

Origination and Innovation in the Vertebrate Limb Skeleton: An Epigenetic Perspective

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ABSTRACT The vertebrate limb has provided evolutionary and developmental biologists with grist for theory and experiment for at least a century. Its most salient features are its pattern of discrete skeletal elements, the general proximodistal increase in element number as development proceeds, and the individualization of size and shape of the elements in line with functional requirements. Despite increased knowledge of molecular changes during limb development, however, the mechanisms for origination and innovation of the vertebrate limb pattern are still uncertain. We suggest that the bauplan of the limb is based on an interplay of genetic and epigenetic processes; in particular, the self-organizing properties of precartilage mesenchymal tissue are proposed to provide the basis for its ability to generate regularly spaced nodules and rods of cartilage. We provide an experimentally based “core” set of cellular and molecular processes in limb mesenchyme that, under realistic conditions, exhibit the requisite self-organizing behavior for pattern origination. We describe simulations that show that under limb bud-like geometries the core mechanism gives rise to skeletons with authentic proximodistal spatiotemporal organization. Finally, we propose that evolution refines skeletal templates generated by this process by mobilizing accessory molecular and biomechanical regulatory processes to shape the developing limb and its individual elements. Morphological innovation may take place when such modulatory processes exceed a threshold defined by the dynamics of the skeletogenic system and elements are added or lost. *J. Exp. Zool. (Mol. Dev. Evol.)* 304B:593–609, 2005. © 2005 Wiley-Liss, Inc.

The vertebrate limb, a conspicuous, stereotypical array of skeletal elements and associated tissues that arose in a nearly modern form approximately 200 million years after the establishment of the chordate body plan, has long held interest for comparative anatomists and evolutionary biologists. Its amenability to surgical manipulation at embryonic stages, particularly in avian species, and the fact that over- and under-expression of many developmentally significant genes have characteristic effects on limb patterning and morphogenesis in chicken and mouse embryos have also made the limb a mainstay of experimental developmental biology. This combination of features in one system would seem ideal for investigations of the origination and evolution of morphological innovations.

The realization of this potential in studies of the developing limb has been constrained by the fact that interpretations of its evolution and development, not surprisingly, have been based on the major gene-centered conceptual frameworks of the second half of the 20th century: neo-Darwinism and the notion of the “genetic program.” The first of these has spurred a quest, with little success

thus far, for incrementalist scenarios for the morphological changes involved in the “fin-limb transition” at the inception of the tetrapods, despite dramatic growth in knowledge of the accompanying phylogenetic genotypic changes. The second has given rise to the repeated assertion and withdrawal of candidate molecules underlying a putative coordinate system in a formalistic “positional information” model that hypothesizes independent specification of skeletal features along the limb’s three Cartesian axes.

In this paper, we suggest that an understanding of the origination of the limb skeletal pattern and the various morphological innovations it has accumulated in different taxonomic lineages over the course of evolution can best be achieved by adopting a wider perspective. Rather than assuming that skeletal pattern is encoded in the embryo’s developmental repertoire by a hierarchy

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of gene–gene interactions, we suggest that it emerges from a complex system in which physical and other conditional, nonprogrammed (“epigenetic” in the broad sense; see Newman and Müller (2000) or Müller and Olsson (2003)) mechanisms of morphogenesis and pattern formation are also at play. In this view, networks of gene interactions regulate inherent system behaviors that depend on properties and mechanisms beyond the strictly genetic.

To avoid misunderstanding, we will be very explicit about what we mean by epigenetic mechanisms. Developmental processes which depend extensively on feed-forward gene regulatory modules—the generation of the *Drosophila* pair-rule gene expression pattern (Lawrence, '92) or the induction of mesoderm in the sea urchin (Davidson et al., 2002), for example—may theoretically form patterns with little participation by extragenetic or epigenetic mechanisms. They are “hierarchical” in the sense of Salazar-Ciudad et al. (2001a). Such mechanisms require an extraordinary level of evolved intricacy (e.g., position-specific promoters, Stanojevic et al., '91; “smart genes,” Davidson, '90) to produce a pattern of any complexity.

In contrast with this mainly genetically programmed mode of development, patterns can readily arise from the reciprocal interaction of a system’s components (e.g., cells, their genes, and gene products) if the regulatory modules are employed in a reciprocal, multidirectional fashion, rather than as strict hierarchies. Such mechanisms, termed “emergent” by Salazar-Ciudad et al. (2001a), can not only be employed to produce simple gradients that can act as positional coordinates, but can also “self-organize” patterns of considerable complexity (see below), that are represented nowhere in the organism’s genome. Dynamical self-organization, moreover, is just one aspect of epigenetic determination of biological form. Discontinuous transitions from one pattern to another in a developing system (and therefore potentially during evolution) can also occur by “morphodynamic” pattern formation in which change in tissue geometry occurs simultaneously with (rather than follows) generation of morphogen gradients (Salazar-Ciudad and Jernvall, 2002; Salazar-Ciudad et al., 2003) as well as by reactivity of developing tissues exceeding certain thresholds as physical stresses act on them in organized contexts (Müller, 2003; see below).

Most developmental processes, of course, make use of both gene regulatory hierarchies and

epigenetic determinants. Indeed, even the paradigmatic case of the *Drosophila* pair-rule hierarchy depends on reciprocal as well as unidirectional causal chains (Clyde et al., 2003). Moreover, any epigenetically based developmental process will typically use sequential episodes of gene expression to prepare the system for the next round of organizational change. The domination of the “positional information” (Wolpert, '71, '94) model in limb studies over the last quarter century (e.g., Capdevila and Izpisúa Belmonte, 2001; Tickle, 2003) has, however, led to the limbs being considered at the more hierarchical end of the spectrum. We suggest below that this view is now dissolving and we propose an alternative framework.

Epigenetic determination requires molecular hardware—genes and their products—every bit as much as hierarchically programmed mechanisms. The biochemical oscillators underlying the cell cycle (Murray and Hunt, '93) and the generation of somites (Pourquié, 2003), for example, are self-organizing epigenetic processes that encompass and animate, rather than replace, molecular-genetic mechanisms. Epigenetic mechanisms are, to a great extent, the way in which molecular and genetic components perform their developmental roles. In a related fashion, since in our epigenetic framework change in gene allelic frequencies and gene expression are part of, but not the sole determining factors of, evolutionary and developmental morphological change, neo-Darwinian incrementalism and the developmental program notion are not rejected but rather assigned more limited roles.

