 1996). So far, it is unclear whether myopia develops from adaptive physiologic responses to visual demands of modern societies or instead develops from physiologically inappropriate processes that may override, rather than exploit, normal regulatory mechanisms (Stone, 2008). Genetic factors have been implicated in the etiology of both myopia and hyperopia (Wojciechowski, Cogdon, Bowie, Munoz, Gilbert & West, 2005). Twin and family studies have long suggested a genetic component in myopia and several chromosomal loci have been linked with human myopia, including high myopia (Hornbeak & Young, 2009, Young, Metlapally & Shay, 2007). While contemporary clinical research supports the notion that myopia represents a "complex" disorder with both environmental and genetic influences (Farbrother, Kirov, Owen & Guggenheim, 2004, Hornbeak & Young, 2009, Klein, Duggal, Lee, Klein, Bailey-Wilson & Klein, 2005, Morgan & Rose, 2005, Saw, Chua, Wu, Yap, Chia & Stone, 2000, Zadnik, 1997), the literature is often contradictory; and the relative importance of genes vs. environment in myopia remains controversial (Lyhne, Sjølie, Kyvik & Green, 2001, Morgan & Rose, 2005, Rose, Morgan, Smith & Mitchell, 2002). *Despite the important public health problem and many clinical and laboratory research initiatives, understanding of the pathophysiologic mechanisms responsible for ametropias is limited; and consequently, no approved and clinically acceptable therapies are available to normalize or reduce abnormal refractive development in children* (Saw, Chua, Hong, Wu, Chan, Chia, Stone & Tan, 2002, Saw et al., 1996).

Refractive development and the retina

Persuasive evidence, initially developed in animals, implicates the visual image in refractive development and has localized the controlling mechanism(s) largely to the retina (Norton, 1999, Stone, 1997, Stone, 2008, Wallman, 1993). The induction of so-called form-deprivation myopia by goggle wear or lid suture in species as varied as chick (Wallman, Turkel & Trachtman, 1978), tree shrew (McBrien & Norton, 1992), and monkey (Raviola & Wiesel, 1985, Smith, Harwerth, Crawford & von Noorden, 1987, Troilo & Judge, 1993) first demonstrated visual feedback in eye growth control. Visual image degradation in young children similarly was found to associate with ipsilateral myopia (Nathan, Kiely, Crewther & Crewther, 1985), as with disorders that obstruct the visual axis like ptosis (Hoyt, Stone, Fromer & Billson, 1981) or a scarred cornea (Twomey, Gilvarry, Restori, Kirkness, Moore & Holden, 1990). In each species, the major anatomical change characterizing form-deprivation myopia is vitreous chamber enlargement, similar to common human myopia.

Another widely studied example implicating visual feedback in the regulation of refractive development, the wearing of defocusing spectacle lenses to shift the image plane in front of

or behind the retina induces compensating changes in eye growth to reposition the retina at the image plane in chicks (Schaeffel, Glasser & Howland, 1988), tree shrews (Norton & Siegwart, 1995, Shaikh, Siegwart & Norton, 1999), marmosets (Graham & Judge, 1999) and monkeys (Hung, Crawford & Smith, 1995, Smith, 1998). The posterior shift of the visual image from concave (minus) spectacle lenses accelerates ocular growth and keeps the focal position of distant images in the photoreceptor plane; conversely, the anterior shift of the visual image from convex (plus) spectacle lenses slows eye growth and accordingly also keeps distance images in the photoreceptor plane. After spectacle lens removal, eyes previously wearing a minus spectacle lens have myopic refractions; those previously wearing a plus spectacle lens have hyperopic refractions.

Much evidence localizes the visual mechanism regulating eye growth largely to the eye itself (Norton, 1999, Stone, 1997, Stone, 2008, Wallman, 1993). As just two examples, form-deprivation myopia in both monkeys and chicks still develops after optic nerve section to separate the eye from the brain (Raviola & Wiesel, 1985, Troilo, Gottlieb & Wallman, 1987, Wildsoet & Pettigrew, 1988); and the wearing of hemi-field defocusing lenses induces local changes in eye growth preferentially in the region of the eye where the retina receives a defocused image (Diether & Schaeffel, 1997). The dual properties of visual (and hence neural) regulation and intrinsic ocular location (as shown by optic nerve section) identify the retina as a major site regulating refractive development (Stone, 1997, Stone, 2008, Wallman, 1993).

Retinal pharmacology as a tool to study eye growth mechanisms

Most efforts to identify retinal mediators that signal eye growth and regulate refractive development have used histochemical, biochemical, pharmacological and tissue culture methods (Stone, 2008). Among retinal transmitters and/or modulators implicated so far in eye growth are dopamine (Iuvone, Tigges, Fernandes & Tigges, 1989, Stone, Lin, Laties & Iuvone, 1989), vasoactive intestinal peptide (Pickett Seltner & Stell, 1995, Stone, Laties, Raviola & Wiesel, 1988, Tkatchenko, Walsh, Tkatchenko, Gustincich & Raviola, 2006), glucagon (Buck, Schaeffel, Simon & Feldkaemper, 2004, Feldkaemper & Schaeffel, 2002, Fischer, McGuire, Schaeffel & Stell, 1999a, Vessey, Lencses, Rushforth, Hruby & Stell, 2005a), γ-aminobutyric acid (GABA) (Chebib, Hinton, Schmid, Brinkworth, Qian, Matos, Kim, Abdel-Halim, Kumar, Johnston & Hanrahan, 2009, Stone, Liu, Sugimoto, Capehart, Zhu & Pendrak, 2003) and acetylcholine (Stone, Lin & Laties, 1991, Stone, Sugimoto, Gill, Liu, Capehart & Lindstrom, 2001b), although whether the latter acts at the retina is unclear (Fischer, Miethke, Morgan & Stell, 1998, Lind, Chew, Marzani & Wallman, 1998, Luft, Ming & Stell, 2003). Other agents also implicated in eye growth regulation include retinoic acid (Fischer, Wallman, Mertz & Stell, 1999b, Mertz & Wallman, 2000, Seko, Shimizu & Tokoro, 1998) and growth factors, such as transforming growth factor and basic fibroblast growth factor (Honda, Fujii, Sekiya & Yamamoto, 1996, Rohrer & Stell, 1994, Rohrer, Tao & Stell, 1997, Seko, Shimokawa & Tokoro, 1995).

Drugs interacting with many of these neural receptors attenuate experimental myopia in visually deprived eyes but do not alter the growth of eyes with non-restricted vision (Stone, 2008). However, drugs interacting with a few of these receptors modify refractive development of both visually deprived eyes and eyes with non-restricted vision (Stone et al., 2003, Truong, Cottriall, Gentle & McBrien, 2002). Why some drugs alter refractive development only under circumstances precluding visual feedback and others influence eye growth under conditions with either restricted or non-restricted vision is speculative.

