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.

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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;ibia 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 Hamburger 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 lateral plate mesoderm (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 knockout [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 induction 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...
that RA might be indirectly influencing Tbx5 [28] and in zebrafish, this regulation is occurring through Wnt2b [12,26].

2.3. Wnt and Fgf signaling in limb initiation

Beads soaked in Fgf8 and Wnts possess the ability to induce ectopic limbs in the embryo flank [30,31], positioning Fgf and Wnt as key molecules in limb induction. Fgf and Wnt signaling components interact with each other to implement the limb initiation program (Fig. 1). The expanded model proposed by Kawakami et al. [30] states that during chick forelimb initiation, fgf8 expressed in the intermediate mesoderm (IM) [31,32] activates wnt2b expression in the IM and LPM, which then induces fgf10 in the LPM (Fig. 1A′). From here, Fgf10 induces wnt3a in the surrounding ectoderm (SE), where it activates fgf8 expression (Fig. 1B′) and this happens in parallel to the appearance of the AER at the distal tip of the limb bud, overlying the fgf10 expressing limb mesenchyme (Fig. 1C′). Wnt3a helps in the maintenance of fgf8 in the AER [30,33]. Expression of fgf10 in chick presumptive hindlimb LPM is regulated by wnt8c [30]. Both Wnt2b and Wnt8c signal through canonical β-catenin pathway [30]. Although Wnt2b is a crucial component for chick and zebrafish forelimb initiation [12,26,30], its participation in mouse limb induction is questioned, since it is not expressed in the mouse limb [8]. Nevertheless, the crucial transcription factors of Wnt/β-catenin signaling, Lef1 and Tcf1 are known to be required for fgf10 expression and limb development in mouse [8,34]. Induction and maintenance of Fgf10 is crucial for proper limb initiation as the knockout of fgf10 generates limbless mice [35]. Also, absence of fgf10 in the prospective forelimb bud mesenchyme of Tbx5 knockout mice [13] and inability of fgf10 knockout mice to maintain Tbx5 expression [35], suggest the existence of a positive feedback loop between these molecules. Fgf10 is also known to maintain Tbx5 expression in the forelimb and pectoral fin of chick and zebrafish, respectively [12]. Unlike Tbx5 knockouts, in
Tbx4<sup>−/−</sup> mouse embryos fgf10 expression in the hindlimb is initiated but not maintained [15], indicating that Tbx4 is not required to initiate fgf10 expression in mouse hindlimb mesenchyme. Among the FGF receptors (FGFR) that are tissue-specifically expressed in the developing limb, FGFR1 and FGFR2 are expressed in the limb bud from very early stages [36]. While the absence of Fgfr1 didn’t block limb initiation [37], Fgfr2 knockout mice lacked limbs [38], emphasizing the importance of Fgfr2 for limb initiation. Activation of fgfr2 isoforms Fgfr2IIC and Fgfr2IIIC in the mesenchyme and ectoderm by the ectoderm- and mesenchyme-expressed Fgf8 and Fgf10, respectively, is crucial for limb initiation [38,39].

3. Proximal-Distal (PD) limb outgrowth and patterning

3.1. The AER and Fgf signaling in outgrowth and patterning

PD outgrowth and patterning is mainly driven by Fgfs produced in the AER. Induction of fgf8 in the SE by LPM-expressed Fgf10 is a crucial step in the establishment of the functional AER [38,40]. In addition to Fgf signaling mediated by Fgfr1IIC [39,41,42], signaling from Wnt/β-catenin [43], Bmp/Bmp1a [44–46], RA [47] and ectoderm expressed-Shh [48] are implicated in the maintenance of the AER.

AER-Fgfs function as cell survival and proliferation factors for the subjacent mesodermal cells [49,50]. The mature chick and mouse AER expresses fgf2, fgf4, fgf8, fgf9, fgf19 and fgf4, fgf8, fgf9, fgf17, respectively [51]. Both in chick and mouse, fgf8 has the longest expression time-window, covering the entire AER tissue and other AER-fgfs appear relatively later in the posterior AER [51,52]. Accordingly, mice with conditional fgf8 deletion displayed defective limbs [53,54] while KO mice for other AER-Fgfs, alone (fgf4, fgf17 and fgf9) or in combination (triple KO for fgf4, fgf9, fgf17; Supplementary Table 1), did not show any limb abnormalities [52], revealing Fgf8 as the key AER-Fgf for normal limb development. But, double fgf8/fgf4 knockouts had more severe forelimb defects and completely lacked hind limbs [50], indicating the requirement of cumulative AER-Fgf8 and Fgf4 action in this process. Furthermore, the triple fgf8/fgf4/fgf9 knockout mice epitomize the contribution made by each AER-Fgf for the total AER-derived signal, by producing even more severe limb phenotypes [52].

