Deciphering skeletal patterning: clues from the limb

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Even young children can distinguish a *Tyrannosaurus rex* from a *Brontosaurus* by observing differences in bone size, shape, number and arrangement, that is, skeletal pattern. But despite our extensive knowledge about cartilage and bone formation per se, it is still largely a mystery how skeletal pattern is established. Much of what we do know has been learned from studying limb development in chicken and mouse embryos. Based on the data from such studies, models for how limb skeletal pattern is established have been proposed and continue to be hotly debated.

Some 360 million years ago our ancestors began to crawl on land with the aid of paired appendages. Evolution has since produced limbs that permit modes of locomotion as distinct as running, flying and swinging through trees. Despite the adaptive changes to the tetrapod limb skeleton, its fundamental organization has been remarkably conserved. Three discrete compartments are evident in most extant tetrapod limbs: proximal (closest to the body wall, that is, upper arm or thigh); middle (forearm or calf); and distal (hand or foot) (Fig. 1). In the generic limb, the proximal compartment (stylopod), contains one bone, the middle (zeugopod) contains two, and the distal (autopod) contains a variable number, including at most five digits. Generally, it is variation in bone length and shape, and bone number in the autopod, that allows limbs to serve markedly different functions.

Vertebrate species can be distinguished from one another by the size, shape, number and arrangement of their bones — properties that collectively define skeletal pattern. Although the process by which skeletal pattern is established is still largely mysterious, what we do know comes primarily from studies of limb development in chicken and mouse embryos. Limb skeletal elements develop from cartilage anlagen, a process known as endochondral bone formation (see review in this issue by Kronenberg, page 332). It begins when chondrocyte progenitors form a densely packed 'condensation', which subsequently develops into a cartilage template that is ultimately replaced by bone. The essential steps in establishing limb skeletal pattern, that is, 'patterning', take place prior to and during the formation of these cartilagenous anlagen.

One of the most informative approaches for exploring the mechanism of limb patterning is to perturb limb development, and then assess the effect on skeletal pattern. Such studies have provided a wealth of intriguing data, on which models for limb skeletal patterning have been based, but much remains to be learned. Here we review what is known about limb skeletal patterning, discuss the leading models of how this process occurs and the controversy surrounding them, and identify areas that must be explored so that we can gain a deeper understanding of skeletal patterning mechanisms.

Key steps in limb skeletal patterning

Each limb arises from a small bud of mesodermal cells covered by surface ectoderm. The mesodermal cell population comprises all the progenitors of the chondrocytes and connective tissues such as tendons and muscle sheaths. Other tissues, such as muscle and blood vessels, develop from cells that migrate into the early limb bud[1–4].

Establishment and outgrowth of the limb bud

One potentially important factor in defining skeletal pattern is the number of cells that constitute the initial limb bud, but little is known about how this number is determined. In part, initial limb bud size is controlled by the signalling system that induces limb bud formation at stereotypic positions along the body axis (Fig. 2a). Such signals, which originate in tissues that lie interior (medial) to the lateral plate mesoderm (LPM) from which the limb bud forms, regulate gene expression in the LPM[5–7]. However, a signal from the ectoderm that covers the limb-forming LPM also seems to be involved. Initial limb bud size is significantly reduced when FGF8, a member of the fibroblast growth factor (FGF) family of secreted signalling molecules, is inactivated in prospective limb ectoderm[8]. The limbs that develop in such *Fgf8*-deficient mice lack several skeletal elements[9,10], defects that could be due to small initial bud size and/or to lack of FGF8 signalling at later stages.

Once established, the limb bud rapidly increases in size (Fig. 2b). Fate mapping studies have shown that expansion of the progenitors of the proximal, middle and distal compartments is completed at different times, in a proximal to distal sequence[11,12]. Little is known about how this is regulated, but it may be a reflection of the timing of condensation, which is correlated with decreased mitotic activity[13].

Condensation

The formation of the condensations that prefigure the skeletal elements is arguably the most critical event in skeletal patterning, so it is surprising how little is known about what determines their size, shape and number. Prechondrogenic condensations are detectable morphologically as local regions of increased cell density (Fig. 2c), and by molecular markers[14]. Cell–cell interactions are important in initiating condensation, perhaps by establishing an 'aggregation centre' that recruits cells from surrounding tissue[15]. At the molecular level, evidence largely from cell-culture studies suggests that several different classes of molecules, including the cell adhesion molecules N-cadherin and NCAM, are important in condensation[14,16].