Even a hypothetical framework integrating these diverse observations to understand the retinal control of refractive development is not available, and many fundamental questions

are unanswered. For instance, it is not known how retinal signals reach the outer coats of the eye to govern its growth, because no neural pathways are known that directly connect the retina with either the choroid or sclera. Visual input might stimulate the retina to elaborate growth regulators directly or may initiate a multi-level biochemical cascade ultimately regulating eye growth, perhaps involving retinal pigment epithelial or choroidal cells (Rymer & Wildsoet, 2005, Stone, 2008). Despite the many uncertainties, the hypothesis of retina control underlies much contemporary research on refractive development. While many contemporary investigations address the scleral responses in experimental ametropia, this review concentrates on retinal mechanisms.

Gene expression profiling and the challenge of understanding ametropia

Hypotheses long dominating the clinical literature unfortunately have provided few unambiguous insights and no clearly effective and clinically acceptable therapies to normalize the refractive development. The more recent introduction of modern biomedical methods to laboratory animal models of eye growth identified some retinal molecules that might potentially signal eye growth and introduced some additional hypotheses about the etiology of refractive error. Clearly lacking, however, is the broad mechanistic framework needed to inter-relate the various clinical and laboratory concepts and to address the clinical problem of refractive error in a practical manner that will lead to effective therapeutic approaches in children.

Altered gene expression characterizes many physiological and pathological conditions. Studies of gene expression are impacting research of many diseases, including disorders where the relative contributions of genetics and environment have been difficult to unravel (Hoheisel, 2006, Slonim, 2002, Strohman, 2002, Wilson, Hobbs, Speed & Rokoczy, 2002). Over the past decade, various gene profiling methods have been applied to the retina seeking mechanisms modulating refractive development. Many have used these methods chiefly to identify specific signaling molecules or proteins otherwise implicated in the regulation of eye size. From our perspective, though, the broader question and promise of studying retinal gene expression in laboratory models of eye growth is whether this approach can provide the conceptual mechanistic framework of refractive development that is both needed and lacking.

A few general caveats are appropriate in reviewing any report profiling gene expression, regardless of the particular method. Tissue isolation must be performed carefully and clearly specified because variable tissue content between samples can induce artifacts in gene expression that reflect tissue identity rather than the underlying biological process. Because methods for gene expression tend to be labor-intensive and/or expensive, pooling of tissues may seemingly offer certain advantages. However, pooling of tissues also reduces statistical power and can confound the results from increased variability and/or unrecognizable outliers in a pooled sample (Peng, Wood, Blalock, Chen, Landfield & Stormberg, 2003). Pooling can be useful if the biological differences are high compared to the technical variation in experiments (Kendziorski, Zhang, Lan & Attie, 2003); but this is not typically the case in the refractive studies discussed below. Finally, profiling methods permit assaying large numbers of genes, and appropriate bioinformatics methods are needed to control for multiple testing/replicate measures and to assess the results in ways that are both biologically and statistically meaningful (Benjamini & Hochberg, 1995, Breitling, 2006, Bretz, Landgrebe & Brunner, 2005, Cui & Churchill, 2003, Ness, 2006).

Another general caveat reflects the inherent cellular complexity of the retina itself and whether it is isolated with or without the retinal pigment epithelium (RPE). Changes in gene expression in a single subtype or small subset of retinal neurons may be masked by stable

expression, reciprocal changes or high variability in other retinal neurons. In addition, a number of the investigations of "retinal" gene expression in experimental models of eye growth include RNA from choroid or other unspecified eye tissues, thereby increasing the challenge of identifying mechanistically informative changes in retinal gene expression.

Conducted mostly but not exclusively in chick, earlier methods to identify changes in retinal gene expression included differential display (Feldkaemper, Wang & Schaeffel, 2000, Fujii, Escaño, Ishibashi, Fujii, Sekiya, Yamamoto & Saijoh, 2000, Morgan, Kucharski, Krongkaew, Firth, Megaw & Maleszka, 2004), subtractive hybridization (Ishibashi, Fujii, Escaño, Sekiya & Yamamoto, 2000, Tkatchenko et al., 2006) as well as candidate gene approaches (Akamatsu, Fujii, Escaño, Ishibashi, Sekiya & Yamamoto, 2001, Bhat, Rayner, Chau & Ariyasu, 2004, Buck et al., 2004, Escaño, Fujii, Ishibashi, Sekiya & Yamamoto, 1999, Escaño, Fujii, Sekiya, Yamamoto & Negi, 2000, Feldkaemper et al., 2000, Fujii, Honda, Sekiya, Yamasaki, Yamamoto & Saijoh, 1998, Honda et al., 1996, Ohngemach, Buck, Simon, Schaeffel & Feldkaemper, 2004, Seko, Shimokawa & Tokoro, 1996). Among the genes identified in these molecular studies that seem relevant to potential signaling pathway(s) are sonic hedgehog (Akamatsu et al., 2001, Escaño et al., 2000), bone morphogenetic proteins (Escaño et al., 1999), neuroendocrine specific-proteins A and B (Fujii et al., 2000), glucagon (Buck et al., 2004, Feldkaemper et al., 2000), vasoactive intestinal peptide (Tkatchenko et al., 2006), retinoic acid receptor-α/β (Bitzer, Feldkaemper & Schaeffel, 2000, Morgan et al., 2004, Seko et al., 1996), transforming growth factor-β (Honda et al., 1996, Simon, Feldkaemper, Bitzer, Ohngemach & Schaeffel, 2004) and inducible nitric oxide synthase (Fujii et al., 1998). Importantly, vasoactive intestinal peptide (Pickett Seltner & Stell, 1995, Stone et al., 1988), transforming growth factor-β (Rohrer & Stell, 1994), glucagon (Feldkaemper & Schaeffel, 2002), nitric oxide (Nickla & Wildsoet, 2004) and retinoic acid (Mertz & Wallman, 2000, Seko et al., 1998) each have been implicated independently as potentially mediating eye growth control with biochemical, histochemical and/or pharmacological methods. Following initial immunohistochemical findings (Fischer et al., 1999a), defocus was found to influence mRNA expression levels of the immediate-early gene ZENK (Simon et al., 2004). These approaches to studying retinal gene expression have used different eye growth models and/or tissues. While providing potentially informative individual genes, these studies have not yet revealed an overall conceptual framework for the signaling cascade(s) that regulate ocular growth or cellular pathway(s) through which retinal activity modulates scleral growth and refraction.