In the distal limb mesenchyme of mouse and chick, Fgf signaling is mediated by Erk/MAPK and Akt/Pi3K intracellular pathways, respectively [55,56]. However, p-Erk expression in chick AER is necessary to preserve AER integrity [56] where Fli13 is involved [57], and to operate the epithelial-mesenchymal loop between the AER-Fgf and ZPA-Shh [58].

A series of Cre-mediated KO studies to delete Fgfr1 or Fgfr2 expression from the mesenchyme or ectoderm of mice [41,59,60] showed their importance for limb mesenchymal cells survival and proliferation, early and late PD-patterning events and for the establishment of proper chondrogenic primordia. Inactivation of Fgfr1IIC and Fgfr2IIC in the limb mesenchyme, either alone or in combination, demonstrated their partial redundancy in transducing Fgf signaling in the early limb mesenchyme [60]. Overall, Fgfr1 and Fgfr2 function as the predominant mesenchymal and ectodermal Fgf receptors, respectively [39,41,42]. The major functional studies carried out to decipher the function of Fgf signaling during limb development are summarized in Supplementary Table 1.

3.2. Wnt and Bmp signaling in outgrowth and patterning

Several Wnt family members are expressed in the limb mesenchyme and in the SE including the AER [61]. Like Fgf signaling, Wnt signaling is also required for cell proliferation and cell fate specification [62] and it negatively regulates chondrogenesis [62,63]. During limb development, the AER serves as the source of Fgf signaling and the ectoderm, including the AER, emanate Wnt signaling. While continuous exposure to Fgf8 or Wnt3A alone provided chondrogenic or connective tissue fate, respectively, their combined application to limb micromass cultures retained the cells in an undifferentiated proliferative state [62]. As per the model proposed by the authors, once the cells in the core of the limb bud escape the influence of both Fgf and Wnt signaling, they begin their chondrogenic differentiation program. But, in the periphery, the cells are still receiving Wnt signal from the SE, which maintains cell proliferation and respecifies them toward soft connective tissue fates. The limb outgrowth is more pronounced distally because of the combined strength of Fgf and Wnt signaling from the AER, compared to the strength of Wnt signaling alone from the non-rind ectoderm [62].

Several Bmp ligands are expressed throughout limb development both in the AER and mesenchyme [64] particularly, Bmp2, Bmp4 and Bmp7. Conditional inactivation of Bmp2, Bmp4 and Bmp7 either alone or in combination revealed that none of these Bmps are involved in limb patterning, but a threshold of Bmp signaling is necessary to form proper chondrogenic condensations [65]. The ubiquitously expressed Bmp receptor, BmpR1a, has high affinity for Bmp2 and Bmp4 [66,67] and BmpR1a mutant mice presented abnormalities in all the limb segments [68]. In these mutants, both AP and DV patterning genes displayed defective expression but not the PD-patterning genes [68]. BmpR1b does not play any role in limb patterning because mouse mutants for BmpR1b only display mild defects in cartilage differentiation [69,70].

3.3. RA signaling in PD patterning

AER-Fgf signaling from the distal limb is counteracted by the proximal, flank-Ra signaling and this antagonism is proposed to instruct limb PD patterning [71–73]. However, whether this antagonism occurs at the limb PD level or in the LPM prior to limb budding is still not conclusive, because no RA activity was detected in the rescued forelimb buds of mouse mutants lacking RA synthesis [28,74]. According to these authors, RA-mediated inhibition of Fgf8 signaling in the presumptive forelimb flank creates a permissive condition for correct spatiotemporal induction of Tbx5 expression and thus normal forelimb initiation [28,74].

RA activity is indicated by two closely related homeobox genes, Meis1 and Meis2, which are expressed in the LPM before limb initiation, then in the entire nascent limb bud and later in the proximal limb region, up to the humerus–radius/ulna boundary [74,75]. Meis1 and Meis2 have been identified as determinants of proximal limb elements, because ectopic distal Meis expression inhibited progressive distalization and formed limbs with proximally shifted identities along the PD axis of chick and mouse [72,75,76]. When RA activity in the limb mesenchyme was distally expanded by inactivation of Cyp26b1, distal limb truncations were observed that were similar to the phenotype obtained by Meis overexpression [77]. Paradoxically, recent studies using Rdh10 and Raldh2<sup>−/−</sup> mutant mice lacking RA activity has suggested that RA signaling is not required to establish Meis1/2 expression during limb development [74]. Whether, RA acts as an instructive or permissive signal to proximalize the limb bone elements calls for further research in the field.

