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Foxe3 is required for morphogenesis and differentiation of the anterior segment of the eye and is sensitive to *Pax6* gene dosage

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Abstract

The *dysgenetic lens* (*dyl*) mouse mutant has mutations in *Foxe3*, which inactivate DNA binding by the encoded forkhead transcription factor. Here we confirm, by targeted inactivation, that *Foxe3* mutations are responsible for the *dyl* phenotype, which include loss of lens epithelium; a small, cataractic lens; and failure of the lens to detach from the surface ectoderm. In contrast to a recent report of targeted *Foxe3*, we found no phenotypic difference between *dyl* and $Foxe3^{-/-}$ mutants when congenic strains were compared, and thus nothing that argues against $Foxe3^{dyl}$ being a null allele. In addition to the lens, most tissues of the anterior segment–iris, cornea, ciliary body and trabecular meshwork–are malformed or show differentiation defects. Many of these abnormalities, such as irido-corneal and irido-lenticular adherences, are present in a less severe form in mice heterozygous for the *Foxe3* mutation, in spite of these having an intact lens epithelium. Early *Foxe3* expression is highly sensitive to a halved *Pax6* gene dosage and there is a striking phenotypic similarity between *Pax6* and *Foxe3* mutants. We therefore propose that many of the ocular malformations associated with *Pax6* haploinsufficiency are consequences of a reduced expression of *Foxe3*.

Keywords: Forkhead; Lens; Cataract; Aphakia

Introduction

For over a century, lens formation has been the classical example of induction and in the last two decades the molecular mechanisms behind differentiation and morphogenesis of the lens have begun to be understood. A master gene of eye development is *Pax6*, and although it is expressed in both the inducing optic vesicle and the responding ectodermal cells, genetic and tissue recombination experiments have demonstrated that it is its ability to confer lens forming competence to the presumptive placode cells that is crucial for its role in lens development (Collinson et al., 2000; Davis-Silberman et al., 2005; Dimanlig et al., 2001; Fujiwara et al., 1994; Quinn et al., 1996). The combined action of Pax6 in pre-placode cells and inducing factors—including Bmp and Fgf (reviewed by Lang, 2004)—from the optic vesicle, triggers activation of a set of transcription factors which mediate the differentiation and morphogenesis that lead to lens formation. These include Prox1, essential for lens fiber differentiation and cell cycle exit (Wigle et al., 1999); Mab21l1, drives proliferation and invagination in the lens placode (Yamada et al., 2003); Sox proteins, activators of crystallin gene expression (Kamachi et al., 2001); Six3, maintains—by mutual activation—expression of *Pax6* (Goudreau et al., 2002); and Foxe3, the subject of this article.

Transcription of *Foxe3* is activated in the early lens placode around E9, stays on in the entire lens vesicle up until about E12.5, when it is switched off in the differentiating primary lens fibers (Blixt et al., 2000). Expression persists in the anterior lens epithelium throughout life and this tissue requires Foxe3 for maintenance, proliferation and survival (Blixt et al., 2000). The *dysgenetic lens* (*dyl*) mouse mutant (Sanyal and Hawkins, 1979) has two amino acid substitutions in the forkhead (DNA binding) domain of Foxe3, which co-segregate with the *dyl* phenotype (Blixt et al., 2000) and destroys the protein's binding to its cognate site in DNA (Ormestad et al., 2002).

In addition to maintaining the lens epithelium in an actively proliferating, undifferentiated state, *Foxe3* is also required for closure of the lens vesicle; in *dyl* mice, the lens pit stays as an urn-

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shaped structure, continuous with the surface ectoderm that will later form the corneal epithelium (Blixt et al., 2000; Sanyal and Hawkins, 1979). A milder version of this defect occurs, with incomplete penetrance, in *Foxe3* heterozygotes in the form of a persistent connection between lens and cornea, or remnants of lens material inside the central corneal stroma (Ormestad et al., 2002). This malformation overlaps with the clinical manifestations of Peters' anomaly, and two human cases of anterior segment dysgenesis have been reported to be heterozygous for mutations in *FOXE3* (Ormestad et al., 2002; Semina et al., 2001).

Here, we confirm that the mutations in *Foxe3* are responsible for the *dyl* phenotype by showing that a targeted deletion of the *Foxe3* forkhead box phenocopies *dyl*. We also investigate the consequences of inactivation of *Foxe3* for development of the anterior segment of the eye, and the response of *Foxe3* expression to altered *Pax6* gene dosage, in the light of similarities between *Pax6* and *Foxe3* haploinsufficiency phenotypes.

