How to Make a Limb?

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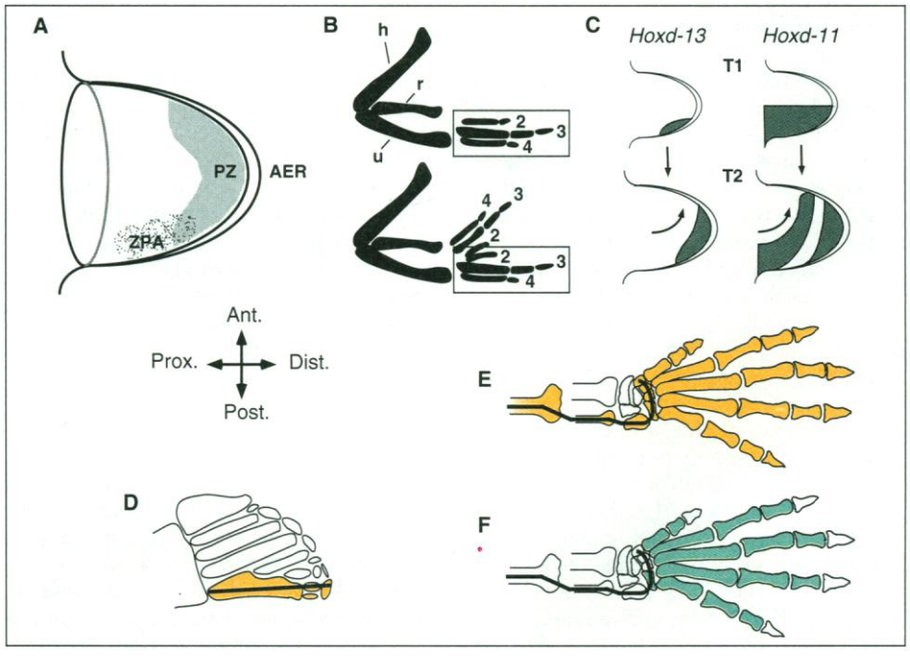
Vertebrate limbs are an amazing example of successful adaptation to various environmental conditions. In higher vertebrates, forelimbs help to fly, swim, walk, dig, grasp, or play the Goldberg variations. Yet their basic structure (the sequence and spatial arrangement of bony elements) is always the same. This implies the existence of a unique developmental strategy for building a limb (a limb plan) that early on imposes a basic scheme, on top of which subsequent species-specific customizations occur. The description of such a universal limb plan, and hence the idea that the genetic and developmental processes that generate this plan are very ancient, has been controversial for about a century (*1*), and it is worth asking whether recent discoveries of key genes in this process can bring new arguments to the debate.

Limb development is characterized by two essential phenomena. First, a structure must be produced, which requires that cells quickly proliferate. Second, this structure has to be organized, a process usually referred to as patterning. Although these two aspects of limb ontogenesis have often been considered as rather independent, it is becoming increasingly clear that they are not. Limb buds are composed of different regions, each with specific functions (*2*): an ectodermal ridge (AER), which maintains the proliferation of cells located underneath in the "progress zone," and a posteriorly located area, the polarizing region (ZPA) (Fig. 1A), which contains organizational properties. When ZPA cells are grafted to the anterior margin of a budding chicken wing, the result is a spectacular mirror-image duplication of the distal wing pattern (Fig. 1B). This effect can be reproduced to different extents by other signaling regions of the developing body such as Hensen's node or by molecules like retinoic acid or the chicken *shh* gene product, which is related to the product of the *Drosophila* gene *hedgehog* (*2*, *3*).

Although this dramatic result has sometimes been interpreted as a respecification or modification of the wing pattern, actually the original wing pattern (digits 2, 3, and 4) remains unmodified (Fig. 1B). Rather, an extra structure is produced. Why does the pattern of this supernumerary structure look like the original one? This could be explained if the anteroposterior asymmetry in the limb arises because cells along this axis are at different stages of one and the same developmental process (anterior being the default value of an anterior to posterior range) and that this process is linked to cellular proliferation. Limb mesenchyme may proliferate faster in the posterior region of that limb than in the anterior, a possibility supported by fate maps and other data (*4*). When a high rate of proliferation is induced in the anterior portion of the limb, such as by the experimental treatments mentioned above, the cells may progress a few steps further in the developmental range and change their fates accordingly to produce posterior structures. If proliferation is resumed at a more distal position, cells of future digit 2 will become more "posterior" with the concurrent suppression of the most anterior digits in the duplicated structure. In such a scheme, proliferation generates patterning, and the duplication of a preexisting structure demonstrates the invariance of the mechanism within a given species. It also illustrates the great difficulty of producing nonpatterned structures.