3.4. PD patterning models

Different models have been proposed to explain limb PD patterning (Fig. 2): the Progress Zone (PZ) model [78]; the Two Signal (TS) model [79] and, more recently, the Integrated Space-Time model for limb PD/AP patterning [80]. For the sake of...
understanding, the later model is provided in Section 5, after the section on limb AP patterning.

3.4.1. The Progress Zone (PZ) model and the limb molecular clock

Microsurgical experiments performed in the 1940s showed that the earlier the removal of the AER, the most proximal limb elements are truncated [81]. The PZ model was built on this foundation and proposed that the positional values in the distal mesenchyme freeze upon AER ablation and the resulting skeletal patterns reproduce the PD information acquired by the mesenchymal cells until AER ablation. The presumptive fate of the distal mesenchyme was further identified by swapping the tissue from younger to older embryos [78,82], leading to the proposal of further identified by swapping the tissue from younger to older AER ablation. The presumptive fate of the distal mesenchyme was specified. Over time, limb outgrowth will displace the distal limb from the flank-RA signaling. Simultaneously, ZPA-Shh permissive signal get well-established, creating the developmental program of a clock that provides the cells the notion of time they spend in the PZ. Due to continuous cell proliferation and outgrowth of the limb, mesenchymal cells will be pushed out of the PZ and escape the influence of the AER. The amount of time each cell spends in the PZ, measured by the intrinsic timer, will determine its PD positional identity.

The first evidence for the existence of such a time counting mechanism was provided in 2007 based on the 6h periodic hairy2 gene (a Hairy-Enhancer-of-split (HES) family member) expression oscillations in stage HH20-28 chick distal limb chondrogenic precursor cells [83,84]. However, further work is required to substantiate the causality between the dynamics of limb bone element formation and the periodicity of the limb molecular clock [85].

3.4.2. The Two Signal (TS) model

The developing hindlimb mesenchymal cells experience the opposing signaling activities of the flank-RA and the AER-Fgf [72,75] and this antagonism is the basis of the TS model [79] (Fig. 2B). As a consequence of limb outgrowth, these signaling gradients get distanced from each other, establishing three distinct domains, representing the three limb segments: the proximal stylopod domain under the influence of RA, the distal autopod domain influenced by Fgf signal and the middle zeugopod domain that is neither under the influence of RA or Fgf signaling [79] (Fig. 2B). These domains express specific markers, namely the proximal Meis1 or Meis2, the middle Hoxa11 and the distal Hoxa13. Except for Meis genes, none of these segment specific markers are directly involved in segment specification [79]. The TS model refers to the distal mesenchymal cells that are maintained in a proliferative undifferentiated state by the AER signal as the Undifferentiated Zone (UZ) [79,86]. Continuous proliferation in the UZ will push the cells out of this zone and from the influence of the AER-Fgf signaling, allowing them to enter the differentiation program. At the time of their exit from the UZ, the cells will only express one of the three limb segment markers which determine their fate. The proximal limit of the AER-Fgf signaling from where cells start their differentiation program is named as the ’Differentiation Front’ (DF) [79]. The main difference of the TS model from the PZ model is that the TS model does not contemplate the operation of a clock mechanism and depends solely on the relative levels of proximalizing-RA vs distalizing-Fgf activity for
cell fate specification. By performing both in vitro and in vivo experiments in chick, a balance between the trunk-RA and the distal-Fgf signals was shown to be the key for limb PD patterning [71,73].

4. Limb Anterior-Posterior (AP) patterning

4.1. The ZPA and Shh signaling

The polarizing region or the ZPA, located at the posterior distal margin of the limb mesenchyme, was identified by grafting this tissue from a donor to the anterior mesenchyme of a host early wing bud which produced mirror-image symmetrical digit duplications [87]. The number and identity of the induceddigits depends both on the strength/concentration and duration of the polarizing signal [87], suggesting that the ZPA signal should be mediated by a morphogen. Later, Shh was found to be this morphogen [88]. There are three digits in chick wing (digit 1, 2 and 3) [89] and five digits in mouse forelimb (digit 1, 2, 3, 4 and 5) [90]. In both species, the anterior most digit (digit 1) is patterned independently of Shh signaling [87].