Microarrays and profiling gene expression to understand refractive development

More recently introduced, microarrays are powerful, versatile tools to study mRNA expression (Churchill, 2002, Hegde, Qi, Abernathy, Gay, Dharap, Gaspard, Hughes, Snesrud, Lee & Quackenbush, 2000, Hess, Zhang, Baggerly, Stivers & Coombes, 2001, Quackenbush, 2001, Schulze & Downward, 2001). Microarrays can be made on glass slides or membranes with thousands of spots, each a DNA sequence of interest. Imaging the hybridization of tissue-derived fluorescent labeled nucleotides to these DNA spots identifies the presence of specific mRNAs and permits estimates of relative mRNA abundances. Microarray methods allow parallel and simultaneous screening for multiple genes, tens of thousands on genome-wide chips now available commercially, with excellent sensitivity, high throughput and with limited amounts of biological material. They can identify expression of individual genes or expression patterns of multiple genes, as well as compare multiple samples. Commercial microarray platforms, such as Affymetrix oligonucleotide GeneChips, are widely used because they can provide broad genome-wide coverage and have full annotation of known genes (obviating the need to sequence informative probes); but they are expensive. Custom spotted arrays have the advantages of flexible design to the

condition under study and improved assessment of genes that have not yet been identified or included into genome databases and lower cost. Custom spotting of microarrays, though, may yield greater variability than commercial production methods (Members of the Toxicogenomics Research Consortium, 2005, Ness, 2006, White & Salamonsen, 2005). Variability can be an important consideration because it can potentially mask small fold changes.

A few laboratories have now adapted microarray technology to investigate the regulation of refractive development, as described below. The justifications for this approach are the ideas that visual stimuli altering eye growth induce transcriptome level changes in the retina and that these molecular signatures can identify not only individual retinal mediators of refractive development but also may provide the overview to formulate useful and testable mechanistic hypotheses for the laboratory and hopefully the clinic.

While it is impractical to validate every gene on the long lists usually resulting from microarray profiling, some independent biological replicates with independent methods are essential to provide an assurance of the reliability of the lists of differentially expressed genes. A subset of genes for validation commonly are selected for biological interest or for pronounced change in expression, and their altered expression is frequently confirmed using qPCR (real-time quantitative reverse transcription-polymerase chain reaction).

Microarrays in eye growth models in chick

Retinal profiling of form-deprivation myopia in the chick

The chicken genome was sequenced relatively recently (http://www.chicken-genome.org/resources/databases.html, International Chicken Polymorphism Map Consortium, 2004, Wallis, Aerts, Groenen, Crooljmans, Layman, Graves, Scheer, Kremitzki, Fedele, Mudd & et al., 2004), and Affymetrix (Affymetrix, Santa Clara, CA) used this resource to introduce Chicken GeneChips (http://www.affymetrix.com/products/arrays/specific/chicken.affx). We initiated our own profiling experiments studying form deprivation myopia in chick and used Affymetrix Chicken GeneChips. Form deprivation induces a very rapid myopic response in newly hatched chicks (Stone, 1997, Wallman, 1993). Because it has the largest existing database on pharmacology and signaling mechanisms among eye growth models (Stone, 2008), we believe form-deprivation myopia in chick can provide a productive biological model for genome-wide profiling techniques.

We induced unilateral form-deprivation myopia in week-old white Leghorn chicks and used Affymetrix oligonucleotide chicken GeneChips to profile the combined retina/RPE after 6 hours or 3 days of unilateral goggle wear and emphasized within-bird, experimental-to-contralateral eye statistical comparisons (McGlinn, Baldwin, Tobias, Budak, Khurana & Stone, 2007). Despite the possibility of contralateral effects in chick eye growth models (Stone et al., 2003, Wildsoet & Wallman, 1995), inter-bird comparisons complicate the statistical approaches to gene profiling because of potential spurious differences between birds; inclusion of cohorts of birds with bilaterally “intact” visual input also raises the cost of GeneChips and reagents if individual rather than pooled eyes are to be studied. Goggle or minus lens wear for 3 hours is sufficient to cause changes in choroidal thickness and subsequent scleral proteoglycan synthesis (Kee, Marzani & Wallman, 2001), an index of altered scleral growth; and plus lens wear for 6 hours is also sufficient to alter subsequent changes in choroidal thickness (Zhu, Park, Winawer & Wallman, 2005, Zhu & Wallman, 2009) and scleral proteoglycan synthesis (Kee et al., 2001). Potential alterations in diurnal rhythms also seem to influence refractive development (Stone, 2008), and the 6 hour time precludes a full intervening diurnal cycle. The 3 day time is adequate for chick eyes to

manifest the growth and refractive effects of goggle and lens wear (Irving, Callender & Sivak, 1991, Kee et al., 2001), and it thus permits characterizing gene expression in established myopia. The 3-day sampling time hopefully minimized more marked secondary effects that might occur with longer-term visual manipulations and more pronounced anatomical growth. Thus, the 6 hour sampling point is a rational profiling time to identify genes associated with the onset of myopia, and 3 days is a reasonable time to sample established myopia. Because of the robust myopic response, we had expected that goggle wear in chicks would induce changes of large magnitude in many genes.

Contrary to these expectations, we found quite small changes in retinal gene expression in form-deprivation myopia despite the representation of over 32,000 genes on Affymetrix chicken GeneChips (McGlinn et al., 2007). Statistically, we analyzed the normalized microarray signal intensities by the Significance Analysis of Microarrays approach (SAM) (Tusher, Tibshirani & Chu, 2001), on using a ≥1.2 fold-change filter, a two-class paired design comparing each goggled eye with its contralateral control eye, and a SAM false discovery rate arbitrarily set at 13% for each time. For goggled to contralateral eye comparisons, the maximum/minimum fold changes in specific gene expression were +1.36/−2.16 after 6 hrs and +1.55/−2.18 after 3 days of goggle wear. Only 15 genes for 6 hours of visual deprivation and a list of 280 genes for 3 days of visual deprivation met these criteria (Figure 1, Table 1) (McGlinn et al., 2007). Most of these genes were found to be differentially expressed at just one of the two sampling times, and only 7 genes were identified as differentially expressed at both times.

Retinal profiling after imposed defocus in the chick

Form deprivation and lens-induced eye growth models, both of which result in enlarged myopic eyes, share similar pharmacological responses at least in terms of available data. Reduced daytime retinal dopamine metabolism develops in both models (Guo, Sivak, Callender & Diehl-Jones, 1995, Schaeffel, Bartmann, Hagel & Zrenner, 1995, Stone et al., 1989); both goggle and (minus lens)-induced myopia are inhibited by local application of the dopamine agonist apomorphine (Schmid & Wildsoet, 2004, Stone et al., 1989), the muscarinic antagonist atropine (Schmid & Wildsoet, 2004, Stone et al., 1991) or $GABA_C$ antagonists (Chebib et al., 2009, Stone et al., 2003). Despite these similarities, lens-induced myopia differs mechanistically from form deprivation myopia not only in the method of perturbing visual input, but also in the electroretinogram (Fujikado, Kawasaki, Suzuki, Ohmi & Tano, 1997), in temporal characteristics of the response, in the effects of altered lighting (Kee et al., 2001).

