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Gradients, waves and timers, an overview of limb patterning models

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ABSTRACT

The vertebrate limb represents one of the oldest and most studied models in developmental and regenerative biology. Starting with classical experimental embryology and regenerative studies, its relevance in understanding biological mechanisms has expanded through the molecular biology era and now leads systems biology approaches in organogenesis. Limb patterning is organized along three main orthogonal axes; proximo-distal (P-D), antero-posterior (A-P) and dorso-ventral (D-V). Considerable heterogeneity has been found for the mechanisms involved in patterning these three axes, including signal gradients, cell-intrinsic timers and Turing-type signalling wave formation. Here we concentrate on reviewing patterning mechanisms along the P-D and A-P axes, in which different mechanisms converge and interact to pattern segmented structures.

Contents

1. Introduction

The tetrapod limb is a complex structure patterned along three main axes; proximo-distal (P-D), antero-posterior (A-P) and dorso-ventral (D-V). Along the P-D axis three main skeletal segments can be distinguished: the most proximal one corresponds to the stylopod (humerus/femur); the intermediate one corresponds to the zeugopod (ulna and radius/tibia and fibula) and the distal one comprises the autopod (hand/foot). Specification and patterning of limb skeletal elements along these axes is largely achieved by the action of signalling centres positioned at the apical ectodermal ridge (AER) for the P-D axis, the zone of polarizing activity (ZPA) for the A-P axis, and the dorsal ectoderm for the D-V axis.

The limb initiates as a swelling from the lateral body wall around stage 16HH in chick and E9.5 in mouse (reviewed in [1]). The limb bud grows mainly along the P-D axis [2,3] with additional A-P growth during autopod generation. The AER is an ectodermal thickening that runs from anterior to posterior along the D-V border of the distal ectoderm. The AER is essential for growth and patterning along the P-D axis through the production of fibroblast growth factors (FGFs) and WNT signals (reviewed in [1]). The ZPA is a posterior mesodermal region lying beneath the ectoderm that expresses *Sonic Hedgehog* (*Shh*) [4,5], which controls growth and patterning along the A-P axis. The ZPA is located near the posterior end of the AER and a positive feedback loop is established between these two signalling centres preserving their function during limb outgrowth. Finally, the D-V pattern of the limb bud is directed by the non-AER ectoderm. ENGRAILED-1 (EN-1) in the ventral ectoderm restricts *Wnt7a* to the dorsal ectoderm. WNT7A at the same time induces *Lmx1b* in the dorsal mesoderm [6–8]. While the AER and ZPA produce, respectively, distal and posterior signals, antagonizing gradients have been proposed to pattern these axes. Additional mechanisms involving time measurement and Turing reaction-diffusion processes have also been proposed to pattern these axes.

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Here we will review P-D and A-P patterning mechanisms, with a special focus on antagonizing gradients and recent advances on non-gradient mechanisms.

2. Proximo-distal patterning

How the P-D pattern is established in the tetrapod limb remains a controversial question. The precursors for all limb P-D segments lie initially beneath the AER and expand and differentiate in a P-D sequence. More than 40 years ago the **progress zone model** (PZM) [9] postulated that beneath the AER and under its influence, a defined-size region of undifferentiated mesenchyme called the progress zone (PZ) is established. As the limb bud grows, cells from the PZ would passively exit this region due to proliferation and start differentiation upon loss of AER influence. While in the PZ, cells would undergo progressive distalization, so that PZ exit time would not only allow differentiation but also determine the P-D fate. This model assumes that cells need to "count" time and was the first to consider time as an important factor in specification.

In contrast to the PZ model, the **two-signal model** (TSM) proposes progressive limb distalization regulated by signals [10–12]. This model exploited the identification of *Meis* genes as proximal limb specification factors [10,12,13] and is based on the observations that retinoic acid (RA), a proximal signal emanating from the flank, activates these genes [12] and AER-FGF signals counteract this activity [12]. Further support to this model was provided by the analysis of mouse mutants for AER-*Fgf*s [11], for the RA-degrading enzyme *Cyp26b1* [14] and for *Shh* [15]. This model reconciles well with the progressive nature of limb development; initially the flank is rich in RA and upon limb induction *Raldh2* expression stops in the prospective limb bud [16] and distal FGF signal builds up, resulting in a temporally and spatially dynamic RA/FGF ratio during limb outgrowth.

Molecular analysis applied to classical and new embryological experiments led to the formulation of the **early specification model** [17], which proposed that the three P-D segments are specified at once in the early undifferentiated limb and just differentiate and expand progressively as the limb bud grows. This model received some support from phenotypes reported in AER-*Fgf* mutants but did not provide a plausible mechanisms or markers for the proposed early segmental specification. Furthermore, it did not account for the progressive nature of P-D limb segment specification [18].

