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Molecular Models for Vertebrate Limb Development

Review

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The three-dimensional form of organisms is achieved through a process called pattern formation. For the most part, general features of animal body plans are initially laid out during embryogenesis in broad strokes. For instance, differential fates are specified along the rostral–caudal axis. During subsequent development, further refinement of these broad distinctions occurs, leading to the formation of semi-autonomous regions, commonly referred to as secondary fields, in which the process of regional specification is repeated anew. Pattern formation in secondary fields can be conceptualized as occurring in four stages: first, the cells that make up the field itself are defined; second, specific signaling centers are established within the field, which serve to provide positional information; third, this positional information is recorded on a cell-by-cell basis; and finally, cells differentiate in response to additional cues according to their already-encoded positional information. The molecular basis for these four events is the subject of much current interest in developmental biology, and in particular, significant progress has recently been achieved in understanding each of these processes in one vertebrate secondary field, the developing limb bud. This system has been particularly informative because it is amenable to several powerful, complimentary approaches, including surgical manipulations, ectopic expression studies in the chick, as well as targeted gene disruption in mice. Together these studies have started to provide a conceptual framework for understanding pattern formation on a mechanistic level.

Developmental Anatomy of the Vertebrate Limb

The vertebrate limb originates from a dual contribution of lateral plate and somitic mesoderm (Figures 1A and 1D; Chevallier et al., 1977; Christ et al., 1977). Through differential proliferation of the flank, specific regions of the lateral plate form buds at presumptive limb levels (Figures 1A and 1B; Searls and Janners, 1971). Shortly thereafter, cells from the lateral edges of nearby somites migrate into the limb. All adult limb muscle derives from these migratory cells. Limb muscle, nerve, and vasculature have their origins in extra-limb regions, while all other limb tissues, including skeletogenic mesenchyme, cartilage, and tendons derive from lateral plate mesoderm. The limb bud is enveloped by an overlying ectodermal jacket, whose distal tip, in most tetrapods, forms a specialized epithelial structure, the apical ectodermal ridge (AER) running along its anterior–posterior (A/P) axis at the interface of dorsal and ventral territories (Figure 1E; Saunders, 1948; Fallon and Kelley, 1977; Todt and Fallon, 1984). Soon after the AER forms, mesenchymal cells aggregate to form cartilage blastemal elements that prefigure skeletal limb components (Hall and Miyake, 1992). Cells directly under the AER remain undifferentiated in the so-called "progress zone," while condensation initiates in proximal limb regions, so that the humeral analage forms first, followed by the radius and ulna, and lastly the digits. The net result of these developmental processes is the establishment of familiar prototypical tetrapod limb features (Figure 1C). The first clues to how this complex shape and form is achieved came from the work of experimental embryologists who identified specific regions of the developing chick limb bud that are essential in directing growth and patterning.

Fibroblast Growth Factors Specify the Limb Field and Promote Initial Outgrowth

A clue to how growth within the limb field may be regulated came from foil barrier and extirpation studies, which indicated that the intermediate mesoderm is required for limb bud initiation (Stephens and McNulty, 1981; Strecker and Stephens, 1983; Geduspan and Solursh, 1992). Limbs will not form when a barrier is placed between lateral plate and intermediate mesoderm. Similarly, removal of intermediate mesoderm results in loss of adjacent limb tissue. One interpretation of these experiments is that the intermediate mesoderm produces a factor that maintains proliferation of the flank in presumptive limb regions. However, it should be noted that a recent reexamination of the role of the intermediate mesoderm in limb initiation has produced conflicting results, hence the role for this tissue in limb bud initiation remains controversial (Fernandez-Teran et al., 1997).

If the intermediate mesoderm is indeed involved in limb bud initiation, the expression pattern of chick fibroblast growth factor 8 (FGF-8), as well as its morphogenetic abilities, make it an excellent candidate for playing a role in this process (Crossley et al., 1996; Vogel et al., 1996). *FGF-8* expression is transiently localized to the intermediate mesoderm at forelimb and hindlimb levels just prior to limb bud outgrowth. This pattern is consistent with the hypothesis that FGF-8 might be responsible for the maintenance of high levels of proliferation of the presumptive forelimb and potentially be the critical factor from the intermediate mesoderm implicated by foil barrier experiments. Supporting this hypothesis is the observation that when beads soaked in FGF-8 or other FGF family members are placed in interlimb regions, new ectopic limb buds form from the lateral plate mesoderm (Cohn et al., 1995; Crossley et al., 1996; Vogel et al., 1996). The experimentally induced limb buds form an AER at their tip and develop quite normally, and at 10 days their morphology is typical of normal limbs. Hence, FGF-8 is sufficient to induce an entire program of limb bud initiation and subsequent patterning. Although the finding that FGF-8 can so dramatically affect the fate of flank tissue is truly remarkable, it is unclear

