Developmental Biology 322 (2008) 56–64

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Eye formation in the absence of retina

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ARTICLE INFO

Article history:
Received for publication 25 March 2008
Revised 20 June 2008
Accepted 3 July 2008
Available online 16 July 2008

Keywords:
β-catenin
Conjunctiva
Crystallin
Development
Eyelid
Foxe3
Lacrimal gland
Lens
Pax6
Periocular mesenchyme
RAX
Retina
Rx
Wnt

ABSTRACT

Eye development is a complex process that involves the formation of the retina and the lens, collectively called the eyeball, as well as the formation of auxiliary eye structures such as the eyelid, lacrimal gland, cornea and conjunctiva. The developmental requirements for the formation of each individual structure are only partially understood. We have shown previously that the homeobox-containing gene *Rx* is a key component in eye formation, as retinal structures do not develop and retina-specific gene expression is not observed in *Rx*-deficient mice. In addition, *Rx*−/− embryos do not develop any lens structure, despite the fact that *Rx* is not expressed in the lens. This demonstrates that during normal mammalian development, retina-specific gene expression is necessary for lens formation. In this paper we show that lens formation can be restored in *Rx*-deficient embryos experimentally, by the elimination of β-catenin expression in the head surface ectoderm. This suggests that β-catenin is involved in lens specification either through *Wnt* signaling or through its function in cell adhesion. In contrast to lens formation, we demonstrate that the development of auxiliary eye structures does not depend on retina-specific gene expression or retinal morphogenesis. These results point to the existence of two separate developmental processes involved in the formation of the eye and its associated structures. One involved in the formation of the eyeball and the second involved in the formation of the auxiliary eye structures.

Introduction

The development of the vertebrate eye and its auxiliary structures has fascinated researches for more than a century. However, in spite of steady progress, a comprehensive understanding of the formation of the eye and its auxiliary structures is still missing.

Eye formation is a complicated process, as the different components of the eye, such as the retina, lens, cornea, conjunctiva, eyelids, eyelid muscles and lacrimal glands, are formed from different tissues. The retina is formed from the neuroectoderm, the lens from the surface ectoderm and the auxiliary tissues are formed from the head surface ectoderm, neural crest cells and the head mesoderm. How these processes are coordinated is a key question of the developmental biology of the eye.

For historical reasons, the retina and the lens together are called the eyeball. The cornea, eyelids, eyelid muscles, conjunctiva, lacrimal glands and other eye structures not derived from the retina or the lens are called the auxiliary eye structures.

Formation of the retina begins with the specification of retinal cells in the anterior neuroectoderm. The first morphological sign of this specification is the formation of two lateral grooves in the anterior neuroectoderm called the optic sulci. The cells of the optic sulci evaginate and form the optic vesicle. The distal portion of the optic vesicle will form the retina and the proximal will develop into the optic stalk. The homeobox-containing gene *Rx* is a key component in formation of retinal structures, as mice lacking *Rx* function do not form optic sulci or optic vesicles and do not display retina-specific gene expression (Mathers et al., 1997; Zhang et al., 2000). Studies in human, medaka, zebrafish and *Xenopus* suggest that *Rx* genes are required for the formation of the vertebrate retina in general (Bailey et al., 2004; Casarosa et al., 1997; Chuang et al., 1999; Furukawa et al., 1997; Kennedy et al., 2004; Loosli et al., 2003; Ohuchi et al., 1999; Voronina et al., 2004).

First morphological signs of lens formation are visible when the evaginating optic vesicle contacts the head surface ectoderm. This head surface ectoderm begins to thicken and forms a lens placode. In species like mouse and chick, the lens placode invaginates forming a lens vesicle. In other species, like zebrafish and *Xenopus*, the lens delaminates from the overlaying ectoderm (Ishibashi and Yasuda, 2001; Soules and Link, 2005). The distal part of the optic vesicle

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doi:10.1016/j.ydbio.2008.07.009

invaginates and forms a cup around the developing lens. Whether the formation of the lens depends on the formation of the retina is one of the oldest questions in the field of eye development. Hans Spemann concluded in 1901 (Spemann, 1901) that in the frog *Rana fusca*, lens induction depends on the optic cup, as upon its removal, the lens does not form. Several investigators came to the same conclusions, but others voiced dissenting views, as they found "free lenses" in several amphibian species upon experimental manipulation (for review see Mangold, 1931). Occasionally, contradictory conclusions were reached when the same species was used for experimental purposes (for review see von Woellwarth, 1961). Interestingly, it was found that the formation of "free lenses" greatly depended on the temperature at which the animals were raised prior to experimental manipulation (Ten Cate, 1956). For example, in *Rana esculenta,* lens formation was dependent on the optic cup if the animals were raised at 12 °C (54 °F), but appeared to be independent of the optic cup if the animals were raised at 25 °C (77 °F). These experiments explained why different investigators reached different conclusions using very similar experimental designs. This also demonstrated the difficulties with interpretation of results obtained from manipulated embryos. While a comprehensive understanding of lens formation in different amphibian species will require a detailed reinvestigation using molecular markers, in mice there is genetic evidence that formation of the lens depends on retinal formation. In *Rx*-deficient mouse embryos, which do not form any retinal structure and do not display any retina-specific gene expression, the lens placode, and consequently, the mature lens do not develop, demonstrating that in this species, retinal cells are necessary for lens formation (Brownell et al., 2000; Mathers et al., 1997; Swindell et al., 2006). It is not certain at this point whether the presumptive retinal cells exert their influence on lens formation through gene expression or through the formation of the optic cup. There is good evidence that signaling from the optic vesicle is essential for the activation of the lens-specific gene network and the formation of the lens placode (Faber et al., 2002; Furuta and Hogan, 1998; Kamachi et al., 1998; Wawersik et al., 1999). However, several investigators presented evidence indicating that it is the mechanical protection of the optic cup against the neural crest cells that is the critical for lens formation (Bailey et al., 2006; Sullivan et al., 2004; von Woellwarth, 1961).

