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Pax6 dosage requirements in iris and ciliary body differentiation

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ABSTRACT

Pax6 is a highly conserved transcription factor that controls the morphogenesis of various organs. Changes in Pax6 dosage have been shown to affect the formation of multiple tissues. *PAX6* haploinsufficiency leads to aniridia, a pan-ocular disease primarily characterized by iris hypoplasia. Herein, we employ a modular system that includes null and overexpressed conditional alleles of Pax6. The use of the *Tyrp2-Cre* line, active in iris and ciliary body (CB) primordium, enabled us to investigate the effect of varying dosages of Pax6 on the development of these ocular sub-organs. Our findings show that a lack of Pax6 in these regions leads to dysgenesis of the iris and CB, while heterozygosity impedes growth of the iris and maturation of the iris sphincter. Overexpression of the canonical, but not the alternative splice variant of Pax6 results in severe structural aberrations of the CB and hyperplasia of the iris sphincter. A splice variant-specific rescue experiment revealed that both splice variants are able to correct iris hypoplasia, while only the canonical form rescues the sphincter. Overall, these findings demonstrate the dosage-sensitive roles of Pax6 in the formation of both the CB and the iris.

Introduction

Organ master regulators are genes that are responsible for the initial commitment of a pluripotent tissue to a particular fate. We now know that besides this fundamental role, such genes can be utilized at later stages for additional functions in the forming tissues. This concept goes along with the genomic data indicating that the actual number of genes is somewhat smaller than initially believed.

Pax6 is an excellent example of a master regulator. This member of the PAX family of transcription factors is highly conserved among metazoans, and is both necessary and sufficient for eye formation in vertebrate and invertebrate species (Chow et al., 1999; Gehring, 2002; Grindley et al., 1995; Halder et al., 1995; Kozmik, 2005; Quiring et al., 1994). Moreover, widespread ocular expression during late stages of eye development implicates later roles for Pax6 in the differentiation and maintenance of distinct ocular cell types (Walther and Gruss, 1991). The aim of this study was to decipher the dosage requirements for Pax6 in the formation of the iris and ciliary body (CB), accessory sub-organs of the eye that play important roles in visual function (reviewed in Beebe (1986); Davis-Silberman and Ashery-Padan (2008); Napier and Kidson (2007)). The lens is attached to the CB by suspensory ligaments; contractions of the ciliary muscle alter lens shape and allow accommodation. In addition, the epithelial layers of the CB secrete aqueous and vitreous humors, which generate intraocular pressure (IOP). The iris mainly regulates the amount of light entering the eye by mydriasis and miosis of the pupil.

The iris and CB develop from the margins of the optic cup (OC). The inner layer of the OC gives rise to the neuroretina, the outer layer to the underlying retinal pigmented epithelium (RPE) and the marginal zone to the eye's anterior structures — the iris and CB. During embryogenesis and early postnatal stages, the margins of the OC extend and differentiate into the iris and CB epithelia and into the smooth muscles of the iris, the sphincter and the dilator. Migratory periocular mesenchymal cells adhere to the iris and CB primordium and create an associated stroma (Smith et al., 2002).

Pax6 is expressed in the iris and CB throughout their development and maturity. At first, Pax6 is uniformly expressed across the OC (Walther and Gruss, 1991). At around mid-gestation, a proximal^{low}–distal^{high} gradient in the protein level is established within the optic cup, with lower expression in the proximity of the optic nerve and higher expression in the distal tips of the optic cup. At this stage, Pax6 expression in the proximal RPE is downregulated (Baumer et al., 2002; Walther and Gruss, 1991). Pax6 levels are highest in the non-neuronal

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progenitor pool, destined for an iris and CB fate (Davis-Silberman et al., 2005). Pax6 expression persists in the iris and CB progenitors, probably functioning in their differentiation and specification. In adults, Pax6 is expressed in the iris musculature and in the epithelial layer of both the iris and CB, and it is required for the stem cell properties of the pigmented layer of the CB (Xu et al., 2007).

