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Limb Development: The Rise and Fall of Retinoic Acid

Retinoic acid was thought to play a key instructive role during limb bud initiation and subsequent patterning. New results argue instead that its role is permissive: retinoic acid is essential only to antagonize early axial Fgf signals that otherwise inhibit the limb field.

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Vitamin A is a nutritional supplement essential for various aspects of adult physiology as well as normal embryogenesis. The primary metabolic derivative of vitamin A is retinoic acid, a small lipophilic molecule acting as a ligand that binds to nuclear receptors of the steroid receptor superfamily. These receptors bind to genomic retinoic acid responsive elements (RAREs) and function either as transcriptional activators or repressors depending on whether or not they are bound by retinoic acid [1]. During embryonic development, retinoic acid levels must be correctly regulated; excess levels are teratogenic, often causing defects in the same embryonic processes that are thought to require retinoic acid signaling during normal development [1]. The rate-limiting step in retinoic acid synthesis from vitamin A is controlled by one of three retinaldehyde dehydrogenases (Raldh 1, 2 or 3), each of which is expressed in restricted temporal-spatial domains in the embryo [2]. Raldh2 plays the most important role in early embryogenesis as it is responsible for nearly all retinoic acid production during early development [3]. Oxidation of retinoic acid, leading to its degradation and removal, is carried out by several cytochrome P450 family members (Cyp26a1, b1 and c1), which are also expressed in spatially restricted domains, frequently complementary to that of the Raldh synthetic enzymes in the early embryo [1] (Figure 1). It is clear that such specific sites of retinoic acid synthesis and degradation result in graded retinoic acid distributions, which have been implicated in the instruction of embryonic patterning by a large body of work spanning three decades. Now focusing on the embryonic limb in a recent issue of *Current Biology*, Duester and colleagues [4] challenge some of these long-accepted models and propose that retinoic acid plays no instructive role, but rather acts entirely permissively in limb initiation and patterning (Figure 1).

Retinoic acid was the first candidate molecule hailed as a 'morphogen' — a diffusible signal that generates pattern in the embryo because cells receiving the signal respond differently at different threshold concentrations of the signal. This hypothesis was proposed based on studies of limb development, one of the most intensely studied aspects of vertebrate morphogenesis [5]. Limbs originate as outgrowths of mesenchyme, jacketed in ectoderm, budding from the lateral plate of the embryo. Once limb bud formation is underway, the bud is a self-organizing system maintained by mutual cross-regulation of several signaling centers that coordinate patterning and growth: the most important of these are the apical ectodermal ridge (AER), a specialized ectoderm ridge along the distal edge of the limb bud secreting Fgf signals that support cell survival and outgrowth of the proximal-to-distal axis (shoulder to digits), and the zone of polarizing activity (ZPA), which secretes Sonic Hedgehog (Shh) to pattern the anterior-to-posterior axis (thumb to pinky) (Figure 1). These signaling centers have been largely conserved across vertebrate evolution — they function in an analogous manner even during early pectoral fin development in fish. The ascendancy of retinoic acid to morphogen status began when it was found to effectively mimic the action of the ZPA and thus was hypothesized to be the long sought for molecular effector of ZPA function. Subsequent work revealed that retinoic acid was not the endogenous ZPA signal but rather induced expression of the transcription factor Hand2, which polarizes the limb AP axis and activates Shh, which then carries out ZPA functions [6].

Besides its possible role in anterior-posterior patterning, retinoic acid may act in patterning the limb proximo-distal axis. Retinoic acid acts as a proximalizing agent in amphibian limb regeneration, which shares some regulatory features with normal limb development. Retinoic acid treatment of amputation blastemas clearly leads to a re-establishment of proximal fates with ensuing outgrowth of a complete set of duplicated proximo-distal structures [7]. In the chick embryo, retinoic acid regulates the proximal expression of the tale-homeobox gene *Meis/Meis2* in the limb [8], noted for its role in specifying 'proximal' segment

identity in the *Drosophila* limb and a possible similar function in vertebrates [9]. In the amniote limb bud, a proximo-distal retinoic acid gradient is generated by the restriction of both synthesis and metabolic turnover. This occurs by high levels of retinoic acid synthesis via Raldh2 activity proximally in the trunk and high levels of retinoic acid metabolism via Cyp26b1 activity in the distal limb. This gradient in normal limb buds is readily visualized [10,11] by staining the limb bud for β-galactosidase activity encoded by a commonly used RARE-LacZ transgene reporter [12]. Likewise, the same technique has been used to visualize the disruption of this gradient due to increased limb bud retinoic acid levels in mouse mutants with an inactivated *Cyp26b1* gene. These mutants display a reduction of distal skeletal elements of the limb, and changes in gene expression consistent with a proximalization of limb elements, including upregulation of Meis/Meis2 [11]. These findings support the idea that a retinoic acid gradient controls the limb's proximo-distal axis. On the other hand, the distal reduction or truncation defects due to excess retinoic acid or ectopic Meis expression in the developing amniote limb do not really recapitulate the proximalizing role of retinoic acid during amphibian limb regeneration, where specification of proximal fates by retinoic acid results in a complete set of limb elements with proximal duplications [7–9]. Also, the RARE-lacZ data only tell us that a gradient exists and that the increased retinoic acid levels in *Cyp26b1* mutant embryos disrupt limb development; however, they do not prove that retinoic acid activity is required for limb development, which is the question the Duester group attempts to answer in a recent issue of *Current Biology* [4].

