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An analysis of anterior segment development in the chicken eye

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ABSTRACT

Precise anterior segment (AS) development in the vertebrate eye is essential for maintaining ocular health throughout life. Disruptions to genetic programs can lead to severe structural AS disorders at birth, while more subtle AS defects may disrupt the drainage of ocular fluids and cause dysregulation of intraocular pressure homeostasis, leading to progressive vision loss. To date, the mouse has served as the major model to study AS development and pathogenesis. Here we present an accurate histological atlas of chick AS formation throughout eye development, with a focus on the formation of drainage structures. We performed expression analyses for a panel of known AS disorder genes, and showed that chick PAX6 was localized to cells of neural retina and surface ectoderm derived structures, displaying remarkable similarity to the mouse. We provide a comparison to mouse and humans for chick AS developmental sequences and structures and confirm that AS development shares common features in all three species, although the main AS structures in the chick are developed prior to hatching. These features enable the unique experimental advantages inherent to chick embryos, and we therefore propose the chick as an appropriate additional model for AS development and disease.

1. Introduction

The vertebrate eye is a complex sensory organ that acts as an extension of the brain to provide visual detail of an organism's surroundings. It has adapted through evolution to enable different species to see in the dark, under water, over great distances, and across a variable spectrum of light wavelengths (Nilsson, 2013), reflecting species habitation in diverse ecological niches. Consequentially, although all vertebrates have camera eyes (composed of lens and retina), the precise structure of the eye varies with the requirements of the individual species.

The front of the vertebrate eye, described as the anterior segment (AS), contains transparent structures that collect and focus light (the lens and cornea), and muscular structures that facilitate this (iris and ciliary body). The anterior segment is lubricated throughout the life course by the aqueous humor, a fluid secreted by the ciliary body into the posterior chamber (the space posterior to the iris but anterior to the lens) which then flows through the pupil to the anterior chamber (the space between the iris and cornea). This humor is removed from the eye via outflow through intertrabecular spaces in the trabecular meshwork in the iridocorneal angle (at the recess between the iris-foot and cornea), into Schlemm's canal and from there into the venous system. The balance between secretion and drainage maintains intraocular pressure (IOP) homeostasis, which is essential for the healthy function of the eye (Fautsch et al., 2006). Malformation, injury, or obstruction to tissues in the AS and drainage structures can lead to raised IOP and subsequent damage to the retina and optic nerve, manifesting as glaucoma (Weinreb et al., 2014). Primary open angle glaucoma (POAG), the most common form, is a complex inherited trait defined by increased resistance to drainage through the trabecular meshwork. POAG may affect both humans and companion animals, with a significant burden on health and morbidity (Foster, 2002; Kanemaki et al., 2013). Primary angle closure glaucoma (PACG), involves narrowing of the iridocorneal angle through contact between the iris and trabecular meshwork. It is likely also a complex inherited trait and is more common in people living in Asia (Quigley and Broman, 2006; Vithana et al., 2012). A form of narrow or closed angle glaucoma is also seen in dogs, associated with goniodysgenesis, a congenital malformation of the pectinate ligament in the drainage angle (Mellersh, 2014).

Genome-wide association studies have been highly successful in identifying several POAG-associated loci in humans, and genetic linkage studies have implicated candidate genes within chromosomal regions in POAG affected families (Liu and Allingham, 2017; Allingham et al., 2009). In addition, genome-wide studies focused on patients of Asian ancestry have identified a number of loci associated with PACG (Vithana et al., 2012; Wiggs and Pasquale, 2017). Yet, the identification of precise causative alleles has been elusive using these approaches. In

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contrast, DNA sequencing efforts have identified specific single-gene causes for POAG (Allingham et al., 2009), however those variants so far identified only account for <10% of all glaucoma cases. Next generation sequencing technologies (whole exome and complete genome) may yet improve this situation (Liu and Allingham, 2017). These are clearly genetically heterogeneous and highly complex diseases, and human genetics approaches are currently limited in their ability to identify their direct causes.

Conditions that affect the structural development of the AS are generally described as anterior segment dysgenesis (ASD) disorders: a heterogeneous group of diseases including aniridia (absence of iris), iris hypoplasia (malformed iris structures), ectopia lentis (subluxation or dislocation of the lens), corneal opacity, congenital cataract, adhesions between the lens and iris or lens and cornea, abnormal drainage angle (including goniodysgenesis), endothelial dystrophia, sclerocornea and megalocornea. Although genetic causes of many ASD have been identified (reviewed in (Sowden, 2007)), many cases still lack a genetic diagnosis. Consequently, there remain major gaps in our understanding of the genetic and molecular mechanisms that guide the development of the AS and drainage structures. Understanding the precise contribution of POAG and PACG candidate genes to development and homeostasis of the AS also requires a thorough understanding of how these structures develop.

Studies of human AS development are limited due to specimen availability, with few reports available illustrating its complete development. The most comprehensive studies have been limited in sample numbers, and biased towards early eye development (McMenamin, 1989; O'Rahilly, 1966), and to date, Ida Mann's robust analysis of human AS development remains the exceptional authoritative reference (Mann, 1969). To augment these, detailed developmental analyses in mice have been performed (Smith et al., 2001), and the mouse has proved to be a useful genetic tool for functional analyses of some ASD genes (Sowden, 2007; Cvekl and Tamm, 2004). However, mouse anterior segments are small and their eyes are highly compact with a large lens and thick retina; for example see Fig. 1 of Tkatchenko et al. (2010). The mouse AS also develops over a long time period, ranging through embryonic stages to several weeks after birth (Hitchcock, 2009). Supportive additional model systems may help improve our understanding of ASD and glaucoma causation, and offer inherent and unique advantages over existing mouse models.