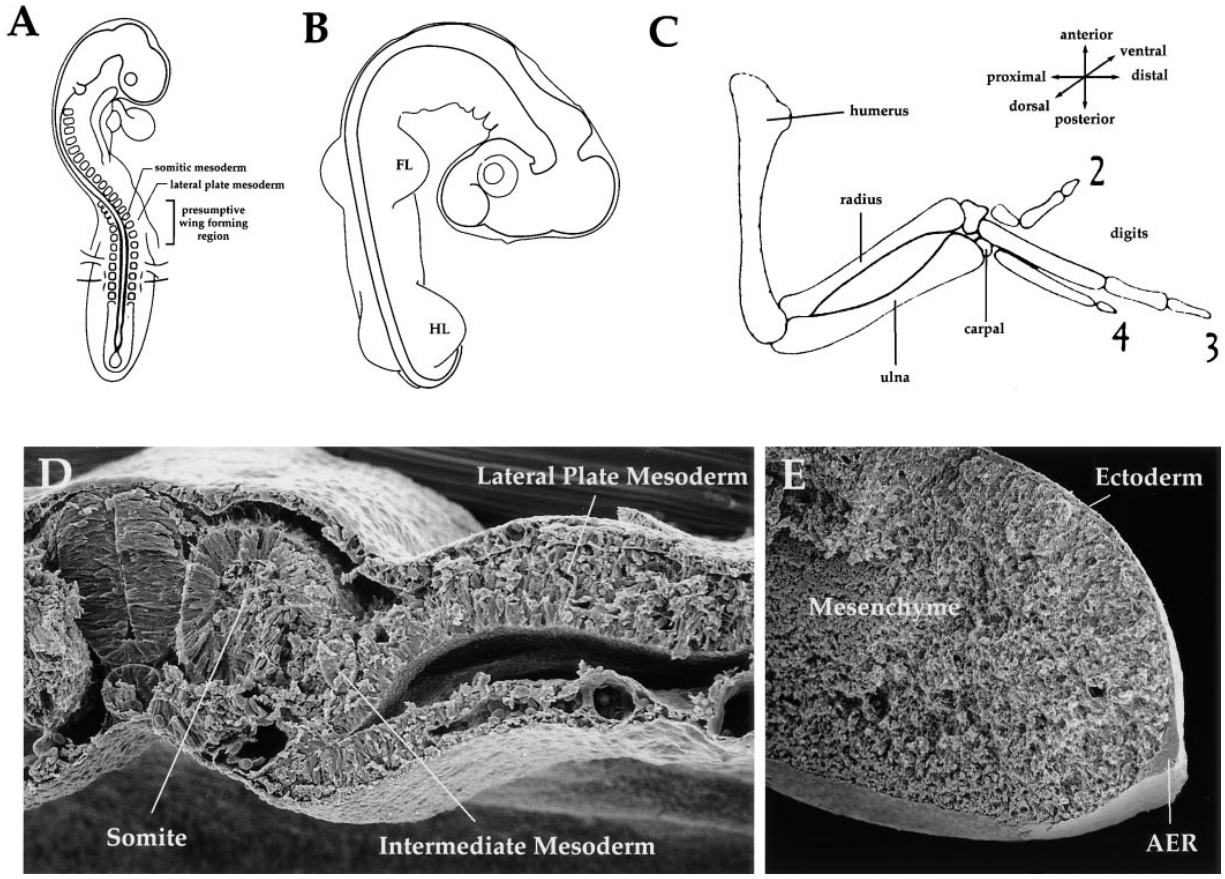

Figure 1. Developmental Anatomy of the Chick Wing

(A) Schematic of a chick embryo at about 50 hr of incubation. The presumptive forelimbs region is located within the lateral plate mesoderm adjacent to somites 16–20. At this stage, the presumptive hindlimb region resides adjacent to paraxial mesoderm that has not yet segmented.

(B) Schematic of a chick embryo at about 72 hr of incubation. At this stage the presumptive limbs appear as buds jutting out from the flank.

(C) Schematic of a chick wing at 10 days. By this stage, the basic adult pattern of the wing has been realized as a cartilaginous model: a single long bone, the humerus, is present most proximally, followed by two long bones, the radius and ulna. At the distal end are the wrist (carpal) and digit (phalanges) elements. The three cardinal axes of the limb are indicated to the right of the schematic. For comparisons between forelimbs and hindlimbs of different species, it is often useful to refer to the homologous regions as stylopod (upper limb), zeugopod (middle limb), and autopod (distal limb).

(D) Scanning electron micrograph (SEM) section of a 50 hr chick embryo. The limbs form from the lateral plate mesoderm, and a migratory contribution from adjacent somites. The intermediate mesoderm lies in between the lateral and somitic mesoderm.

(E) SEM section of the forelimb of a 72 hr chick wing bud. At this stage, limb buds have a relatively simple histological profile with a mesenchymal core surrounded by an ectodermal jacket. The apical ectodermal ridge (AER) is located at the distal tip of the bud. Micrographs in (D) and (E) courtesy of Gary C. Schoenwolf, University of Utah School of Medicine.

whether FGF-8 is necessary for limb bud initiation or that the endogenous signaling factor(s) include other as yet unidentified FGF family members, or other signaling molecules entirely. One model, which could account for the apparently long distance between the source of FGF-8 in the intermediate mesoderm and the ultimate target tissue, the forming limb bud, is that FGF-8 could act in a relay, inducing a second signal in the lateral plate mesoderm that in turn acts on cells within the limb field. A strong candidate for such a secondary factor is FGF-10, which is expressed within the early limb field and can be induced by ectopic application of FGF-8 (Ohuchi et al., 1997). FGF-10 is also capable of inducing an AER in ectoderm directly adjacent to a carrier bead, while FGF-8-derived ectopic limbs only form an AER after significant growth away from the carrier bead.

Once limb buds form, their continued proliferation depends on the AER. If the AER is removed, outgrowth is affected, resulting in distal truncations (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). The exact level of truncation depends on when the AER is excised: early removals lead to proximal truncations, while later removals allow for more distal outgrowth. Once again, FGFs figure prominently during this phase of limb development. At least three FGFs are expressed in limb ectoderm: *FGF-2*, *-4*, and *-8*. *FGF-8* is the first to be expressed, prior to AER formation, in a broad stripe of cells along the distal tip of the limb. *FGF-8* expression later restricts to the AER once the ridge has fully formed (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). In addition to *FGF-8*, the mature

AER also expresses *FGF-2* across its entire A/P extent and *FGF-4* in its posterior two-thirds (Niswander and Martin, 1992; Suzuki et al., 1992; Savage et al., 1993; Dono and Zeller, 1994). Any of these FGFs can substitute for the AER in terms of maintaining distal outgrowth; however, the relative importance of individual FGFs in regulating distal limb outgrowth has not been clearly evaluated (Niswander et al., 1993; Fallon et al., 1994; Crossley et al., 1996).

Both Common and Independent Mechanisms Regulate AER Position and Dorsal–Ventral Cell Fates

How is a single AER formed at a defined location? Somewhat surprisingly, while the mature AER is a narrow band of cells located between the dorsal and ventral limb ectoderm, it is derived from the convergence of a broad area of ectoderm. Fate-mapping studies using chick–quail chimeras have shown that prior to limb outgrowth, a 150 μm wide domain of ectoderm situated above the lateral plate mesoderm is fated to form the AER. At this time, the prospective dorsal limb bud ectoderm overlies the somites, and the future ventral limb bud ectoderm derives from cells above the lateral somatopleural mesoderm (Michaud et al., 1997). One interpretation of these observations is that the presumptive limb ectoderm is initially divided into two broad domains, a dorsal domain covering the somites and extending to the middle of the lateral plate and a ventral domain extending laterally. The AER then arises along this border, recruiting cells from a broad region on either side during its morphogenesis.