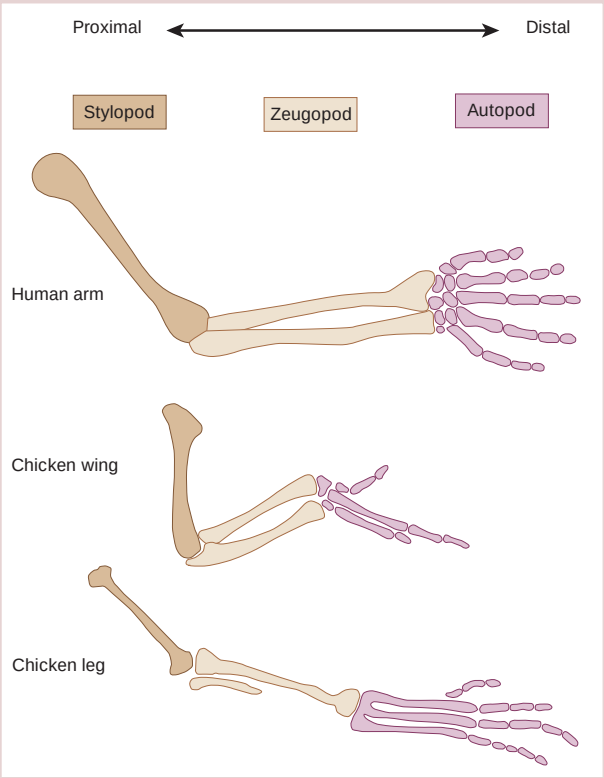

Figure 1 Comparison of human and chicken limb skeletons, illustrating the three compartments in tetrapod limbs and the variation in autopod skeletal elements in the forelimb (wing) and hindlimb (leg).

It appears that condensations form at specific stages of limb development, in a proximal to distal sequence, irrespective of how many chondrocyte progenitors are present. If the timing of condensation is indeed fixed, then alterations in cell number in the limb bud should affect skeletal pattern. For example, if fewer chondrocyte progenitors are present at the time of condensation, one might expect smaller elements to form. In fact, there is evidence suggesting that when limb bud cell number is reduced, the skeletal elements that form are smaller than normal and misshapen, and when it drops below a certain threshold, condensations do not develop[17,18]. If more chondrocyte progenitors are present at the time of condensation, one might expect larger elements to form. Interestingly, however, mutants with limb buds that are larger than normal, and thus presumably contain more cells than normal, develop limbs with more elements in the autopod[19].

A few genes required for condensation have been identified by mutational analysis in mice. For example, null mutations in *Bmp5*, a member of the bone morphogenetic protein (BMP) family of signalling molecules, result in the absence or alteration in the shape of specific condensations at various locations in the body (but not in the limbs)[20]. More global effects, leading to the absence of all condensations, are observed when *Sox9*, an *Sry*-related transcription factor gene, is inactivated in the early mouse limb bud[21]. However, it is not yet known what role *Sox9* plays in condensation.

One of the many unresolved questions about the mechanism of condensation in the limb bud is whether multiple focal condensations form at different locations, or if an initial focal condensation forms and then elongates at its distal end, branching at various points to increase the number of skeletal element precursors across the width of the limb (for example, at the stylopod/zeugopod boundary, where there is a transition from one to two elements; see Fig. 1)[15]. The argument against the latter model is that there are several experimental situations in which distal structures form, apparently in the absence of any proximal structures from which they could be derived by branching[22].

Joint formation

Joint formation is another process crucial for patterning. It occurs in several stages[23], either at the boundary between two adjacent condensations or within a single condensation. For example, in the autopod, individual digital rays are divided by joints into hand or foot elements and the phalanges that comprise the digits (Fig. 2d). Therefore, proper positioning of joints within a condensation influences the number and size of skeletal elements.

Joints become morphologically detectable when chondrocytes in the prospective joint-forming region become denser and flatten, and chondrogenic differentiation is inhibited. This creates an 'interzone', the site of the future cell death that creates the joint space. Two genes, *Gdf5* and *Nog*, which encode a BMP-related protein and BMP antagonist, respectively, are crucial in joint formation. Loss of *Gdf5* function results in several skeletal abnormalities including absence of specific joints in the autopod[24]. Further analysis has suggested that *Gdf5* has multiple roles in skeletal development, including restriction of joint development to the appropriate locations[25]. Loss of *Nog* function causes complete failure of joint formation in the autopod, at least in part via an effect on *Gdf5* expression[26]. Ectopic expression of *Wnt14* in chicken limb buds induces morphological and molecular manifestations of early joint formation, including expression of *Gdf5*, suggesting that this member of the Wingless family of intercellular signalling molecules also has a role in normal joint formation[27]. These and related studies have provided a good start towards understanding the molecular mechanism of joint formation and how they are localized to specific sites.

Cartilage and bone formation

Much of the patterning process is complete by the time that condensations have differentiated into cartilagenous templates (Fig. 2e) and cartilage markers such as Alcian blue are detectable. However, the remaining steps in endochondral bone formation do influence final skeletal pattern, and patterning defects are observed as a consequence of experimental manipulations or mutations that perturb these late-stage processes. For example, mutations in *Ror2*, a receptor-like tyrosine kinase, cause limb skeletal defects as a result of abnormal cartilage development[28].