Neo-Darwinian mechanisms, in which biological forms typically change in an incremental fashion due to genes of “small effect,” may only pertain to systems sufficiently evolved so that numerous genes and gene–gene interactions have come to serve canalizing and fine-tuning functions (Newman and Müller, 2000). Moreover, while such “autonomized” (Müller and Newman, '99) forms indeed develop in modern-day embryos with dependence on sequences of hierarchically linked gene expression events, it is not necessarily true that the same genes were part of the structure’s originating process at its evolutionary inception (Newman, '94; Newman and Müller, 2000). (For our use of “origination” see Müller and Newman (2003) and this volume.) This implies that a central focus of evolutionary developmental biology should be on “core” sets of cellular and molecular interactions likely (on the basis of cell

biological and phylogenetic inferences) to have participated in the origination of the structure or pattern of concern (see Müller and Newman, 2003; Newman, 2003). We elaborate on these ideas for the limb example in the remainder of this article.

LIMB SHAPE VS. LIMB SKELETAL PATTERN

The *shaping* of the developing limb—quantitative alterations in size and shape of the limb buds—is one area in which there has been a substantial increase in knowledge despite the conceptual constraints mentioned above. First, with no morphological innovation being involved, the evolution of limb bud shape can be well-accommodated within the neo-Darwinian paradigm. Second, the conceptual and experimental issues involved in tissue shaping involve cell division and death, viscoelastic behavior of tissues (see Dillon and Othmer, '99), and reciprocal interactions between different tissue types. As elaborate as the patterns of gene expression regulating (and regulated by) these processes may be, there is little complexity in the outcome: a mass of tissue of a particular size and shape. Limb bud shaping, like many other developmental and evolutionary processes not involving abrupt morphological transitions and novelties, can be understood on the basis of continuous incremental change in tissue mass and form, driven by an interplay of signaling among cells of different populations and tissue types. It is no surprise, therefore, that many of the factors involved in vertebrate limb outgrowth and shaping, e.g., the *Dlx*, *Lmx*, *Hox*, *R-fng*, *Fgf*, *Shh*, and *Wnt* gene products (see Tickle (2003), for a review), can be traced back to ancestral metazoans where they were also (based on their functions in modern invertebrates) involved in tissue outgrowth and shaping.

Patterning of the limb skeleton has been slower to yield to an approach based on mapping gene expression patterns during development. The bones of the limb are discrete arrays of elements that individually differ in size and shape and vary in number in a discontinuous fashion between limb region, limb type, and species. In contrast, expression patterns of transcription factors tend to be continuous, as are profiles of released morphogenetic and growth factors.

For the past three decades, much work on the development of the limb skeleton has tacitly

assumed that once a molecular “coordinate system” is set up across the limb bud, its “interpretation” as specific arrays of skeletal elements would be just a downstream effect: a readout of the appropriate portion of each cell’s genomic representation of the entire pattern (Wolpert, '71, '94; Capdevila and Izpisúa Belmonte, 2001; Tickle, 2003). The determinants of the presumed coordinate system or its main axes have been proposed, at various points, to be time spent within a critical distance of the apex (Summerbell et al., '73), distance from the posterior “zone of polarizing activity” (Wolpert and Hornbruch, '81), retinoic acid (Tickle et al., '85), retinoid receptors (Maden et al., '88), *Hoxd-11* (Izpisúa Belmonte et al., '91), combinations of Hox proteins (the “*Hox* code”) (Tabin, '92; Morgan and Tabin, '94), Sonic hedgehog (*Shh*) (Riddle et al., '93), and BMP (Dahn and Fallon, 2000). Simple axis-specific informational roles for each of these variables and factors have been rejected (e.g., Noji et al., '91; Wanek et al., '91; Davis and Capecchi, '94; Graham, '94; Dudley et al., 2002; Sun et al., 2002; Ahn and Joyner, 2004). Recent work (e.g., Innis et al., 2002; Sun et al., 2002; Chen et al., 2004; Harfe et al., 2004; Scherz et al., 2004; Zakany et al., 2004) suggests that Hox gene products, *Shh*, and time of exposure to these and other factors such as fibroblast growth factors (FGFs) act on mesenchymal cells not as variables of some informational coordinate system, but as modulators of the inherent property of these cells to generate discrete skeletal structures. Indeed, randomized limb mesenchymal cells with disrupted gradients of Hox proteins, *Shh*, etc., give rise to digit-like structures *in vivo* (Ros et al., '94) and discrete, regularly spaced cartilage nodules *in vitro* (Downie and Newman, '94; Kiskowski et al., 2004). Moreover, simultaneous knockout of *Shh* and its inhibitory regulator *Gli3* in mice yields limbs with numerous extra digits (Litingtung et al., 2002). If anything, such gradients limit and refine the inherent capacity to produce skeletal elements rather than being necessary to it.

EPIGENETIC ORIGINATION OF THE LIMB SKELETON

The ancestral fish in which the tetrapod limb originated was capable of producing discrete bony skeletal elements (i.e., the sarcopterygian basal and metapterygial elements) but not the autopod (wrist bones and digits), which is considered the key innovation in tetrapod limb

evolution. A popular model for fin-limb transition, first presented in the 1980s (Shubin and Alberch, '86), focused, in part, on mechanisms capable of producing the discontinuous patterns of the limb skeleton. This step forward, however, was undercut by attempts of other investigators to meld this model with then current notions of positional information.

It had earlier been suggested that the endoskeletal fin radials of sarcopterygian fish might be homologous to tetrapod digits (Gregory and Raven, '41). Like digits, the radials extend to either side of a roughly defined central axis along the proximodistal dimension of the limb. Shubin and Alberch ('86) proposed that the mesenchymal tissue mass (mesoblast) of the limb could generate preskeletal primordia by three different processes: de novo formation, segmentation, and branching. They also suggested that the central axis, rather than being an arbitrary anatomist's convention, was a real coordinate system axis that had "bent" anteriorly during evolution, so that the digits represented only "post-axial" extensions. The morphological novelty of the digits was thus proposed to be a result of deformation of the putative coordinate system. A popular idea at the time was that gradients of Hox gene products were positional information coordinates (Izpisúa-Belmonte et al., '91; Tabin, '92). When Coates ('91) noted that the expression pattern of *Hoxd*-class genes was displaced anteriorly and reversed in the tetrapod limb bud relative to the fish fin bud, the case for the evolutionarily bent axis was seemingly clinched.