Several morphogenetically important genes are expressed along this border prior to AER differentiation, including those that encode the homeobox-containing transcription factor Engrailed-1 (En-1) and the secreted factor Radical fringe (r-Fng), a member of a vertebrate family of secreted factors related to Drosophila Fringe (Fng), which are believed to modulate signaling through the Notch pathway (Panin et al., 1997). *En-1* is expressed solely in the ventral ectoderm, including the ectoderm above the splanchnic mesoderm, while *r-Fng* expression is restricted to dorsal ectoderm (Davis and Joyner, 1988; Davis et al., 1991; Gardner and Barald, 1992; Laufer et al., 1997; Rodriguez-Esteban, et al., 1997). Recent evidence indicates that both *En-1* and *r-Fng* play prominent roles in AER positioning. The interface between *r-Fng*-expressing and *r-Fng*-nonexpressing cells is precisely where the AER forms. Indeed, when that normal interface is disrupted, either by ectopic expression of *r-Fng* or by eliminating portions of endogenous *r-Fng* expression, new AERs form at boundaries of *r-Fng*-expressing and -nonexpressing cells. En-1 plays a role in restricting *r-Fng* expression to the dorsal ectoderm, ensuring that a sharp ventral boundary of r-Fng is maintained. Hence, En-1 assists in AER positioning at the dorsal–ventral (D/V) interface. It is important to note that although the information specifying the proper D/V location of the AER lies within the ectoderm, the initial stimulus leading to AER formation in fact derives from the underlying mesoderm (Saunders and Reuss, 1974). Therefore, interactions within the ectoderm may specify exactly where an AER forms, but a signal from the mesoderm is necessary to trigger the differentiation of limb bud ectoderm to form an AER. The molecular nature of this signal is currently unknown.

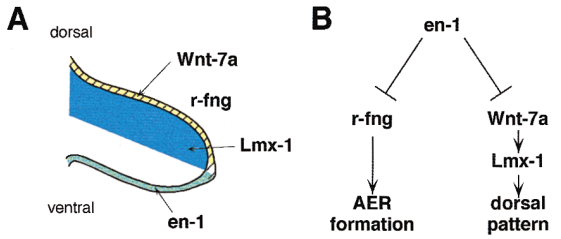

Figure 2. Mechanisms of D/V Patterning and AER Positioning

(A) Gene expression along the limb bud D/V axis. *Wnt-7a* and *Radical fringe* (*r-Fng*), which encode secreted factors, are expressed in the dorsal ectoderm. The homeodomain-containing factors encoded by *Lmx-1* and *Engrailed-1* (*En-1*) localize to the dorsal mesoderm and ventral ectoderm, respectively.

(B) Genetic interactions involved in AER formation and specification of dorsal pattern. *En-1* expression in the ventral ectoderm restricts the expression of *r-Fng* and *Wnt-7a* to the dorsal ectoderm. Interaction between *r-Fng*-expressing and *r-Fng*-nonexpressing cells leads to the specification of the AER. Wnt-7a instructs the dorsal mesoderm to adopt dorsal characteristics, such as *Lmx-1* expression, which in turn specifies dorsal pattern. *En-1* has a dual function in AER positioning and dorsal specification and hence acts to coordinate the two processes.

Since the AER forms at the interface between dorsal and ventral ectoderm, an appropriate question to ask is whether the same factors might serve to pattern cell fates along the D/V limb axis. As with AER formation, ectodermal signaling has been implicated in D/V patterning of both ectodermal and mesodermal tissues. Reversal of the ectoderm 180° about its D/V axis results an inversion of D/V polarity of distal limb mesoderm (MacCabe et al., 1974; Pautou, 1977). Hence, ectodermal signal(s) must specify mesodermal cell fates along the D/V limb axis. At least for the distal limb bud, the nature of these signals and the mechanism by which they act are beginning to be understood. At least four genes are known to be differentially expressed along the D/V axis (Figure 2A). These are *En-1* expressed in the ventral ectoderm, *r-Fng* and the secreted factor Wnt-7a expressed in the dorsal ectoderm, and the LIM-homeodomain protein Lmx-1, expressed in the dorsal mesoderm (Dealy et al., 1993; Parr et al., 1993; Riddle et al., 1995; Vogel et al., 1995). The function of each of these factors has been addressed by ectopic expression in the chick limb bud or by targeted disruption in mice or by a combination of these two complimentary methods. Either Lmx-1 or Wnt-7a can dorsalize the ventral mesoderm in the distal portion of the limb bud (Riddle et al., 1995; Vogel et al., 1995). A similar dorsalization also occurs in loss-of-function *En-1* mutants (Loomis et al., 1996). Conversely, loss of *Wnt-7a* leads to the acquisition of ventral characteristics in the distal, dorsal limb regions (Parr and McMahon, 1995). Taken together, these findings have been incorporated into a model (Figure 2B) in which Wnt-7a represents a dorsal signal that results in the expression of *Lmx-1* in dorsal mesenchyme. The function of En-1 as a ventral regulator is to repress the expression of *Wnt-7a* in ventral ectoderm.

Together En-1 and Wnt-7a ensure that *Lmx-1* expression is confined to the dorsal mesenchyme. In some way, Lmx-1 instructs dorsal patterning in limb bud mesenchyme. An interesting idea is that the ventral pattern is the default and that Lmx-1 modifies that default pathway. Given that Lmx-1 is a transcription factor, it should regulate the expression of other factors which themselves contribute to dorsal fates. The identification of these factors will help define precise functions for Lmx-1 in dorsal pattern specification.