Regulatory genes affecting pattern

A process as intricate as limb skeletal patterning obviously depends on the deployment of numerous regulatory genes, including homeobox transcription factor (Hox) genes related to the *Drosophila* homeotic genes. Vertebrate Hox complexes arose by two sequential duplications of an ancestral cluster, and therefore genes at corresponding positions in the different complexes are similar in sequence (paralogues)[29]. Their distinctive expression patterns in the developing limb prompted investigators to undertake mutational analysis in mice, which has shown that genes in paralogue groups 9–13 of the *Hoxa* and *Hoxd* clusters are crucial in patterning the limb skeleton. At present, the view is that *Hoxa* and *Hoxd* group 9 and 10 genes influence stylopod size and shape, group 11 genes are essential for normal development of zeugopod elements, and group 11, 12 and 13 genes are involved in patterning the autopod, which is extremely sensitive to the level of expression of these genes[30]. But interpretation of the data is complicated by the fact that *Hoxd*-null mutant phenotypes vary markedly depending on whether the allele was produced by insertion or deletion. These differences can be explained by the finding that gene deletion, but not insertional mutation, causes alterations in the expression of nearby genes that have profound effects on skeletal element number, size and shape[31].

Because *Hoxa* and *Hoxd* genes are expressed in dynamic patterns throughout limb development[32], an important question is when do

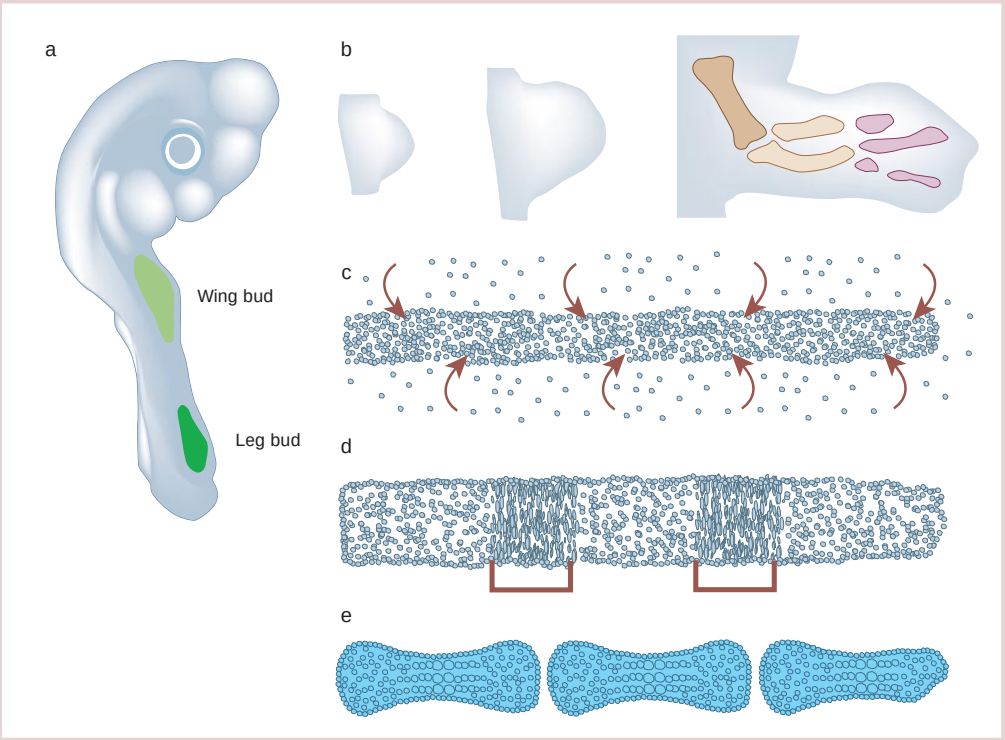

Figure 2 Key stages in limb skeletal development. **a**, Limb formation initiates in the chicken embryo at ~3 days of development, when the primary embryonic axis (head to tail) is elongating. First the forelimb (wing) buds and then the hindlimb (leg) buds begin to protrude from the sides of the embryo. **b**, Substantial outgrowth and patterning occurs over the course of the next three to four days, culminating in the establishment of the cartilage templates that prefigure the bones (illustrated on the right). **c**, During this time, chondrocyte progenitors aggregate (brown arrows) and form prechondrogenic condensations (a schematic digital ray is illustrated); **d**, joint formation begins (brown brackets); and **e**, the chondrocytes differentiate and begin secreting cartilage extracellular matrix molecules, which can be detected by Alcian blue staining.

they act to affect limb pattern? Several studies have pointed to effects on condensation or later events. For example, misexpression of *Hoxd13* in chicken limb buds leads to a decrease in cell proliferation in the growth plate of the tibia[33]. Moreover, in mice lacking both *Hoxa11* and *Hoxd11* function, the cartilage templates that will form zeugopod elements are nearly normal in size at early stages of their development, but in the final skeleton the zeugopod is dramatically reduced[34].