Following 24 hours of binocular spectacle lens wear of +6.9 diopters OU, 123 retinal transcripts were found to be differentially expressed (6% false discovery rate; $p<0.5$; ≥ 1.5 fold-change in either direction) compared to untreated control chicks, also using the Affymetrix Chicken GeneChip platform (Table 1) (Schippert, Schaeffel & Feldkaemper, 2008). Other methods had previously implicated two of these differentially expressed genes following positive lens wear: glucagon and the immediate early gene ZENK (ERG1). Sixteen of the identified genes also were tested by qPCR, and nine of these were confirmed to be differentially expressed. These nine genes were also assayed by qPCR in retinas from chicks reared under two other conditions: 4 hours of +6.9 diopter spectacle lens wear, and 24 hours of −7 diopter spectacle lens wear. None of these genes were found to be altered after only 4 hours of plus spectacle lens wear, despite all of them being differentially expressed after 24 hours. Comparing the +6.9 to −7 diopter lens after 24 hours of wear, two patterns of differentially expressed genes were identified. In one pattern, 6 genes were up- or down-regulated in the same direction with either the plus or minus lens. In the other pattern, three of the differentially expressed genes following plus lens wear were not affected following minus lens wear.

Three differentially expressed genes in form deprivation myopia (McGlinn et al., 2007) were also affected following plus lens wear (Schippert et al., 2008). Perhaps the most interesting is prepro-urotensin II-related peptide, the precursor to the recently discovered biologically active peptide urotensin II-related peptide (URP) (McGlinn et al., 2007). Prepro-URP was down-regulated in both conditions. Biochemically similar to urotensin II, URP activates the urotensin II receptor and, among other effects, may stimulate growth signaling pathways (see McGlinn et al., 2007). Of the other two common genes, LOC424393 (homolog to the human BAT2 domain containing 1) was upregulated in both conditions; and the expressed sequence tag (ChEST955o8) was up-regulated following goggle wear but was down regulated following plus lens wear.

Recovery from myopia

If non-restricted visual input is restored to young chicks after myopia induction, the eye "recovers" from myopia with slowed growth and resultant emmetropia (Wallman & Adams, 1987). Optically, this paradigm is equivalent to placing a plus lens in front of an eye; but it differs biologically from a simple imposed defocus experiment because the eye is myopic at the initiation of "recovery." Following 10 days of monocular goggle wear to induce form deprivation, goggles were removed and combined retina/RPE/choroid preparations were assayed by a 4000 gene chicken immune system glass slide microarray after either one or four days of recovery (Rada & Wiechmann, 2009). Samples were pooled for microarray analysis, and a fold-change ≥ 2.5 of the pooled specimens was the parameter used to identify differentially expressed genes, with no evident statistical analysis described. After one day of recovery, only one gene was differentially expressed, avian thymic hormone at a +12.3 fold-change increase. After 4 days of recovery, 1 gene was up-regulated and 10 genes were down-regulated, by the authors' 2.5 fold-change criterion (Rada & Wiechmann, 2009). None of these genes overlap with those identified in form-deprivation myopia (McGlinn et al., 2007) or growth inhibition from plus lens wear (Schippert et al., 2008).

Microarrays in mammalian models of eye growth

Degrading image contrast and retinal gene expression in mice

To identify retinal genes influenced by the altered visual conditions affecting eye growth, the transcriptome of neurosensory retina without RPE was analyzed with Affymetrix GeneChip Mouse Genome 430 2.0 arrays after unilateral retinal image degradation by frosted goggles in mice (Brand, Schaeffel & Feldkaemper, 2007). Three mice were evaluated after one of three times of visual degradation: 30 minutes, 4 hours, and an extended course of two periods of 6 hours separated by a 12-hour dark phase. Compared to contralateral eyes, no differentially expressed genes were identified when the data were evaluated by the Benjamini/Hochberg method (Benjamini & Hochberg, 1995) to establish a false discovery rate. Instead, the authors applied paired t-test's on individual animals, without stated correction for multiple comparisons, and used a p-value of < 0.05 and a fold-change ≥1.5 as criteria for differentially expressed genes. With these criteria, the expression of 16 genes was altered after 30 minutes (13 up-regulated, 3 down-regulated); 27 genes, after four hours (4 up-regulated, 23 down-regulated); and 21 genes after more extended visual deprivation (10 up-regulated, 11 down-regulated). Fold-changes in these identified genes were low, with both up- and down-regulated genes falling in the 1.50–2.27 fold-change range. Another significant finding is that identified genes were largely different after the different periods of visual degradation, with the exception of the down-regulation of the early growth response 1 (Erg-1) gene.

Eye growth and expression profiling in *Egr-1* knockout mice

In the chick retina, the transcription factor ZENK is upregulated in conditions that reduce eye growth and downregulated in conditions that stimulate eye growth (Bitzer & Schaeffel, 2002, Fischer et al., 1999a), although there is some inconsistency in this bidirectional response (Schippert, Schaeffel & Feldkaemper, 2009). The mammalian othologue to ZENK, *Egr-1*, is downregulated in the retina of mice wearing a diffuser of the sort that induces form deprivation myopia in other species (Brand, Burkhardt, Schaeffel, Choi & Feldkaemper, 2005). Analogous patterns of *Egr-1* expression have also been observed in subpopulations of retinal neurons of rhesus monkeys under conditions that optically modify eye growth (Zhong, Ge, Smith & Stell, 2004). In an investigation of a potential role for this transcription factor, the refractions of homozygous *Egr-1* knockout mice were some 4–5 diopters less hyperopic (but without true myopia) relative to the wild-type (Schippert, Burkhardt, Feldkaemper & Schaeffel, 2007). The eyes of the *Egr-1* knockout mice were only transiently elongated at 42 and 56 days compared to wild-type mice, after which the axial lengths were comparable despite the persistent reduced hyperopia in the knockout mice. By optical modeling, changes in the properties of the mouse crystalline lens seemingly contribute to the reduced hyperopia. These results further suggest that activation of the transcription factor *Egr-1* (ZENK, in chick) may inhibit eye growth and that its suppression may stimulate eye growth. While *Egr-1* functions in a variety of physiologic processes and influences many genes (Schippert et al., 2009), the downstream messengers for regulating eye growth are as yet not clearly elaborated.

To identify genes that might be involved in this transient eye growth effect, microarray analysis of retinal gene expression was assessed in homozygous *Egr-1* knockout mice at 30 and 42 days of age, corresponding to the ages before and during the time of enhanced axial eye growth (Schippert et al., 2009). Using a false discovery rate of 5% and fold-change of at least 1.5, 73 differentially expressed genes were identified at 30 days (34 downregulated genes, maximum fold-change = −2.55; 39 upregulated genes, maximum fold change = +4.10), and 135 differentially expressed genes were identified at 42 days (22 downregulated genes, maximum fold-change = −2.88, except for one gene at −17.48; 113 upregulated genes, maximum fold change = +2.73), compared to wild-type mice. Only 13 altered genes were common to both gene lists, and 12 of these were differentially expressed in the same direction at each time.

