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Developmental Biology 294 (2006) 119–132
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Optic cup and lens development requires Pax6 expression in the early optic vesicle during a narrow time window

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Received for publication 23 December 2005; revised 15 February 2006; accepted 21 February 2006
Available online 27 March 2006

Abstract

Pax6 mutations cause complex ocular malformations, but it is uncertain whether early eye development normally requires Pax6 function in both the optic vesicle (OV) and the lens epithelium, or only in the latter. To investigate this question, we electroporated the OV with anti-Pax6 or control morpholinos before the onset of lens placode formation. Pax6 downregulation was already detectable in the OV 10 h after anti-Pax6 treatment, and was accompanied by a significant increase in the death of OV cells. A *small eye*-like phenotype developed thereafter, whose severity was developmental stage-dependent. When treatment was applied at Hamburger Hamilton (HH) stage 10, there was no optic cup formation, and lens development was abortive despite normal Pax6 expression in the lens epithelium. Treatment at HH stage 11 resulted in structurally normal lens and optic cup, although the latter showed abnormal expression domains for several transcription factors. Early eye development therefore requires cell-autonomous Pax6 function not only in the lens but also in the optic vesicle, where it plays a hitherto unknown role in cell survival. The results, moreover, indicate that there is a critical stage during which Pax6 expression in the OV is necessary for normal lens development.
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Keywords: Pax6; Morpholino; Small eye; Aniridia; Cell death; Retina; Transcription factors; Loss-of-function

Introduction

The vertebrate eye develops through a complex series of morphogenetic events requiring the differentiation and coordinated assembly of structures with different embryonic origin. The initial eye primordium is the optic vesicle (OV), a neural tube evagination surrounded by cephalic mesenchyme and ectoderm. The optic vesicle invaginates and becomes patterned into the optic stalk (OS), the retinal pigment epithelium (RPE) and the neural retina (NR); the surface ectoderm originates the lens and the external epithelium of the cornea and the conjunctiva, and the mesenchyme forms the corneal and conjunctival stroma, the choroid and the sclera. A transient period of close contact between the optic vesicle and the presumptive lens ectoderm, during which they exchange inductive signals, is critical for normal eye development (reviewed by Chow and Lang, 2001; Fitzpatrick and van Heyningen, 2005).

Mutations in several genes can cause congenital ocular abnormalities, through mechanisms that are still incompletely understood (Ferda Percin et al., 2000; Glaser et al., 1994; Jordan et al., 1992; Voronina et al., 2004; review: van Heyningen and Williamson, 2002; Fitzpatrick and van Heyningen, 2005). Among these genes is Pax6, whose heterozygous mutations cause human *aniridia* and Peter's anomaly (Glaser et al., 1992; Hanson et al., 1994; Jordan et al., 1992), and the *small eye* phenotype in mice and rats (Hill et al., 1991; Matsuo et al., 1993). Anophthalmia can result from either loss-of-function

Abbreviations: afi, average fluorescence intensity; au, arbitrary units; α-Pax6 embryos, embryos treated with Pax6-MP; C-MP, control morpholino; C-MP embryos, embryos treated with C-MP; ED, embryonic day; HH, Hamburger–Hamilton; MP, morpholino; OV, optic vesicle; Pax6-MP, morpholino against Pax6; PBS, phosphate-buffered saline; RPE, retinal pigment epithelium; St, stage.

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0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.02.033

mutations (Glaser et al., 1994; Hill et al., 1991) or over-expression of the Pax6 gene, suggesting dosage-dependent Pax6 effects (Schedl et al., 1996).