Other transcription factor genes, including members of the T-box family, also are important in limb skeletal patterning[35–39]. In fact, based on overexpression studies in chicken limb buds, it has been suggested that forelimb and hindlimb identity are specified by *Tbx5* and *Tbx4*, respectively[5]. However, analysis of *Tbx5*- and *Tbx4*-null mutant mouse embryos has not provided any insight into this issue, because forelimbs do not develop in the absence of *Tbx5* function[40], and without *Tbx4* function, although limb buds form, embryos do not survive long enough to determine limb skeletal pattern (V. Papaioannou, personal communication).

Sources of signals affecting pattern

Over the past half-century, experimental manipulations, primarily on chicken limb buds, have provided evidence that specific regions of the limb bud produce signalling molecules that influence limb skeletal pattern[41–43]. The knowledge that such regions exist in the early limb bud has led to the hypothesis that key steps in skeletal patterning occur as limb bud outgrowth proceeds. Here we highlight basic information about these signalling regions; more comprehensive treatments can be found in recent reviews[5,44].

Apical ectodermal ridge

The apical ectodermal ridge (AER) is a morphologically distinct ectoderm that rims the distal tip of the limb bud (Fig. 3a). Interest in AER function began in 1948, when Saunders[45] removed the AER from the chicken limb bud at successive stages of development and found that this caused abnormalities in skeletal pattern. The experimental limbs looked as though they had been severed (truncated) at different distances from the shoulder. When the AER was removed early, zeugopod and autopod were absent, but when it was removed later, only autopod was absent (Fig. 3b), showing that signals from the AER are essential for skeletal development. Given the importance of the AER, the mechanism by which it is established and maintained has been the subject of intense study[5,6,42,46].

Numerous genes expressed in the AER have been identified, including several members of the FGF and BMP families[5,6,42] (Fig. 3c). Application of beads containing recombinant FGF protein to the distal tip of AER-excised limb buds rescues skeletal development[47,48], and no other molecules have been reported to have such activity. Thus, FGFs are considered to be the key mediators of AER function.

Recent studies have provided an explanation for the phenotypes caused by AER removal and the ability of FGFs to functionally compensate for it. Following up on earlier experiments showing that AER removal causes cell death[47,49], Dudley *et al.*[12] found that the domain of cell death remains constant in size (extending inwards ~200 μm from the distal tip) when the AER was removed at early and mid limb bud stages. When it was removed at much later stages, distal cells survived but had a significantly lower than normal mitotic index. This suggests that the truncations are progressively less severe when the AER is removed at later and later stages, because over time, the domain of cells affected contains a smaller and smaller proportion of distal limb skeletal progenitors. It was also found that FGF protein rescued skeletal development only when applied before cells died, and moreover when cells were dye-labelled in the region where cell death was observed, they failed to contribute to skeletal elements. These data support the hypothesis that AER removal causes distal truncations because it results in death of the distal cells at early stages or reduces their proliferation rate at late stages, and that FGF rescues limb skeletal development by preventing these effects.

To determine the function of FGFs produced specifically in the AER (AER-FGFs) during normal limb development, gene knock-out experiments have been performed in mice. When two of the four AER-FGFs, FGF4 and FGF8, are eliminated in the limb bud using a conditional gene inactivation approach[8–10,50,51], no limb forms. However, when AER-FGF function is present early and then eliminated, limbs develop with abnormal skeletal pattern, and the defects,