An obvious approach to address this question would be to analyze embryos lacking Raldh activity and, therefore, retinoic acid itself. Raldh inhibitors block limb bud formation in chick [13] and the zebrafish mutant *no fin* was found to be a loss-of function allele of *Raldh2* [14]. Mouse mutants lacking *Raldh2* do not form limb buds, but it is possible that this is not an effect on limb development *per se* but rather due to growth arrest [3]. This impediment can be overcome by providing the pregnant mothers with dietetic retinoic acid supplements [2,3]. When a low
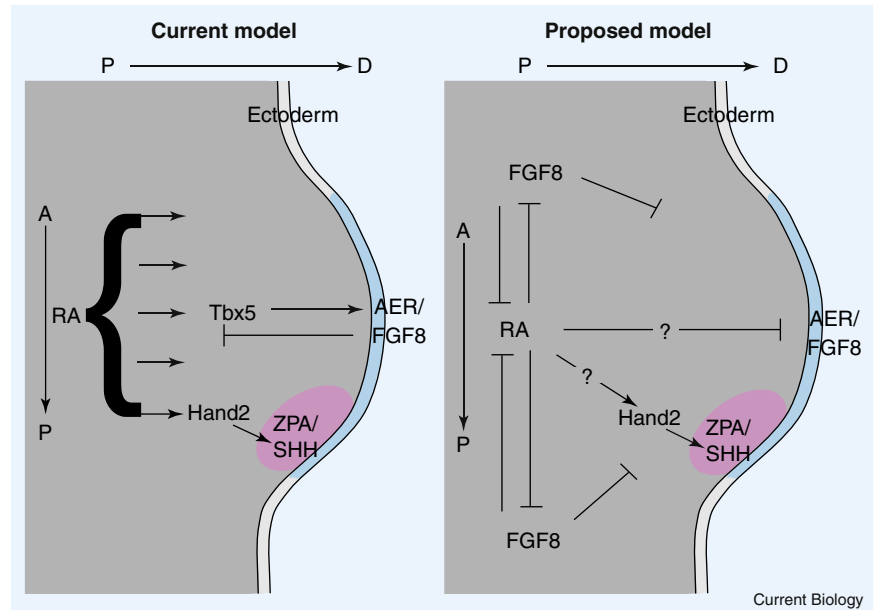

Figure 1. Old and new models of retinoic acid function in limb development.

In the current model (left), retinoic acid (RA), generated in the lateral flank mesoderm, induces the expression of *Tbx5*, which is required for forelimb initiation, as well as *Hand2*, required for proper induction of *Sonic Hedgehog* (*Shh*) in the zone of polarizing activity (ZPA). Additionally, opposing activities of proximal retinoic acid and distal fibroblast growth factor (Fgf), generated from the apical ectodermal ridge (AER), pattern the proximal distal (P-D) axis of the limb. Thus, retinoic acid plays an instructive role in limb development. The new model (right) proposed that retinoic acid is only required permissively to inhibit the spread of *Fgf8* expression that, if unchecked by retinoic acid, would inhibit limb induction. (A-P: anterior-posterior axis.)

retinoic acid dose is supplied, the forelimbs of such 'rescued' embryos are smaller than normal, and display abnormal expression domains of *Shh* and *Fgfs* [10,15]. In contrast, their hindlimbs develop rather normally [10,15]. Furthermore, limb bud-specific retinoic acid activity was very low or undetectable using the aforementioned RARE-lacZ reporter. However, near the hindlimb bud, reporter activity, presumed to be induced by retinoic acid that was produced via Raldh3 activity, was detected nearby in the mesonephros (embryonic kidney) near the hindlimb, leading to speculation that this activity may be required for the observed comparatively normal hindlimb [2,3].

Writing in *Current Biology*, Zhao *et al.* [4] followed up on this by generating *Raldh2$^{-/-}$;Raldh3$^{-/-}$* double mutant embryos which lack all endogenous retinoic acid in the trunk. When these embryos were rescued with low doses of supplemental retinoic acid, normal hindlimb buds still formed that displayed relatively normal expression of genes involved in limb patterning. Because the authors found no detectable expression from the

RARE-lacZ transgene in the hindlimb buds, nor in the lateral plate mesoderm, they concluded that retinoic acid activity is not required for hindlimb development.

Analysis of the smaller forelimb buds that develop in these mutants generated more complex data. Again, using the same RARE-lacZ transgene as a reporter of retinoic acid activity, the authors found that maternally supplemented retinoic acid induced transgenic β-galactosidase activity only in the neural tube, relatively far away from the emerging forelimb. Realizing that potential limb-bud specific retinoic acid activity may be present but not high enough to induce the reporter transgene, they also examined the expression of two endogenous genes that contain regulatory RAREs and respond to retinoic acid signaling: *RARβ*, which is expressed in the lateral plate mesoderm prior to limb bud induction, and *Cdx1*, which is expressed in the limb bud mesenchyme. Neither gene was expressed in their respective domains in rescued *Raldh2$^{-/-}$* limbs, supporting the authors' conclusion that retinoic acid signaling within or

near the limb bud is dispensable for limb development.