Although the molecular mechanisms that lead to D/V cell fate specification seem tightly linked to those that position the AER, they are in fact separable. For example, Eudiplopodia mutant chick embryos have ectopic AERs that do not form at the interface between dorsal (*Wnt-7a*-expressing) and ventral (*En*-expressing) cells. Instead they form from dorsal ectoderm, which expresses only *Wnt-7a* (Laufer et al., 1997). As a consequence, these ectopic limbs are double-dorsal (Goetinck, 1964). Since these observations demonstrate that AERs can form from tissue that does contain both *Wnt-7a*-expressing and -nonexpressing cells, it follows that localized Wnt-7a activity might be dispensable for AER positioning. Indeed, *Wnt-7a* homozygous mutant mice, whose limbs are ventralized, have defined AERs in the proper location (Parr and McMahon, 1995). Moreover, interfering with the normal D/V boundary of *Wnt-7a* by ectopic expression in ventral ectoderm does not alter the normal positioning of the AER (Riddle et al., 1995; Vogel et al., 1995). Hence, the D/V cell fate specification pathway involving *Wnt-7a* and the AER-inducing pathway involving *r-Fng* are separate. However, the spatial restriction of both *Wnt-7a* and *r-Fng* to the dorsal ectoderm is maintained through the action of a single gene, *en-1*. En-1 represses *Wnt-7a* in the ventral ectoderm and also likely simultaneously represses r-Fng (Loomis et al., 1996; Laufer et al., 1997; Rodriguez-Esteban et al., 1997). *En-1* clearly plays an essential role in AER positioning since in *En-1* mutant mice, the AER flattens and spreads into ectopic ventral locations (Loomis et al., 1996). Further insight into the interactions of *En-1*, *Wnt-7a*, and *r-Fng* in AER specification will come from examination of mice carrying mutations in several of these genes.

Sonic hedgehog Regulates Anterior–Posterior Pattern and Is Required for Distal Outgrowth

Unlike the D/V axis, positional information along the A/P axis is already present in lateral mesoderm prior to limb bud formation (Harrison, 1918, 1921; Hamburger, 1938). An important advance in understanding A/P positional specification came from heterotopic grafting of small blocks of posterior mesenchyme into ectopic anterior locations (Saunders and Gasseling, 1968). In these pioneering experiments, Saunders and coworkers found that in response to these grafts, whole mirror-image duplications of the distal limb were readily obtained. When the grafted posterior mesenchyme was of quail origin, it became clear that duplicated structures were not derived from the graft itself, rather the graft induced surrounding host tissue to proliferate, change its fate, and become a mirror-image of the posterior limb. Because the graft reorganized A/P order of structures within the limb, this region was termed the zone of polarizing activity (ZPA), or polarizing region.

Soon after the initial reports of polarizing activity, a mechanistic model for the function of the ZPA was proposed (Wolpert, 1969). According to this model, ZPA cells secrete a morphogen which sets the identity of cells along the A/P limb axis. Cells near to the ZPA would receive a high concentration of the morphogen, and adopt posterior fates, while cells further from the ZPA would sense a lower morphogen concentration and adopt anterior fates. Several lines of evidence support the gradient model for ZPA function. First, the number and morphology of ectopic digits formed in the ZPA transplantation assay is a function of the number of ZPA cells transplanted (Tickle, 1981). A saturating number of cells results in a full mirror-image duplication with the digit pattern 4-3-2-2-3-4 (a chick wing has three morphologically distinct digits, which in the wild-type wing are designated from posterior to anterior as 4-3-2), while transplantation of limiting number of cells progressively leads to a 3-2-2-3-4 and finally to a 2-2-3-4 pattern. The number of cells required to form a single digit 2 is approximately 30 (and in some experiments as few as 9). Second, transplanting ZPA cells to the apex, as opposed to the anterior, of a host limb bud results in the generation of a more complex set of ectopic digits in a pattern consistent with the superposition of ectopic and endogenous gradients of a signal (Tickle et al., 1975). Third, when the endogenous ZPA signal is attenuated by reducing the number of ZPA cells in the posterior mesenchyme, the number of endogenous digits is reduced accordingly (Smith et al., 1978). Finally, the ZPA can act over a considerable distance, on the order of 150–200 μm (10–20 cell diameters; Honig, 1981). Since this is close to the size of the digit field in the early limb bud, the activity of the ZPA could be responsible for patterning the entire A/P axis.

The molecule responsible for the morphogenetic properties of the ZPA is Sonic hedgehog (Shh; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). *Shh*'s expression colocalizes with ZPA activity in the chick limb bud, and its expression can be induced by application of retinoic acid, an agent known to induce polarizing activity (Riddle et al., 1993). Definitive proof that Shh can mediate the activity of the ZPA came from grafting experiments in which Shh-expressing cells were implanted along the anterior margin of the limb bud. Mirror-symmetric duplications identical to those of ZPA grafts can be obtained either with cells expressing *Shh*, as in the original experiments or by purified recombinant protein loaded onto beads (Riddle et al., 1993; Chang et al., 1994; Lopez-Martinez et al., 1995).

Transplantation of the ZPA, or application of Shh, to ectopic locations can reorganize A/P limb pattern and can concurrently induce ectopic proliferation of limb bud mesenchyme. Therefore it has been inferred that the normal function of the ZPA is to promote proliferation of posterior limb bud mesenchyme and to specify the identity of the limb along the A/P axis (Saunders and Gasseling, 1968; Wolpert, 1969; Tickle et al., 1975). This implication has not been without considerable controversy. In fact, some reports claimed that the endogenous ZPA has no function at all, at least once limb buds

have formed (Fallon and Crosby, 1975). The availability of a specific molecular marker for polarizing activity has allowed for a reinvestigation of the requirement for Shh (and the ZPA) in normal limb patterning. Careful ZPA deletion experiments (Pagan et al., 1996) clearly demonstrate that if the entire ZPA is deleted in an early limb bud, pattern is truncated along the A/P and proximal–distal (P/D) axes. The discrepancy between these findings and earlier reports is almost certainly due to the fact that prior to the cloning of Shh, ZPA cells could not be accurately followed and hence the purported ZPA removals were in fact incomplete. Removal of the polarizing signal by genetic methods confirms these findings, conclusively demonstrating that *Shh* is essential for polarizing activity, and polarizing activity is essential for proper A/P and P/D limb patterning. The limbs of *Shh* mutant mice have a single spike-like proximal bone—a femur in the more severely affected hindlimb, or a humerus with a short, continuous distal bony extension in the forelimb (Chiang et al., 1996). The fact that ectopic *Shh* can induce complete A/P patterning, and that the loss of *Shh* results in A/P truncations, does not however imply that in the absence of Shh there is no A/P information. At least on a molecular level, some Shh-independent A/P patterning has been shown by analysis of the limb buds of the chick mutant *limbless*, which exhibits posteriorly nested expression of *Hox* genes, in spite of producing no detectable *Shh* (Ros et al., 1996; Noramly et al., 1996; Grieshammer et al., 1996).