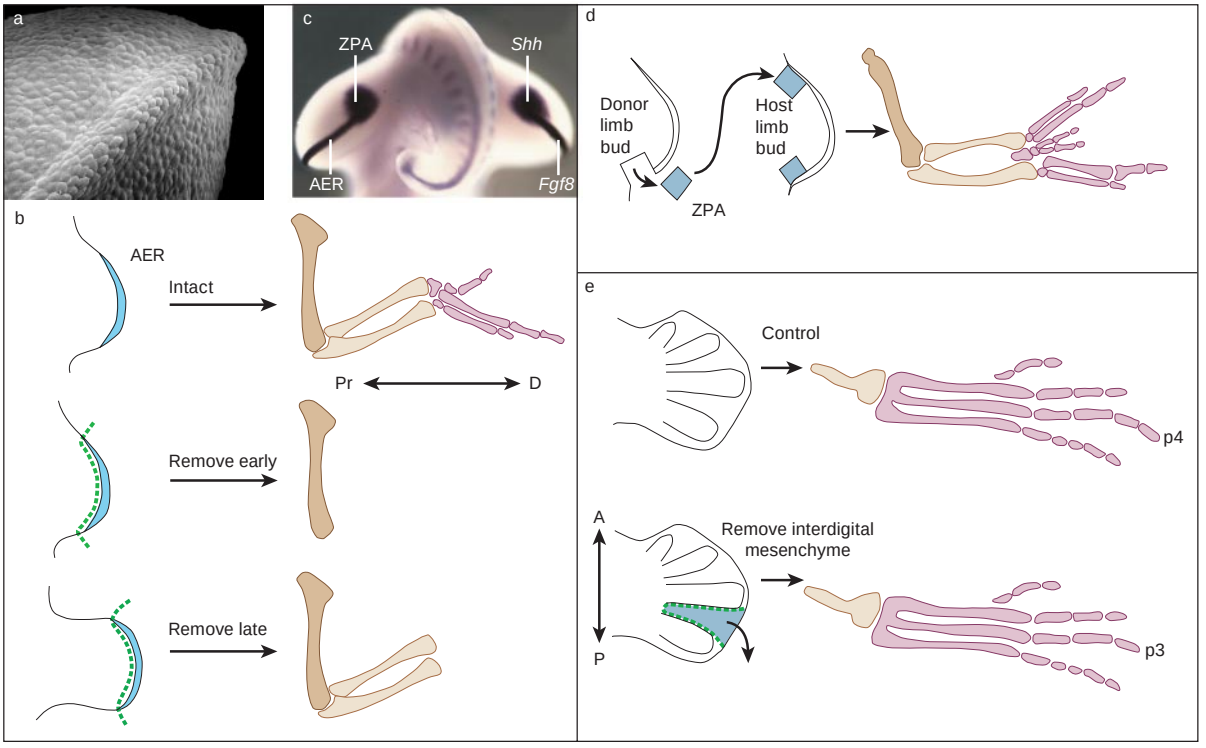

Figure 3 Signalling centres in the limb bud. **a**, Scanning electron micrograph showing the distinctive morphology of the apical ectodermal ridge (AER) that is localized at the distal tip of the limb bud (photograph courtesy of K. W. Tosney). **b**, Removal of the chick wing bud AER at an early stage results in limbs that appear severed (truncated) at the level of the elbow. AER removal at a later stage results in truncation at a more distal level[45]. **c**, Expression of genes encoding secreted signalling molecules, as visualized by RNA *in situ* hybridization in this posterior view of the leg buds of a chicken embryo. *Fgf8* is expressed in the AER and *Shh* in the zone of polarizing activity (ZPA). The tail is marked by *Shh* expressing cells along its length. (Photograph courtesy of P. Crossley.) **d**, When the ZPA is removed from its normal location on the posterior side of a donor limb bud and grafted to the anterior side of a host limb bud, the resulting limb has a mirror image duplication of the autopod skeletal elements[52] (compare with normal pattern of wing skeletal elements in panel **b**). **e**, Removal of the interdigital mesenchyme at early post-condensation stages alters the number of phalanges in the digit anterior to the deleted region[62]. In this case a digit that normally has four phalanges (p4 in control) now has only three.

including a decrease in skeletal element number or size, are traceable to pre- or early condensation stages[8]. Based on a detailed analysis of the mutant phenotypes, AER-FGF signalling seems to influence skeletal pattern, at least in part, by regulating the number of chondrocyte progenitors available for condensation via an effect on cell survival.

Zone of polarizing activity

The zone of polarizing activity (ZPA) resides in mesenchyme at the posterior distal margin of the limb bud[52]. Grafting a ZPA to the anterior distal margin of a chicken limb bud causes mirror-image duplications of skeletal elements (Fig. 3d), suggesting that this region controls patterning along the anterior–posterior axis (thumb to little finger)[5,53]. The secreted protein Sonic hedgehog (SHH) is produced in the ZPA (Fig. 3c), and *Shh* expression can mimic the effects of ZPA grafts, suggesting that it can be equated with ZPA activity[54]. It is thought that SHH functions as a morphogen, possibly through an inductive effect on *Bmp2* expression[55], and that the distance it diffuses depends upon post-translational modifications[56]. The molecular mechanism by which SHH signalling regulates its target genes involves effects on GLI3, the vertebrate homologue of *Drosophila* Cubitus interruptus, which functions either as transcriptional activator (GLI3A) or repressor (GLI3R). High levels of SHH prevent the formation of GLI3R and promote GLI3A function. Thus the local concentration of SHH regulates target gene expression by controlling the balance of GLI3 repressor and activator forms[57,58].

Mutational analysis in mice has provided clues about the normal function of SHH in limb skeletal development. $Shh^{-/-}$ mutant limbs have a normal stylopod, but exhibit severe defects distally[59,60]. The presence of an extensive domain of abnormal cell death in $Shh^{-/-}$ limb buds[59] suggests that, like AER-FGFs, SHH may influence skeletal pattern by controlling cell survival. The ectopic apoptosis in $Shh^{-/-}$ limb buds is caused by the abnormally high level of GLI3R produced in the absence of SHH, because removing one functional copy of *Gli3* in $Shh^{-/-}$ mice reduces the amount of ectopic apoptosis, and removing both functional copies of *Gli3* in $Shh^{-/-}$ mice eliminates it[61]. When *Gli3* dosage is reduced in $Shh^{-/-}$ mice, zeugopod and autopod skeletal elements develop, presumably because there are now sufficient chondrocyte progenitors available to form the condensations. As expected, $Shh^{-/-}$,$Gli3^{+/-}$ mice have fewer digits (3–4) than $Shh^{-/-}$,$Gli3^{-/-}$ mice (6–11 digits)[57,61]. The digits formed in $Shh^{-/-}$ limbs in the absence of one functional copy of *Gli3* all resemble digit 1 (as judged by phalanx number), whereas those that form in the absence of both *Shh* and *Gli3* function are morphologically indistinguishable from one another, and difficult to categorize. These observations show that in addition to regulating cell survival, SHH also has a role in patterning the autopod.