A network of molecular signals functions to initiate \textit{shh} expression in the ZPA (Fig. 3A). A \textit{cis}-regulatory region known as the ZPA regulatory sequence (ZRS), located about 800 Kb up-stream of \textit{shh} gene is also involved in this process [91]. Before \textit{shh} induction, the limb is pre-patterned by mutually antagonizing anterior Gli3 and the posterior-\textit{Hand2} expression (basic helix-loop-helix transcription factor) [92]. \textit{Hand2} expression in the posterior limb is positively regulated by the concerted activity of all four Hox9 genes (\textit{Hoxa9}, \textit{Hoxb9}, \textit{Hoxc9}, and \textit{Hoxd9}) [93] and RA signaling [94]. The expression of 5’\textit{HoxA} and \textit{HoxD} genes is also restricted to the posterior limb by Gli3 [95–97]. Together, the 5’Hox and \textit{Hand2} initiate ZPA-\textit{shh} expression, by directly interacting with the ZRS [98,99].

Establishment of ZPA-\textit{shh} also requires AER-Fgf signaling through Fgfr2 [32,38] and dorsal ectoderm-produced Wnt7a is necessary to maintain \textit{shh} in the ZPA [100] (Fig. 3C). Shortly after AER starts to express \textit{fgf8}, it induces \textit{shh} expression in the ZPA [32] (Fig. 1C), which is then maintained by the positive ZPA-\textit{Shh/Grem/AER-Fgf} module throughout limb development [45,101] (Fig. 3A and B). This module comprises an initial fast loop (2 h), where Bmp induces its own antagonist, Grem, in the distal limb mesenchyme and a slower loop (12 h), where Grem antagonizes Bmp signaling allowing the rise of AER-Fgf, ZPA-Shh, and Grem activities [45] (Fig. 3A and B). This loop explains why AER-\textit{fgf4} and -\textit{fgf8} expression are abrogated in mouse and chick limbs developed in the absence of Shh signaling [102]. Accordingly, AER-\textit{fgf8}/\textit{fgf4}

![Fig. 3.](A) Establishment of ZPA-Shh: Molecular interactions involved in ZPA-Shh establishment are shown in an early stage limb bud (HH17 in chick or E9.5 in mouse). The limb is pre-patterned by mutually antagonizing anterior Gli3 (blue) and the posterior \textit{Hand2} (yellow). Positive cooperative regulations from RA, AER-Fgfs, \textit{Hand2} and 5’\textit{Hox} genes facilitate \textit{shh} induction in the ZPA and, in turn, \textit{Shh} induces \textit{fgf4} expression in the posterior AER. (B) The positive and negative modules ensuring limb outgrowth: In the early limb bud, Bmp signaling induces Grem expression in the mesenchyme through a fast module (2 h – [45]). In subsequent stages (as represented here), Grem expression will be ZPA-Shh dependent. By antagonizing Bmp signaling, Grem mediates the propagation of the positive ZPA-Shh/Grem/AER-Fgf module (12 h – [45]), enabling the rise of all its components. Simultaneously, a negative AER-Fgf/Cyp26B1/RA module will also be functional to ensure a RA free distal limb mesenchyme [47]. (C) Limb Dorsal-Ventral (DV) patterning: Wnt7a in the dorsal ectoderm induces \textit{Lmx1b} exclusively in the dorsal mesenchyme while BMP signaling in the ventral ectoderm induces \textit{En-1} expression and these players underlie limb DV patterning. Besides, Wnt7a is also necessary for proper ZPA-Shh expression and Bmp signaling for the establishment of the AER. All limbs are represented anterior on top and proximal to the left. The interactions that are not active are represented by dotted lines, arrows indicate positive transcriptional interaction, “T” shaped lines represent inhibition.)
double mutants have no ZPA-shh expression [50]. Along with the propagation of the positive ZPA-Shh/Grem/AER-Fgf module, ZPA-Shh signaling also indirectly enables the establishment of the antagonistic AER-Fgf/Cyp26B1/RA module in the distal limb mesenchyme (Fig. 3B), which eliminates the teratogenic activity of RA in the distal limb mesenchyme and promotes distal propagation [47]. In order to properly pattern the limb AP axis, Shh should be produced at the right level and its production must be strictly restricted to the posterior distal mesenchyme. The level of Shh produced at the right level and its production must be strictly restricted [47].

Canonical Shh signaling acts through Gli transcription factors (Gli1-3 in vertebrates). Gli1 is a target of Shh signaling and functions as an activator. Gli2 and Gli3 can either be activators or repressors, depending on the presence or absence of Shh [107]. Across the limb field Gli1 and Gli2 mediate the activator function while Gli3 mainly functions as a repressor [107,108]. Genetic analysis shows that Gli1 and Gli2 are dispensable for limb AP patterning [109,110], but inactivation of Gli3 resulted in severe polydactyly [111], emphasizing the importance of Gli3 in specifying the number and identity of digits. While shh mutant limbs produce only one digit [102], the limbs of double Gli3/shh mutant mice are polydactyly, identical to single Gli3 mutants [92,112], suggesting that Shh patterns the AP axis almost solely through Gli3 processing. Supportively, the non-processed full length Gli3 that functions as an activator was able to considerably rescue shh mutant limb phenotype [113]. Recently, Gli3 was reported to inhibit the expression of G1–S transition cell-cycle genes and Grem1 in the anterior limb to ensure pentadactyly [114].