Does Shh function as a diffusible morphogen, as suggested by the gradient model of ZPA function or does it act exclusively by local mechanisms? The currently available evidence is conflicting. Like the ZPA, Shh can produce not only full duplications including an ectopic digit 4, but also other duplications including a single ectopic digit 2 suggesting that the Shh signal might be dose-dependent (Riddle et al., 1993). However, arguing against the gradient model is the observation that, within limits of detection, Shh protein does not appear to diffuse significantly from its site of synthesis along the posterior limb bud margin (Lopez-Martinez et al., 1995; Marti et al., 1995). Moreover, when expressed in either insect or mammalian cell lines, both Drosophila Hedgehog (Hh) and mouse Shh protein is tightly bound to the cell surface (Chang et al., 1994; Lee et al., 1994; Bumcrot et al., 1995; Lopez-Martinez et al., 1995). The nature of this strong adherence appears to stem from a novel posttranslational addition of a cholesterol moiety executed during processing of the propeptide (Porter et al., 1996a, 1996b). This data suggests that in vivo, the Shh signal may be restricted to neighboring or near-adjacent cells. If this is the case, the long-range effects of Shh would have to be mediated by other Shh-dependent signaling molecules. For example, *bone morphogenetic protein 2* (*Bmp-2*) is expressed in a broader domain than that of *Shh* in posterior limb bud mesenchyme (Francis et al., 1994) and can be induced by Shh (Laufer et al., 1994); also, there is evidence that it possesses weak polarizing activity (Duprez et al., 1996).

Resolution of these important questions regarding the range and mechanism of action of Shh will be facilitated by the definition of the Shh signaling pathway in vertebrate embryos and the isolation of cDNAs whose transcription is directly regulated by Shh. One likely biochemical target of Shh is the transmembrane protein patched (Ptc). In Drosophila, Ptc antagonizes the activity of Hh, and it is possible that Hh functions by inhibiting the activity of Ptc (Ingham et al., 1991). Indeed, it has been suggested that *Ptc* encodes for a receptor for Hh and biochemical evidence supports this hypothesis (Ingham et al., 1991; Marigo et al., 1996a; Stone et al., 1996). In addition to being a potential receptor for Shh, *Ptc* is possibly a transcriptional target of Shh signaling. Transcription of *Ptc* is up-regulated in cells responding to Hh in both Drosophila and vertebrate embryos (Ingham et al., 1991; Capdevila et al., 1994; Concordet et al., 1996; Goodrich et al., 1996; Marigo et al., 1996b). If up-regulation of *Ptc* transcription is indeed a direct response to Shh, then its high-level expression pattern defines a minimal range of Shh signaling. Based on the difference in expression domains of *Shh* and *Ptc*, the range of action of Shh would be at least on the order of 100–200 μm in the chick limb, consistent with the range of action of the ZPA, but at odds with anti-Shh antibody staining. This disparity most likely reflects an inability to detect low levels of Shh protein, but it remains possible that *Ptc* transcription is not a direct readout of Shh concentration. Whether Shh acts via a long-range mechanism or via a short-range mechanism in the limb thus remains an important unresolved issue.

The Initiation of Anterior–Posterior Positional Information in the Limb and the Role of Retinoic Acid

The discovery of Shh as the key mediator of ZPA function allows the issue of A/P limb patterning to be pushed further back: what mechanisms lead to restricted *Shh* expression in the posterior limb bud? Pre-limb bud flank mesoderm has polarizing activity (Hornbruch and Wolpert, 1991) even though *Shh* is not expressed in the flank at these stages. However, when that same tissue is transplanted to a permissive environment underneath the anterior AER, *Shh* expression is indeed activated in the transplanted flank cells (Yonei et al., 1995). These results suggest that the region of cells capable of expressing *Shh* far exceeds that which will form the ZPA. Part of the restriction of *Shh* expression to the posterior appears to be due to an active suppression mechanism operating in the anterior limb. This has been revealed by the observation of ectopic *Shh* expression in the anterior limb bud of several polydactylous mouse mutants (Chan et al., 1995; Masuya et al., 1995). Some of these genes may normally function to directly suppress inappropriate *Shh* expression. Alternatively, some may eliminate *Shh* expression indirectly by inducing programmed cell death. There are a number of regions that undergo programmed cell death during limb development. Cells at the anterior margin (the anterior necrotic zone) are the first, and one of the largest of these regions, encompassing much of the anterior 25% of the midstage chick bud in the chick (Saunders and Fallon, 1966). This may serve to remove cells that otherwise would activate *Shh* expression. An important later location of programmed cell death is between the digits that serve to produce the free digits of the mature limb. Mutations affecting cell death in both the anterior necrotic zone and in the later interdigital necrotic zone might explain the frequent cooccurence of polydactyly and syndactyly

in the same syndromes. While the molecular nature of the mutation in most of these mice is not known, *Xt* mice have a deletion that abolishes the expression of the zinc finger–containing factor *Gli-3* (Hui and Joyner, 1993). This mutation may directly affect regulation of *Shh* expression, rather than indirectly via a programmed cell death mechanism, as Gli-3 is a member of a family of transcription factors implicated in mediating hedgehog signaling. An interesting parallel can be drawn with the Drosophila homolog of Gli-3, Ci, whose functions include repression of *hh* expression in the anterior wing compartment (Dominguez et al., 1996).

For *Shh* to be expressed properly in the posterior distal margin of the limb mesenchyme, there additionally appears to be a requirement for positively acting factors. One particularly intriguing candidate in this regard is the transcription factor Hoxb-8. Its endogenous graded expression pattern in the lateral plate mesoderm of the flank correlates well with the A/P domain of cells with potential to activate *Shh* expression when transplanted into a limb bud (Yonei et al., 1995; Lu et al., 1997). Ectopic expression of *Hoxb-8* results in the formation of ectopic ZPA tissue along the anterior margin of the limb bud (Charité et al., 1994). In keeping with the known role of *Hox* genes in specifying A/P positional information along the main body axis, Hoxb-8 might normally play a role in localizing the ZPA to discrete limb bud cells. It might function by interfering with a *Shh* repressor, by directly activating *Shh* transcription, or by giving competence to respond to other positive regulators of *Shh* transcription. Hoxb-8 is not, however, sufficient to induce *Shh* expression, as it is only the *Hoxb-8*-expressing cells at the distal margin that produce Shh, either in the endogenous or ectopic situation. It is very likely that this reflects a requirement for signals from the AER for *Shh* expression, most probably members of the FGF family that are necessary for maintenance of *Shh*, as discussed above. Besides *Hoxb-8*, there is evidence to suggest that other *Hox* genes are required for establishing the early limb bud. The expression domains of *Hox-9* genes in the lateral plate mesoderm correlate with the hindlimb and forelimb fields (Cohn et al., 1997), and mice lacking *Hoxb-5* have a rostral shift in the position of their shoulder girdle (Rancourt et al., 1995).

