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Review

Getting a handle on embryo limb development: Molecular interactions driving limb outgrowth and patterning

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ABSTRACT

Development of the vertebrate embryo involves multiple segmentation processes to generate a functional, articulated organism. Cell proliferation, differentiation and patterning involve spatially and temporally regulated gene expression and signal transduction mechanisms. The developing vertebrate limb is an excellent model to study such fine-tuned regulations, whereby cells proliferate and are differentially sculptured along the proximal–distal, anterior–posterior and dorsal–ventral axes to form a functional limb. Complementary experimental approaches in different organisms have enhanced our knowledge on the molecular events underlying limb development. Herein, we summarize the current knowledge of the main signaling mechanisms governing vertebrate limb initiation, outgrowth, specification of limb segments and termination.

Contents

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1. Introduction

The limb bud initiates from the lateral body wall as a small protrusion of mesenchymal cells within an ectodermal jacket, and is transformed into a three dimensional, functional adult limb. Spatiotemporally coordinated cellular and molecular interactions from the embryo flank, apical ectodermal ridge (AER), zone of polarizing activity (ZPA) and the non-ridge ectoderm sculpt the limb bud along the proximal–distal (PD), anterior–posterior (AP) and dorsal–ventral (DV) axes. The limb is a segmented structure [1], with the basic skeletal architecture of the proximal stylopod, middle zeugopod and distal autopod that are laid down in a PD sequence. While the number of bone elements in the stylopod (humerus or femur) and zeugopod (ulna and radius; tibia and fibula) is conserved across species, the autopod (carpals, metacarpals and phalanges) has been phylogenetically tweaked to adapt specific abilities [2–4]. The overall limb architecture across species, however, is set by conserved mechanisms and the key players are, the fibroblast growth factors (Fgfs), Wnts, sonic hedgehog (Shh), retinoic acid (RA) and bone morphogenetic proteins (Bmps). With the purpose of providing an overview on limb development and promoting its use as a model system for specialized studies, here we review the major molecular events during limb development, namely its initiation, PD, AP and DV outgrowth/patterning and termination.

2. The intermingled process of limb initiation and identity

The presumptive limb territory is molecularly specified at Hamburguer and Hamilton (HH) [5] stage HH13-HH14 in chick (48–50 h of egg incubation) although it only becomes visible to the eye at HH17 (after 53–60 h), or at embryonic day 9.5 in mouse (E9.5). After molecular specification of the forelimb (between somites 15–20 in chick and 7–12 in mouse) and hindlimb regions (somites 26–32 in chick and 23–28 in mouse) at precise AP positions, epithelial-to-mesenchymal transitions (EMT) and intense proliferation of the somatopleural lateral plate cells will cause the limb bud mesenchyme to protrude outward, enveloped into an ectodermal layer of cells [6,7]. The essential role of EMT in limb initiation was recently demonstrated in chick embryo [6]. These authors showed that at HH13, the somatopleure that eventually gives rise to the limb bud, is epithelial in nature, which in later stages become mesenchymal and generate the limb primordium. In addition to the two genes that control limb initiation, Tbx5 and Fgf10 [6], it is possible that more players are involved in the EMT of the somatopleure epithelium and this awaits further research.

2.1. Tbx genes in limb initiation

Although tetrapod fore- and hindlimb pairs look alike in early stages of development, they soon become morphologically and functionally distinct. This starts with the conserved expression of T-box transcription factors *Tbx5* and *Tbx4* in the LPM of prospective fore- and hindlimbs, respectively, and the expression of a paired-like homeodomain factor, *Pitx*, in the hindlimb mesenchyme. Misexpression studies of *Tbx5*, *Tbx4* and *Pitx1* in mouse, chick and zebrafish have corroborated their indispensable roles in limb initiation [8–14]. In *Tbx5* conditional knockout mice, forelimb buds were not formed [8,13]. Inhibition of *Tbx5* or *Tbx4* activity in the prospective fore- and hind-limb fields in chick also produced limbless embryos and their misexpression in the chick embryo flank produced ectopic limbs [14]. In both these scenarios, *Tbx* genes functioned through Fgf and Wnt signaling components, namely *fgf10*, *fgf8* and *wnt2b* or *wnt8c* [14], suggesting that *Tbx* genes function upstream of *fgf* and *wnt* expression (Fig. 1A, A′). However, in zebrafish, Wnt2b is reported to act upstream of *Tbx5* during limb induction [12], signifying that there might be variations in the molecular hierarchy between species.

Unlike *Tbx5* knockouts [8,13], *Tbx4*$^{-/-}$ mouse embryos displayed normal hindlimb induction and initial patterning, although they failed to develop further [15]. Subsequent studies revealed that while Tbx5 and Tbx4 are not necessary for limb outgrowth and skeletal element patterning [16,17], they are required for patterning of the limb muscles and tendons [18]. Employing limb-rescue assays, Minguillon et al. [11] showed that *Tbx4* is capable of replacing *Tbx5* in the forelimb without changing forelimb identity. This ability questions *Tbx5* and *Tbx4* as the molecules that provide limb-specific morphologies. However, the difference in the phenotypes observed in *Tbx5* [8,13] and *Tbx4* [15] null mutants still argues against the possibility of one *Tbx* gene being substituted by the other. Thus, the involvement of *T-box* genes in limb-specific morphologies is still elusive.

Pitx1 is reported to regulate *Tbx4* expression in the hindlimb [19] and contribute to hindlimb specific morphologies when misexpressed in place of Tbx5. Consistent with *Pitx1*'s role in hindlimb identity [19], its misexpression in mouse forelimb region transformed it into a hindlimb at the level of gene expression, bones, muscles and tendons [20].

2.2. Retinoic acid (RA) signaling in limb initiation

RA, the active derivative of vitamin A, has been shown to be critical in many aspects of limb development including its initiation. Although it is difficult to detect its precise location in the embryo, the distribution of RA synthesizing (Retinaldehyde dehydrogenases: *Raldh1-3*) and catabolizing (cytochrome P450 family members: *Cyp26a1*, *b1*, *c1*) enzymes is an approach to infer the location and relative amounts of RA. *Raldh2* is expressed in the somites and in the LPM during limb initiation stages [21,22]. Inserting an impermeable barrier between the somites and the presumptive forelimb LPM inhibited forelimb formation [23], suggesting the importance of somite-produced RA for limb initiation. In zebrafish, transplantation of wild-type paraxial mesoderm cells into *Raldh2* mutant embryos was able to rescue the absence of pectoral fins, further indicating the requirement of RA synthesized in the somitic mesoderm for pectoral fin induction [24].

While perturbation of RA signaling in chick, mouse and zebrafish prevented limb budding [25–27], maternal dietary RA supplementation rescued the absence of forelimbs in *Raldh2* null mice [27,28], clearly showing the involvement of RA in limb initiation. Both in mouse and zebrafish, RA is proposed to have an early role of inducing *Tbx5* expression [27,29]. Accordingly, *Tbx5* is absent in the forelimb field of mouse and zebrafish embryos lacking RA synthesis, and was rescued by RA supplementation [25,27–29]. Nevertheless, a RARE-lacZ reporter failed to detect RA activity in the presumptive limb mesenchyme of the rescued *Raldh2* mutant mouse, suggesting

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