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REVIEW

Activator-Inhibitor Dynamics of Vertebrate Limb Pattern Formation

Stuart A. Newman* and Ramray Bhat

The development of the vertebrate limb depends on an interplay of cellular differentiation, pattern formation, and tissue morphogenesis on multiple spatial and temporal scales. While numerous gene products have been described that participate in, and influence, the generation of the limb skeletal pattern, an understanding of the most salient feature of the developing limb—its quasiperiodic arrangement of bones, requires additional organizational principles. We review several such principles, drawing on concepts of physics and chemical dynamics along with molecular genetics and cell biology. First, a ''core mechanism'' for precartilage mesenchymal condensation is described, based on positive autoregulation of the morphogen transforming growth factor (TGF)-β, induction of the extracellular matrix (ECM) protein fibronectin, and focal accumulation of cells via haptotaxis. This core mechanism is shown to be part of a local autoactivation-lateral inhibition (LALI) system that ensures that the condensations will be regularly spaced. Next, a ''barebones'' model for limb development is described in which the LALI-core mechanism is placed in a growing geometric framework with prediffer-entiated ''apical,'' differentiating ''active,'' and irreversibly differentiated ''frozen'' zones defined by distance from an apical source of a fibroblast growth factor (FGF)-type morphogen. This model is shown to account for classic features of the developing limb, including the proximodistal (PD) emergence over time of increasing numbers of bones. We review earlier and recent work suggesting that the inhibitory component of the LALI system for condensation may not be a diffusible morphogen, and propose an alternative mechanism for lateral inhibition, based on synchronization of oscillations of a Hes mediator of the Notch signaling pathway. Finally, we discuss how viewing development as an interplay between molecular-genetic and dynamic physical processes can provide new insight into the origin of congenital anomalies. **Birth Defects Research (Part C) 81:305–319, 2007.** © 2008 Wiley-Liss, Inc.

INTRODUCTION

Defects of the limb skeleton are among the most frequent human congenital anomalies. Mutations and teratogens can dramatically affect the structure of the limb skeleton without otherwise impairing survival, reproduction, and other bodily functions. This has led to the presence of a wide-ranging set of limb variations throughout the human population as well as providing the opportunity, using experimental systems, to explore the bases of normal and abnormal limb formation. The limb skeleton, an array of jointed bone or cartilage elements, has a stereotypical pattern that (as Charles Darwin noted) is only modestly altered by adaptations for functions as varied as walking, swimming, flying, and grasping (Darwin, 1859). The transmission and molecular genetics of limb variations have been extensively studied in humans and mice, while the developing appendages in embryos of egg-laying species have lent themselves to experimental analysis by surgical manipulation. In fishes and amphibians the paired limbs, or related structures, exist with variant anatomical characteristics and regenerative properties, enabling informative comparative studies. Finally, limb bud mesenchymal cells from avian and mammalian species can be grown in culture, where they undergo differentiation and pattern formation, though simplified, with a time-course and on a spatial scale similar to that in the respective embryos. These features have made the limb a highly favorable system for studying the generation of multicellular form.

Over the past three decades the protein products of scores of genes have been identified as participating in patterning of the limb skeleton (reviewed in Tickle, 2003; Newman and Müller, 2005). Genes and the interactive networks in which they participate, however, are only part of the story in the generation of biological structures (Nijhout, 1990; Newman and Comper, 1990; Newman,

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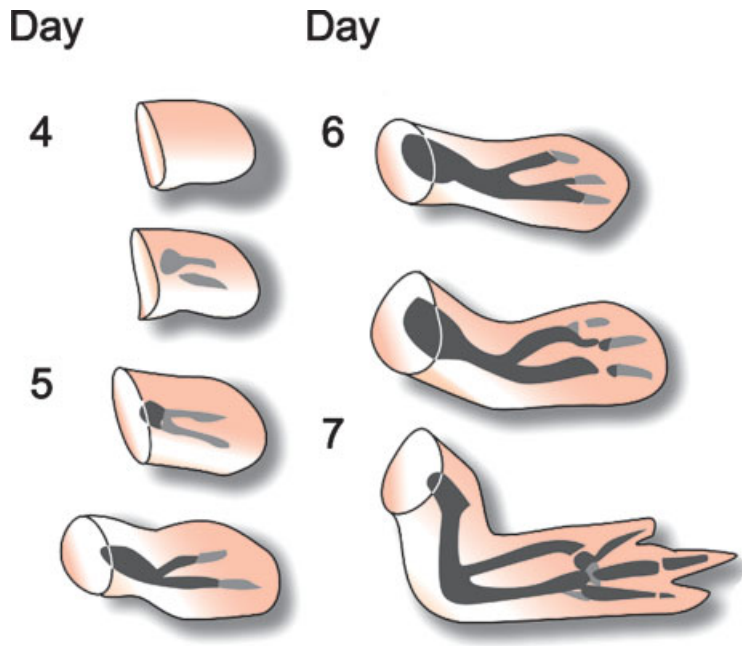

Figure 1. Progress of limb skeletal development in a chicken forelimb (wing) between four and seven days of embryogenesis. The skeletal elements emerge in a proximodistal order. Gray represents precartilage condensation and black definitive cartilage. This developmental period corresponds to human limb development between approximately 25 to 50 days of gestation. (Adapted from Newman and Frisch (1979) and Forgacs and Newman (2005)).