What determines the proper expression domains of *Hox* genes in the lateral plate, and in particular the domain of *Hoxb-8* expression? At least one key upstream determinant appears to be retinoic acid. When retinoic acid is introduced into the anterior of a limb bud, it produces mirror-image limb duplications by inducing formation of an ectopic ZPA (Noji et al., 1991; Wanek et al., 1991) and *Shh* expression (Riddle et al., 1993). *Hoxb-8* is rapidly induced in the anterior limb cells as a direct response to retinoic acid, in the absence of protein synthesis (Lu et al., 1997), thus likely giving them the competence to express *Shh* in the presence of FGFs from the AER. This likely recapitulates part of the process by which the endogenous ZPA is established since inhibitors of retinoid synthesis (Stratford et al., 1996) or activity (Helms et al., 1996) applied to the flank prevent the initial induction of Shh and block endogenous expression of *Hoxb-8* (Lu et al., 1997). Thus, by regulating *Hox* genes and influencing the future domain of *Shh* expression, retinoids play a key role in establishing the initial A/P asymmetries of the limb bud.

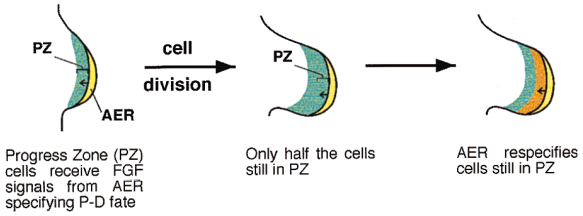

Figure 3. The Progress Zone Model and Progressive Proximal–Distal Specification

(Left) Progress zone (PZ) cells lie subjacent to the apical ectodermal ridge (AER, yellow). Under the influence of AER signals, the PZ cells acquire a P/D positional address (green).

(Middle) As cells within the progress zone proliferate, some of these cells leave the progress zone and are displaced proximally. Cells outside the influence of the progress zone retain their positional address when they exit the progress zone.

(Right) Cells that remain in the progress zone have their positional address adjusted to a more distal value (indicated by the orange color). Through repeated application of this mechanism, distal enlargement of the limb and P/D patterning could be coordinated.

The Progress Zone: Proximal–Distal Patterning and the Integration of Axial Patterning Mechanisms

Of the three cardinal limb axes, the mechanisms that lead to cell fate specification along the P/D axis are least understood. One paradigm, largely unmodified since its conception more than 20 years ago, is the progress zone model (Summerbell et al., 1973). According to this model, cell fate along the P/D axis is specified by the time spent in the progress zone, a region of distal mesenchyme in close proximity to the AER. Cells that exit the progress zone early adopt proximal fates, while cells that remain in the progress zone longer adopt progressively more distal fates. How these fates might be specified and recorded remains largely unknown. One possibility is that signals from the AER differ during proximal cell fate specification and distal cell fate specification. Alternatively, a constant signal may be summed over time resulting in the specification of progressively more distal fates. The former possibility is unlikely since heterochronic transplants demonstrate that the AER does not provide specific positional information with respect to the P/D axis (Saunders and Reuss, 1974). The strongest support for a constant AER signal comes from experiments that demonstrate that ectopic application of a single factor can substitute for the P/D signaling activities of the AER. Application of FGF beads to AER-denuded limb buds restores both outgrowth and patterning to a remarkable degree and in the best examples afford near complete limb development including digits (Niswander et al., 1993; Fallon et al., 1994). Thus, one plausible model for P/D specification is that progress zone cells receive a constant, perhaps saturating FGF signal and that the time progress zone cells receive this signal is recorded and summed over time (Figure 3).

When cells exit the progress zone, they carry with them positional information not only along the P/D axis,

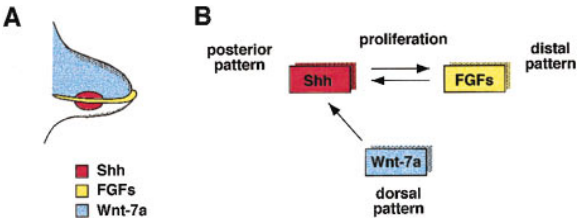

Figure 4. Three Axes and Three Signals: Shh, FGFs, and Wnt-7a Orchestrate Limb Pattern

(A) Schematic of a limb bud viewed from the posterior–dorsal aspect showing the localization of Shh to the ZPA, FGFs to the AER, and Wnt-7a to the dorsal ectoderm.

(B) Codependence of Shh, FGF, and Wnt-7a signaling and axial patterning. While each secreted factor can be associated with patterning along a single axis, affecting the expression of any single factor will lead to modulation of the other two. For example, reduction of Wnt-7a signaling will lead directly to dorsal patterning defects, but indirectly to posterior defects through a diminution of Shh signaling, and to proliferation defects via a subsequent effect on *FGF* expression.

but also along the A/P and D/V axes. Microsurgical manipulation of axial development, either by grafting a ZPA or by ectodermal reversals, yields different results depending on the age of the host limb mesenchyme (MacCabe et al., 1974; Summerbell and Lewis, 1975). If the graft is carried out early, both proximal and distal fates are altered. Conversely, if the grafts are performed on older limbs, only distal elements are affected. What these experiments indicate is that only progress zone cells are plastic with respect to their positional information along all three cardinal limb axes. By limiting A/P and D/V cell fate specification to cells within the progress zone, axial specification along all three axes is temporally and spatially coordinated.

