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Changes in retinal αB-crystallin (cryab) RNA transcript levels during periods of altered ocular growth in chickens

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ABSTRACT

Changes in retinal crystallin gene expression have been implicated in the development of myopia in animal models. We therefore investigated the expression of αB-crystallin (cryab) in the chicken retina during periods of increased ocular growth induced by form-deprivation and negative lens-wear, and during periods of decreased ocular growth induced by diffuser removal from previously form-deprived eyes, and plus lens-wear. Cryab RNA transcript levels in the chicken retina were measured using semi-quantitative real-time RT-PCR, at times between 1 h and 10 days after the fitting of diffusers or negative lenses, and at times between 1 h and 3 days following the removal of diffusers from previously form-deprived eyes, or the addition of plus lenses. Changes in expression for each condition at each time-point are analysed relative to expression in retinas from age-matched untreated control birds. No change in relative expression of cryab RNA transcript was detected 1 h after fitting diffusers to induce form-deprivation myopia. A transient increase in cryab RNA transcript expression was detected around 1 day later ($p = 0.02$), but expression returned to control levels after three days. After 7 ($p = 0.005$) and 10 ($p = 0.001$) days, retinal cryab RNA transcript expression progressively increased relative to controls. After removal of the diffusers, to initiate recovery, cryab RNA transcript expression remained elevated, with only a slight return to control levels. During the development of lens-induced myopia, no changes in cryab RNA transcript expression relative to controls were seen on day 1, but increases were seen at 10 days ($p = 0.004$). No significant changes in retinal cryab RNA transcript expression were seen in response to plus lenses compared to either contralateral control values (MANOVA; $F = 0.60$, $p = 0.48$) or age-matched untreated values (MANOVA; $F = 4.10$, $p = 0.08$). Changes in retinal cryab RNA transcript expression were not systematically related to changes in the rate of eye growth. The role of the transient increase in cryab expression observed after 1 day of form-deprivation, which was not seen after fitting negative lenses, is unclear. The later increases in relative cryab expression seen during the development of form-deprivation and lens-induced myopia occur too late to have a major role in the differential regulation of eye growth between experimental and control eyes. Given that cryab is a member of the small heat shock protein family, the later increases may reflect the emergence of cell damage related to high myopic pathology in the experimentally enlarged eyes and retina.

1. Introduction

Myopia has emerged as a major epidemic in urban East Asia over the past 3–4 decades, with the prevalence of myopia in those finishing secondary or high school now around 80–90%, while around 20% have high myopia (<−6 D) (Morgan and Rose, 2005). Considerable attention has been devoted to the relative contributions of genetic (Young et al., 2007) and environmental (Morgan and Rose, 2005) variation to differences in human refractive development, and their role in the emergence of this epidemic. The current evidence suggests that high myopia, at least in many parts of the world, may be of substantially genetic origin, whereas more moderate myopia may be more related to environmental exposures.

Animal models of experimental myopia provide a prime example of environmental impacts on eye growth (Irving et al., 1992; Schaeffel et al., 1988; Wallman et al., 1978), since they involve experimental manipulation of eye growth through form-deprivation, or the imposition of myopic or hyperopic defocus. Irrespective of the links between variation at the genomic level and variation at

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the phenotypic level, changes in gene expression in these paradigms must underpin the changes in eyes growth observed, providing insight into the cellular and molecular pathways involved, and possibly identifying candidate genes for involvement in human myopia. Perhaps the strongest evidence relates to the potential role of Egr-1 and glucagon (Feldkaemper and Schaeffel, 2002; Fischer et al., 1999), which show bi-directional changes related to increases or decreases in the rate of eye growth. But a number of other molecules have also been implicated (Brand et al., 2007; Frost and Norton, 2007; Ishibashi et al., 2000; Iuvone et al., 1989; McGlinn et al., 2007; Morgan et al., 2004; Ohngemach et al., 2004; Schippert et al., 2008; Stone et al., 1988, 1989).

In a differential gene display analysis, we previously reported evidence for changes in αB-crystallin (cryab) RNA transcript expression in the retina during the early stages of the development of FDM in the chicken (Morgan et al., 2004), which supports previous reports of changes in the expression of other crystallins in form-deprivation myopia (Ishibashi et al., 2000).

Cryab is a 175-amino-acid-long polypeptide and as a member of the small heat shock protein (sHSP) family, its expression can be induced by a variety of physiological stresses including heat stress, osmotic stress, pH extremes, metal toxicity, as well as other cell traumas (Klemenz et al., 1991; Dasgupta et al., 1992; Aoyama et al., 1993; Head et al., 1994; Ito et al., 1997; Kato et al., 1997). Work on lens epithelial cells has suggested that alpha crystallins may be involved in the regulation of cellular growth and genomic stability (Andley et al., 1998, 2001; Bai et al., 2003). Mutation and alterations in cryab expression have also been associated with a number of neurological diseases such as Creutzfeldt-Jacob disease, Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Iwaki et al., 1989, 1992; Renkawek et al., 1994; Sax and Piatigorsky, 1994; van Noort et al., 1995). Recent animal studies have suggested that cryab is involved in remodelling of damaged neurons and protecting against neuronal toxicity (Lowe et al., 1992; Smith et al., 2005). In the retina, stress, induced by intense light exposure or retinal tearing, up-regulates the expression of members of the α-, β- and γ-crystallin gene families (Sakaguchi et al., 2003; Schmeer et al., 2008; Vazquez-Chona et al., 2004).