4.2. Limb AP patterning models

Several models have been proposed to explain Shh-mediated limb AP patterning (Supplementary Fig. 1). The very first is the French flag model proposed based on the spatial gradient of Shh across the chick wing bud [115] (Supplementary Fig. 1A), where each color represents a particular threshold of Shh that will give rise to a digit. Then, a Gli activity-based model was proposed showing that the anterior-most and posterior-most digits are specified by high Gli3-R and by the absence of Gli3-R activities, respectively [107] (Supplementary Fig. 1B). The Shh temporal gradient model, revealing the importance of both the spatial and temporal requirement of Shh signaling for limb AP patterning was proposed by Harfe et al. [90] (Supplementary Fig. 1C). According to this model, the whole digit 2 and half of digit 3 are formed by cells that experienced paracrine Shh signaling through diffusion while the other half of digit 3, digit 4 and digit 5 are created by cells that underwent high, autocrine Shh signaling, progressively for longer duration.

Patterning of the limb distal mesenchyme by Shh is also linked with cell proliferation [116,117]. Work performed in mouse allowed the proposal of the biphasic model, as per which Shh has an early transient role in the specification/patterning of digit progenitors and a later prolonged role in proliferative expansion of the specified progenitor pool [117] (Supplementary Fig. 1D). Both the proliferative role of Shh and its transient requirement in the early limb bud to pattern limb AP axis was validated in chick [116,118]. More recently, digit patterning was also explained by a Turing-type mechanism based on the dosage of distal Hox genes [119].

4.3. The role of Bmp and RA signaling in shaping the digits

Bmp signaling and its intracellular mediators – phosphorylated-SMADs – are implicated in digit specification [66,120,121]. Moreover, Bmp signaling has prominent role in shaping the digits through interdigital apoptosis [122,123]. RA signaling also accelerates cell death in the interdigital domain [124,125]. Although RA receptor Rarb deficient limbs were normal, Rarb/Rar double mutants showed interdigital webbing [126], supporting the role of RA signaling in interdigital cell death.

5. The Integrated Space-Time model for limb PD/AP patterning

The expression of the limb molecular clock gene hairy2 is regulated by the key limb signaling molecules Fgf, RA and Shh [127,128]. Fgf and Shh are instructive and permissive signals for limb hairy2 expression, respectively [127], whereas RA can have both instructive and permissive functions [128]. Since this regulatory network brings together the crucial components of both the PZ and TS models, a new model conciliating the previous ones was proposed, called the “Integrated Space-Time Model” [80] (Fig. 2C). According to this model, the early limb mesenchyme presents non-oscillatory hairy2 expression due to simultaneous influence of flank-RA and AER-Fgf signaling and this would specify the proximal-most stylopod. Over time, the distal limb mesenchyme is distanced from flank-RA signaling and will be progressively influenced by combined AER-Fgf and ZPA-Shh signaling. Varying posterior–anterior gradients of Gli-activator to Gli-repressor ratio (Gli-A/Gli-R) established by ZPA-Shh signaling will allow on/off hairy2 expression, constituting a time-counting mechanism underlying the progressive establishment of cell positional information for zeugopod and autopod specification [80] (Fig. 2C). The Integrated Space-Time model positions the limb molecular clock Hairy2 transcription factor as a crucial molecular component that integrates spatial morphogenetic gradients with temporal precision along limb PD and AP axes, ensuring coordinated PD and AP limb outgrowth and patterning.

6. Limb Dorsal-Ventral (DV) patterning

DV axis specification in vertebrate limb occurs through a complex series of epithelial-mesenchymal interactions [129] (Fig. 3C). It has been suggested that the signals from the somitic mesoderm specify a dorsal fate to the neighboring LPM [130], which is transferred to the SE prior to limb budding and this results in the expression of wnt7a in the presumptive dorsal limb ectoderm. En-1 is induced in the ventral ectoderm by Bmp signaling through BmpR1a. In En-1 KO limbs, when Bmp expression is impaired, wnt7a is misexpressed in the ventral ectoderm and the distal structures develop with bi-dorsal character [44,131]. In the absence of Wnt7a, the limb acquires bi-ventral identity at the expense of the dorsal pattern [132]. Wnt7a induces the expression of the UM-homeodomain transcription factor Lmx1b specifically in the dorsal mesenchyme of the limb bud. Experiments in the chick and mouse indicated Lmx1b as necessary and sufficient to specify dorsal limb pattern [133,134].

