Developmental Biology 320 (2008) 140–148

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Impairing retinoic acid signalling in the neural crest cells is sufficient to alter entire eye morphogenesis

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ARTICLE INFO

Article history:
Received for publication 13 February 2008
Revised 7 April 2008
Accepted 30 April 2008
Available online 11 May 2008

Keywords:
Retinoic acid receptor
Neural crest cells
Eye development
PITX2
Extraocular muscle

ABSTRACT

Retinoic acid (RA) is known to be required at various levels of eye patterning via Retinoic Acid Receptors (RAR); however the molecular and cellular mechanisms triggered by these nuclear receptors are still obscure. The genetic studies performed here enable us to present a new model to study RA action during eye development. By inactivating the three RARs, specifically in the periocular mesenchyme, we discriminate the individual contribution of each RAR during eye development and describe a new function for RARs during the formation of the optic nerve. We demonstrate that RARα is the only receptor that mediates RA signalling in the neurectoderm during ocular development. Surprisingly, and despite a sophisticated pattern of RA-activity in the developing retina, we observed that RA signalling is not autonomously required in this tissue for eye formation. We show that the action of RA during eye morphogenesis is occurring specifically in neural crest-derived periocular mesenchyme and is mediated by all three RARs. Furthermore, we point out that *Pitx2*, which encodes a homeodomain transcription factor, is a key RA-responsive gene in neural crest cells during eye development. Interestingly, we observed that RA is required in the neural crest cells for normal position of the extraocular muscle.

Introduction

During vertebrate development, the eye is constructed from neural and surface ectoderm as well as from the periocular mesenchyme (POM). This latter tissue derives from neural crest cells (NCCs) and mesoderm. The primary function of ocular mesenchyme is to provide multiple mature cell lineages that are necessary for the development of normal anterior segments (including the corneal endothelium, anterior chamber and iris stroma). A second essential function is that cells originating from the surface epithelium need to interact with POM cells for proper eyelid development (Le Lievre and Le Douarin, 1975). Finally, the POM provides essential signals for the specification of retinal pigmented epithelium (RPE) and the differentiation of the optic stalk, both structures arising from neural ectoderm (Evans and Gage, 2005; Fuhrmann et al., 2000). Failure of proper interactions between these ectodermal tissues and the POM results in developmental disorders such as Peters' anomaly, Axenfeld-Riegers's syndrome or aniridia (Lines et al., 2002).

Vitamin A (retinol) is known to be critical for vertebrate eye development as demonstrated by severe ocular defects occurring after gestational vitamin A deficiency, such as microphthalmia and coloboma of the retina (Dickman et al., 1997). Administration of Retinoic acid (RA), the active metabolite of vitamin A, can rescue these eye defects indicating that RA mediates the developmental actions of vitamin A (Dickman et al., 1997). RA signalling is transduced by specific nuclear retinoic acid receptor heterodimers (RXRα/RARα, RXRα/RARβ and RXRα/RARγ) that act as transcription factors and regulate specific target genes during development. During the last decade, extensive work has been done to decipher the molecular mechanisms triggered by RA during vertebrate development and notably in eye patterning (for a review see Mark et al., 2006). In particular, numerous gene knockout studies have demonstrated that the three RARs (RARα, RARβ and RARγ) play important and overlapping roles during eye development (Ghyselinck et al., 1997; Lohnes et al., 1994). Embryos carrying a null mutation of only one RAR display relatively minor defects, but loss of function of two RARs results in several ocular abnormalities, including malformation of the eyelids, shortening of the ventral retina, coloboma and severe malformation of the anterior segment (absence of the iris stroma, the corneal stroma and the anterior chamber).

Although obvious redundancy between RARs has been documented, accurate comparison of phenotype between various combinations of RAR knockout mice has led to the attribution of specific function to a given RAR. For instance, while all three RARs are expressed in

Abbreviations: E(n), embryonic day; NCC, neural crest cell; POM, periocular mensenchyme; RA, retinoic acid; ALDH1A, retinaldehyde dehydrogenase; RAR, retinoic acid receptor; RPE, retinal pigmented epithelium; RXR, 9cis–RA receptor, VAD, vitamin A-deficient.