Interdigital mesenchyme

Although the potential patterning function of signals produced at early stages has been the subject of numerous studies, little is known about how signals produced at later stages, after initiation of condensation, affect skeletal pattern. Dahn and Fallon[62] demonstrated that the mesenchyme between the digital rays (interdigital mesenchyme or IDM) produces signals that influence the number of phalanges

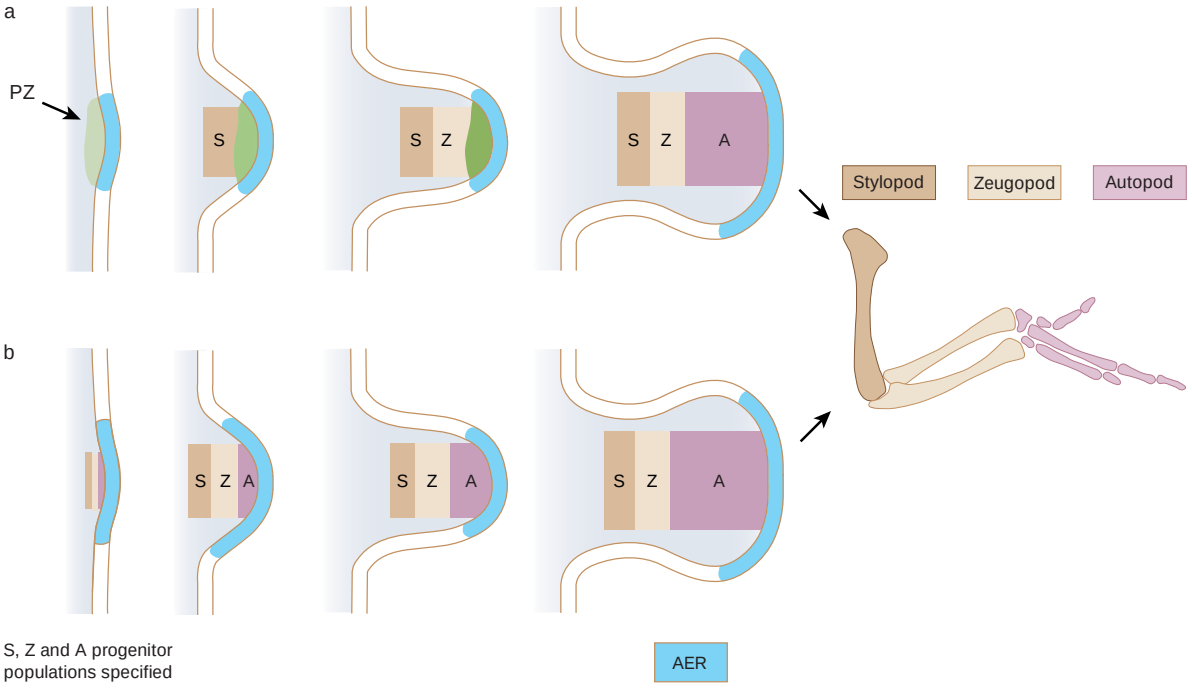

Figure 4 Two models for proximodistal limb skeletal patterning. **a**, The progress zone model proposes that the mesoderm at the distal tip of the limb bud constitutes a 'progress zone' (PZ, in green) of fixed dimensions, in which cells are receiving progressively more distal positional information over time (as indicated by the change in intensity of green colour from one stage to the next). When cells exit the progress zone they lose their lability, as they are no longer under the influence of signals from the AER (blue). Thus, the first to leave become stylopod (S) progenitors, whereas those that leave later become zeugopod (Z) and even later, autopod (A) progenitors[65]. **b**, The early specification model[12] proposes that at an early limb bud stage, cells are broadly specified to form the three compartments of the limb. The cells then undergo considerable expansion before becoming determined to form the different skeletal elements, as indicated.

that ultimately develop (Fig. 3e). These signals apparently include BMPs, because application of Noggin protein causes alterations in phalanx number similar to those observed when the IDM is removed. These effects on digit pattern underscore the importance of local signalling during cartilage template development, and raise the possibility that signals from cells surrounding more proximal structures may also influence skeletal pattern. Such local sources of signalling molecules at late stages may be established as a consequence of signalling at early stages by SHH or other factors.