These studies suggest that elevated levels of cryab may be a critical marker of neuronal stress or damage. We have now examined the expression of cryab during the regulation of refractive development of the eye, to determine whether retinal expression of cryab changes in a systematic way. We used two paradigms to promote the development of myopic refractive error – form-deprivation myopia (FDM) (Wallman et al., 1978; Wiesel and Raviola, 1977) and negative lens-induced myopia (LIM) (Irving et al., 1991; Schaeffel et al., 1988). We also used two paradigms to inhibit the growth of eye – one based on the removal of the diffusers after induction of FDM, when recovery from myopia is observed due to inhibition of axial elongation in the myopic eye (Wallman and Adams, 1987). The other involved fitting positive lenses, which induces lens-induced hyperopia (LIH), by inhibiting axial elongation in a normal eye (Irving et al., 1991; Schaeffel et al., 1988). Early changes in the expression of cryab RNA transcripts might indicate an involvement in the regulation of the rate of eye growth, whereas later changes, particularly in the growth-promoting paradigms, might indicate involvement in pathological responses.

2. Methods

2.1. Animal housing

One day-old male Australorp chickens were obtained from Barter & Sons Hatchery, Luddenham, NSW. Chickens were maintained in temperature-controlled rooms under a 12:12 h light/dark cycle, with incandescent illumination of ∼200 lux during the light phase, and <1 lux in the dark phase (lights on at 6am and off at 6pm). Chickens had access to unlimited amounts of food and water, and were given three days to become accustomed to their surroundings before experiments were started. All experiments were approved by the Australian National University Animal Experimentation Ethics Committee (Protocols R.VS.14.03 and R.VS.18.05) and conformed to the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research.

2.2. Experimental manipulation of refractive development

The methods used for induction of FDM, which results from increased axial elongation of the eye, have been previously described in detail (Ashby et al., 2007; McCarthy et al., 2007). Briefly, FDM was induced monocularly by fitting a translucent diffuser over the left eye of the chicken, using Velcro mounts. LIM, which also results from increased axial elongation of the eye, was induced by fitting −10 D PMMA lenses over the left eye, using similar Velcro mounts, while LIH was induced with +10 D PMMA lenses, using similar techniques. In all experiments, the right eye was untreated and acted as a contralateral control.

For the analysis of changes in gene expression during the development of FDM ($n = 90$) or LIM ($n = 90$), chickens were exposed monocularly to diffusers or negative lenses for periods of 1 h or 1, 3, 7 or 10 days. For examination of changes in gene expression during the development of LIH ($n = 48$) or recovery from FDM ($n = 54$) after 10 days of form-deprivation, chickens were followed for periods of 1 h, 1 or 3 days. For FDM, LIM and recovery from FDM, nine samples were collected, per experimental condition, at each time-point. Each sample contained two retinas from experimental eyes, one from each of two animals. For LIH, eight samples were collected per time-point. Retinas from contralateral eyes were also collected, and processed separately as internal contralateral controls. In parallel, pooled retinas were collected from age-matched untreated control animals ($n = 9$) at each time-point for all experimental conditions.

2.3. Tissue preparation

Chickens were euthanased with CO_2. Each eye was removed and hemisected equatorially, with the anterior portion of the eye and vitreous body discarded. The posterior eye cup was floated in chilled phosphate-buffered saline (NaCl, 137 mM; KCl, 2.7 mM; Na_2HPO_4, 11.3 mM; KH_2PO_4, 1.5 mM; pH 7.4), allowing collection of the retina free of RPE, choroidal and scleral tissue. Tissue was immediately frozen on dry ice then stored at −80 °C until use.

Preliminary experiments (Fig. 1) showed that there were no significant diurnal rhythms in the expression of cryab RNA transcript, sampling at 4 h intervals over a 24 h period, whereas rhythms in the expression of per 2 RNA transcript, similar to those previously reported (Yoshimura et al., 2000), were detected. This suggests that time of collection of the samples was not an important factor, but, as an additional precaution, all samples were collected between 12 noon and 2 pm.

2.4. Preparation of RNA and reverse transcription to cDNA

The methods used for the preparation of RNA, reverse transcription and quantification by real-time RT-PCR using the combined Trizol®/Qiagen RNeasy method have been previously described in detail (Ashby et al., 2007; Kucharski and Maleszka, 2005). Total RNA purity was checked using gel electrophoresis, and