7. Termination of limb outgrowth

Together with the inhibitory AER-Fgf/Grem loop [135], the ZPA-Shh/Grem/AER-Fgf positive module has been shown to terminate limb outgrowth in both chick and mouse, following different sequences [135,136] (Supplementary Fig. 2). In the early limb bud (chick: HH18-23 and mouse: E9.5–10.5), the level of AER-Fgf signaling is too low to inhibit Grem expression. Instead, the positive module of ZPA-Shh/Grem/AER-Fgf will facilitate limb outgrowth by increasing the strength of AER-Fgf signaling. By stage HH23-27 in chick and E10.5–12 in mouse, the strength of AER-Fgf signaling is...
high enough to inhibit Grem expression in the mesenchyme [135]. As a consequence of this inhibition and continuous growth of the distal limb, Grem negative domain expands and triggers a sequence of termination mechanisms that differ in mouse [135] (Supplementary Fig. 2A) and chick [136] (Supplementary Fig. 2B). In mouse, the termination sequence starts with the inability of Grem negative domain to relay ZPA-Shh signal to AER-Fgf, which in turn will reduce ZPA-shh transcription and ultimately Shh-mediated induction of Grem [135]. Whereas, in chick, the Grem negative domain will first be out of range to receive ZPA-Shh signal because of the refractory nature of Shh producing cells to express Grem [136]. By stage HH27 in chick, cells competent to express Grem will be located too far from the ZPA to receive Shh signaling, terminating the loop from ZPA-Shh/Grem [136] (Supplementary Fig. 2B).

Recently, two other molecules were added to the limb termination loop, namely Twist and Tbx2. Overexpression of Twist in the chick hindlimb caused premature termination of limb outgrowth by repressing Grem [137]. Similarly, in mouse hindlimb, misexpression of Tbx2 also resulted in premature termination of the ZPA-Shh/Grem/AER-Fgf module [138].

8. Conclusions and perspectives

Although a long standing model for tissue patterning and outgrowth studies, the developing vertebrate limb continues to present exciting challenges to developmental biologists of all ages and specific fields of interest. Whether you are focused on structure formation, cell dynamics, stem cell properties, regeneration capacity, intricate gene expression regulation, ionic exchanges or mathematical modeling – you name it – the vertebrate limb continues to be an excellent model to pursue unexplored, daring paths. We hope the knowledge herein summarized will challenge the curious reader to embrace it!

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.semcdb.2015.01.007.

References


**Supplementary figure 1. Models for limb anterior-posterior (AP) patterning.**

(A) The French flag model: Each color represents different threshold concentrations of ZPA-Shh morphogen established by diffusion across the chick wing bud mesenchyme, which will pattern the wing digits, D1-3 [1]. (B) Gli activity based model: The anterior digit (D1) is specified purely by high Gli-R and the most posterior digit (D5) is specified due to total absence of Gli-R in this domain. The opposing Gli-R and Gli-A gradients derived from Shh signaling will specify intermediate digits D2, D3 and D4 [2]. (C) The temporal gradient model for AP patterning proposes that the digits are specified both by Shh concentration as well as duration of exposure. D1 is Shh-independent; D2 is specified by cells that were exposed exclusively to long-range Shh signaling by diffusion (low concentrations); D3 is specified by a combination of cells exposed to low Shh concentration and by descendant cells derived from former shh-expressing cells shortly after shh expression; D4 and D5 are
specified solely by Shh-descendants derived from cells that expressed *shh* for progressively longer duration of time [3]. (D) The biphasic model: Shh is required for both AP digit patterning and distal mesenchymal cell proliferation. Within the first 12h of *shh* induction, it patterns the entire AP axis. After this initial phase, Shh is necessary for survival and proliferation of the already specified progenitor pool to form digit primordia. This expansion occurs in the order of D4, D2, D5 and D3 (indicated by increasing number of asterisks) [4]. All limbs are represented anterior on top and proximal to the left.

**Supplementary figure 2**

**A. Mouse limb bud**

![Mouse limb bud diagram](image)

**B. Chick limb bud**

![Chick limb bud diagram](image)

**Supplementary figure 2. Outgrowth termination mechanisms in mouse and chick.** Both in mouse and chick, high level of AER-Fgf signaling inhibits *Grem* expression in the distal mesenchyme [5]. (A) As a consequence of this inhibition, in mouse, the distance between
Grem positive domain and the AER-Fgfs will be widened, not allowing Grem to maintain AER-\textit{fgf} expression. This leads to the overall fall of AER-\textit{fgfs} first and then to the sequential loss of ZPA-\textit{shh} and mesenchymal-Grem expression \cite{5}. (B) In chick, the distance between \textit{Grem} positive domain and ZPA-Shh increases as a result of the refractory nature of Shh-descendants to express \textit{Grem}. The reduction in Grem leads to increased Bmp signaling that will in turn downregulate AER-\textit{fgfs} and, ultimately, ZPA-\textit{shh} expression by stage HH27 \cite{6}. Numbers 1-3 represent the order of events. All limbs are represented anterior on top and proximal to the left.