Comparing the retinal gene expression at 42 days versus 30 days within the two groups of mice, 215 genes were differentially expressed in the older knockout mice compared to the younger knockout mice (39 downregulated genes, maximum fold-change = −4.01; 176 upregulated genes, maximum fold-change = +2.49); and 54 genes were differentially expressed in the older wild-type mice compared to the younger wild-type mice (17 downregulated genes, maximum fold-change = −2.40; 37 upregulated genes, maximum fold change = +2.62). Only 8 common genes were differentially expressed at the two ages in knockout and wild-type mice, and these genes were altered in the same direction in both types of mice.

This report emphasized comparisons between 30 and 42 days of age in the knockout and wild-type mice. It is unclear whether the differential gene expression between 30 and 42 days could account for the small, transient difference in eye growth comparing the *Erg-1* knockout and wild-type mice (Schippert et al., 2007, Schippert et al., 2009). Considering all differentially expressed genes in this study, the mean old-change was 1.48±0.41, consistent with the modest changes in gene expression identified in most other investigations described here. While the authors did not define specific roles for the differentially expressed genes in the *Erg-1* knockout mice, they do emphasize the potential value of microarray technology in identifying novel signaling candidates (Schippert et al., 2009).

Spectacle lens effects in marmosets

Comparative gene expression was studied in the choroid/RPE in 4 marmosets wearing a +5 diopter spectacle lens over one eye and a −5 diopter spectacle lens over the contralateral eye for an extended period of 92 days (Shelton, Troilo, Lerner, Gusev, Brackett & Rada, 2008). Given this sampling time, the identified genes reflect well-established growth differences. Human 12K cDNA plastic arrays were used. Based on a p-value ≤0.05 from the Student's t-test without stated correction for multiple comparisons, 204 genes were differentially expressed in minus-lens wearing eyes compared to plus-lens wearing eyes,183 genes being up-regulated and 21 genes being down-regulated. The magnitude of the fold-changes varied considerably between genes: for up-regulated genes, the fold changes were found to be in the 1.73–134.26 range; for down-regulated genes, in the 0.07–25.00 range. While assaying choroid/RPE and not the sensory retina proper, the study identified many cell receptors, signaling molecules and other potentially informative proteins.

Form-deprivation myopia in the primate

After unilateral lid fusion for approximately 2–4 months as a means to induce form deprivation myopia in rhesus and green monkeys, the retinal gene expression in the closed and contralateral open eyes were evaluated by cDNA subtractions to create cDNA libraries of potentially differentially expressed retinal genes; selected sequenced cDNAs were spotted onto glass slides for use as custom arrays (Tkatchenko et al., 2006). These custom arrays were screened using mRNA from rhesus and green monkeys with unilateral eyelid fusion that had not been used in generating the arrays. A total of 119 genes were found to be differentially expressed, some of which correlated positively and others of which correlated negatively with axial elongation. One of the identified genes, that for vasoactive intestinal peptide (VIP), had previously been implicated in experimental primate form-deprivation myopia by immunohistochemistry (Stone et al., 1988) and also has been implicated in form-deprivation myopia of the chick (McGlinn et al., 2007, Pickett Seltner & Stell, 1995).

Some 69% of differentially expressed genes in this study were involved in cell proliferation and nucleic acid metabolism, based on Gene Ontology classifications. Follow-up investigations to the arrays revealed that the periphery of the primate retina contains mitotically active neuroprogenitor cells and that the number of these cells increases in proportion to the increased depth of the vitreous chamber. The role of these proliferating retinal neurons in myopic eye growth requires further research, but the authors suggest that VIP might stimulate the proliferation of these neuroprogenitor cells (Tkatchenko et al., 2006). These findings also provide an excellent illustration of how critical analysis of gene expression profiling data can lead to novel biological insights - in this case, the proliferation of retinal cells in experimental primate myopia.

DISCUSSION

General methodologic considerations

Only a limited number of microarray studies have been described in full publications so far; and these differ in profiling platform (Affymetrix GeneChips, specialized arrays or custom arrays), species (chick or mammals), experimental approach to perturb normal refractive development and sampling time. Importantly, gene expression platforms compare differences in RNA abundance and are not suited to the direct study of post-transcriptional or post-translation modifications, e.g., activation of receptor tyrosine kinases by tyrosine phosphorylation (Schlessinger, 2000), which may be central to the signaling pathways governing eye growth control. For example, retinal dopamine has been previously implicated in refractive development (Stone, 2008, Stone et al., 1989), and post-translational

protein phosphorylation controls the activity of the rate limiting enzyme in its synthesis (Fujisawa & Okuno, 2005).

Analysis of microarray results requires complex statistics, suited to both the underlying design of the biological experiments and to the many technical complexities of microarray hybridization. Some of the analytical issues include the needs to normalize output signals, to adapt statistical approaches suited for the large number of data points derived from a quite limited number of biological samples and the inclusion of biological (as opposed to technical) validations of the statistical results. In this context, designing biological experiments from the outset with bioinformatics input can insure future suitability of stringent, independently recommended statistical approaches to develop a useful list of differentially expressed genes, including those at low expression levels, with techniques that emphasize controlling the false discovery rate (Allison, Cui, Page & Sabripour, 2005, Benjamini & Hochberg, 1995, Breitling, 2006, Bretz et al., 2005, McLachlan, Do & Ambroise, 2004, Tusher et al., 2001).

To insure that microarray experiments can be properly interpreted and verified independently, the Minimum Information About a Microarray Experiment (MIAME) standard has been developed (Brazma, Hingamp, Quackenbush, Sherlock, Spellman, Stoeckert, Aach, Ansorge, Ball, Causton, Gaasterland, Glenisson, Holstege, Kim, Markowitz, Matese, Parkinson, Robinson, Sarkans, Schulze-Kremer, Stewart, Taylor, Vilo & Vingron, 2001, Burgoon, 2006). Depositing primary data in publicly available databases, such as the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), also is recommended for efficient dissemination of data. Public access allows future exploration of past data as methods evolve and permits detailed comparisons of expression data between studies to resolve differences that can occur between expression profiling, for instance, with different species and experimental paradigms.

The interested reader is advised that not all published gene profiling applications to refractive development have adhered to this widely accepted MIAME standard and that the primary data for a number of the studies are not accessible in public databases such as GEO that would enable the interested reader to perform direct comparisons between studies. In fact, only three of the now available profiling studies of refractive development (McGlinn et al., 2007, Schippert et al., 2009, Tkatchenko et al., 2006) can be accessed in GEO at the present time (Accession Numbers: GSE3300, GSE6543 & GSE16974). Adhering to these recognized approaches should be a goal in designing future microarray experiments addressing refractive development.