Interestingly, the correct levels of Pax6 expression are essential for proper development of the eye, particularly of the iris. Mutations in human *PAX6* lead to aniridia, a pan-ocular dominantly inherited disease, associated primarily with iris hypoplasia (Glaser et al., 1994; Jordan et al., 1992). Other features of aniridia include corneal opacity, cataract formation, foveal dysplasia and late-onset glaucoma (Elsas et al., 1977; Guercio and Martyn, 2007; Rush, 1926). The Small-eye condition in mice and rats (*Sey*), caused by heterozygosity for mutations in *Pax6*, constitutes an animal model that closely resembles human aniridia (Baulmann et al., 2002). Interestingly, overexpression of Pax6 in mice also causes ocular defects, including microphthalmia, microcornea, iris and CB cysts, cataracts and retinal abnormalities (Manuel et al., 2008; Schedl et al., 1996).

To investigate the tissue specific roles of Pax6 within the anterior eye structures, we previously employed the *Cre/loxP* approach to inactivate one allele of *Pax6* in the OC (Davis-Silberman et al., 2005). This local reduction in Pax6 dosage interrupts iris development in several ways: first, it leads to a significant decrease in the size of the iris progenitor pool; second, it causes a delay in the expression of muscle-specific markers, and finally, it impedes the morphogenesis of the sphincter muscle and the stroma (Davis-Silberman et al., 2005).

In vertebrates, Pax6 has two major splice variants that differ in the structure of their paired domain, due to an alternative insertion of 42 bp between exons 5 and 6. This alternative splice variant, designated Pax6-5a, exhibits unique DNA-binding properties and probably regulates a different set of downstream targets (Chauhan et al., 2004b; Kozmik et al., 1997). Unlike other Pax domains that interact through the amino terminus ("PAI") of the paired domain, Pax6-5a binds to DNA through the carboxyl terminus ("RED") (Chauhan et al., 2002; Kozmik et al., 1997). Based on this property, it has been suggested that Pax6-5a is related to Eyegone, one of the Pax6 paralogs in *Drosophila*, which also binds to DNA through the RED half-domain and recognizes the same consensus DNA-binding site (Dominguez et al., 2004).

As been noted above, Pax6 proteins are widely expressed throughout the eye. Most of the data, nevertheless, does not distinguish between the specific expression patterns of the different splice variants. Few studies, performed on a variety of organisms, did took the alternative splicing into consideration and demonstrated that both proteins are expressed in the brain (Pinson et al., 2005) and in the ocular system, including the lens, iris, cornea and retina (Azuma et al., 2005; Jaworski et al., 1997). Within the retina, Pax6-5a was found to be restricted to an area between the optic nerve head and the fovea in marmosets (Azuma et al., 2005). RT-PCR performed on bovine eye demonstrated that the ratio between the two splice variants varies within different eye parts, with preference of the lens to the canonical form while the iris yields higher levels of the alternative variant (Azuma et al., 2005; Jaworski et al., 1997). In primate lens, cornea and macula, nevertheless, both proteins were found in similar proportion (Zhang et al., 2001). Finally, the correct ratio between the two transcripts has been shown to be essential for the proper regulation of specific targets, such as some of the lens crystallins (Chauhan et al., 2004a).

The deletion of exon 5a in *e5a/e5a* mice leads to an ocular phenotype that includes defects in the iris, cornea, lens and retina (Singh et al., 2002). Moreover, there is a clear requirement for correct Pax6-5a dosage, as *e5a/+* mice display iris hypoplasia, whereas lens-specific overexpression of Pax6-5a leads to cataracts (Duncan et al., 2000). In humans, heterozygous missense mutations within exon 5a lead to a variety of ocular phenotypes, including Peters' anomaly, Axenfeld's anomaly, congenital cataracts and foveal hypoplasia (Azuma et al., 1999; Epstein et al., 1994).