Although the bent axis notion was ultimately found to have little embryological support (Vargesson et al., '97), the aspect of Shubin and Alberch's model that focused on de novo formation of skeletal elements, and segmentation of pre-existing elements, remains credible. Unfortunately, however, while the bent axis is no longer referred to in most discussions of limb origination, neither is discontinuous mesenchymal morphogenesis (Coates et al., 2002; Shubin, 2002). This may, in part, be due to the specific underlying mechanism Shubin and Alberch proposed to account for discontinuous patterning: mechanical instabilities resulting from traction of cells on the extracellular matrix (Oster et al., '83). It was subsequently found that limb mesenchymal cells do not exert traction on the ECM in vitro (Markwald et al., '90) and that the dependence of chondrogenic patterns in vitro on matrix density is not consistent with a traction/mechanical instabil-

ity model (Miura and Shiota, 2000a). Furthermore, the model implies that branching bifurcations should be a major mode of development in the limb (Oster et al., '83). But while bifurcated condensations are sometimes observed, they do not appear to be formed by a branching mechanism, i.e., one that requires a pre-existing element to produce a new bifurcated one (Cohn et al., 2002). It is also significant that the number of elements that eventually appear in specific regions of the limb mesoblast is specified before mesenchymal condensation actually takes place (Wolpert and Hornbruch, '90). This indicates that pattern formation is a molecular process independent of the mechanical changes that produce condensations, contrary to the model of Oster et al. ('83).

The inability of the mechanical model to account for skeletal pattern formation has led some investigators to suggest that the only alternative is the positional information idea (Cohn et al., 2002). This is not the case. As we will describe below (see also Newman, 2002), processes based on Turing-type reaction-diffusion instabilities (Turing, '52; Meinhardt and Gierer, 2000; Miura and Maini, 2004a) can produce discrete elements de novo and by segmentation. This class of mechanism thus provides a plausible epigenetic basis for skeletal patterning in the generic sense of production of rods and nodules of cartilage, subject to the modulatory and fine-tuning effects outlined above.

In the following sections we explore the question of how, if not by interpretation of positional values in a coordinate system, digits or other discrete skeletal structures may form within limb mesenchyme. We outline a core skeletogenic mechanism consisting of a set of cell and molecular interactions involved in controlling cell movement and relative affinity (Newman and Frisch, '79; Miura and Shiota, 2000a, b; Hentschel et al., 2004). The physical system thus constituted self-organizes spatiotemporal patterns of mesenchymal condensations (Hentschel et al., 2004; Kiskowski et al., 2004; Chaturvedi et al., 2005). A variety of factors, including the gradient molecules described above, but also biomechanical effects (Müller and Streicher, '89), will influence the number, shaping, and "identity" (Wagner and Gauthier, '99) of individual elements. When continuously varying modulatory effects cause the pattern forming system to exceed a threshold developmentally or evolutionarily (Müller, '90), a novel skeletal structure may be added or lost.

A CORE SET OF CELLULAR AND MOLECULAR PROCESSES IN CHONDROGENIC PATTERNING

The limb buds emerge from the body wall, or flank, at four discrete sites: two for the forelimbs and two for the hindlimbs. The paddle-shaped limb bud mesoblast, which gives rise to the skeleton and muscles, is surrounded by a layer of simple epithelium, the ectoderm. The skeletons of most vertebrate limbs develop as a series of precartilage primordia in a *proximodistal* fashion: that is, the structures closest to the body form first, followed, successively, by structures more and more distant from the body. For the forelimb of the chicken, for example, this means the humerus of the upper arm is generated first, followed by the radius and ulna of the mid-arm, the wrist bones, and finally the digits. (Urodele salamanders appear to be an exception to this proximodistal progression; Franssen et al., 2005.) The order in which the primordia actually chondrify may differ from the order in which the condensations appear (Blanco and Alberch, '92). The cartilage is mostly replaced by bone in species with bony skeletons.

Before the cartilages of the limb skeleton form, the mesenchymal cells of the mesoblast are dispersed in a hydrated ECM, rich in the glycosaminoglycan, hyaluronan. The first morphological evidence of the skeletal primordia are precartilage mesenchymal condensations (Hall and Miyake, '95, 2000; Newman and Tomasek, '96). Condensation involves the transient aggregation of cells within a mesenchymal tissue. This process is mediated first by the local production and secretion of ECM glycoproteins, such as fibronectin, which act to trap the cells at specific locations (Frenz et al., '89a,b). The aggregates are then consolidated by direct cell-cell adhesion which depends on the temporary expression by the condensing cells of cell-surface molecules such as NCAM (Widelitz et al., '93), N-cadherin (Oberleidner and Tuan, '94), or cadherin-11 (Kimura et al., '95; Luo et al., 2005) (see also Chimal-Monroy and Diaz de Leon, '99). The final step in this process of spatiotemporally controlled cartilage development (chondrogenesis) is the expression of cartilage-associated gene products (reviewed in Okazaki and Sandell, 2004) and production of a cartilage-specific extracellular matrix (reviewed in Ratcliffe and Mow, '96). The molecular components of this matrix (discussed in the section on the Role of Biomechanics, below) include type II collagen and

a major proteoglycan, aggrecan, consisting of several glycosaminoglycans linked to a cartilage-specific core protein.

Because all the precartilage cells of the limb mesoblast are capable of producing fibronectin and cadherins, but only those at sites destined to form skeletal elements actually do so, there clearly must be communication among the cells to divide the labor. This is mediated in part by secreted, diffusible factors of the TGF- β family of growth factors, which promote the production of fibronectin (Leonard et al., '91) and N-cadherin (Tsonis et al., '94). As in other connective tissues (Van Obberghen-Schilling et al., '88), TGF- β is positively autoregulatory in limb bud mesenchyme (Miura and Shiota, 2000b).

Limb bud ectoderm performs several important functions. First, it is a source of FGFs (Martin, '98). Although the entire limb ectoderm produces FGFs, the particular mix of these factors produced by the apical ectodermal ridge (AER), a narrow band of columnar ectodermal cells running in the anteroposterior direction along the tip of the growing limb bud in most amniotes, is essential to limb outgrowth and pattern formation. The AER keeps the precondensed mesenchyme of the "apical zone" in a labile state (Kosher et al., '79) and its removal leads to terminal truncations of the skeleton (Saunders, '48).

The FGFs produced by the ectoderm affect the developing limb tissues through one of three distinct FGF receptors. The apical zone is the only region of the mesoblast-containing cells that express FGF receptor 1 (FGFR1) (Peters et al., '92; Szebenyi et al., '95). In the developing chicken limb, cells begin to condense at a distance of approximately 0.3 mm from the AER (Summerbell and Lewis, '75). In this "active zone," FGFR1 is downregulated and cells that express FGFR2 appear at the sites of incipient condensation (Peters et al., '92; Szebenyi et al., '95; Moftah et al., 2002). These are the same cells that produce and experience high levels of fibronectin. Activation of these FGFR2-expressing cells by FGFs releases a laterally acting (i.e., peripheral to the condensations) inhibitor of cartilage differentiation (Moftah et al., 2002). Although the molecular identity of this inhibitor is unknown, its behavior is consistent with that of a diffusible molecule (Moftah et al., 2002). Finally, differentiated cartilage in the more mature region (frozen zone) proximal to the condensing cells expresses FGFR3, which is involved in the growth control of this tissue (Ornitz and Marie, 2002). The ectoderm, by

virtue of the FGFs it produces, thus regulates growth and differentiation of the mesenchyme and cartilage.