Low fold-changes in most identified genes

Particularly for genome-wide profiling of eye growth models in chick, most altered genes in retina/RPE (McGlinn et al., 2007) or retina (Schippert et al., 2008) are differentially expressed at low fold-change level (Table 1). While not unique to refractive perturbations in the chick, the low fold-changes intuitively seem surprising because of the robust eye growth response to either goggle or spectacle lens wear. In mice under experimental conditions seeing insights into refractive development, microarray profiling also has identified low fold-changes of the differentially expressed retinal genes (Brand et al., 2007, Schippert et al., 2009). Commonly in microarray experiments, an arbitrary fold change of 2.0 is selected for future analysis, but most of the statistically changed genes in the genome-wide chick studies fell below that level. Low fold-changes create challenges for designing independent biological validations of the profiling results, but they also put high demands on investigators in the biological and statistical design of experiments to achieve informative and mechanistically meaningful results. High fold changes have been detected in some studies of chick (Rada & Wiechmann, 2009) or marmoset (Shelton et al., 2008). However,

the identified genes have not been detected in other studies of chick. For the marmoset data, the animals were reared with a binocular plus/minus defocus paradigm not generally used in other studies, the experiment extended for several months, and the possibility of species differences cannot be excluded.

Gene expression and the duration of altered visual input

In the genome-wide profiling studies in chick, the earliest times assayed were at either 4 hours (Schippert et al., 2008) or 6 hours (McGlinn et al., 2007) of altered visual input. As described above (see "Retinal profiling after imposed defocus in the chick"), the early time points in these microarray studies should reveal retinal genes involved in the onset of the growth response to visual perturbation in the chick. Because of the manifest growth changes after 3 days of visual deprivation in chick (Kee et al., 2001), gene changes at 3 days in chick or at later times in mammalian experiments should reflect more established growth responses to altered visual input.

Whether viewed in terms of the results of individual studies that included multiple time points or in terms of comparisons between studies with different time points, the identity of differentially expressed retinal genes is highly dependent upon the duration of altered visual experience; and only a small minority of genes seem to be common to multiple times. To illustrate from this conclusion from two studies in chick, unilateral goggle wear for 6 hours (Figure 1) resulted in altered gene expression in 15 retinal/RPE genes at 6 hours, 280 genes at 3 days and only 7 genes common to both times compared to the contralateral eye (McGlinn et al., 2007). Twenty four hours of bilateral +6.9 diopter lens wear for 24 hours induced changes in the expression of 123 retinal genes compared to a control group (Schippert et al., 2008); 9 of 16 selected genes could be validated by qPCR. None of these latter 9 genes were found to be differentially expressed after +6.9 diopter lens wear for 24 hours, confirming differences in these genes between 4 and 24 hours of defocus from plus lenses.

Interpreting these temporal patterns is speculative at present. As examples, they could represent detection genes responding to blur or defocus, reflect retinal adaptations to continuing visual distortions and/or identify changes in growth signaling mechanisms as refractive development progresses. Even in wild-type mice, retinal gene expression varies between two time points in early development (Schippert et al., 2009). Resolving the basis of the temporal changes in gene expression likely will provide important clues to understand both normal eye development and the pathogenesis of refractive errors. As a potentially useful hypothesis, the genes responsible for the onset of eye growth disturbances may differ at least in part from those responsible for their progression. From a clinical perspective, it would seem essential to learn if and how the mechanisms underlying the onset of myopia or hyperopia parallel or diverge from those mechanisms underlying their progression.

The presumed genetic-environmental interaction in clinical myopia

Genetics—Particularly for myopia pathogenesis, the clinical literature has long asserted mechanisms involving the interactions of heredity and environment. While modern genetic studies have linked human myopia to various chromosomal locations and identified some candidate genes (Hornbeak & Young, 2009), the extent to which purely genetic contributions contribute to refractive error development has remained controversial (Morgan & Rose, 2005). A widely discussed and plausible hypothesis is that genes contributing to myopia may prove to be susceptibility genes to environmental factors, rather than being causative genes *per se*. The experimental models of eye growth all use an environmental manipulation (i.e., modification of visual input) to induce quite robust shifts in refractive development. Particularly in the genome-wide microarray experiments in chick that have

used the more stringent statistical analytical approaches (McGlinn et al., 2007, Schippert et al., 2008), the modest fold-changes and limited number of differentially expressed genes suggest that major changes in retinal gene expression may not be necessary to alter refractive development after environmental perturbations. Thus, polymorphisms in specific human genes with only mild effects on the activity of protein products may account for the environmental effects on refractive development.

Besides such general hypotheses about how gene variations might contribute to refractive error development in children, several microarray studies have identified genes that can be considered candidate genes for future investigation because they lie within chromosomal intervals linked to human myopia (McGlinn et al., 2007, Tkatchenko et al., 2006). Reviewed elsewhere (Stone, 2008, Wallman & Winawer, 2004), available results imply a broad phylogenetic conservation of the neural mechanisms regulating eye growth and refractive development, and translational research is demonstrating potential clinical relevance of many laboratory findings. Thus, gene expression profiling data from experimental animals can provide valuable potential leads for clinical genetics.

Environment—The clinical literature, accumulating over centuries, has sought the environmental parameters that account for the onset and/or progression of refractive errors. Despite these efforts, modern clinical investigations have generally found that conventional clinical ideas at best account for only a small fraction of the refractive variability within populations (Stone, 2008). Laboratory investigations are pointing to some leads for potential environmental parameters possibly influencing refractive development. As one example, photoperiod has long been known to influence profoundly eye development in the chick (Jensen & Matson, 1957, Lauber & McGinnis, 1966, Li, Troilo, Glasser & Howland, 1995, Stone, Lin, Desai & Capehart, 1995, Stone, Lin, Sugimoto, Capehart, Maguire & Schmid, 2001a). Altered photoperiod has only minor effects on refractive development in monkey, though the extent to which these represent inter-animal variability in small experimental series is unclear (Smith, Bradley, Fernandes, Hung & Boothe, 2001, Smith, Hung, Kee, Qiao-Grider & Ramamirtham, 2003). The basis of these effects on refractive development may reflect the involvement of retinal dopamine in refractive development in chicks and monkeys, given dopamine's role in linking intra-retinal rhythms to the light:dark cycle (Iuvone, Tigges, Stone, Lambert & Laties, 1991, Iuvone, Tosini, Pozdeyev, Haque, Klein & Chaurasia, 2005, Stone, 1997, Stone, 2008, Stone et al., 1989). Accumulating association studies in children now also suggest that photoperiod or some other aspect of light exposure may influence refractive development in children (Dirani, Tong, Gazzard, Zhuang, Chia, Young, Rose, Mitchell & Saw, 2009, Loman, Quinn, Kamoun, Ying, Maguire, Hudesman & Stone, 2002, Mandel, Grotto, El-Yaniv, Belkin, Israeli, Polat & Bartov, 2008, McMahon, Zayats, Chen, Prashar, Williams & Guggenheim, 2009, Quinn, Shin, Maguire & Stone, 1999, Rose, Morgan, Ip, Kifley, Huynh, Smith & Mitchell, 2008, Vannas, Ying, Stone, Maguire, Jormanainen & Tervo, 2003). Whether these findings relate to light exposure *per se*, light intensity (Ashby, Ohlendorf & Schaeffel, 2009) or light effects on diurnal or circadian rhythms, for instance, is presently unclear. As a second example, nicotinic receptor activity affects refractive development in chick (Stone et al., 2001b); and clinical association studies have found relationships between refractive distributions and passive exposure to cigarette smoking (Saw, Chia, Lindstrom, Tan & Stone, 2004, Stone, Wilson, Ying, Liu, Criss, Orlow, Lindstrom & Quinn, 2006). Whether the association with cigarette smoke derives from nicotine or some other component of tobacco smoke is not known.