What could be the molecular basis of such a progressive, anterior-to-posterior and unidirectional process? Part of this mechanism may rely on a precisely choreographed activation of the *Hox* gene family. Vertebrate *Hox* genes are clustered (*5*) in the genome, and their expression is initiated in a sequence that follows the gene order within the clusters (*6*). The first part of the limb in which a subset of *Hoxa* and *Hoxd* genes are activated is the posterior part. Subsequently, the expression domains extend anteriorly, in the most distal parts (Fig. 1C, arrows). As the activation of the colinear sequence of *Hoxd* genes occurs exclusively in progress zone cells (*7*), it is possible that a more rapid rate of cell division may allow more *Hox* genes to be activated in posterior than in anterior cells. If so, then any treatment of anterior cells that will promote mitosis in situ may automatically activate additional *Hox* genes, which in turn would induce production of more



Limb development. (**A**) Schematic view of a developing vertebrate limb at a late bud stage with an apical ectodermal ridge (AER), a progress zone (PZ), and a zone of polarizing activity (ZPA). (**B**) A normal chicken wing skeleton (top) and a wing skeleton after graft of a ZPA at the anterior margin (bottom). The rectangles define the original skeleton. h, humerus; r, radius; u, ulna. Digits are numbered from posterior (4) to anterior (2). (**C**) Expression domains of the mouse *Hoxd-13* and *Hoxd-11* genes at an early bud stage (T1, top) and during handplate formation (T2, bottom). After an initial expression restricted posteriorly (T1), the domains extend anteriorly, in the most distal part (6). In the case of *Hoxd-11*, the domain splits into two parts, one in the presumptive digits, the other one in the future carpus. This anteriorization results in skewed boundaries (see arrows) that may reflect the bending of the major limb axis (the formation of the digital arch) as shown under (E). (**D** and **E**) The proposed positions of the metapterygial axes (bold line) in sturgeons (*Acipenser*) (D) and in a mouse forelimb (E). Yellow areas in (D) and (E) show the distributions of postaxial elements. (**F**) The same hand as in (E) with, in green, those bones affected in the limbs of animals with a mutant *Hoxd-13* gene. Data are adapted from (*8*, *11*).

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posterior structures. In this model, it would be difficult (if not impossible) to produce an extra structure that does not look like the original one.

Evidence that proliferation and patterning are two sides of the same coin also comes from the inactivation of the *Hoxd-13* gene. The absence of this very late and posterior product (the last gene in the *HoxD* complex) leads to a general delay in limb morphogenesis. The bones are smaller in all digits, indicating that the limb had not been properly finished (*8*). Such a reduction in the global size of the skeletal pattern is likely due to a decreased capacity to recruit enough cells to condense the future cartilage, an expected consequence of a decreased rate of proliferation. This phenotype shows that the patterning information for the limb is not distributed only by gradients and coordinates defined along the proximodistal and anteroposterior axes, in which case local changes in identities would be expected. It also demonstrates that the identity of a given digit is not fixed by the presence or absence of a particular *Hox* product but rather by small shifts in the balance of information within the same plan of construction (*8–10*). Here, we do not play on a chess grid, but rather on a dynamic referential with three dimensions plus time.