The interdependence of axial specification goes beyond a simple limitation of a competence to respond to patterning signals to the progress zone. Two examples help to illustrate this point. First, the primary signals for A/P patterning and distal outgrowth, namely Shh and FGFs, are codependent (Laufer et al., 1994; Niswander et al., 1994). Microsurgical removal of the AER results in rapid loss of *Shh* expression in posterior mesenchyme. Similarly, genetic attenuation of AER signaling, as occurs in the mouse mutant limb deformity, can initially result in normal *Shh* initiation, but subsequently leads to a loss of *Shh* due to an inability of the mutant AER to maintain *Shh* expression (Chan et al., 1995; Haramis et al., 1995). Since FGFs are the predominant outgrowth signal for the developing limb bud, it has been argued that Shh controls proliferation in the posterior mesenchyme by the induction of *FGF-4* (Laufer et al., 1994; Niswander et al., 1994). A further integration of FGF-4 and Shh signaling is revealed by the dependence of posterior mesenchyme gene expression on both FGF-4 and Shh (Laufer et al., 1994). Coexpression of *FGFs* and *Shh*, while not required for all Shh targets (Marigo et al., 1996b, 1996c), is necessary for the induction of *Bmp-2* and the *HoxD* cluster genes in mesodermal tissues. In this manner, positional information and proliferation are coordinated (Figure 4).

A second example of the interdependence of signaling centers and the molecules they produce involves Wnt-7a, the primary dorsalizing signal for the limb, and Shh. In *Wnt-7a* mutants (Parr and McMahon, 1995), the limbs are shorter and often lack posterior-most skeletal elements. This phenotype is readily explained by the observation that *Shh* expression is reduced significantly in *Wnt-7a* homozygotes. As a result, the most posterior digit is not specified, and at the same time *FGF-4* expression in the posterior AER is reduced. The combined reduction of *Shh* and *FGF-4* expression leads to posterior and distal truncations. Complementary parallel experiments also demonstrate a functional role for the dorsal ectoderm in maintenance of *Shh* expression (Yang and Niswander, 1995). In these experiments, removal of the dorsal ectoderm leads to a reduction of the level of *Shh* transcription, an effect that can be rescued by grafting of cells that express *Wnt-7a*. Since one demonstrated activity of Wnt-7a is to induce the expression of *Lmx-1* (Riddle et al., 1995; Vogel et al., 1995), a possibility is that Lmx-1 might participate in *Shh* initiation and/or maintenance. Indeed, ectopic expression of *Lmx-1* can result in *Shh* induction in approximately 30% of injected embryos. As a result, ectopic digits form in the anterior of the limb. A clear explanation for this latter observation is not yet at hand, but these results indicate a reciprocal interaction between D/V pathways mediated by Wnt-7a and Lmx-1 and A/P pathways mediated by Shh (Figure 4).

The Readout of Positional Signaling: *Hox* Genes and Growth Factors

If early coordinated signals serve to provide positional cues to developing limb bud cells, how might these signals act to change cellular properties so that subsequent growth and differentiation lead to predictable and stereotyped limb morphogenesis? One component of this process must be the activation of key regulatory genes that mediate the instructions encoded by early patterning signals. As discussed above, one such gene is *lmx-1*, which is induced by the dorsal Wnt-7a signal, and appears to encode dorsal positional information in the distal mesenchyme. Another important group of genes in this regard are the clustered *Hox* genes (reviewed in Krumlauf, 1994).

The expression domains of 5′-members of the *HoxD* cluster, *Hoxd-9-Hoxd-13*, are initially established in a nested set centered around the posterior of the limb bud (Dolle et al., 1989). These genes can be ectopically activated by the combined influence of Shh and FGFs. This suggests that these factors play an endogenous role in the regulation of the *Hoxd* genes, although their normal initiation may involve other factors besides FGFs and Shh since at least some of these genes are expressed prior to that of *Shh* or AER formation (Nelson et al., 1996). Subsequently, *HoxD* gene expression patterns are not strictly correlated with the A/P or P/D axes, but rather are quite dynamic and go through several distinct phases (Figures 5A and 5B; Duboule, 1994a; Nelson et al., 1996). The early nested expression of *HoxD* genes is in the presumptive zeugopod (forearm and lower leg). At a slightly later stage, the same genes are expressed across the anlagen of the autopod (hand and

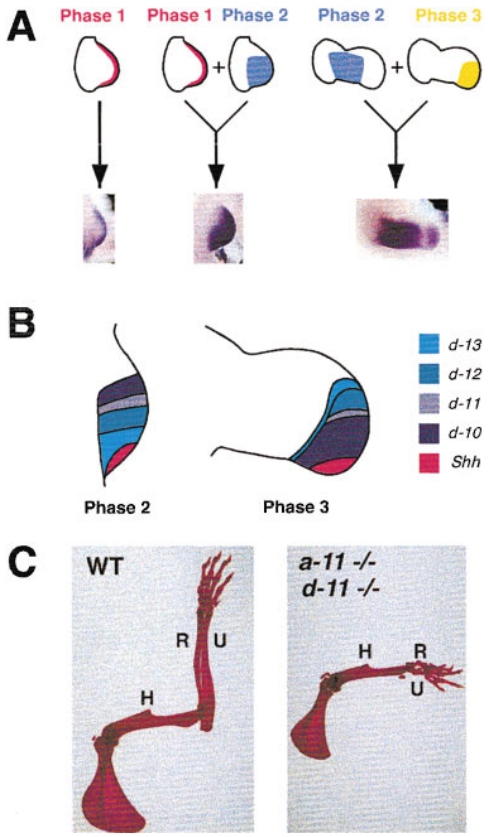

Figure 5. *Hox* Genes and the Specification of Limb Bud Positional Information

(A) *Hox* gene expression in the chick wing bud is quite dynamic, with several independently regulated phases of expression (shown here for *Hoxd-10*). In phase 1, *Hox* genes are expressed across the entire distal limb bud, during the time that the upper wing is specified. Subsequently in phase 2, *Hox* genes are expressed in a posteriorly nested order. A limb bud at the time the lower wing is specified shows overlapping expression in both phase 1 and 2 patterns. Finally, in phase 3 the *Hox* genes are expressed in a more distal pattern. At the time the digits are specified, the wing bud expresses the *Hox* genes in both phase 2 and phase 3 patterns.

(B) In the chicken limb bud, the relative order of expression of the *Hox* genes reverses between phase 2 and phase 3. Although the order of the *HoxD* genes is different in the zeugopod from that in the autopod, Sonic hedgehog (Shh) is able to induce these genes in the proper temporal and spatial order within each segment. Thus, the order in which *Hox* genes are activated in response to Shh is dependent upon the P/D segment of the limb bud on which Shh is acting. It is important to note that even though the *Hox* genes are centered around the Shh–expressing cells and can be activated by Shh, their expression is initiated in a posteriorly biased manner even in the absence of Shh (see text).