Chicken embryos are an excellent model for the study of vertebrate development and are particularly useful for the study of eye diseases (Hocking, 2013), retinal development (Vergara and Canto-Soler, 2012), developmental gene expression, patterning and morphogenesis (Peters and Cepko, 2002; Schook, 1980), and cell-fate mapping (Johnston et al., 1979). However, to our knowledge the histological sequence of chick AS development has not been clearly described. In the present study, we explored the anatomy and structural development of the chick AS - with focus on the drainage structures - to provide an assessment of its suitability as a model for future in-depth studies.

2. Methods

2.1. Chick embryos

Fertile eggs (wild-type Hy-Line Brown layers) were obtained from The National Avian Research Facility (Roslin Institute) and incubated at 37 °C until they reached the desired stage, using Hamburger Hamilton (HH) staging (Hamburger and Hamilton, 1992) as a guide. A minimum of three eyes from independent chicks were used for each stage analyzed.

2.2. Histological staining

Enucleated eyes were fixed in 4% paraformaldehyde (PFA) for 12–24 h on a roller shaker at 4 °C, and then quickly rinsed in PBS before they were dehydrated in an ethanol-PBS series of 30%, 50%, 70% and 100% (1 h each at room temperature). Samples were paraffin-embedded in a Leica ASP200 tissue processor. Once in paraffin, eyes were cut manually in the sagittal plane using a microtome setting the thickness between 8–10 μm. All slides were stained using a Leica Autostainer XL with a conventional H&E program. All histological data was captured with a Hamamatsu Slide Scanner using a 40× air objective, and analyzed using NPD View.2 Software (Hamamatsu).

2.3. Gene expression analysis

Expression of known ASD genes was analyzed in the developing chick using reverse transcriptase PCR (RT-PCR). We dissected anterior segments from chicks at embryonic days (E) 9, 12, 14 and three days post-hatch (P3). For E7 embryos we used whole eyes. For each stage, we used a minimum of six eyes from different chicks. Samples were pooled and RNA was extracted using TriZol Reagent (Thermo Scientific) according to the manufacturer's published protocol. We performed cDNA synthesis using 2.0 μg total RNA input with Superscript III reverse transcriptase (Thermo Scientific) with random hexamers and following the manufacturer's instructions to a total reaction volume of 20 μl (negative controls were performed with no reverse polymerase added). For RT-PCR reactions, oligonucleotide primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) using transcript data obtained from Ensembl (Gallus_gallus-5.0; http://www.ensembl.org/Gallus_gallus/Info/Index). All primers were designed to amplify across multiple introns, except for the single-exon genes *FOXC1* and *FOXE3*. RT-PCR reactions were performed in 25 μl volumes containing final concentrations of: 0.2 μM primers, 0.2 μl Faststart Taq Polymerase (5 U/μl; Roche), 1× PCR reaction buffer (2 mM $MgCl_2$), 200 μM of each dNTPs and 1.0 μl input cDNA. Cycle conditions were: 95 °C for 4 min; then 35 cycles of 95 °C for 45 s, 56 °C for 45 s, 72 °C for 1 min; then 72 °C for 10 min. Amplicons were run on 1% agarose gels with 0.5% TBE running buffer. A list of specific oligonucleotide primers is provided in the Key Resources Table.

2.4. Mouse gene expression analysis

A developmental time course of mouse eyeball from C57BL/6J mice of mixed sexes was available through the FANTOM5 project (Kawai et al., 2001; Forrest et al., 2014; Okazaki et al., 2002). Gene expression levels, based on cap analysis of gene expression (CAGE), which quantifies expression based on detection of the initial 27 nucleotides of transcripts, were estimated using the Zenbu browser (http://fantom.gsc.riken.jp/zenbu/) by recording the total tags per million across the whole gene region. Accession numbers were E15.CNhs10593.426-16C9, E17.CNhs11023.1261-18D4, N02.CNhs11205.1551-44G8, N16.CNhs11188.777-19A2, adult.CNhs10484.31-12G4. Expression profiles of individual genes mentioned can be viewed on the Zenbu browser by entering the gene name in the search box. Data is supplied in Supporting Table S1.

2.5. Immunofluorescence analysis for PAX6

Wild type fertilized eggs were collected and incubated for the required days (E9 & E11) at 37 °C. Eyes were resected and the entire anterior region was dissected and fixed in 4% PFA for 2 h at room temperature. Samples were then rinsed once in PBS and then immersed in 15% sucrose-PBS solution on a rotating shaker overnight at 4 °C. Samples were then equilibrated in 7% gelatin:15% sucrose-PBS for 4 h in a waterbath at 37 °C before cooling and embedding in blocks. Sections were cut at 10 μm, mounted on Superfrost plus (Thermo-Fisher) slides and air-dried for 1 h at room temperature. Sections were then frozen at −20 °C until required. For immunofluorescence, sections were thawed and rinsed twice in PBS (for 5 min each), then blocked with 1% BSA in a 0.25% Triton-X-100 PBS solution. Monoclonal antibodies