Here, we studied the sensitivity of the CB and iris to changing dosages of Pax6. A complete absence of Pax6 precludes the formation of both organs, while haploinsufficiency leads to a milder phenotype of the iris but does not affect the CB. The canonical splice variant of Pax6 was found to be more potent than the alternative in correcting this phenotype, and upon overexpression leads to CB defects. Overall, this study exposes the late and splices variant-specific roles of Pax6 in the development of the iris and CB and expands our knowledge on the phenomenon of Pax6 dosage sensitivity.

Material and methods

Mice

Pax6$^{flox/flox}$, *Z/AP*, *JoP6* and *JoP6-5a* mouse lines were established as described previously (Ashery-Padan et al., 2000; Berger et al., 2007; Lobe et al., 1999) (Figs. 1A and 4A). The *Tyrp2-Cre* transgene contains regulatory sequences of tyrosinase-related protein-2 (*Tyrp2*) (−754 bp upstream to +194 bp downstream of the transcription start codon) followed by the coding sequence of the *Cre* recombinase gene (Fig. 1B).

Genotyping was performed by PCR using the following forward and reverse primers: Cre 5′-ATGCTTCTGTCCGTTTGCCG-3′, 5′-CCTGTTTTGCACGTTCACCG-3′, Tm = 57 °C; Flox 5′-GCGGTTGAGTAGCTCAATTCTA-3′, 5′-AGTGGCTTGGACTCCTCAAGA-3, Tm = 58 °C; LacZ (for genotyping of the *JoP* and *Z/AP* mice) 5′-CGTCACACTACGTCTGAACGTCG-3′, 5′-CAGACGATTCATTGCCACCATGC-3′, Tm = 65 °C. The genetic backgrounds were: ICR for *Z/AP* mice; C57BL6J for *Pax6$^{flox/flox}$* mice, and either C57BL6J or mixed ICR/C57BL6J for *Tyrp2-Cre* mice.

Pupil constriction, histology and EM

The day on which the copulatory plug was observed was defined as embryonic day (E) 0.5, and the day of birth was referred to as postnatal day (P)1. For pupil constriction, the enucleated eyes were incubated for 7 min in 4% pilocarpine (Sigma) in 0.9% NaCl. Eyes and embryos were photographed using an MZFLIII fluorescent stereomicroscope.

Specimens were fixed overnight in 4% paraformaldehyde and embedded in paraffin, according to standard protocols. For semithin sectioning, eyes were fixed overnight in 0.1% cacodylate-buffered fixative containing 2.5% paraformaldehyde and 2.5% glutaraldehyde and embedded in Epon (Roth, Karlsruhe, Germany). For scanning electron microscopy (SEM), eyes were fixed overnight in 0.1% cacodylate-buffered fixative containing 2.5% paraformaldehyde and 2.5% glutaraldehyde. After 30 min incubation in 1% osmium tetroxide and dehydration in ascending ethanol and acetone series, specimens were critical-point dried, sputter-coated with gold and examined under a JSM 840A scanning electron microscope (JEOL). For histological staining, 10-μm paraffin sections were stained with hematoxylin-eosin and 1-μm semithin sections were stained with toluidine blue, according to standard protocols.

Immunofluorescence analysis, β-galactosidase staining and TUNEL assays

For immunofluorescence analysis, 10-μm paraffin sections were stained as previously described (Ashery-Padan et al., 2000). Primary antibodies used were αPax6 (1:1000, Chemicon), αSma (α smooth muscle actin, 1:250, Sigma), αCaveolin3 (1:100, Santa Cruz), αhAP (human alkaline phosphatase, 1:100, Santa Cruz), αVC1.1 (1:500, Sigma), αPhospho-histone3 (1:500, Santa Cruz) and αCyclinD1 (1:250, Thermo Scientific). For all antibodies but αSma, sections were boiled twice in unmasking solution (Vector) prior to blocking. Secondary antibodies were conjugated to rhodamine red-X or to Cy2