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Retinoic acid signaling in perioptic mesenchyme represses Wnt signaling via induction of *Pitx2* and *Dkk2*

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ABSTRACT

Morphogenesis during eye development requires retinoic acid (RA) receptors plus RA-synthesizing enzymes, and loss of RA signaling leads to ocular disorders associated with loss of *Pitx2* expression in perioptic mesenchyme. Several Wnt signaling components are expressed in ocular tissues during eye development including *Dkk2*, encoding an inhibitor of Wnt/β-catenin signaling, which was previously shown to be induced by *Pitx2* in the perioptic mesenchyme. Here, we investigated potential cross-talk between RA and Wnt signaling during ocular development. Genetic studies using *Raldh1*/*Raldh3* double null mice deficient for ocular RA synthesis demonstrated that *Pitx2* and *Dkk2* were both down-regulated in perioptic mesenchyme. Chromatin immunoprecipitation and gel mobility shift studies demonstrated the existence of a DR5 RA response element upstream of *Pitx2* that binds all three RA receptors in embryonic eye. *Axin2*, an endogenous readout of Wnt/β-catenin signaling, was up-regulated in cornea and perioptic mesenchyme of RA deficient embryos. Also, expression of *Wnt5a* was expanded in perioptic mesenchyme of RA deficient eyes. Our findings demonstrate excessive activation of Wnt signaling in the perioptic mesenchyme of RA deficient mice which may be responsible for abnormal development leading to defective optic cup, cornea, and eyelid morphogenesis.

Introduction

Retinoic acid (RA) is a vitamin A derivative essential for embryonic development. Vitamin A deficiency is known to cause various ocular malformations including retinal defects, microphthalmia, coloboma and vision impairment. Ocular morphogenesis during embryonic development is a multi-step process involving induction, proliferation, differentiation and migration of cells. The vertebrate eye is formed from neural ectoderm, surface ectoderm and perioptic mesenchyme, which is derived from neural crest cells. An essential function of perioptic mesenchyme is to contribute multiple mature cell lineages that are required for the normal ocular anterior segment and eyelid development (Le Lièvre and Le Douarin, 1975). In humans, defects in survival, migration and differentiation of perioptic mesenchymal cells can lead to anterior segment dysgenesis and increased risk of glaucoma (Gould et al., 2004). Also, mutation of human *PITX2* leads to Axenfeld–Rieger syndrome characterized by ocular anterior segment defects (Semina et al., 1996; Mears et al., 1998; Kozlowski and Walter, 2000; Vieira et al., 2006; Weisschuh et al., 2006).

A role for RA has been well established in various stages of eye development based upon the overlapping expression patterns of RA-synthesizing enzymes (retinaldehyde dehydrogenases; RALDHs) and retinoic acid receptors (RARs) in ocular tissues, combined with corresponding loss-of-function studies in mouse (Lohnes et al., 1994; Ghyselinck et al., 1997; Wagner et al., 2000; Mic et al., 2004; Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008). RA synthesis is carried out by three RALDH enzymes (encoded by *Raldh1*, *Raldh2*, and *Raldh3*) with ocular *Raldh1* expression limited to the dorsal retina and ocular *Raldh3* expression limited to the ventral retina (Duester, 2008). RA serves as a ligand for nuclear RA receptors (RARα, RARβ, and RARγ) which bind to DNA as heterodimers with retinoid X receptors (RXRα, RXRβ, and RXRγ) (Mark et al., 2006). RA signaling is transduced when RA binds to the RAR component of RAR/RXR heterodimers that are bound to RA response elements (RAREs) of specific target genes (Mic et al., 2003). Loss of ocular RA synthesis in mouse embryos carrying mutations of both *Raldh1* and *Raldh3* does not disrupt eye dorsoventral patterning as originally proposed, but it does disrupt anterior segment morphogenesis leading to excessive perioptic mesenchyme growth associated with dysgenesis of cornea and eyelid and rotation of the optic cup along the dorsoventral axis (Matt et al., 2005; Molotkov et al., 2006). As *Raldh1* and *Raldh3* are expressed in the retina but not the perioptic mesenchyme, it is now clear that RA signaling for eye morphogenesis occurs in a paracrine fashion (Molotkov et al., 2006).