The limb ectoderm is also involved in shaping the limb bud. By itself, the limb mesenchyme, being an isotropic tissue with liquid-like properties, tends to round up (Foty et al., '96). When ensheathed by the ectoderm, however, it assumes a paddle shape. This is evidently due to the constraining biomechanical influence of the epithelial sheet and its underlying basal lamina (Borkhvardt, 2000).

A schematic representation of the components and interactions of the proposed core mechanism for limb skeletal pattern formation is shown in Fig. 1.

ADDING THE PHYSICS OF SELF-ORGANIZATION

A “bare-bones” mechanism for limb skeletal pattern formation

Chemical systems of any type in which there is a slow-spreading, self-enhancing “activator” (of any process or reaction) that directly or indirectly induces the production of a slower spreading “inhibitor” of the same process are capable of giving rise to spatial patterns of the reaction product. Turing was among the first to discuss this “symmetry-breaking” process (Turing, '52) and the first to suggest that it might have a role in embryonic pattern formation. This class of mechanism was proposed on theoretical grounds alone as the basis of several developmental phenomena (Gierer and Meinhardt, '72; Kauffman et al., '78) including skeletal patterning in the limb (Newman and Frisch, '79). Subsequently, the Turing instability (designated as such because the situation in which the distribution of reaction product is uniform is dynamically unstable) was demonstrated experimentally in chemical systems (Castets et al., '90; Ouyang and Swinney, '91), and experimental work in conjunction with computational analysis in a number of biological systems supported the idea that such mechanisms operate during development. The systems studied included development of pigment patterns in fish (Kondo and Asai, '95), spacing of feather germs in avian skin (Jiang et al., '99), formation of vertebrate body axis (Meinhardt, 2001), and precartilage condensations in mouse limb mesenchyme *in vitro* (Miura and Shiota, 2000a,b; Kiskowski et al., 2004; Miura and Maini, 2004b).

In Turing-type systems (also commonly referred to as “reaction–diffusion” systems, because the usual way in which the activator and inhibitor spread in a chemical system is by simple diffusion), the formation of a spatial pattern (typically spots and stripes of a chemical substance; Alber et al., 2005a) is not an inevitability, but depends on the “tuning” of the values of various kinetic constants and diffusion coefficients (see Meinhardt and Gierer, 2000; Miura and Maini, 2004a). For biological systems the reacting components are cells, in their capacity to produce various molecules and alter their differentiated states, and the diffusing components are released signaling molecules—gene products, nucleotides, lipids, etc. Mutations in genes specifying transcription factors, morphogens, and so forth will typically affect the rates of molecular–genetic processes, so that natural selection can tune the associated reaction–diffusion systems. It is significant that even if changes in the parameters mentioned are incremental, particular balances and ratios among factors will induce discontinuous changes in the pattern formed by the system. The system can go from no pattern to a pattern of spots or stripes, for example, undergo a transition from spots to stripes, or to patterns with different numbers of spots and stripes.

The core set of cellular and molecular processes described in the previous section and depicted in Fig. 1 fulfills the formal requirements of a reaction–diffusion mechanism. The role of the locally diffusible, positively autoregulated (Miura and Shiota, 2000b) activator of condensation formation (by virtue of its induction of fibronectin production; Leonard et al., '91) is served by TGF- β . A laterally acting inhibitor of condensation is produced from sites of activation in response to peripherally supplied FGFs (Moftah et al., 2002). Production of this inhibitor depends on the demonstrated localization of FGFR2c at condensation sites (Moftah et al., 2002). The simplest Turing circuit requires a causal link between the activator and the inhibitor. While elevated levels of FGFR2c colocalize with downstream effects of TGF- β , i.e., elevated fibronectin, in early condensations, we have no evidence that TGF- β upregulates FGF2c; it is possible that the nonuniform FGFR2c pattern is established independently by a parallel set of interactions. We also do not know at what level (e.g., TGF- β production, fibronectin production or secretion) the lateral inhibitory effect on condensation acts.