Formation of the auxiliary eye structures takes place upon the invagination/delamination of the lens. The corneal epithelium forms where head surface ectoderm is overlying the lens. The ectoderm surrounding the eye proliferates and folds over the developing cornea, forming a conjunctival sack. The ectoderm of this sack will form the ectoderm of the cornea, conjunctiva, lacrimal gland and eyelid. To what degree the development of these auxiliary eye structures depends on the development of the eyeball, or its individual components, i.e. the retina and the lens, is an interesting question that was intensely studied in the first half of the last century. Is the formation of auxiliary eye structures a consequence of gene expression in the developing retina and lens, or is the formation of these structures a craniofacial process, in which the development of the retina and the lens play only a limited role?

Otto Mangold attempted to address this question at the beginning of last century by the ablation of the eye rudiment in amphibians (Mangold, 1931). Since he found that the ablation of the eye rudiment did not prevent eyelid formation, he proposed that the specification of auxiliary eye structures either takes place at very early stages of development, before the experimental ablation of the eye is possible, or that the eyeball does not control the development of the auxiliary eye structures. Others attempted to answer this question by analyzing accidental and hereditary anophthalmia. However, no definitive conclusions were reached, as it was impossible to determine when the ablation of the eyeball took place and whether it was complete (Recordon and Griffiths, 1938; Rogalski, 1926; Voronina et al., 2004; Woolard, 1926). A good example demonstrating the nature of this problem is present in the anophthalmic individual lacking normal *RAX (RX)* function identified by Voronina et al. (2004). In this study, an anophthalmic child is presented that is missing a wild type copy of the *RAX* gene. This child has apparently normal eyelids. However, a detailed analysis shows that the mutated RAX genes of this child encode partially functional proteins. The activity of these proteins might be sufficient to initiate the development of a rudimentary eye and lead to normal development of auxiliary eye structures. Therefore, no firm conclusions can be made about the relationship between the formation of the eyeball and the formation of auxiliary eye structures in this case.

In this study, we took advantage of the *Rx*-deficient embryos to investigate the dependence of the formation of the auxiliary eye structures on the development of the eyeball and its components, the retina and the lens. *Rx*-deficient embryos do not develop eyeballs, and importantly, while the extent of brain defects varies in these embryos, they never develop any retinal or lens structure and they do not display any retina or lens-specific gene expression.

Materials and methods

Mouse lines

P6 5.0 LacZ reporter mice were used and genotyped as previously reported (Williams et al., 1998; Makarenkova et al., 2000). *Rx*−/− mice were used and genotyped as previously reported (Mathers et al., 1997).

LacZ reporter staining and histology

Embryos were fixed in 4% paraformaldehyde, washed in PBS and then stained for lacZ activity using X-gal. Stained embryos were then dehydrated, embedded in paraffin and sectioned. Sections were dewaxed and counterstained with eosin.

In situ hybridization

In situ hybridizations using *Pitx2*, *Foxl2* and *keratocan* riboprobes were performed using standard protocols (Wilkinson, 1992).

Skeletal staining

Newborn pups were fixed in 95% ethanol and then stained with Alcian blue and Alizarin red to visualize cartilage and bone.

Results

Formation of auxiliary eye structures

In order to directly address the question of whether the development of auxiliary eye structures depends on the formation of the retina and the lens, we analyzed the formation of auxiliary eye structures in *Rx*-deficient embryos. *Rx*-deficient embryos do not form any retina or lens structure and consequently do not form an eyeball (Mathers et al., 1997; Zhang et al., 2000). This phenotype is 100% penetrant. Furthermore, *Rx*-deficient embryos do not display any retina or lens-specific gene expression (Brownell et al., 2000; Zhang et al., 2000). Serial sections of *Rx*−/− newborn pups indicated the presence of eyelids and conjunctival sacks (Fig. 1A). The *Pax6-lacZ* reporter (Williams et al., 1998) was crossed into the *Rx*−/− strain because in wild type E16.5 embryos, this reporter is expressed in the lens, as well as in the ectoderm of the conjunctival sack, which includes the ectoderm of the cornea, conjunctiva and eyelid (Figs. 1B, C, F, G) (Kammandel et al., 1999; Makarenkova et al., 2000). This reporter is also expressed in the lacrimal gland that forms from the conjunctival sack by evagination and multiple branching (Fig. 1C)