References

\begin{enumerate}
\item Ahn S, Joyner AL. Dynamic changes in the response of cells to positive hedgehog signaling during mouse limb patterning. Cell 2004;118:505-16.
\item Scherz PJ, Harfe BD, McMahon AP, Tabin CJ. The limb bud Shh-Fgf feedback loop is terminated by expansion of former ZPA cells. Science 2004;305:396-9.
\end{enumerate}
**Supplementary table 1.** Limb phenotypes, gene expression alterations and conclusions obtained from Fgf signaling components functional studies performed in chick and mouse models.

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Organism</th>
<th>Manipulation strategy</th>
<th>Limb phenotypes and main conclusions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>fgf2</em></td>
<td>Chick</td>
<td>Morphogen soaked bead implantation in the absence of AER</td>
<td>Ectopic limb formation from the flank upon respective bead implantations, revealing the importance of AER-FGFs for normal limb development;</td>
<td>[1]</td>
</tr>
<tr>
<td><em>fgf4</em></td>
<td>Chick</td>
<td>FGF4- soaked bead implantation</td>
<td>FGF4 can substitute the AER and provide all the signals necessary for limb outgrowth and patterning</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>Chick</td>
<td>FGF4- soaked bead implantation</td>
<td>FGF4 is a chemoattractant and is required for distal limb outgrowth</td>
<td>[3]</td>
</tr>
<tr>
<td><em>fgf4</em></td>
<td>Mouse</td>
<td>C-KO by Msx2-Cre (AER) or RAR-Cre (IM, LPM &amp; AER)</td>
<td>No limb defects; normal <em>shh, Bmp2, Bmp4, fgf8</em> &amp; <em>fgf10</em> expression - suggesting a combination of two or more AER-FGFs are required for limb development; Other AER-FGFs could compensate the absence of FGF4</td>
<td>[4, 5]</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Fgf8 was substituted by Fgf4 by concomitantly activating conditional <em>fgf4</em> GOF allele and inactivating <em>fgf8</em> loss-of-function allele in the same cells via Msx2-Cre</td>
<td>FGF4 rescued limb defects caused by the loss of FGF8 - demonstrating that FGF4 can functionally replace FGF8; <em>fgf4</em> GOF produced limb polydactyly &amp; syndactyly - suggesting the importance of controlled FGF expression for normal limb development.</td>
<td>[6]</td>
</tr>
<tr>
<td><em>fgf8</em></td>
<td>Chick</td>
<td>Morphogen soaked bead implantation</td>
<td><em>fgf8</em> expression in the IM is necessary for limb induction; FGF8 induce <em>shh</em> expression in stage HH17 limb mesenchyme</td>
<td>[7]</td>
</tr>
<tr>
<td></td>
<td>Chick</td>
<td>RCAS virus mediated OE</td>
<td>Reduction of proximal skeletal limb structures; digit abnormalities resembling the phocomelic phenotypes - suggested FGF8 as a key signaling molecule involved in limb initiation, outgrowth and patterning</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>C-KO by Mxs2-Cre or RAR-Cre</td>
<td>Limb skeleton was severely affected; caused late <em>shh</em> expression; anteriorly expanded AER-<em>fgf8</em> expression - suggesting the importance of AER-<em>fgf8</em> in limb patterning and outgrowth</td>
<td>[9, 10]</td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>C-KO by Lefty-Cre (expressed in the IM)</td>
<td>Lefty-Cre-<em>fgf8</em> KO: no limb defects</td>
<td>[11]</td>
</tr>
<tr>
<td><em>fgf8</em></td>
<td>Mouse</td>
<td>C-KO by RAR-Cre or AP2-Cre</td>
<td>RAR-Cre-<em>fgf8</em>/<em>fgf4</em> DKO: Absence of forelimb and formation of defective hind limbs;</td>
<td>[11]</td>
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</table>