Besides RA signaling, many components of the Wnt signaling pathway are also expressed in ocular tissues. A role for Wnt signaling during embryonic eye development has been established in

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regulation of distinct processes including eye field specification, morphogenetic movements, proliferation, differentiation and apoptosis (Fuhrmann, 2008). In short, canonical Wnt/β-catenin signaling is activated by binding of Wnt ligands to frizzled and LRP5/6 co-receptors followed by stabilization and translocation of β-catenin to the nucleus where it regulates transcription of target genes by interacting with TCF/LEF transcription factors (Van de Wetering et al., 1997; Logan and Nusse, 2004; Angers and Moon, 2009). Among the several Wnt ligands and signaling components expressed in the developing eye, *Dkk2* (an antagonist of canonical Wnt/β-catenin signaling) is highly expressed in the perioptic mesenchyme, and recently it has been shown that *Dkk2* is a critical downstream target of *Pitx2* in neural crest derived perioptic mesenchyme (Gage et al., 2008). *Pitx2* encodes a homeodomain transcription factor which plays an essential role during ocular anterior segment patterning and development (Gage et al., 1999; Hjalt et al., 2000; Gage et al., 2005). Heterozygous mutations in human *PITX2* result in Axenfeld–Rieger syndrome characterized by anterior segment dysgenesis and high risk of developing glaucoma (Semina et al., 1996; Mears et al., 1998; Kozlowski and Walter, 2000; Vieira et al., 2006; Weisschuh et al., 2006). Global and neural crest specific *Pitx2* knockout mice exhibit a similar eye phenotype including abnormal anterior segment differentiation, vasculogenesis, eyelid defects and coloboma (Gage et al., 1999; Kitamura et al., 1999; Lu et al., 1999; Evans and Gage, 2005). The eye defect of *Pitx2* knockout mice is quite similar to that reported for *Raldh1*/*Raldh3* and *RARb*/*g* double knockout mice (Matt et al., 2005; Molotkov et al., 2006; Matt et al., 2008). Previous studies in embryos lacking either ocular RA synthesis or RA receptors demonstrate that *Pitx2* is down-regulated in the perioptic mesenchyme (Matt et al., 2005; Matt et al., 2008; See and Clagett-Dame, 2009). The observation that *Pitx2* functions as an inducer of *Dkk2* in perioptic mesenchyme provides a new paradigm for inhibition of canonical Wnt signaling through a critical role of *Pitx2* during eye development (Gage et al., 2008).

Here, we investigate potential cross-talk between RA and Wnt signaling during eye development. We report that the *Pitx2* gene contains a DR5 RARE located 4.3 kb upstream. We also demonstrate that RA activity in the perioptic mesenchyme is required for expression of not only *Pitx2* but also *Dkk2* which affects Wnt/β-catenin signaling, and for repression of *Wnt5a*. RA inhibition of Wnt signaling in the perioptic mesenchyme provides a mechanism for proper anterior segment formation, eyelid development, and optic cup orientation along the dorsoventral axis.