Remarkably, some data suggest that the effects of light and/or tobacco smoke may be acting in the perinatal period in children (Fotedar, Mitchell, Burlutsky, Rose, Morgan, Wang & Study, 2008, Mandel et al., 2008, Quinn et al., 1999, Stone, 2008, Stone et al., 2006). These evolving findings suggest that environmental factors acting during very early childhood or

even during fetal life may have persistent effects on eye development that require years to become manifest. The notion of that environmental effects on pregnant women or during early childhood could effect refraction later in life is an inadequately explored clinical area but seems promising. These ideas are presently controversial, and designing clinical research besides association studies to address these relationships is both challenging and difficult.

Because they provide such broad biological sampling, microarrays could contribute to hypotheses about potential environmental effects on refractive development. While they have not made such contributions as yet speaks more to the limited number of studies and the methodological peculiarities of several of the available reports, rather than the potential of the method. For instance, implicating circadian genes in experimental models of eye growth could provide important justification to initiate experimental studies of circadian rhythms in children developing refractive errors.

Gene identities—In comparing eyes with experimentally altered refractive development to controls, differential gene expression could result from genes governing refractive development. Instead, though, altered genes also could result from the effects of image degradation on visual processing in the retina, from genes that relate to the growth of tissues that line the growing eye, or from genes mediating or responding to endogenous diurnal rhythms know to be affected at least in some eye growth models. Differences between reports in identified genes likely involve variations in microarray type, species, experimental model, duration of vision disruption, sampling methods, statistical criteria or other technical differences.

A large number of genes have been identified so far in retinal gene profiling studies, too many to review here individually. Based on our own research perspective of retinal signaling that might modulate eye growth, some of the more interesting include the genes for vasoactive intestinal peptide, bone morphogenetic protein 2, connective tissue growth factor, prepro-urotentsin II-related peptide and the urotensin 2 receptor, β1 $GABA_A$ receptor subunit, glucagon, growth factor receptor-bound protein 2, oxysterol-binding protein 2 and arginine vasopressin. Potential roles of some of these gene products are discussed in the original references (McGlinn et al., 2007, Schippert et al., 2008, Tkatchenko et al., 2006). Each protein product of these genes has the potential to influence visual signaling within the retina, but many also have known general effects on extracellular matrix proliferation, cell proliferation and tissue morphogenesis that might contribute refractive development. Thus, each needs further investigation in studies designed specifically to understand their biological role in refractive development.

The direction of gene changes vs. the direction of eye growth—A visual feedback regulatory mechanism is generally accepted as regulating eye growth (Wallman & Winawer, 2004). Of the widely used experimental models, goggle or minus spectacle wear may activate mechanisms stimulating eye growth and plus lens wear or goggle removal may activate mechanisms inhibiting eye growth that occur during the emmetropization process. Based on presently available microarray profiling, the patterns of altered gene expression do not clearly parallel the direction of eye growth in a simple manner. Comparisons are most easily performed within or between studies in chick, despite differences in sampled tissues (McGlinn et al., 2007, Schippert et al., 2008, Shelton et al., 2008). Here, the disparity between gene identifications in the various models is a predominant feature. Further arguing against simplicity in the signaling patterns regulating eye growth, some retinal genes are altered in the same direction while others are altered in the different direction following plus or minus lens wear (Schippert et al., 2008). Of the three differentially expressed genes common to both goggle and plus lens wear which would stimulate or inhibit eye growth respectively (McGlinn et al., 2007, Schippert et al., 2008), the expression of two genes is

changed the same direction in both conditions; and only the expression of only gene occurred in opposite directions. Similarly, 9 retinal genes validated by qPCR as differentially expressed following 24 hours of plus lens wear, six of these genes were altered in the same direction after 24 hours of minus lens wear; the other 3 genes were altered only following 24 hours of plus lens wear, not minus lens wear (Schippert et al., 2008). It is possible that retinal genes changing in the same direction regardless of whether growth is stimulated or inhibited may be involved in "priming" the regulatory pathway or otherwise influencing general adaptations to altered eye size. The limited number of genes so far identified that are differentially expressed in opposite directions under growth stimulatory and inhibitory conditions may indeed have roles in influencing the direction of eye growth. Such ideas are speculative, though, and require direct studies.

As a simplified hypothesis to understand the mechanism of eye growth control, a “stop-go signaling model” for eye growth control can be constructed that proposes that peptides (i.e., gene products) that inhibit eye growth (“stop” signal) will be elevated in conditions with slowed eye growth (e.g., + lens wear) and/or reduced in conditions with accelerated eye growth (e.g., goggle or − lens wear); and peptides that stimulate eye growth (“go” signal) will be elevated in conditions with accelerated eye growth and/or reduced in conditions with slowed eye growth.(Bertrand, Fritsch, Diether, Lambrou, Müller, Schaeffel, Schindler, Schmid, van Oostrum & Voshol, 2006, Feldkaemper & Schaeffel, 2002, Rohrer & Stell, 1994, Vessey, Rushforth & Stell, 2005b, Wallman, 1990) Besides the transcription factor *Erg-1* or ZENK (Bitzer & Schaeffel, 2002, Fischer et al., 1999a, Schippert et al., 2009), a number of peptides have been identified (e.g., glucagon, basic fibroblast growth factor and transforming growth factor-β) that may act in this capacity (Feldkaemper & Schaeffel, 2002, Rohrer & Stell, 1994, Vessey et al., 2005b). Because microarrays sample the transcriptome so broadly, the gene expression patterns in established eye growth models could be informative in pointing to molecules and/or pathways specifically implicated in either inhibiting or stimulating eye growth. The only available pertinent data are the limited comparisons in the retinal profiling of form deprivation myopia and imposed defocus in the chick, already discussed above (McGlinn et al., 2007, Schippert et al., 2008). Questions raised by these comparisons include the extent to which reciprocal or parallel transcriptome changes occur in conditions that inhibit or stimulate eye growth, the extent to which goggles and minus lens wear induce common transcriptome changes, the extent to which the transcriptome is altered independent of the direction of eye growth, which genes or pathways might be responses to alter eye growth and, most importantly, which genes or pathways might be responsible for the altered ocular development.