Materials and methods

Targeting Foxe3

A targeting construct containing a total of 8.9 kb of the *Foxe3* locus was made from overlapping 129/Sv genomic λ clones. The forkhead box of *Foxe3* (from *Nco*I to *Not*I) was replaced by a *lacZ-PGK-Neo*^{R} cassette and the HSV-*tk* gene was appended to the short arm of the construct for negative selection. The construct was linearized at the end of the long arm and electroporated into R1 ES cells. Colonies resistant to 300 μg/ml G-418 and 2 μM ganciclovir were screened by Southern blot with two probes. The first probe is located outside the short (2.2 kb) arm of the targeting construct (5′ of *Foxe3*) and identifies fragments beginning at an external *Sac*I site and ending at *Sac*I sites downstream of the forkhead box (*wt* allele, 8.7 kb) or within the *lacZ-PGK-Neo*^{R} cassette (targeted allele, 6.5 kb). The other probe is located within the long (6.7 kb) arm and identifies the same *Sac*I fragment as the short arm probe (wt allele, 8.7 kb), and a fragment from a *Sac*I site within the *lacZ-PGK-Neo*^{R} cassette to a site within the long arm (targeted allele, 7.4 kb). Homologous recombination between the targeting construct and the *Foxe3* locus occurred with a frequency of 7% of Neo^R colonies. Targeted cell clones were used to generate chimeras through injection into C57Bl/6 blastocysts. A knockout line was established following germline transmission of targeted ES cells and made congenic with the *dyl* (Sanyal and Hawkins, 1979) strain by crossing with Balb/c for five generations. Genotyping of tail biopsies or embryos was performed by Southern blot, or by PCR using a common primer located upstream of the forkhead box, combined with either a primer specific for the *wt* allele, or a primer in *lacZ*, specific for the targeted allele.

Mouse strains

The *dyl* mutant, which arose spontaneously in the Balb/c strain (Sanyal and Hawkins, 1979), was obtained from The Jackson Laboratory (Bar Harbor, Maine). The *Small eye* (*Sey*) mutant was kindly provided by Dr H. Edlund (Umeå, Sweden), but comes originally from Dr V. van Heyningen (Edinburgh, UK). We obtained it on an impure C57Bl/6 background and have transferred the allele to Balb/c; the animals used here have at least five generations on this genetic background. PCR genotyping of the *Pax6*^{Sey} and *Foxe3*^{dyl} alleles was performed as previously described (Blixt et al., 2000; Grindley et al., 1995). Balb/c mice as wild-type controls and for breeding of mutants were purchased from Charles River Inc. (Germany).

Histology, in situ hybridization and immunohistochemistry

In situ hybridization of whole-mount mouse embryos and cryosections (8 μm) with *Foxe3* and *Cryaa* probes were performed as previously described (Blixt et al., 2000). A minimum of four embryos of each genotype and developmental stage were analyzed by whole-mount *in situ* hybridization. For immunohistochemistry and hematoxylin–eosin staining, eyes were fixed in 4% paraformaldehyde and processed for paraffin embedding and sectioning (5 μm). Glutaraldehyde fixation, followed by epoxy resin embedding, was used for thin (1 μm) histological sections (stained with Richardson's methylene blue/azur II) and electron microscopy (TEM). The rabbit polyclonal anti-Foxe3 antiserum was directed against a peptide corresponding to the carboxyterminal 19 amino acids of murine Foxe3. N-cadherin was stained with a mouse monoclonal antibody (Clone 3B9; Zymed, 33-3900) using "The Ark" mouse-on-mouse kit from Dako and antigen retrieval by treatment with Tris–EDTA buffer in a pressure boiler. ZO-1 was detected with a rabbit polyclonal antiserum (Zymed, 61-7300) after antigen demasking with Type XIV protease (Sigma), 2 mg/ml, 10 min, 37°C. Binding of antibodies was visualized with streptavidin–HRP, DAB and signal amplification with a TSA kit (Perkin Elmer).

Volume measurements

Embryos with 33±1 somites were selected from E10.5 litters of different genotypes, paraffin embedded and sectioned. Lens tissue volume was calculated by integration of area measurements from serial sections, using an Zeiss Axioplan 2 microscope and the area measurement function of the AxioVision software.

Results

Targeted deletion of Foxe3 phenocopies dysgenetic lens (dyl)

To settle if the mutation in *Foxe3* that we previously found in the *dysgenetic lens* (*dyl*) strain (Blixt et al., 2000; Sanyal and Hawkins, 1979) is responsible for the mutant phenotype and, if so, whether it represents a null mutation, we created a targeted deletion of the forkhead box in *Foxe3* (Fig. 1). To minimize the effects of differences in genetic background, the *Foxe3*^{tm} line was made congenic with the *dyl* strain by crossing with Balb/c for five generations. Mice homozygous for the targeted allele (*Foxe3*$^{tm/tm}$) showed the characteristic fusion between cornea and a small, severely cataractic lens, as previously described for the *dyl* mutant. Offspring from crosses between *dyl* and knockout mice (*Foxe3*$^{tm/dyl}$) were phenotypically indistinguishable from the (homozygous) parental strains at all embryonic and postnatal stages examined (Fig. 2), which formally proves that *dyl* is an allele of *Foxe3*. It also supports our prediction, based on inability of the Foxe3^{dyl} protein to bind DNA, that *Foxe3*^{dyl} is a null allele (Blixt et al., 2000; Ormestad et al., 2002).

Immunohistochemistry with an antiserum directed against the carboxyterminal end of Foxe3 showed intense nuclear staining in the lens epithelium of wild-type and *dyl* E14.5 embryos, but no detectable staining in the knockout (Figs. 2C, F, I). Beside confirmation of successful inactivation of Foxe3 in the targeted allele, this indicates that the Foxe3^{dyl} protein is stable and nuclear, which supports the notion that it is the failure to bind DNA that is responsible for the loss-of-function phenotype.

Foxe3 is important for differentiation of anterior segment mesenchyme

Transplantation experiments in chick embryos showed the anterior surface of the lens, i.e., the lens epithelium, to be important for corneal development (Beebe and Coats, 2000; Genis-Galvez, 1966; Genis-Galvez et al., 1967; Zinn, 1970).