Models of limb skeletal patterning

Efforts to explain the mechanism of limb patterning have been based on two fundamental concepts in developmental biology — specification and determination[63]. A cell or group of cells is defined as 'specified' when it has acquired molecular information that allows it to differentiate into a particular cell or tissue type, and, when placed in 'neutral' environments, will differentiate into that particular cell or tissue type. Specified cells are still labile, because they can respond to new signals by altering their differentiation path, that is, they can be 're-specified' to form a different cell or tissue type. As development proceeds, cells lose this lability, and finally become 'determined'. Determined cells differentiate into the specified cell or tissue type, even when they are exposed to new signals.

The prevailing view of limb patterning is that at early stages cells become specified to form different parts of the limb skeleton. Although there are obvious mechanisms for specifying a particular cell or tissue type, the mechanism for specifying structure is less clear. To address this problem, it has been proposed that cells acquire 'positional information', which tells them where they are with respect to certain boundary or reference regions. 'Positional values' in a three-dimensional coordinate system acquired at early stages are later interpreted by appropriate cytodifferentiation[64]. Specification to form a particular cell type and acquisition of positional information may occur independently. Furthermore, anterior–posterior and proximodistal patterning mechanisms are thought to be separable, but obviously need to be coordinated in some way.

The 'progress zone' model proposed by Wolpert and colleagues[65,66] postulates that cells acquire proximodistal positional information progressively, in a proximal-to-distal sequence, by measuring time spent in a 'progress zone' (PZ) ~300 μm deep from the limb bud tip. The longer the time spent in the PZ, the more distal the positional values they acquire. As all cells in the PZ are dividing, but PZ size is constant, cells must be continually exiting the PZ, at which time the specification clock stops. Cells that exit early are specified to form proximal limb structures, whereas those that exit late are specified to form distal ones (Fig. 4a). According to this model, the AER facilitates specification by maintaining PZ cells in a labile state. Because FGFs can functionally replace the AER[47,48], they are assumed to be the factors that maintain such lability. However, some of the phenotypes observed when AER-FGFs are inactivated do not match what would be expected if the proximodistal specification clock were slowed or stopped[8].

Early efforts to test the PZ model involved grafting the distal tip of an 'old' limb bud to the proximal stump of a 'young' limb bud. This model predicts that the composite bud should develop into a limb missing middle structures (zeugopod), because old distal tip cells should have already distalized beyond the point of producing zeugopod, and there would be no stimulus for cells in the stump to form a zeugopod. Although this result was obtained by one group of investigators[65], the opposite result — a complete proximodistal axis

including zeugopod elements — was obtained by others[67,68]. The reason for these different outcomes is not known.

One popular misconception about the PZ model is that cells in the hypothetical PZ drive outward growth of the limb bud by proliferating more rapidly than cells elsewhere in the limb bud. But even before the PZ was proposed, Hornbruch and Wolpert[69] showed that the mitotic index is relatively uniform throughout early chicken limb buds, and the same has been found in mice[8]. Another misconception is that the PZ model is a stem cell model, which postulates that when individual PZ cells divide, they give rise to one daughter cell that exits the PZ proximally and one that remains within it. If so, descendants of individual PZ cells should contribute to skeletal elements along the length of the proximodistal axis. However, the PZ model was never envisaged by its authors as a stem cell model; they hypothesized that at any stage, cells localized in the deepest part of the PZ soon find themselves outside the PZ and therefore cease changing positional values. Consistent with this view, fate-mapping studies show that descendants of small groups of cells at different depths within the proposed PZ contribute almost exclusively to one skeletal compartment[11,12].

This last observation prompted Dudley *et al.*[12] to propose that specification to form proximal, middle or distal limb structures does not occur progressively, but instead occurs very early, perhaps even before limb bud outgrowth has begun. According to this 'early specification model' (Fig. 4b), the specified populations subsequently expand as the limb bud grows, and become determined in a proximal-to-distal sequence. This concept has been criticized on the grounds that there are not enough cells in the early limb bud to comprise a miniature limb skeleton that will simply expand to full size over time[70]. But such criticism is based on the assumption that the early specification model proposes that all positional values are assigned early; in contrast, Dudley *et al.*[12] suggest that cells are specified more broadly, as progenitors of stylopod, zeugopod or autopod, and that over time they expand and become determined to form particular structures in response to cell–cell interactions and signalling.

The PZ and early specification models have different explanations for the results of various studies (see Box 1), but debate continues as to whether one or the other — or neither — model is correct. This issue could be resolved if molecular markers were found that could distinguish cells specified to form one set of limb skeletal elements from another. Such markers could then be used to determine the timing of specification.