In most tetrapods, establishing the limb prechondrogenic pattern (the future skeletal pattern) follows a conserved and well-defined sequence of branching and segmentation, whereby branching (the production of two elements out of one, as the radius and ulna are produced from the distal humeral condensation) tends to occur in postaxial mesenchyme only. On the basis of a detailed analysis of these branching patterns in several vertebrates, Shubin and Alberch (*11*) proposed that the major limb axis (the basic axis of the limb plan) follows the humerus, ulna, and ulnare, and is then skewed anteriorly to produce the digital arch, which will further match with the distal row of the carpal bones (Fig. 1E). Branching occurs only in condensations produced on the posterior side of this axis (postaxially), including branching for all digits (Fig. 1E). Coates (*12*) pointed out a striking correspondence between Shubin and Alberch's view of the bending of this major axis and the posterior-to-distal transition in the expression domains of those *Hoxd* genes activated during limb outgrowth (*6, 13*) [Fig. 1; compare (C) and (E)], which raises the possibility that these *Hoxd* genes are specific for postaxial development. In *Hoxd-13* mutant limbs, the alterations were restricted to this compartment (Fig. 1F) (*8*). In terms of cellular proliferation, this supports the idea that posterior (postaxial) cells toward the distal end of the developing limb (the autopod) tend to drive most of morphogenesis. Although the association between *Hox* genes and proliferation has some experimental support, the relation between proliferation and the branching mechanism is speculative (*11*). It is possible, however, that positive and negative regulation of local growth rates by *Hox* gene products could dictate the pathway of condensations. In this view, a local increase in the number of cells available to condense may induce a prechondrogenic condensation to split in this particular way (by branching). Likewise, a local decrease in the number of cells may result in the termination of a condensation.

Can this help us to understand the evolution of vertebrate appendages? The passage from aquatic life to a terrestrial environment was accompanied by important modifications of the appendicular skeleton. In fossil lobe-finned fish and surviving basal taxa such as the dipnoan lungfish, *Neoceratodus*, the skeletal pattern of the pectoral fins shows a major axis extending from the proximal metapterygium to the distal tip of the endoskeletal part, a situation somewhat similar to the pectoral fins of living cartilagenous fishes (such as *Squalus*) or primitive actinopterygians (such as sturgeons) (Fig. 1D) (*11*). The homology between this straight metapterygial axis and the bent axis of higher vertebrates (Fig. 1, D and E) has provided new insights into the ontogenic relation of such diverged appendages (*1, 11*), suggesting either that the entire distal part of the limb is homologous to the distal but posterior part of an ancestral fin or that it has no homologous structures in ancient fishes (Fig. 1, D and E; compare the yellow areas). In the latter case, the transition of fin to limb may have involved an enhanced postaxial proliferation of the developing fin, preceding (or concomitant with) the subsequent formation of a limb autopod.

The absence of a structure homologous to the tetrapod autopod is striking in teleost fishes, such as trout and zebrafish, in which the bony part of the pectoral fin (endoskeleton) is small when compared to the fin rays (dermal skeleton), which make up most of the fin and have a different developmental origin. During fin development, the future endoskeletal portion stops proliferating soon after budding, probably as a result of changes in the surrounding ectodermal layer, which then folds on itself to serve as a support for fin rays. In this context, an attractive hypothesis was proposed (*14*), whereby the time at which the developmental transition from a ridge to a fold occurs may decide the extent of development of endoskeletal elements. An early transition, soon after budding, generates the typical teleost pectoral fin, whereas a late transition would allow more proliferation within the future endoskeletal part and hence more elements to differentiate. In this view, the tetrapod limb represents an extreme case with an extended proliferation of the endoskeletal part and a concurrent absence of dermal skeleton. Consequently, morphological and molecular similarities between anterior and posterior appendages may reflect comparable relative extents of cell proliferation rather than the effect of a sudden transformation of one structure into the likeliness of the other one (*15*).

Hoxa and *Hoxd* genes could be involved in this evolutionary scheme in either of two ways. On the one hand, *Hox* genes may not be causally linked to variations in the proliferation of the endoskeletal part but may simply have adapted to such variations such that enhanced cell proliferation promotes and maintains *Hox* gene activation postaxially. Alternatively, *Hox* genes could control the growth of the bud by indirectly acting on the overlaying ectoderm, and thus be directly involved in generating the variations of this phenomenon. For example, strong and sustained *Hox* expression posteriorly may stimulate proliferation as controlled by the ectoderm. The analyses of the corresponding genes in the relevant species, whenever possible, will help to distinguish between these alternatives.

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16. I thank P. Sordino, J. Beckers, P. Kahn, and J. Rickard for discussions, comments, and suggestions on the manuscript and N. H. Shubin for his explanations, corrections, and comments. Supported by funds from the Swiss National Research Foundation, the Canton de Genève, the Claraz Foundation, and the Human Frontier Science Program.