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Analysis of *Cyp26b1*/*Rarg* compound-null mice reveals two genetically separable effects of retinoic acid on limb outgrowth

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ABSTRACT

The role of retinoic acid (RA) in limb development is unclear, although it has been suggested to be a proximalizing factor which plays a morphogenetic role in pattern formation. Exogenous RA produces a teratogenic effect on limb morphology; similarly, changes in the endogenous distribution of RA following genetic ablation of the RA-metabolizing enzyme, CYP26B1, result in phocomelia accompanied by changes in expression of proximo-distal (P–D) patterning genes, increased cell death, and delayed chondrocyte maturation. Here we show that disruption of RA receptor (RAR) gamma in a *Cyp26b1*$^{-/-}$ background is able to partially rescue limb skeletal morphology without restoring normal expression of proximo-distal patterning genes. We further show that embryos deficient in CYP26B1 exhibit early localized domains of mesenchymal cell death, which are reduced in compound-null animals. This model reveals two genetically separable effects of RA in the limb: an apoptotic effect mediated by RARγ in the presence of ectopic RA, and a P–D patterning defect which is uncovered following the loss of both CYP26B1 and RARγ. These data provide genetic evidence to clarify the roles of both RA and CYP26B1 in limb outgrowth and proximo-distal patterning.

Introduction

Retinoic acid (RA), the principally active metabolite of vitamin A, is required for normal embryonic development. However, maintaining appropriate levels of RA during development is essential, as inappropriate exposure of RA-sensitive tissues to excess levels results in teratogenicity. RA can elicit transcription through nuclear hormone receptor complexes consisting of retinoic acid receptor (RARα, β, γ) and retinoid X receptor (RXRα, β, γ) heterodimers, which act, in general, as ligand-activated transcription factors at RA response elements (RAREs) (Mark et al., 2006; Niederreither and Dolle, 2008). The receptor isoforms are widely expressed throughout the embryo during all stages of development (Dolle et al., 1989; Dolle et al., 1990; Ruberte et al., 1991; Ruberte et al., 1990), and although there is some tissue-specificity for the various isoforms, the receptors exhibit considerable functional redundancy (reviewed in Mark et al., 2009). Thus, essentially all cells within the embryo are able to respond to RA, in principle. This means that proper regulation of RA distribution during embryonic morphogenesis is necessary to limit tissue exposure. Regulation of RA distribution is accomplished primarily via strict localized RA synthesis and degradation by the RALDH enzymes (RALDH1-3) and CYP26 enzymes (CYP26A1, B1, and C1), respectively (Duester, 2008; Niederreither and Dolle, 2008).

Genetic inactivation of each of the RA receptor isoforms and isotypes, *Raldh's*, and *Cyp26s*, either alone or in combination, has revealed some specific roles for each of these proteins in controlling RA signalling and has uncovered tissues where RA is required, active, and harmful. One such tissue that is acutely sensitive to changes in RA availability is the developing limb. The limb is an important heuristic model for studying the molecular and cellular biology of pattern formation and signal transduction, ultimately leading to morphogenesis of a highly complex but systematically reproducible structure. The limb bud originates as a protrusion of mesenchymal cells encased by ectoderm and proceeds to grow until each of the characteristic limb segments (the stylopod – humerus or femur, zeugopod – radius/ulna and tibia/fibula, and autopod – wrist or ankle and digits) are established.

Within the limb bud, there are two integrated signalling centers which act to direct development along the anterior-posterior (A–P; or thumb little finger, radius to ulna/tibia to fibula) and proximal-distal (P–D; or body wall to fingers/toes) axes. These signalling centers are known as the zone of polarizing activity (ZPA), located at the posterior limb bud mesenchyme and expressing sonic hedgehog (Shh), and the apical ectodermal ridge (AER) located at the limb bud distal tip which expresses fibroblast growth factors (Fgf8, Fgf4, Fgf9, and Fgf17). It is clear that the interplay between signals from the ZPA and AER ultimately results in the formation of the stylopod, zeugopod, and

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autopod (Laufer et al., 1994; Niswander et al., 1994); however, there remains significant difficulty in reconciling the various genetic data generated in recent years with an appropriate model of vertebrate limb development, and the signals involved in regulating P–D limb outgrowth (Mariani et al., 2008; Tabin and Wolpert, 2007). More specifically, although the AER-derived FGFs have been recently shown to each play a component role and function instructively in limb outgrowth (Mariani et al., 2008), the need for an opposing proximal signal remains undefined. It has been suggested that this signal may be RA (Mariani et al., 2008; Mercader et al., 2000), which is able to diffuse into the limb bud from the adjacent flank region, and can oppose FGF signalling following exogenous application (Mercader et al., 2000).

RA has long been implicated in limb development. Early studies on regenerating amphibian limbs showed that RA had proximalizing activity (Maden, 1982; Niazi and Saxena, 1978), and it was the first candidate morphogen described based on studies in chick limbs, where RA was found to elicit polarizing activity leading to digit duplications (Summerbell and Harvey, 1983; Tickle et al., 1982). Moreover, ablation of the main RA-synthesizing enzyme, RALDH2, results in an absence of limb buds (Niederreither et al., 1999), and RA excess, either pharmacologically or by disruption of the main CYP26 expressed in the limb, *Cyp26b1*, results in various limb malformations including shortening of the P–D axis (Yashiro et al., 2004). Taken together, these early data led to the paradigm that RA acted as an instructive proximalizing morphogenetic factor in limb development; functioning in a graded manner to activate transcription of target genes and thus provide positional information to limb cells. However, using a genetic model that lacks RA synthesis, it has recently been shown that RA is, in fact, dispensable for normal limb P–D patterning but permissive for forelimb induction (Zhao et al., 2009).