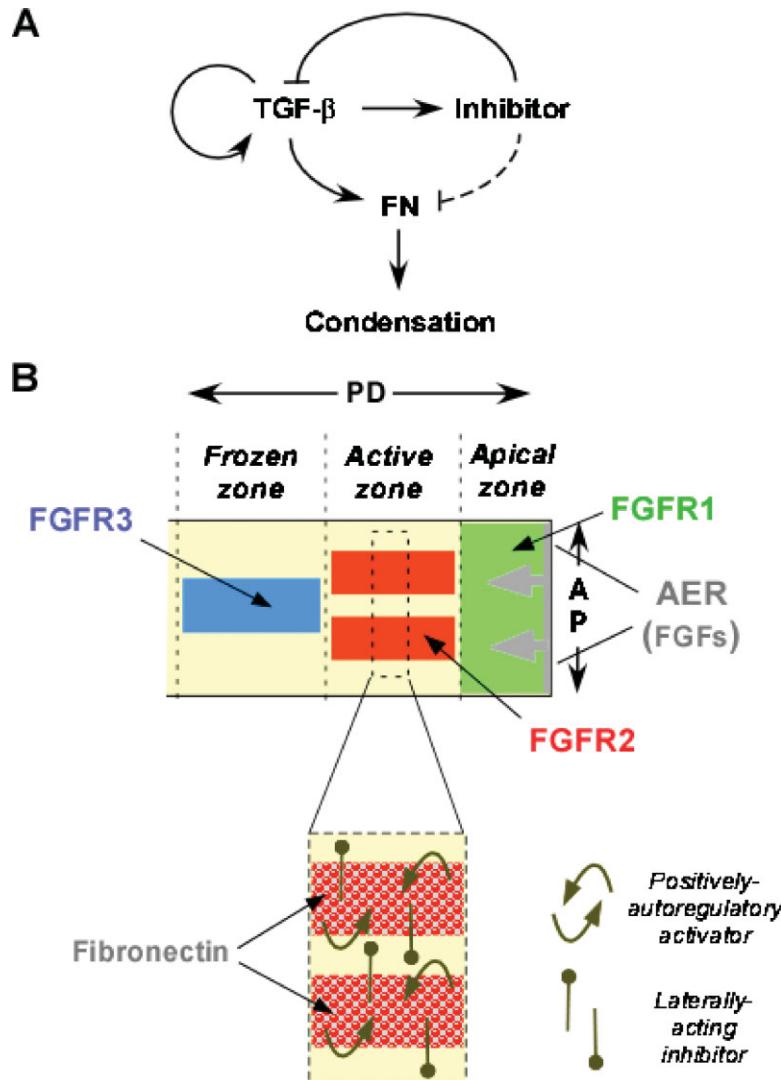


Fig. 1. (A) Schematic representation of the biochemical–genetic circuitry underlying the self-organizing pattern forming core mechanism (Hentschel et al., 2004; Kiskowski et al., 2004). Positive autoregulation of TGF- β , induction of fibronectin by TGF- β , promotion of precartilage condensation by fibronectin, and FGF-dependent elicitation of a lateral inhibitor of cartilage development from sites of condensation are all supported by experimental evidence. The model assumes the inhibitor acts directly on TGF- β . (B) Interactions of the core mechanism are superimposed on a two-dimensional schematic limb bud organized into zones defined by experimentally determined expression patterns of FGF receptors 1, 2, and 3. In the apical zone, cell rearrangement is suppressed by the FGFs emanating from the AER. The active zone, a detailed view of which is shown below, is the site of spatiotemporal regulation of mesenchymal cell condensation (i.e., pattern formation). When cells leave the proximal end of the active zone and enter the frozen zone, they differentiate into cartilage and their spatiotemporal pattern becomes fixed. The length of the dorsoventral axis is collapsed to zero in this simplified model. PD: proximodistal; AP: anteroposterior.

A relatively simple version of the system shown in Fig. 1 was formulated (making the assumption that TGF- β directly induces FGFR2c and the lateral inhibitor acts directly to suppress fibronectin gene expression) in a set of eight coupled nonlinear partial differential equations representing the influences of the various mentioned genes on one another via their products, and on the various cell types, as well as the diffusion of

released signal molecules (morphogens) such as TGF- β and FGF through the ECM, and growth of the different tissue domains (see Hentschel et al., 2004). In this initial model, the limb bud was represented as a two- rather than three-dimensional structure (the dorsoventral thickness of the limb was collapsed to zero), with a rectangular rather than curvilinear contour. Moreover, cell density was represented as a continuous variable

rather than a collection of discrete space-filling objects that could become packed to different extents (Hentschel et al., 2004).

As simplified as this system is relative to the cellular and molecular interactions in the actual developing limb, computer simulation of a set of equations of this complexity to determine the morphogen and condensation patterns it can produce is not feasible. It is possible, however, to use mathematical techniques to determine whether physically realistic solutions to these equations exist which correspond to nonuniform patterns of cell density. We have confirmed that this is indeed the case (Alber et al., 2005b).

We also explored the patterns formed in a system of four equations, which were derived from the original eight by applying some biologically motivated estimates of timescales of various processes modeled by the system (Hentschel et al., 2004):

$$\begin{aligned} \nabla^2 c &= \kappa^2 c, \\ \partial c_a / \partial t &= [J_a^1 \alpha(c, c_a) + J_a(c_a) \beta(c, c_a)]R \\ &\quad + D_a \nabla^2 c_a - k_{c_i} c_a \\ \partial c_i / \partial t &= D_i \nabla^2 c_i - k_{a c_i} c_a + J_i(c, c_a) \beta(c, c_a)R \\ \partial R / \partial t &= [D_{\text{cell}} - (\lambda + \lambda_2 \gamma(c, c_a))R] \nabla^2 R \\ &\quad - \lambda_2 \partial \gamma / \partial c_a R^2 \nabla^2 c_a - \lambda_2 \partial \gamma / \partial c R^2 \nabla^2 c \\ &\quad + rR(R_{\text{eq}} - R) - k_{23} \gamma(c, c_a)R. \end{aligned}$$

In these equations, c , c_a , c_i , and R represent, respectively, the position- and time-dependent concentrations of FGF, TGF- β and the hypothesized inhibitor, and the density of mobile cells. The J 's are reaction terms governing the production, and the D 's are diffusion constants governing the transport, of these molecules, while ∇^2 and $\partial / \partial t$ are differential operators over space and time. With the incorporation of the cell division rate r , the fractions α , β , γ of the total mobile cell density R in the categories R_1 (FGFR1-expressing), R_2 (early FGFR2-expressing) and R'_2 (FGFR2-expressing, fibronectin-secreting), and constants κ and the k 's, this set of equations defines a streamlined network representing the core mechanism for spatiotemporally regulated chondrogenesis.

Using mathematical simplifications based on biological expectations concerning the behavior of the functions involved, we were able to simulate the system under realistic growth dynamics for

the various (apical, active, frozen) domains (Hentschel et al., 2004). The pattern of "bones" that this system predicts is decidedly limb-like (given the constraints noted above) (Fig. 2). It is particularly notable that the number of parallel elements increased in number in a proximodistal order as development progressed, corresponding to the typical developmental sequence in tetrapods. This is a robust property of the core mechanism operating in an elongated tissue mass in which the unorganized region gets progressively narrower (see also Newman and Frisch, '79). Significantly, the system exhibited somewhat different patterns when different initial values of morphogens were used (Fig. 2). The generative system for the limbs in modern-day organisms almost certainly lacks this maladaptive property.

While joints, i.e., discontinuities between the stylopod-, zeugopod- and autopod-like domains, or within the digit-like elements, do not appear in these particular simulations, slight changes in the parameter values in the core mechanism can produce such gaps. As noted in relation to an earlier version of the model (Newman and Frisch, '79), periodic changes in the proximodistal length of the apical zone (Summerbell, '76) can lead to interruptions in the pattern. Another factor that may contribute to the formation of joints is the suggested existence of an underlying developmental clock in the chondrogenic response of limb mesenchymal cells to TGF- β (Leonard et al., '91).

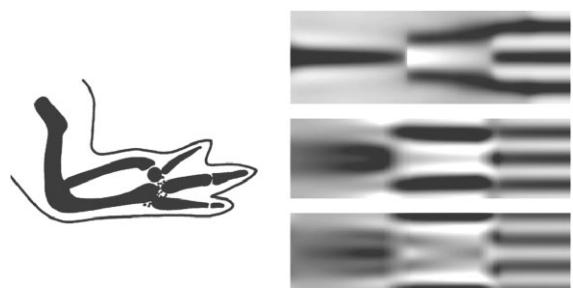


Fig. 2. Simulations of limb skeletal development in the model of Hentschel et al. (2004). Typical examples of skeletal structures generated by the model, using different initial conditions, are shown in comparison to a longitudinal section of the skeleton of the chicken limb at 7 days of development. The distribution of cartilage is shown in a continuous grayscale in the simulation panels, with black representing highest cartilage density. Skeletal form in the model is dependent on parameter values and time-dependent changes in the active zone (which were the same for all three simulations shown) and initial conditions (which differed, yielding slightly different patterns). See Hentschel et al. (2004) for details.

Such temporal oscillations are readily generated by reaction–diffusion dynamics like that of the core mechanism (Boissonade et al., '94).