Overall, the main difference between these models is that in the PZM signals only have a permissive role and P-D identities are instructed by an intrinsic cell-autonomous mechanism, whereas in the TSM signals play a direct instructive role. Therefore, the debate is centred on whether P-D specification occurs in an autonomous or non-autonomous manner. In support of a non-autonomous mechanism, the signalling environment has been shown to be essential for the specification of the main transition from stylopod to zeugopod specification [19,20]. Cooper et al. reached this conclusion through experiments in which they treated cultured limb cells with combinations of FGF8, RA and WNT3A and maintained them in culture for different times. By performing recombinant limbs (RL) with these cells, they were able to test their potential to form the different P-D skeletal elements. Cells exposed to FGF8 + RA + WNT3A retained the ability to develop the whole P-D pattern even after 36 h in culture, while cells exposed only to FGF8 + WNT were only capable of generating autopod elements [19]. Roselló-Díez et al. reached the same conclusions by performing RLs with cells from distal tips of 20HH limb buds that were grafted to a RA-free or RA-rich region. Only RLs transplanted to a naturally RA-rich environment developed the three P-D segments, whereas in the RA-free areas, they just formed the zeugopod and autopod. Furthermore, RLs in RA-free environments supplemented with exogenous RA did develop the three P-D segments and RLs transplanted to RA-rich region treated with RA-antagonist only formed zeugopod and autopod. Despite these results, controversy has been raised on the role of RA in limb P-D specification (reviewed in [21]). *Rdh10*$^{trex/trex}$ RA-deficient mice show stunted forelimbs but hindlimbs are normal [22,23] and retain *Meis* expression in both FLs and HL [23,24]. Based on these observations, it has been proposed that RA does not play any role in P-D limb patterning or limb induction. An alternative **one-signal model** has been proposed [21] in which MEIS activity and the proximal program is a default state present before the limb appears and progressive distalization is achieved by the sole action of the distal signals. According to this model, RA needs to be removed from the distal limb bud by AER-FGF-mediated *Cyp26b1* activation to allow distal gene expression, but it would not play any role in promoting the proximal limb program. However, this model does not take into account that a P-D RA gradient does exist in WT limb buds and that RA proximalizes the limb bud expression profile when increased distally [14,25]. Alternatively, given that the complete *Rdh10* KO still retains some RA-synthesizing ability and that the *Rdh10*$^{trex/trex}$ mutation is hypomorphic [22,26], it could be argued that these mutants produce enough RA for limb patterning. This possibility would highlight a remarkable ability of limb patterning to adapt to variable levels of RA availability. In our opinion, further experiments in which endogenous RA is fully removed from the proximal limb would be required to either accept or discard RA as a proximal signal in P-D limb patterning.

Results supporting the TSM, however, only apply so far to the establishment of the stylopod–zeugopod transition and did not explain further limb distalization. While a low RA/FGF ratio is needed for the zeugopod-autopod transition, this is not sufficient to induce this transition [27]. Studies in the chick have discarded as well that temporal AER-FGF signal integration plays a role in activating *Hoxa13*, a marker of the autopod [20,28]. Results indicate that once the RA/FGF ratio is low enough to allow *Hoxa13* expression, time measured autonomously by distal limb cells is in addition needed for *Hoxa13* transcriptional activation [27,28]. Given that HOXA13 suppresses the zeugopod program, the time between RA/FGF ratio dropping below the *Hoxa13* activation threshold and actual *Hoxa13* activation has been postulated as essential for allowing enough zeugopod cells to be specified [27]. These results suggested a two-phase model in which the signalling environment would control the stylopod–zeugopod transition and further distalization would require a permissive signalling environment and an instructive cell-autonomous timing mechanism [27].

A similar model has been proposed based on elegant transplantation experiments in the chick [29]. In this study GFP-expressing grafts from distal region of a stage 20HH limb were placed under the AER of a 20HH host (homochronic graft) or to stage 24HH hosts (heterochronic). In homochronic grafts, *Hoxa13* expression started at the same time and in the same domain as in the host, however, in heterochronic grafts *Hoxa13* behaved according to the donor age, being activated later than in the host. Interestingly, AER-*Fgfs* expression, which normally decays with limb age, lasted longer in host ectoderm covering the heterochronic grafts, indicating crosstalk from the mesenchyme to AER to instruct on limb bud age. Age-dependent cell cycle and cell adhesive properties also behaved autonomously in these experiments, indicating temporal autonomy of the grafts [29]. Despite these observations, 20HH to 24HH heterochronic grafts did not produce the proximal structures they would have generated in their limb of origin, but developed into digits, according to, and integrating with the host skeletal elements. The environment thus prevented the ectopic differentiation of proximal structures, indicating incomplete ability of intrinsic mechanisms to autonomously generate skeletal elements [29].

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