At early stages of limb development, RA is locally produced in the flank mesoderm by RALDH2, and restricted from diffusing into the limb bud by the activity of the RA-metabolizing enzyme CYP26B1. In the absence of CYP26B1 embryos exhibit defects of both fore- and hindlimbs, caused by local increases in RA, resulting in phocomelia and hypodactyly (Yashiro et al., 2004; and this work). This phenotype was previously suggested to be the result of misexpression of genes involved in P–D patterning, including expansion of proximal *Meis* genes and loss of distal *Hox* genes, along with increased cell death in chondrogenic precursors, and delayed chondrocyte maturation (Yashiro et al., 2004). However, the observed phenotype – severe reduction/fusion of the stylopod and zeugopod along with loss of wrist elements and some digits – was not consistent with what is predicted following an increase in *Meis* expression, namely a selective shortening of the zeugopod and minor effects on autopod formation, but minimal effects on stylopod development (Capdevila et al., 1999; Mercader et al., 1999, 2009). Here we expand upon and reinterpret these data by showing that further disruption of all isotypes of *Rarg* in a *Cyp26b1*-null background is able to rescue formation of the stylopod and some digits, but not the zeugopod. We show that there is increased limb mesenchymal cell survival in the compound-null limbs, but that regulation of P–D patterning genes remains unchanged between *Cyp26b1*$^{-/-}$ and *Cyp26b1*/*Rarg* compound-null limbs. This model provides genetic evidence to suggest that there are two discrete effects of ectopic RA in the limb following the loss of CYP26B1, a "teratogenic" effect mediated by RARγ, and a patterning defect typified by the expansion of *Meis2*, which does not require RARγ.

Materials and methods

Creation of mouse strains

Generation of the mice lacking *Cyp26b1* and all isotypes of *Rarg*, along with genotyping methods by PCR, have been previously described (Iulianella and Lohnes, 1997; MacLean et al., 2007). The mice used in this study were of a mixed genetic background and all embryonic genotypes were generated by intercrossing *Cyp26b1*$^{+/-}$/*Rarg*$^{+/-}$ animals. All animal experiments were performed in ethical accordance with protocols approved by the University's Animal Care and Use Committee.

Skeletal analysis

Cartilage and bone were stained with Alcian blue and Alizarin red, respectively, at E18.5 following standard procedures (Jegalian and De Robertis, 1992). A total of 19 fetuses, including 3 *Cyp26b1*$^{-/-}$/*Rarg*$^{-/-}$ embryos, were scored for skeletal abnormalities (see Table 1 for limb analysis).

Whole-mount in situ hybridization

Staged embryos were pooled according to genotype and whole-mount *in situ* hybridization was performed according to standard protocol (Chotteau-Lelievre et al., 2006). For each probe, embryos were processed concurrently and staining reaction times were maintained between genetic groups to control for variations in signal intensity. At least 3 *Cyp26b1*$^{-/-}$ and control, and 2 *Cyp26b1*$^{-/-}$/*Rarg*$^{-/-}$ embryos were examined for each gene and developmental stage. There was no variation observed within the genetic groups and results depict representative staining. Plasmids for making antisense riboprobes were described previously (*Cyp26b1*) (MacLean et al., 2001) or kindly provided by Drs. P. Dollé (*Meis2*, *Hoxa13*, *Hoxd13*, *Rarg*) Y. Chen (*Shox2*), S. Potter (*Hoxa11*), G. Martin (*Fgf8*), and A. McMahon (*Shh*).

Analysis of apoptosis in whole limb buds

Embryos were collected into warm PBS and stained with 5 μM LysoTracker Red (Invitrogen Canada Inc.) in PBS at 37 °C for

Table 1
Summary of limb skeletal abnormalities observed at E18.5.[a]

	B1$^{-/-}$ ($n=4$, embryos)	γ$^{-/-}$ ($n=4$)	B1$^{-/-}$/γ$^{+/-}$ ($n=8$)	B1$^{-/-}$/γ$^{-/-}$ ($n=3$)
Forelimbs				
Clavicle formed	0/4 (0%)	4/4 (100%)	0/8 (0%)	3/3[b] (100%)
Humerus formed	0/4 (0%)	4/4 (100%)	0/8 (0%)	3/3 (100%)
Radius/ulna formed	0/4 (0%)	4/4 (100%)	0/8 (0%)	
Partial				3/3 (100%)
Digit number				
One	1/4 (25%)			
Two	3/4 (75%)		4/8 (50%)	
Three			4/8 (50%)	
Four				3/3 (100%)
Five		4/4 (100%)		
Hindlimbs				
Pelvic bone formed		4/4 (100%)		3/3[c] (100%)
Partial	1/4[d] (25%)		8/8[e] (100%)	
Femur formed	0/4 (0%)	4/4 (100%)	0/8 (0%)	3/3 (100%)
Tibia/fibula formed	0/4 (0%)	4/4 (100%)	0/8 (0%)	0/3 (100%)
Digit number				
One	1/4[f] (25%)			
Two	3/4 (75%)		4/8 (50%)	
Three			3/8 (37.5%)	
Four			1/8 (12.5%)	3/3 (100%)
Five		4/4 (100%)		

[a] All phenotypes were observed to be bilateral.
[b] Smaller than normal.
[c] Ilium and pubis nearly normal but no ischium.
[d] Partial formation of pubis.
[e] Formed normally with reduction in length of ilium.
[f] Two digits but fused at terminal phalanx.