(C) *Hox* genes seem to function, in part, to drive the proliferation of the limb elements. There is a correspondence between the limb segments regulated by the *Hox* genes and both the order of the genes within the cluster and phase of *Hox* expression. For example, The lower wing is specified during phase 2, when *Hoxa-11* and *Hoxd-11* are broadly expressed. Due to "posterior prevalence" these genes have a greater role in this segment than more 3′ *Hox* genes; and the expression of more 5′ *Hox* genes, such as *Hoxa-13* and *Hoxd-13*, is confined to the extreme posterior margin during this phase and hence does not have a major impact on the development of the lower wing. Thus, the double mutant lacking both *Hoxd-11* and its paralog *Hoxa-11* has an approximately normal upper limb and foot, but the lower limb exhibits little growth after the initial cartilage condensations form, and hence the lower limb segment is nearly missing.

wrist, ankle and foot), with a spatial order along the A/P axis that is, at least in the chicken, actually the reverse of that initially seen in the zeugopod. The expression patterns of a second set of *Hox* genes, *Hoxa-9-Hoxa-13*, are also dynamic, primarily defining domains along the P/D axis (Yokouchi et al., 1991).

To understand their role in limb patterning, the dynamic expression patterns of clustered *Hox* genes need to be interpreted in the context of a general phenomenon known as "posterior prevalence" (Duboule, 1994b). When two or more *Hox* genes are coexpressed in the same cell, the more 5′ gene(s) of the *Hox* cluster will exert a dominant effect. Thus, as the dynamic pattern of *Hox* genes unfolds during limb bud outgrowth, different *Hox* genes play dominant roles in different limb bud regions. This leads to differential growth of limb elements, since different members of each *Hox* cluster have distinct effects on proliferation and differentiation. In contrast to *Hox* genes within a single cluster, paralogous *Hox* genes from different clusters appear to be largely redundant in function. Hence, the loss of any single *Hox* gene results in subtle, spatially limited defects in limb pattern. For example, *Hoxa-13* and *Hoxd-13* mutations each result in relatively minor autopodal defects. However, analysis of compound mutants shows that these genes are partially redundant in function with each other and act in a dosage-dependent manner: removal of increasing numbers of copies of these genes results in progressively more severe defects and in an almost complete lack of chondrogenesis in the autopod of the double homozygote (Dolle et al., 1993; Fromental-Ramain et al., 1996). The importance of the *Hox-13* paralogs specifically for the autopod is consistent with the fact that they are widely expressed in the distal limb during the phase when the autopod is specified; and since they are the most 5′ members of the cluster, they exert a dominant effect. These 5′-most *Hox* genes are not, however, widely expressed in the presumptive zeugopod or stylopod, rather other *Hox* genes play major roles within these more proximal tissues. For example, individual *Hoxd-11* and *Hoxa-11* mutations result in minor defects in the distal portions of the radius and ulna (Small and Potter, 1993; Davis and Capecchi, 1994; Favier et al., 1995). However, *Hoxd-11/Hoxa-11* double mutants exhibit an almost complete loss of the zeugopod in the forelimb (Figure 5C; Davis et al., 1995). This dramatic phenotype is attributable to the total loss of 11th paralog function from the forelimb (there is no *Hoxb-11* gene and *Hoxc-11* is exclusively expressed in the hindlimb). Nonetheless, these data do not support the idea that the 11th paralogs together provide positional information specifying the zeugopod because even in their absence rudiments of the radius and ulna still form. Thus, the essence of a zeugopod, two long bones, is not defined solely by the expression of *Hoxd-11* and *Hoxa-11*. What does appear to be true is that the 11th paralog genes preferentially act to promote growth within this segment. It is important to note in this regard that *Hoxa-11* and *Hoxd-11* are not the only *Hox* genes affecting the zeugopod, since the significant defects in that segment are also seen in *Hoxa-11/Hoxd-12* (Davis and Capecchi, 1996) and *Hoxd-11/Hoxa-10* double mutants (Favier et al., 1996).

Analysis of such skeletal defects, produced by gain or loss of *Hox* gene function, has indicated that a primary role of the *Hox* genes is to regulate the rate and timing of cartilage proliferation and differentiation (Dolle et al., 1993; Davis et al., 1995; Duboule, 1995; Goff and Tabin, 1997). However, *Hox* genes also influence limb patterning at several other stages of development, including proliferation of the undifferentiated mesenchyme, condensation of the mesenchyme into blastemal primordia, and organization of the cartilage cells within the skeletal elements (Davis et al., 1995; Duboule, 1995; Yokouchi et al., 1995; Goff and Tabin, 1997). The specific target genes regulated by *Hox* genes in carrying out these cellular effects are currently completely unknown. In spite of this sizable gap in our knowledge, we are starting to know the identity of various local signals, including members of the TGFβ, FGF, and Hedgehog families, which influence further growth and patterning of individual skeletal elements (see Yamaguchi and Rossant, 1995; Hogan, 1996; Vortkamp, 1997), and it is likely that *Hox* genes act to regulate either the production or the interpretation of these signals.

Conclusions and Future Directions

The process of limb patterning can be conceptualized as taking place in four phases: establishment of the limb field, production of signals from defined centers that globally pattern the field, the recording of positional information within the field in the form of specific gene expression, and finally the regulated differentiation of the limb structures on the basis of the encoded positional information. Great progress has been made in understanding each of these steps in the last five years, such that the patterning of the limb is arguably the best understood vertebrate model of morphogenesis. Yet, the task of understanding limb development is far from complete: for example, mechanisms that pattern the soft tissues, muscle, tendons, and ligaments remain largely unexplored as do phylogenetic mechanisms that lead to the impressive diversity of limb anatomy among specialized tetrapod taxa. However, since we now know the identity of many key molecular players that regulate limb pattern in a few model organisms, one might expect that these questions are now approachable. There is, therefore, reason for genuine optimism that the current heady pace of discovery in this field will continue for the next five years, and that the day is not so far off that we will be able to trace all the steps from the initial induction of the limb field to the detailed morphogenesis of the tissues of the mature limb.

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