Further reductions in complexity of the system of Hentschel et al. (2004) to only two equations, describing simply the interaction and diffusion of the chemical activator and inhibitor of condensation, permitted it to be analyzed in a more realistic three-dimensional computational framework (Chaturvedi et al., 2005; Cickovski et al., 2005). Here the cells were represented as extended, discrete objects that reacted to the morphogen gradients generated by two different simplifications of the system of Hentschel et al. (2004), according to simple rules of their own (i.e., as “cellular automata”; Merks and Glazier, 2005). In the version of Chaturvedi et al. (2005), all four cell types of the model of Hentschel et al. (2004) (apical zone mesenchyme, early active zone mesenchyme, late, fibronectin-producing active zone mesenchyme, frozen zone chondrocytes) were retained, but the zonal organization was imposed *a priori* rather than via a proximodistal FGF gradient. In the version of Cickovski et al. (2005), there are only “condensing” and “noncondensing” cells (determined by levels of TGF- β) but the

zonal arrangement arises in a more realistic fashion by distance from a distal source of FGF. In both versions, cells responded to elevated levels of activator (i.e., TGF- β) by producing additional activator and upregulating adhesive matrix (i.e., fibronectin) in their local environment. These simulations also yielded limb-like skeletal patterns (Chaturvedi et al., 2005; Cickovski et al., 2005) (Fig. 3).

Many of the molecules known to be involved in limb development are not part of the core mechanism. For example, the products of genes such as *Dlx*, *Lmx*, *Hox*, *R-fng*, *Shh*, and *Wnt*, mentioned above as involved in limb bud shaping, are not incorporated into the core mechanism, although the members of the one gene family of this class, that is, the *Fgf*'s, are modulated in their spatiotemporal expression by interaction with some of the others (e.g., *Shh*, *Wnt*; reviewed in Tickle, 2003). Furthermore, some of the omitted molecules (*Shh*, various *Hox* proteins) have changing nonuniform distributions across the limb bud, the dynamics of which are not part of the core mechanism, and influence digit identity, a property the core mechanism also does not purport to explain. This suggests that in a more elaborate

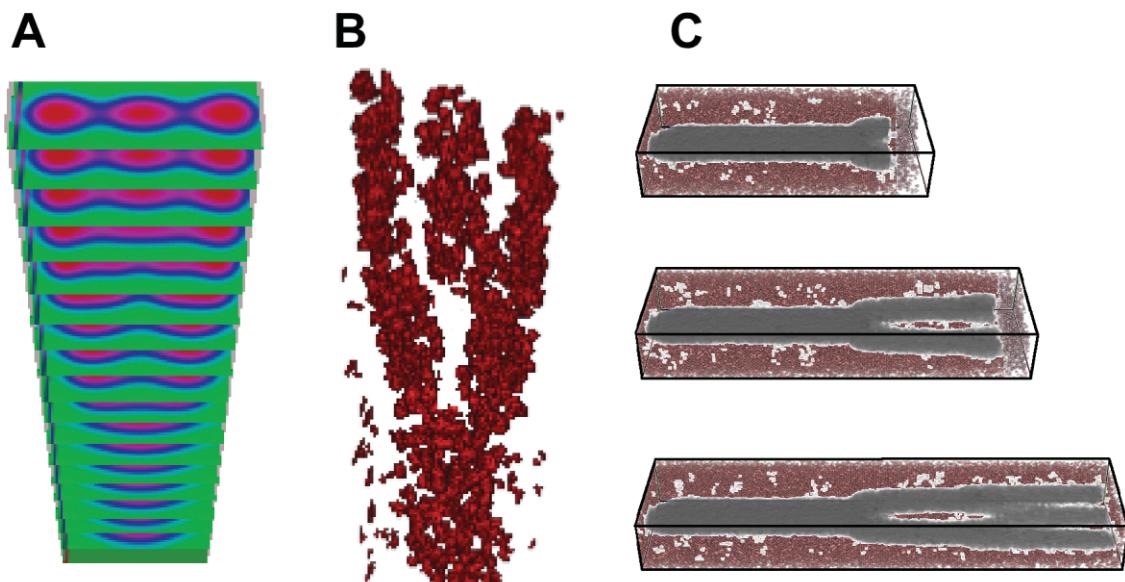


Fig. 3. Three-dimensional simulations based on different approximations of the model of Hentschel et al. (2004) in which simplified versions of the reaction–diffusion system for the activator and inhibitor of chondrogenesis were used, and the cells were modeled as extended, three-dimensional autonomous objects capable of responding to the gradients according to simple rules of their own. (A) Time series of the concentration of the diffusible morphogen TGF- β in cross-sections of the active zone, with time increasing in the upward direction (Chaturvedi et al., 2005). (B) Final pattern of cell condensation obtained by Chaturvedi et al. (2005) with the same parameter set as in (A). (C) Patterns of cell condensation obtained by Cickovski et al. (2005), using a different simplification of the model of Hentschel et al. (2004) from that in (A) and (B), at three successive stages of simulated development. See cited articles for details. (C) Courtesy of T. Cickovski and J.A. Izaguirre, University of Notre Dame.

model that includes both limb bud shaping and skeletogenesis, processes that in simulations of the core mechanism by itself occur uniformly within the active zone (e.g., the emergence of the digits in the autopod) would instead occur at different rates along the anteroposterior axis, in conformity with experimental findings (e.g., Morgan and Tabin '94).

Also not included in the core patterning mechanism are the BMPs, members of the TGF- β superfamily that act downstream from TGF- β itself to promote chondrogenesis (Roark and Greer, '94; Chimal-Monroy et al., 2003), influence digit identity (Dahn and Fallon, 2000), promote interdigital apoptosis (Zuzarte-Luis et al., 2004), as well as participating in the limb-shaping circuitry mentioned above (Wang et al., 2004). Similarly, other members of this superfamily, the GDF proteins, which are variously expressed in specific skeletal elements and in joints (Settle et al., 2003), are also omitted. This reflects our hypothesis that many of the molecular players in this developmental process were acquired later in evolution in the course of integration and autonomization of the limb bauplan (Müller and Newman ('99) and below).

Computational models based on the core mechanism of Hentschel et al. (2004), but in which all the cells are simultaneously subject to self-organizing interactions, generate synchronously appearing nodules in simulations (Zeng et al., 2003; Kiskowski et al., 2004), similar to randomized limb cells cultured in the absence of ectoderm. In contrast, the ability of the two-dimensional model of Hentschel et al. (2004) and its three-dimensional variants (Chaturvedi et al., 2005; Cickovski et al., 2005) to produce, over time, proximodistally increasing numbers of elements depended entirely on the apical/active zone distinction. In the versions of Hentschel et al. (2004) and Cickovski et al. (2005), the zonal organization was established by an FGF gradient whose source is in the AER, in agreement with experiment. The ratios of length to width (and in the three-dimensional versions, to depth) (aspect ratios) in the active zone were also determinants in the order of appearance of rows of specific numbers, a feature seen in an earlier prototype of these models (Newman and Frisch, '79). These models would predict that the proximodistal order could be reversed by alterations of the active zone aspect ratios, a hypothesis that could be tested by comparative measurements in urodele and anuran amphibians (Franssen et al., 2005).