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doi:10.1016/j.ydbio.2008.04.039

developing ocular structures (Ghyselinck et al., 1997; Mori et al., 2001), several lines of evidence suggest that RXRα/RARβ and RXRα/RARγ are the main heterodimers which are instrumental in ocular morphogenesis (Kastner et al., 1997; Zhou et al., 2001). Therefore, a clearly established function for RARα in the eye has not yet emerged.

During development, RA is generated by specific cells in a unique spatio-temporal pattern (Rossant et al., 1991). The first enzymatic step, the oxidation of retinol to retinal, is performed by the retinol dehydrogenase RDH10 (Sandell et al., 2007). Then, three RA–synthesizing enzymes (ALDH1A1, ALDH1A2 and ALDH1A3) are involved in RA synthesis during mouse eye development (Matt et al., 2005; Mic et al., 2004). They have distinct tissue-specific expression patterns from the optic vesicle to the optic cup as ocular formation proceeds. The distribution of ALDH1As and the RA-degrading enzymes within the retina produces a dorsal and a ventral domain of RA separated by a central zone lacking RA activity (Wagner et al., 2000). This particular dorso-ventral pattern of RA activity observed during neural retina formation was initially thought to be crucial for eye development (Wagner et al., 2000). However, so far no conclusive data has been reported to support such a role for RA (Halilagic et al., 2007; Matt et al., 2005; Molotkov et al., 2006). ALDH1A2 which is the first enzyme expressed during ocular development is required between E8.5 and E9.5 for the initial formation of the optic pit (Mic et al., 2004; Niederreither et al., 1999; Ribes et al., 2006). *Aldh1a3*-null embryos display only discrete ocular malformations despite a loss of ventral RA signalling (Dupé et al., 2003) and partial loss of dorsal RA signalling in *Aldh1a1*-null mice does not induce any abnormal eye development (Fan et al., 2003; Matt et al., 2005). Finally, despite a non-overlapping expression pattern of their genes, a functional compensation between ALDH1A1, ALDH1A2 and ALDH1A3 has been demonstrated (Halilagic et al., 2007; Matt et al., 2005; Molotkov et al., 2006). To date, it has been established that during eye morphogenesis, RA is provided by the ALDH1A1 and ALDH1A3 enzymes expressed in the epithelial compartments of the eye (*i.e.,* the retina, retinal pigment epithelium and corneal ectoderm) (Matt et al., 2005; Molotkov et al., 2006). This RA signal diffuses in the mesenchymal compartment (*i.e.,* the POM) to activate RXRα/RARβ and RXRα/RARγ heterodimers. Thus, RA acts in a paracrine process to pattern the anterior segment. It has also been shown that RXRα/RARβ and RXRα/RARγ heterodimers control the extent of cell–death involved in POM remodelling and the expression of *Foxc1* and *Pitx2* genes (Matt et al., 2005), which are crucial genes for the development of the anterior eye segment in mice and humans (Cvekl and Tamm, 2004). Beside this relatively good knowledge of RA action during anterior segment development, its function during optic cup formation is still largely obscure.

The data obtained with various RA-deficient mouse models demonstrate unambiguously that RA is required for several morphogenetic processes during eye organogenesis. By using conditional mutations to inactivate all three RARs in the NCCs, we have generated a new mouse model where RA activity is entirely absent in the NCC-deriving POM. Not only does our work allow us to interpret the disparity of eye phenotypes previously observed in various models of RA deficiency, but definitely demonstrates that RA signalling plays key roles in eye development exclusively through the POM.