Materials and methods

Generation of Raldh1;Raldh3 double null mutant embryos

Raldh1$^{-/-}$;*Raldh3*$^{-/-}$ double homozygous embryos were generated by crossing the two single mutant lines as previously described (Molotkov et al., 2006). Following mating, noon on the day of vaginal plug detection was considered embryonic day 0.5 (E0.5). Embryos were genotyped by PCR analysis of yolk sac DNA. All mouse studies conformed to the regulatory standards adopted by the Animal Research Committee at the Burnham Institute for Medical Research.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to the manufacturer's ChIP protocol (Active Motif, Carlsbad, CA). In separate experiments, ten E8.5 wild-type mouse embryos or eighteen E12.5 wild-type mouse eyes were dissected, pooled, and cross-linked with 1% formaldehyde at room temperature for 15 min; eye tissue included the whole eye and surrounding perioptic mesenchyme. Isolated nuclei (in 650 µl of shearing buffer) from E8.5 mouse embryos were sonicated for twelve pulses of 10 s each (Duty cycle-6, Output 30%) using a Branson Sonifier 450 (or in the case of nuclei from E12.5 eye samples for twenty pulses of 10 s each at 40% power output) using a microtip probe from Misonix Digital Sonicator 4000 (Cole-Parmer Instrument Company, Vernon Hills, IL, USA). Samples were sonicated on ice to shear DNA to an average size of 500 bp followed by centrifugation at 13,000 rpm for 10 min. At this point, a small portion of supernatant was stored as input control. For immunoprecipitation, 150 µl of sheared chromatin mixed with 3 µg of either anti-RARα (sc-551, Santa Cruz Biotechnology), anti-RARβ (Affinity Bioreagents), anti-RARγ (sc-550 Santa Cruz Biotechnology), or control IgG antibodies was used for each ChIP reaction incubated with 25 ml pre-blocked protein G-coated magnetic beads (Active Motifs, Carlsbad, CA) for 4 h at 4 °C. Beads were washed and eluted DNA–protein complexes were reverse cross-linked and purified. The immunoprecipitated DNA was analyzed by PCR. ChIP analysis was performed at least in three independent experiments. PCR products were separated by 3% agarose gel electrophoresis and visualized using ethidium bromide staining. RARE specific and non-specific primer sequences for mouse *Pitx2*, *RARβ*, and *Raldh1* (*Aldh1a1*) genes used in this study were:

Pitx2 DR5a-F: 5′-CAAGATACTGGTCTGTTACCTTCC-3′
Pitx2 DR5a-R: 5′-GTTTCCGAATTACCTATCTGAGAGG-3′
Pitx2 DR5b-F: 5′-CATTTTAAGTCCCTCTCTGACAACC-3′
Pitx2 DR5b-R: 5′-GTGCAAGAGCCTGGTAATCCCT-3′
Pitx2 NS-F: 5′-GAAATTTGTTCCACTCTGGAGAACC-3′
Pitx2 NS-R: 5′-GGTAATGATGGGAAGGGGCTAATC-3′
RARb DR5-F: 5′-TGGCATTGTTTGCACGCTGA-3′
RARb DR5-R: 5′-CCCCCCTTTGGCAAAGAATAGA-3′
RARb NS-F: 5′-AGTACAGACCTTCCAAGAGTGCCT-3′
RARb NS-R: 5′-GTCATGGGAAAGAGAGGTTGAGC-3′
Raldh1 DR5-F: 5′-TGCACACACACCCTTAGCACAG-3′
Raldh1 DR5-R: 5′-CAGGTGACAGGCTCAGCAAATTG-3′

Electrophoretic gel mobility shift assay (EMSA)

Sixteen wild-type E12.5 embryo eyes including surrounding perioptic mesenchyme tissue were dissected out and nuclear protein extracts were prepared as described (Dignam et al., 1983). Biotin-labeled double-stranded oligonucleotide probes containing RARE sequences (DR5a and DR5b) were bound to nuclear extracts. Binding reactions were performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Reaction mixtures were incubated for 20 min at room temperature. Binding reactions were subjected to EMSA on 6% non-denaturing polyacrylamide gels in 0.5 Tris-Borate–EDTA buffer, and detection was performed using a LightShift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's instructions. For super-shift analysis, nuclear extracts were incubated with a 3 µg of anti-RARα (sc-551, Santa Cruz Biotechnology), anti-RARβ (Affinity Bioreagents), or anti-RARγ antibodies (sc-550 Santa Cruz Biotechnology) for 20 min on ice before adding probe. Sequences of wild-type and mutated oligonucleotides (underlined bases indicate mutations introduced) containing the DR5 RA response elements used in gel shift reactions are as follows (the complementary strand was also synthesized and annealed to the strand shown here which was biotin-labeled):

Pitx2 RARE DR5a WT: 5′-TTAGGTAATTCATTAGAAAGTCAATACA-GAC-3′
Pitx2 RARE DR5a Mut: 5′-TTAGGTGATAAATTAGAGAGAAAATACA-GAC-3′
Pitx2 RARE DR5b WT: 5′-TTTAAATCAGATCATCGAAGAGTCACCA-GAAA-3′
Pitx2 RARE DR5b Mut: 5′-TTTAAATCGGAAAATCGAACAGAAACCA-GAAA-3′