Interestingly, small variations in values of parameters (rate constants, diffusion coefficients) in each of these computational models could lead to large changes in morphology similar to those seen with certain genetic variations—fused elements, polydactyly, etc. This capacity to undergo qualitative morphological change as a result of quantitative changes in system parameters, seen in these pathological cases as well as in the modeling of the normal developmental transition from stylopod (single upper limb bone) to zeugopod (double mid-limb bones) to autopod, is a generic property of mechanisms based on reaction-diffusion instabilities (Meinhardt and Gierer, 2000; Miura and Maini, 2004a). Most significantly with regard to the question of morphological innovation in the evolution of the limb skeleton is the capacity of such mechanisms, including the skeletogenic core mechanism hypothesized here, to generate new individual elements and, in a context of changed limb bud growth and shaping such as could be brought on by altered domains of Hox gene expression, key novelties like the autopod.

THE ROLE OF BIOMECHANICS

Once the initial arrangement of precartilage condensations is set during early development by pattern-forming processes such as those described in the previous section, chondrogenesis occurs by a sequence of steps that, according to the categories of gene regulatory network described in the introductory section (see also Salazar-Ciudad et al., 2001a), are more hierarchical than emergent (Chimal-Monroy et al., 2003). This does not mean, however, that it is no longer subject to epigenetic influences. Mechanical factors are ever-present and take on specific relevance when it is recognized that embryos are motile and skeletogenesis is sensitive to mechanical stresses. Indeed, the absence of adequate biomechanical stimuli during embryonic and postembryonic development leads to skeletal malformations (see below) and functional neurological deficits (Bos et al., 2001).

Motor activity begins very early in development. In the chick embryo the first muscle contractions occur on the third day of incubation and follow a characteristic pattern of increasing and decreasing activity (Wu KC et al., 2001). The activity of the embryo, in turn, is influenced by environmental (e.g., behavioral, physical, and chemical) factors (overview in Romanoff, '60). Changes in these parameters can strongly affect the motility patterns and hence skeletogenesis. A rise in the

intensity of ambient light, for instance, leads to an increase in embryonic motility (Wu KC et al., 2001), whereas the decrease of temperature diminishes motor activity (Oppenheim and Levin, '75; Nechaeva and Turpaev, '91).

Both phases of chondrogenesis—cartilage cell differentiation and cartilage matrix production—include mechano-sensitive interactions among several genes and gene products. Central in these interactions are *Sox9* and *IL-1 β* , respectively a transcriptional activator and a transcriptional repressor of type II collagen. *IL-1 β* downregulates the glycosaminoglycan–aggrecan system, whereas it upregulates the cartilage-degrading matrix metalloproteases. Static compression of three-dimensional culture systems containing chondrocytes both upregulates *Sox9* and downregulates *IL-1 β* , leading to an increase of glycosaminoglycan synthesis and of type II collagen mRNA, whereas the metalloproteinase production is diminished. Together, these effects result in an overall two–three-fold increase of type II collagen and aggrecan in compressed chondrogenic cells *in vitro* (Takahashi et al., '98) (Fig. 4). Tensile forces, in contrast, act on the BMP system (Sato et al., '99; Ikegami et al., 2001), affecting the arrangement and proliferation of chondrogenic cells (Bard, '90). In the mechano-transduction pathway involved in chondrocyte proliferation, Indian hedgehog is one of the key signaling molecules acting upstream of BMP (Wu Q et al., 2001).

Through these and other molecular mechanisms (Chiquet, '99; Nomura and Takano-Yamamoto, 2000; Chiquet et al., 2004), movement-dependent mechanical stimulation is responsible for cartilage and bone remodeling during embryogenesis and postembryonic life (Carter et al., '87). A reduction of embryonic movement results in reverse effects. Reduced pressure regimes lead to a drop in type II

collagen, aggrecan, and glycosaminoglycan expression and a consequential reduction of cartilage matrix synthesis. The arrest of embryonic movement by pharmacological paralysis strongly affects long bone growth and joint development (Hall and Herring, '90; Bertram et al., '97). These results highlight the modulatory role of embryonic movement in skeletogenesis and indicate that much of the consolidation, individuation, and refinement of the basic skeletal pattern laid down by locally acting core pattern-forming mechanisms is also subject to an epigenetic physical feedback influence from the motile developing organism.

Although mechano-sensitive regulation of chondrogenesis has a primary role in the shaping of the skeletal elements, these mechanisms are also involved in secondary modifications of the limb pattern that are realized through the loss or addition of elements. Such changes often take place during generation of the adult limb pattern. While deletions occur through fusions of the skeletal primordia during different stages of the skeletogenic process (Müller, '91), the addition of new elements during later stages is often movement dependent. Paralysis leads to the reduction and aplasia of such “sesamoid cartilages” (Wu, '94; Müller, 2003). The de novo formation of cartilage elements can also be experimentally elicited in fetal and postfetal connective tissues when certain pressure regimes are applied (Vogel and Koob, '89; Tagil and Aspenberg, '99). In the course of normal development, the added cartilages become incorporated into one of the existing bones or are elaborated into major independent elements.

There are several evolutionary implications of the mechano-sensitivity of skeletogenesis. On the one hand, it provides an epigenetic link between the environment and development, since chemical or physical changes of environmental parameters can strongly affect embryonic motility and hence the plasticity of skeletogenesis. Natural selection can exploit the variation that results from such environmentally dependent phenotypic plasticity (West-Eberhard (2003) and this volume). But as we noted for the proposed core mechanism of limb patterning, most relevant to the problem of morphological innovation is the potential source of skeletal novelty inherent to a mechanism that has the capacity to form de novo elements. Like the core mechanism, these biomechanical effects can elicit morphological novelty as a “saltatory” byproduct of continuous natural selection. Continuous selection on size, shape, or proportions of

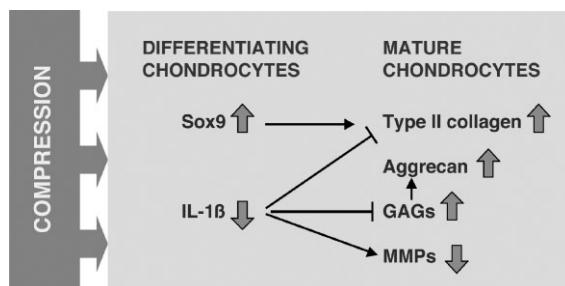


Fig. 4. Molecular mechanisms involved in compression-regulated chondrogenesis. IL: interleukin; GAG: glycosaminoglycan; MMP: matrix metalloproteases. From Müller (2003), after Takahashi et al. (1998).

body parts, such as the relative size of skeletal elements in the limb, will alter the biomechanical conditions in their development and result in new pressure and tension loads. Tissues that have a chondrogenic capacity will respond by producing cartilage matrix and begin cartilage cell differentiation at certain threshold levels of mechanical load. A new skeletal element can result. Skeletal novelties that arise from such a mechanism could remain selectively neutral for long periods of time, since their existence in every generation merely depends on the maintenance of the same biomechanical conditions. But eventually such structures can become subject to selection and, through canalizing and stabilizing evolution (Waddington, '42; Schmalhausen, '49), gradually become integrated into the developmental repertoire (Müller and Newman, '99; Newman and Müller, 2000).