How normal were these rescued forelimbs? The earliest forelimb marker is an expression domain of *Tbx5*, which encodes a T-box transcription factor, in the lateral plate mesoderm in the forelimb "field" prior to visible budding. This *Tbx5* induction does not take place in *Raldh2* [-/-] embryos, but occurs in rescued embryos, albeit with a delay. Furthermore, genes that mark or play a role in patterning the limb axes were all activated in rescued embryos. However, three of these expression domains (*Shh*, *Hand2* and *Hoxa11*) were shifted distally. The authors suggest that this alteration may be due to growth retardation of the limb bud. These distal shifts in expression domains are likely to prefigure a distortion of the final forelimb skeletal pattern [15], leading to the possibility that retinoic acid signaling may be required for formation of a normal skeletal pattern. Nevertheless, the authors' main point that is retinoic acid is not required for induction of these genes *per se*, as had been previously hypothesized [10,15].

If the authors are correct, then retinoic acid plays no instructive role in limb patterning in contradiction to prevailing models of limb development [8,11]. Instead, Zhao *et al.* suggest that retinoic acid may provide a permissive environment locally for limb bud induction (Figure 1). A similar idea was proposed by Gilbert *et al.* [16], who found that axial retinoic acid signals played a permissive role in the induction of zebrafish pectoral fins. Zhao *et al.* [4] advance this idea by suggesting that the role of retinoic acid is to limit *Fgf8* expression, which they hypothesize might otherwise inhibit limb bud induction. Previous work by the Duester lab and others has demonstrated that, in the early embryo, retinoic acid activity restricts two different *Fgf8* expression domains: one at the posterior body axis [17] and one in the cardiac lateral plate mesoderm [18]. Zhao *et al.* [4] suggest that the resulting Fgf8-free zone is necessary for forelimb bud induction. To support this idea they showed that fin development in retinoic acid-deficient zebrafish embryos can be rescued by chemically inhibiting Fgf signaling. They also found aberrant *Fgf8* expression in the intermediate mesoderm near the forelimb in *Raldh2* [-/-] mouse embryos. The brief

maternal retinoic acid dose that allows the formation of small forelimbs in these mutants only slightly altered this *Fgf8* domain; whereas an increased dose that resulted in more normal sized limb buds abolished the ectopic *Fgf8* domain. However, for this level of rescue, RARE-lacZ is activated in the lateral plate mesoderm, suggesting that some level of retinoic acid signaling may be required for normal forelimb budding. In regard to hindlimb development, Zhao *et al.* suggest that, independent of retinoic acid signaling, the normal extension of the body axis results in the regression of the tailbud *Fgf8* domain to a position too posterior to interfere with limb budding.

Zhao *et al.* [4] thus propose that retinoic acid signaling within the limb bud is not required for patterning, but acts in the body axis as a permissive signal that allows limb bud initiation. As the authors point out, this model of limb development requires a reinterpretation of the distalization defects observed in mouse *Cyp26b1 -/-* mutants [11]. From this new perspective, *Cyp26b1*-mediated retinoic acid metabolism does not occur in the distal limb bud to generate a required retinoic acid gradient but simply to cancel retinoic acid activity from the body axis, which would otherwise have teratogenic effects in the limb bud. Such a mechanism seems like a very poor 'design' but clearly may provide a system for natural selection to act upon, thereby varying limb pattern; this speculation implies retinoic acid signaling may play an instructive role in some species and not others.

The model presented by Zhao *et al.* [4] is tantalizing, yet likely to be controversial as sections of it are built upon negative data — the lack of an experimental retinoic acid responsive signal that would have indicated that retinoic acid is active in the limb bud; this is the classic conundrum of 'proving a negative'. As the authors point out, they are assuming that their reporters have very responsive RAREs and, therefore, a lack of a signal is meaningful. Also, it should be noted that this is a standard approach to detect which cells are responding to retinoic acid in the embryo. It may be helpful for the community to generate a wider range of retinoic acid-reporter mice, as has been done in the WNT signaling field [19].

Nevertheless, the authors have provided an intriguing model that is certain to generate discussion and that provides clear predictions that can be tested via conditional genetics [20]. For example, inactivation of *Fgf8* specifically in the intermediate mesoderm of *Raldh2 -/-*-mutants may rescue the forelimb field. On the other hand, if the RARE in the *Fgf8* region is necessary for its repression by retinoic acid, as the authors suggest, then deletion of this element in otherwise wild-type embryos, specifically in the intermediate mesoderm, may inhibit forelimb development. Considering the critical role that Fgf signaling plays later in normal limb bud patterning, it will important to sort out this potential inhibitory role of Fgf in limb induction. Once again, the limb is full of surprises and points the way, via the new work reported in this journal, towards rethinking how regulatory hierarchies govern patterning.

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