The elucidation of the mechanisms leading to the *small eye* phenotype has been challenging due to the reciprocal inductive interactions between the optic vesicle and the lens ectoderm, and to the complex patterns of Pax6 expression in both structures. Pax6 is initially expressed throughout the optic vesicle, but it becomes subsequently restricted to the presumptive RPE and neural retina, and eventually to differentiating ganglion, amacrine and horizontal cells (Walther and Gruss, 1991; Li et al., 1994; Grindley et al., 1995; Belecky-Adams et al., 1997; review: Chow and Lang, 2001). Pax6 mRNA expression is also extensive in the presumptive lens ectoderm, but becomes subsequently restricted to the lens placode (Li et al., 1994; Grindley et al., 1995; Chow and Lang, 2001). A still unanswered question is whether normal eye development requires cell autonomous Pax6 activity in both the optic vesicle and the lens ectoderm, or in only one of them. Some notions that have received extensive (but not universal) acceptance in this regard are: (i) that the primary defect leading to the small eye phenotype is the failure of the surface ectoderm to form a lens placode, (ii) that lens development requires Pax6 activity in the prospective lens ectoderm but not in the optic vesicle and (iii) that Pax6 is not essential for optic vesicle formation, although it does play a role in subsequent steps of retinogenesis (review: Mathers and Jamrich, 2000; Ashery-Padan and Gruss, 2001; Ogino and Yasuda, 2000; Lang, 2004). These notions are supported by descriptive studies of the *small eye* mutation (Hill et al., 1991; Hogan et al., 1986), by recombination experiments with normal and mutant embryonic tissues (Fujiwara et al., 1994), by comparative analysis of Pax6 mutants and Rx-deficient mice (Zhang et al., 2000) and by cre-lox-based Pax6 inactivation in the lens ectoderm (Ashery-Padan et al., 2000). Grindley et al. (1995), however, observed that early *Sey/Sey* mouse optic vesicles are abnormally broad and fail to constrict proximally, prior to the time of lens placode formation; suggesting a requirement for Pax6 in neural ectoderm. In addition, analysis of Pax6 chimeric mice suggested that Pax6 is required in the OV for maintenance of contact with the overlying lens epithelium, allowing subsequent inductive interactions (Collinson et al., 2000). More recently, expression of a dominant-negative version of Pax6 in developing chick embryo OV led to the conclusion that Pax6 expression in neural tissue plays an important role in lens development (Reza and Yasuda, 2004a). Further experimentation with methods allowing Pax6 inactivation with high temporal and spatial resolution appears necessary for solving these discrepancies. The electroporation of antisense morpholino oligonucleotides has been shown to be suitable for experiments of this type (Corey and Abrams, 2001; Heasman, 2002; Kos et al., 2003).

In this study, we have used electroporation of anti-Pax6 morpholinos to investigate whether downregulation of Pax6 in the optic vesicle has cell-autonomous effects on its development, and can trigger a small eye-like phenotype even when Pax6 is normally expressed in the lens. The treatment was initiated at Hamilton–Hamburger (HH) stages 9–12, before the onset of lens placode formation (Kamachi et al., 1998; Reza and Yasuda, 2004b). Pax6 downregulation was already detectable in the OV 10 h after electroporation, when a significant increase in cell death was also observed. When treated at HH stage 10, the embryos developed a severe small eye-like phenotype, with lack of optic cup formation; lens development was abortive, despite normal Pax6 expression in the prospective lens ectoderm. When electroporation was done 7 h later (at HH St 11), however, the lens developed fairly normally and the optic cup was well formed, but had defects in dorso-ventral polarity reflected in the abnormal expression patterns of several transcription factors. Taken together, the results demonstrate that Pax6 has cell-autonomous functions in optic vesicle development, which include a hitherto unknown role in the regulation of cell survival, and that normal lens development requires Pax6 expression in the optic vesicle during a critical time window.

Materials and methods

Experimental animals

White Leghorn chick embryos from B and E Eggs (York Spring, PA), staged as in Hamburger and Hamilton (1951), were used.

Morpholino antisense oligonucleotides

Fluorescein-labeled morpholinos were obtained from GeneTools LLC (Philomath, OR). Two Pax6 morpholino antisense oligonucleotides (Pax6-MP) were designed to target sequences shared by the two chicken Pax6 isoforms (Epstein et al., 1994); one morpholino encompassed the translation start codon (5′CACGCCGCTGTGACTGTTCTGCATG3′), while another corresponded to the 5′ untranslated region (5′CTCGCCTGGAGCGGTCTGGAAGCCG3′). A standard control morpholino (C-MP) provided by GeneTools was used as a negative control; BLAST analysis showed that it had no target sequences in the chicken genome.

In ovo electroporation

Embryonic day (ED) 2 chick embryos were windowed as described (Selleck, 1996). Under blue light illumination generated with a blue dichroic filter (Edmund Industrial Optics, Barrington, NJ) attached to a fiber optic lamp, morpholino solution (0.4–0.6 μl, 1 mM in sterile 1× PBS) was injected in the forebrain ventricular cavity of HH St 9–12 embryos with a Harvard Apparatus PLI-100 picoinjection system using glass capillary needles. Electroporation was performed immediately after injection as in Nakamura and Funahashi (2001), with some modifications. In brief, platinum-iridium electrodes (FHC Inc, Bowdoinham, ME) were positioned at each side of the embryo head; the [−] electrode was consistently placed adjacent to the left optic vesicle, and the anode next to the right optic vesicle. The distance between electrodes was ca. 1.5 mm. Five square pulses (18 V, 50 ms length, 950 ms interval) were delivered using an ECM 830 electroporator (BTX, Holliston, MA). A digital multimeter (Multimaster 560, Extech Instruments, Waltham, MA) connected to a resistor (1.0Ω 1%) was inserted between the electroporator and the electrodes to monitor the peak current (40–80 mA) through the embryo head during electroporation. After electroporation, 10 μl of sterile PBS was dispensed over the embryo head, windows were closed with transpore tape and the embryos were returned to the incubator.

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde in PBS for 2–4 h at room temperature, embedded and frozen for cryosectioning as in Barthel and Raymond (1990). Immunohistochemistry was carried out in 10 μm sections as

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