Many examples of skeletal novelties that have arisen from *de novo* "sesamoids" are known in vertebrate evolution. These include the famous "thumb" and the "seventh digit" of the giant panda and the falciform bone in the mole's hand. A case studied in more detail is the origin of the fibular crest on the tibia of theropod dinosaurs and birds (Müller and Streicher, '89). Here the progressive reduction of the fibula, a characteristic trend in the evolution of bipedal locomotion in reptiles and birds (Streicher and Müller, '92), must have led to an increasing mechanical load on the connective tissue between the tibia and the fibula during embryonic movement. At a certain threshold, point, a stress-induced cartilage arose and can still be observed as a transient structure in extant avian embryos, in which it fails to form in the absence of embryonic movement. The cartilage element may have existed long before its ossification and integration into the tibia in theropods, serving as the probable source of the sudden phylogenetic appearance of the prominent fibular crest in that group of dinosaurs. Several other movement-dependent embryonic cartilages underlie osseous innovations in the avian limb skeleton (Müller, 2003).

CONCLUSIONS

The existence of epigenetic processes of self-organization of mesenchymal condensation patterning and biomechanical modulation of chondrogenesis must inform both developmental and evolutionary hypotheses about the vertebrate limb. In particular, there is no need for a "universal" or even limb-specific system of posi-

tional information for cells to interpret during development. Rather, species-characteristic limb skeletons develop, in our view, by means of modulatory and fine-tuning interactions acting on skeletal templates that are in part self-organized and in part "autonomized" by accumulation of reinforcing genetic pathways over the course of evolution (see below). The protean nature of the core skeletogenic mechanism described here creates an entirely new context for inferences concerning whether limb skeletons of closely related species are primitive or derived based on whether they have more or fewer elements (e.g., Shubin, 2002) and concerning the evolutionary implications of variations in the order of development of digits (Shubin and Alberch, '86; Blanco and Alberch, '92; Hinchliffe and Vorobyeva, '99; Wagner and Gauthier, '99; reviewed in Hinchliffe, 2002).

The new model of origination and innovation of limb patterning we have proposed herein consists of a self-organizational reaction-diffusion-type system of core chondrogenic processes which are modulated by several epigenetic parameters. We do not have enough information at present to say that the hypothesized core processes and related "bare-bones" mechanism were indeed the originating mechanism by which limbs first appeared in vertebrate ancestors approximately 400 million years ago. What our mathematical analyses and computational simulations tell us, however, is that the components and interactions in the developing limb captured by our model, or any biologically equivalent version of these components and interactions, constitute a mechanistic basis for the self-organization of a rough skeletal pattern. The patterns formed by such processes, without additional constraints, are somewhat unreliable, undergoing relatively large-scale changes with variations in initial conditions or parameters. Either type of alteration could produce missing elements, fused elements, extra elements, and so on.

Autonomization (i.e., the establishment of developmental and evolutionary independence from the initiating generative mechanisms; Müller and Newman, '99) of the skeletal pattern and its individual elements may be achieved by natural selection in which there is a premium on stabilizing the morphological outcome (Waddington, '42; Schmalhausen, '49). This involves employing molecular and biomechanical cues beyond the core set to reinforce the generation of the pattern by parallel means: secreted factors or cell-surface components that regulate growth and shaping of

the mesoblast (e.g., Shh, R-fng), transcription factors that control responsiveness to morphogens (e.g., Hox proteins; Gli3; dHand; McFadden et al., 2002), and anabolic tensile forces arising from tissue contraction and embryonic movement acting on forming cartilage elements. Eventually, the underlying circuitry by which the pattern or its elements is achieved may change in a fashion that dissociates them from the originating dependence on self-organizing processes (Salazar-Ciudad et al., 2001a,b).

In terms of the mechanisms discussed above, we suggest the following scenario for the origination and subsequent refinement of the vertebrate limb:

- (i) FGF-dependent cues for outgrowth of fin-fold mesenchymal masses were accentuated as a result of mutationally or environmentally induced changes in the activation spectrum of one or more growth- and shape-influencing genes (van Eeden et al., '96) in an ancestral sarcopterygian fish.
- (ii) The larger size and novel shape of the resulting limb buds provided an opportunity for pre-existing determinants of discrete mesenchymal condensations—e.g., the positively autoregulatory factor TGF- β and a factor limiting its activity—to self-organize several tiers of skeletal rods and nodules in temporal succession rather than just one or two.
- (iii) The temporal separation of the self-organization of the different tiers permitted natural selection of variants in which timing of expression of shaping factors (e.g., Shh) and of factors affecting tissue responsivity to the “core morphogens” (e.g., Hox proteins) determined the numbers and sizes of the elements in the different tiers in a relatively independent fashion.
- (iv) Eventually, specific element identities arose by “autonomizing evolution.” Based initially on differences in Hox and other transcription factor (Barna et al., 2005) combinations at sites at which the various elements arise, leading to distinct responsivities to commonly present morphogens (Dahn and Fallon, 2000), such autonomization may eventually be reflected in element-specific Hox gene regulatory circuits (Herault et al., '97; Spitz et al., 2003) and utilization of distinct morphogen variants by different elements (Settle et al., 2003).
- (v) Over time, the increasingly autonomized skeleton continues to be reshaped and embellished by novelties that arise from the

continued responsivity of chondrogenic tissues to the biomechanical environment.

Although these points are interdependent, each of them is subject to separate tests, refinement, and revision. We emphasize, however, that any model for the origination and development of the vertebrate limb must address the central requirement of evolutionary developmental biology: accounting at the same time for the continued generative stability of a structure, or organized complex of structures (homology), and for the novelties that deviate from it (Müller, 2005). We suggest that the epigenetic framework presented here, drawing on the self-organizing properties and biomechanical responsivity of limb bud mesenchymal tissue, and combining it with the reasonable assumption that the genetic circuitry involved in limb development in contemporary vertebrates was recruited in stages over the course of evolution, accomplishes these goals more successfully than informational coordinate system-based models.

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