The potential of gene expression profiling

Besides the need for optical correction, the blinding ocular diseases associated with either myopia or hyperopia represent serious public health problems. The prevalence of myopia is high and increasing world-wide. Clinically acceptable therapies to normalize eye growth and refractive development in children have been long-sought but remain elusive. The absence of therapeutic approaches to normalize childhood refractive development in large part derives from our limited understanding of the biological mechanisms governing normal refractive development or the mechanisms responsible for ametropias. The need to understand these underlying control processes underlies the rationale for much contemporary refractive research.

While many hypotheses are being generated from by contemporary refractive research, the lack of a comprehensive mechanistic framework for understanding refractive development represents a major unmet need in the field. Microarray profiling is new tactic to understand both normal and abnormal refractive development. Most recent laboratory approaches to study refractive development have typically involved directed experiments addressing

specific mechanistic hypothesis. In contrast, microarrays can survey for genome-wide changes in gene expression in tissues involved in regulating ocular development. While the results are restricted to RNA changes and do not identify protein products or post-translational mechanisms, the broad sampling strategies offer the possibility of uncovering pathways or concepts not easily addressed in more directed initial experiments. Properly performed microarray experiments, however, are expensive, technically difficult and statistically challenging. Microarray profiling is just beginning to be applied to refractive development, and reconciling the available studies is in part limited by their varied experimental design and analytical/statistical features. We believe that continued application of these methods, as well as adoption of proteomic and metabolomic strategies in the future, offers promise to provide the much-needed mechanistic framework that can ultimately be translated into the clinical investigations and hopefully can lead to effective clinical approaches to normalize refractive development in children.

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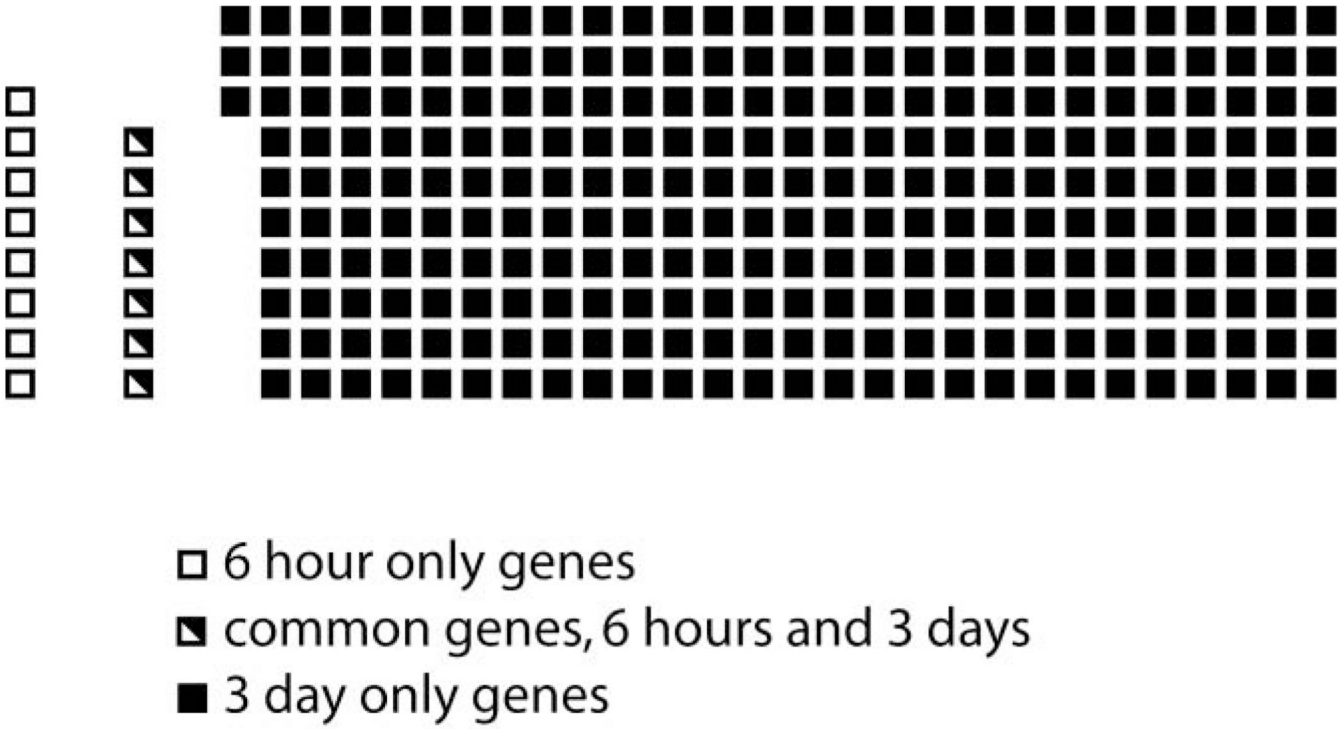

Figure 1. Differentially expressed genes in chick retina after two times of goggle wear

The differentially expressed genes are shown for 6 hours only (n=8 genes, left column), 3 days only (n=273 genes, right) and the overlap of the two times (n=7 genes, second column from left). Gene changes at myopia onset are modeled by the 6-hour time; those in established myopia, by the 3-day time. From McGlinn, et al. (McGlinn et al., 2007), copyright by the Association for Research in Vision and Ophthalmology.

Table 1

Summary of genome-wide microarray studies in chick.

Eye growth model	Tissue	Sampling time	Statistical Criteria	Mean fold-change: all genes *	Up-regulated genes *			Down-regulated genes *			GEO accession number **
					Fold-change		N	Fold-change		N	
					Mean	Maxi-mum		Mean	Maxi-mum		
Form-deprivation myopia, monocular (McGlinn, et al., 2007).	Retina/ RPE	6 hours	FDR 13%	1.35 ± 0.28	+ 1.25 ± 0.04	+1.36	13	− 2.06 ± 0.14	− 2.16	2	GSE 6543
Form-deprivation myopia, monocular (McGlinn, et al., 2007).	Retina/ RPE	3 days	FDR 13%	1.27 ± 0.09	+ 1.26 ± 0.06	+ 1.55	162	− 1.36 ± 0.28	− 2.18	18	GSE 6543
+ 6.9 diopter lens wear, binocular (Schippert, et al., 2008).	Retina	24 hours	FDR 6%	1.97 ± 1.16	+ 2.05 ± 1.36	+ 11.82	67	− 1.85 ± 0.86	− 7.70	56	GSE 11439

* Genes meeting statistical criteria as defined in each individual study; ± S.D.

** Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/)

N = number of differentially expressed genes