If one accepts the premise that there is a mechanism for specifying cells in the early limb bud to form particular skeletal elements, then the key question — which is almost never discussed — is what is the link between the patterning information acquired at early stages and the morphogenetic processes, such as condensation and joint formation, that realize skeletal pattern? One possibility is that patterning information influences the timing of condensation. The earlier it occurs, the smaller the number of cells that will be available for condensation. Conversely, the later it occurs, the greater the number of cells that will be available. And, as discussed above, this could profoundly affect skeletal pattern by influencing the final size and number of elements that form. It is also possible that patterning information acquired at early limb bud stages influences condensation location. In considering the various possibilities, it is important to bear in mind that it is not necessarily the chondrocyte progenitors, but other mesodermal cell types in the limb bud that acquire patterning information. In turn, they might produce signals that influence condensation, cartilage development or joint formation, and therefore skeletal pattern.

A radically different view is that cells in the early limb bud, although perhaps specified to form chondrocytes, have no information about what particular skeletal elements they or their descendants will ultimately form. In this view, the events that occur during early limb development are permissive rather than instructive, producing a bud of appropriate size and shape, within which patterning is initiated as condensations begin to form. Skeletal element size and shape are roughly established as a function of basic cellular processes such as aggregate formation by chondrocyte progenitors[71] and the dimensions of the domains from which cells are recruited to aggregates. Their final form is then determined by local cell–cell interactions and signalling[62,72].

Box 1
Progress zone versus early specification models

For 30 years the 'progress zone' (PZ) model (Fig. 4a) has been the prevailing hypothesis to account for proximodistal limb patterning. According to this model, the apical ectodermal ridge (AER) is a source of permissive signals that keep cells in the PZ labile so they can autonomously acquire progressively more distal positional information. Recently, Dudley *et al.*[12] have proposed an alternative model, in which specification to form the different compartments of the limb is not progressive, but instead occurs at a very early stage of limb bud development (Fig. 4b). The two models propose different explanations for the results of various experimental manipulations, as indicated in the table below.

Box 1 Table **Explanations of experimental manipulations**

PZ model[70,73]	Early specification model[12]
AER removal at successive stages causes distal truncations at increasingly more distal positions along the length of the limb[45] (see Fig. 3b)	
In the absence of the AER, cells lose their lability and therefore can no longer change positional values. The later the AER is removed, the more distal the structures that have been specified before the distalizing clock stops	AER removal causes rapid cell death or a significant decrease in cell proliferation in a domain of fixed size (around the size of the hypothetical PZ). Over time, the domain of cells affected contains a progressively smaller proportion of distal limb skeletal progenitors. (It is noteworthy that unlike all the other explanations listed in this Table — on both sides of the argument — this is the only one that is based on experimental observations rather than hypothesis.)
In limb buds exposed to X-irradiation, proximal skeletal elements are severely reduced, whereas distal ones are less affected[18].	
To compensate for X-ray-induced cell death, the surviving cells must spend extra time in the PZ, while they proliferate and restore the PZ to normal size. They therefore undergo more distalization than normal, and consequently fail to form proximal elements.	Because the specified cells differentiate in a proximal-to-distal sequence at specific developmental stages, proximal cells have less time to replenish the population in the interim between X-irradiation and the onset of condensation than do distal cells. Thus proximal cells are less likely than distal cells to form normal elements.
When a sliver of the tip of a young limb bud (including the AER) is grafted to another location, it gives rise only to digit-like skeletal elements[12].	
To compensate for the small number of cells in the graft, the cells proliferate, stay longer in the PZ, and thus become specified to form only digits.	Cells at the distal tip of the early limb bud are already specified to form autopod elements and realize their developmental potential in the graft.
When distalmost cells from several limb buds are dissociated, reaggregated, placed in a limb bud ectodermal jacket, and grafted to a host embryo, different outcomes are obtained depending on the developmental age of the donor limb buds: stage 20 distal cells formed stylopod, zeugopod and autopod elements; stage 22 distal cells formed zeugopod and autopod elements; stage 24 distal cells formed only autopod elements[12].	
A new PZ is established in the reaggregate limb buds. The different outcomes are due to the different specification states of cells taken at progressively later stages.	Although already specified, distal cells taken at early stages can be respecified in the reaggregates, but become progressively refractory to respecification.

Perspectives and future directions

However valuable the models proposed to date might be, none of them provides more than a rudimentary sketch of how skeletal pattern is achieved. Although progress will doubtless be made by continuing to study the early limb bud, equal emphasis needs to be

placed on acquiring a deeper understanding of cellular and molecular events at later stages. For example, using very early markers of chondrocyte identity and new techniques for studying cell behaviour *in vivo*, in both normal and mutant embryos, it should be possible to answer vital questions such as what controls the timing of condensation, and the dimensions and locations of the condensations. Likewise, it will be important to explore how condensations are transformed into cartilage templates, and joints are positioned.

Using sophisticated genetic approaches that allow for gene inactivation at different developmental stages, it should be possible to determine whether genes that regulate skeletal pattern, such as the *Hoxa* and *Hoxd* genes, are required at early limb bud stages, or if they act only at later stages when skeletal pattern is being realized. With so many avenues to explore, we should soon gain more extensive knowledge of how limb skeletal pattern is established in model organisms. In turn, such information should further our understanding of how variation in skeletal pattern among different species is achieved. □

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