Methods

Mice

Mice, with a mixed C57BL/6–129/Sv (50%:50%) genetic background, were housed in an animal facility licensed by the French Ministry of Agriculture (agreement N°B67–218–5) and all animal experiments were supervised by NBG who is qualified to experiment with mice, in compliance with the European legislation on care and use of laboratory animals (agreement N°67–205). Heterozygous mice were mated overnight, and animals with a vaginal plug at noon of the next day were considered as embryonic day (E) 0.5. The generation of *lox*P-flanked (floxed) *Rara* (*Rara*$^{+/L2}$), *Rarb* (*Rarb*$^{+/L2}$), *Rarg* (*Rarg*$^{+/L2}$) and *Rara*-null mice have been previously described (Chapellier et al., 2002a; Chapellier et al., 2002b; Chapellier et al., 2002c; Lufkin et al., 1993). *Wnt1-Cre* mouse which carrying a transgene containing a *Cre* cassette under the control of the *Wnt1* promoter was obtained from A. McMahon (Danielian et al., 1998). The *R26R* transgenic mice have been previously described (Soriano, 1999). The *Wnt1-Cre* and *Rara*$^{+/L2}$, *Rarb*$^{+/L2}$, *Rarg*$^{+/L2}$ parental lines were intercrossed to generate mutant foetuses lacking RARα, RARβ and RARγ in NCCs (hereafter designated *Rara/b/g*$^{NCC-/-}$ mice). The resulting embryos were genotyped for *Cre*, *Rara*, *Rarb* and *Rarg* using PCR-based methods. The embryos of a littermate who does not carry the *Wnt1-Cre* transgene were used as controls. *Rara/b/g*$^{NCC-/-}$ mice were obtained at a mendelian ratio, but newborns died at birth from respiratory distress.

Histology, staining and in situ RNA analysis

For histology experiments, samples were fixed in Bouin's fluid for 5 days, embedded in paraffin, serially sectioned and stained with Groat's hematoxylin and Mallory's trichrome. For β–galactosidase activity detection, staining was performed as described (Rossant et al., 1991). In situ RNA hybridization was carried out as described (Dupé et al., 2003). Briefly, the digoxigenin–labeled antisense riboprobes were synthesized using cDNA as templates (references upon request). *In situ* hybridizations were performed on serial histological sections along the entire anteroposterior axis of the head. Terminal transferase–mediated dUTP-Nick-End-Labeling (TUNEL) was performed using the Apoptag® kit (Chemicon International).

Results

RARα is the only mediator of RA signalling in the retina

During early eye development, immunolocalization experiments suggest that *Rara*, *Rarb* and *Rarg* genes are all expressed in the cells surrounding the developing optic cup (or POM for Peri Ocular Mesenchyme) whereas RARα would be the only receptor located in the developing retina and the retinal pigmented epithelium (RPE) (Mori et al., 2001).

In order to verify this observation, we examined the RA signalling activity in absence of RARα by crossing a mouse carrying a *RAR-lacZ RA-reporter* (Rossant et al., 1991) gene with the *Rara*-null mutant. The resulting mouse allowed us to follow the RA signalling activity spatiotemporally. We have tested *RARE-lacZ* activity at E10.5, when RA activity is strong in the retina, RPE and corneal ectoderm and present as a weaker signal in the POM (Figs. 1A, C). Compared to this characteristic *lacZ* expression, *Rara*-null mutant mice carrying the *RARE-lacZ RA-reporter* transgene exhibited an alteration of the reporter activity. On external view, RA signalling activity in the developing *Rara*-null eye was slightly reduced in ocular area whereas a significant decrease was observed at the level of the forebrain (Figs. 1A and B). To further explore *lacZ* activity in the developing eye, these embryos were sectioned. We discovered that RA activity was completely lost specifically at the level of the neural retina, the RPE and lens whereas RA activity was normal at the level of the surface ectoderm and the POM. Identical lacZ activity was observed at E11.5 and E13.5 (data not shown). These observations strongly support that *Rara* was the only RAR implicated in the transduction of RA activity in the neurectoderm of the developing eye.

Retinal morphology in Rara-null mouse is normal

The previous experiment revealed that *Rara* was the only receptor capable of transducing RA signalling in the retina. Consequently, *Rara*-null mutant gives a unique model to study the role of an autonomous-RA signal during retina development. We thus undertook an histological analysis of the retina in newborn and adult *Rara*-null mutants. As shown in Fig. 1E, newborn mouse retina have a defined ganglion cell layer and the development of other layers is still under way. There were no significant morphological differences in the retina of control and *Rara*-null newborn mice, either in terms of retinal thickness or with respect to the presence of a ganglion cell layer (Figs. 1E, F). In addition, no morphological differences were observed in the adult retina, as *Rara*-null retina developed normally, displaying the expected lamination (ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer and photoreceptors segments) with the same degree of thickness as that of