<table>
<thead>
<tr>
<th>fgf10</th>
<th>Chick OE by RCAS BP(A)</th>
<th>Mesenchymal FGF10 can induce fgf8 expression and a complete ectopic limb; it is the endogenous inducer of limb bud formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>KO</td>
<td>Perinatal lethality associated with complete absence of lungs; limb bud formation was initiated but outgrowth did not occur; Fgfr2b was expressed in presumptive limb region; no fgf8 expression was detected; AER and ZPA were not established</td>
</tr>
<tr>
<td>fgf17</td>
<td>Mouse KO</td>
<td>No limb defects;</td>
</tr>
<tr>
<td>fgf9</td>
<td>Mouse KO</td>
<td>No limb defects</td>
</tr>
<tr>
<td>AER-fgfs (fgf8,4,9,17)</td>
<td>Mouse C-KO of different combinations by Msx2-Cre</td>
<td>AER-fgf4 fgf9 &amp; fgf17 triple KO: No limb defects &amp; normal shh expression; fgf8fgf17 or fgf8fgf9 or fgf8fgf4 double KO: Mild limb defects; fgf8fgf4fgf9 triple KO: Severe limb truncations - suggesting the contribution of each AER-FGF the total AER-FGF signal</td>
</tr>
<tr>
<td>FgfR1</td>
<td>Mouse KO by chimeric embryo generation</td>
<td>Malformed limb buds - suggesting Fgfr1 mediated signaling in the PZ for cellular proliferation and patterning; also suggest that Fgfr1 signaling is dispensable for AER establishment and limb initiation</td>
</tr>
<tr>
<td></td>
<td>Mouse C-KO by Ap2-Cre and Hoxb6-Cre (LPM at E8.5)</td>
<td>Short &amp; distorted AER; downregulated shh &amp; mkp3 expression; defective autopod development - suggesting the role of FGFR1-mediated signaling in AER formation; cell survival; autopod formation and digit patterning</td>
</tr>
<tr>
<td></td>
<td>Mouse C-KO by T-Cre (in all limb mesenchyme) and Shh-Cre (in posterior limb mesenchyme)</td>
<td>T-Cre-Fgfr1: mutants die at birth &amp; they presented severely affected fore and hind limb skeletons with defect in all the 3 limb segments; abnormal expression of shh, Bmps and Hox13 genes. Shh-Cre-Fgfr1:</td>
</tr>
</tbody>
</table>
mutants miss one digit in the autopod.
- suggesting Fgfr1’s role in PD patterning, cell survival and expansion of progenitor cell population.

| Fgfr1IIIc | Mouse | C-KO by Prx1-Cre (expressed in both fore & hindlimb mesenchyme) | Mild limb skeletal defects
- suggesting a unique role for Fgfr1IIIc in distal limb skeletal formation | [22] |
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<tbody>
<tr>
<td>FgfR2</td>
<td>Mouse</td>
<td>KO by chimeric embryo generation</td>
<td>No limb buds form; fgf10 and msx1 were downregulated in presumptive limb mesenchyme; fgf8 expression was not detected</td>
<td>[23]</td>
</tr>
</tbody>
</table>
| Mouse      | C-KO by Msx2-Cre | Defective autopod; expression of mkp3, shh & Gremlin1 was downregulated
- suggesting the requirement of FgfR2 signaling for the survival and maintenance of AER cells, which in turn affects autopod development | [24] |
| Mouse      | C-KO by Msx2-Cre | Absence of hind limb & severely truncated forelimb (without autopod); also reduced cell proliferation
- accounting decreased cell proliferation, sustained cell death and failure of mesenchymal differentiation for limb truncations resulting from the loss of AER-FGF signal | [22] |
| Mouse      | KO | Perinatal lethality between E10.5-E11.5; no limb formation; absence of fgf8 and downregulated fgf10 expression
- suggesting the importance of FGFR2 signal for the reciprocal regulatory loop between FGFR8 and FGFR10 during limb induction | [25] |
| Mouse      | KO by generating hypomorphic mutants | Produced limbless mice; limb bud initiates but fail to grow; fgf8, fgf10, msx1 & Bmp4 were expressed but shh & fgf4 were not.
- suggesting an essential role for FgfR2IIIB in ZPA-shh and AER-fgf4 induction, AER maintenance, cell survival and limb outgrowth | [26] |
| Fgfr2IIIC | Mouse | C-KO by Prx1-Cre | No limb skeletal defects | [22] |
| Fgfr1IIIc/Fgfr2IIIC | Mouse | C-KO by Prx1-Cre | Severe skeletal hypoplasia in forelimbs and hindlimb-
suggesting that AER-FGF signaling is mediated in the mesenchyme by both FgfR1 & Fgfr2. This facilitates SOX9 function and ensure progressive establishment of chondrogenic primordia along the PD axis. | [22] |

KO: Knockout; C-KO: Conditional knockout; C-GOF: Conditional gain of function; OE: Overexpression; M: Mouse; C: Chick
References