2002; Newman and Bhat, in press). Tissue pattern formation and morphogenesis involve switching of cells between alternative physiological states, redistribution, rearrangement and reshaping of cells and the chemical gradients that act on them (Forgacs and Newman, 2005). Each of these processes is mediated by chemical dynamics and/or the physics of condensed materials. Indeed, physics and chemical dynamics are the means by which gene expression changes tissue form (Forgacs and Newman, 2005) and the linear information of the genotype is implemented to construct a three-dimensional (3D) organismal form.

In this work, we focus on the question of whether, given what is known about the biochemical and cellular bases of limb development, physical and chemical-dynamic organizational mechanisms utilizing these ingredients can account for the general pattern of the skeleton. If so, we will have a ''universal'' mechanism of limb development, in addition to a plausible scenario for the origination of the limb from the fin-appendage of an ancient fish-like ancestor. We will also have improved our understanding of the vulnerabilities of the developing human limb to mutational and teratogenic disturbances.

SKELETAL PATTERN FORMATION: THE CENTRALITY OF PERIODICITY

The limb buds of vertebrates protrude 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* (PD) fashion: that is, the cartilaginous elements destined to be 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 (stylopod) of the upper arm is generated first, followed by the radius and ulna (zeugopod) of the mid-arm, the wrist bones, and finally the digits (autopod) (Fig. 1) (Saunders, 1948; reviewed in Newman, 1988). Urodele salamanders appear to be an exception to this PD progression (Franssen et al., 2005). Cartilage is mostly replaced by bone in species with bony skeletons.

Much work on limb skeletal pattern formation over the past two decades has occurred within the ''positional information'' paradigm (Wolpert, 1971, 1989). This view assumes that limb skeletal elements are established by cells autonomously responding to a system of molecular coordinates along each of three relatively independent axes and thereby assuming precise roles in the developing structure. The anteroposterior (AP) coordinate has been proposed to be concentration values along gradients of retinoic acid or its receptors (Tickle et al., 1985; Dollé et al., 1989), *Hox* gene products (Duboule, 1992), or Sonic hedgehog (Riddle et al., 1993), but each of these suggestions has encountered disconfirming evidence (see Wanek et al. (1991) for retinoic acid; Luo et al. (1995) for retinoid receptors; Davis et al. (1995) for Hox gene products; Kraus et al. (2001) for Sonic hedgehog). The PD coordinate has been proposed to be determined by the length of time cells spend in an apical ''progress zone'' comprising approximately 300 μm (Summerbell et al., 1973), or specified early within the apical zone by an unknown mechanism (Dudley et al., 2002). There is no evidence, however, for an internal biochemical clock that stops when cells move out of the progress zone, nor have spatially determined bands of gene expression been identified within the progress zone that are related to skeletal element identity as postulated by the early specification model (Dudley

et al., 2002; Tabin and Wolpert, 2007).

The positional information notion implicitly assumes that the identities of cells in the developing limb are specified along separable geometric coordinate axes. But, in fact, the three limb axes themselves are not independently specified (Bowen et al., 1989), and factors such as the *Hox* gene products, which clearly influence the details of the skeletal pattern, do so in a fashion that cannot be accounted for by the simple combinatorial codes implied by the positional information framework (Graham, 1994; Newman, 1996).

Most importantly, the positional information model, devised to represent a recurrently employed set of molecular signals that could produce the pattern of a French flag as readily as the whorls of a fingerprint, if only the organism's genome were capable of providing the proper readout (Wolpert, 1971), is indifferent to the fact that the vertebrate limb, the segmental plate, and epithelial appendages such as bristles, hairs and feathers, to take a few examples, have quasiperiodic patterns. In the limb the periodicity is twofold—there is a segmental pattern along the PD axis, where successive elements are separated by joints, and structural repetition across the zeugopod and autopod, where parallel skeletal elements are separated by intervening soft tissue, or by free spaces created by apoptotic removal.

While it is clear that position-related identities are indeed imprinted on limb skeletal elements by gradients of molecules, including those of retinoic acid, bone morphogenic protein (BMP)- and Wnt-family morphogens, and homeodomain proteins including those of the Hox, Tbx, Prx, and Meis families (reviewed in Tickle, 2003; Tickle, 2006), the roles of such determinants vary from species to species and from one region of a developing limb to another. They can most productively be treated as second-order fine-tuning effects acting on the stripe- and spot-like elements of developing cartilage as the skeleton takes form (Tickle, 2006; Newman, 2007). Indeed, attempts to discern positional informational grids in these patterns of gene expression independently of an underlying generator of spatial periodicities of precartilage primordia is increasingly reminiscent of the pre-Copernican program of employing epicycles to account for the retrograde motion of the planets around the Earth.

A CORE GENETIC NETWORK FOR PRECARTILAGE CONDENSATION

The limb mesoblast consists of mesenchymal cells distributed uniformly within a hyaluronan rich extracellular matrix (ECM). Before these cells differentiate into chondrocytes (cartilage cells), they transiently condense into tight aggregates at discrete sites where the cartilaginous elements will ultimately form. Precartilage condensations form when the ECM changes locally in composition, becoming richer in glycoproteins such as fibronectin, thus trapping (Frenz et al., 1989; Cui, 2005) the cells and modifying their movement. These aggregations are further consolidated through cell-cell adhesive interactions mediated by cell-surface attachment molecules (CAMs), such as N-CAM (Widelitz et al., 1993), N-cadherin (Oberlender and Tuan, 1994), and possibly cadherin-11 (Luo et al., 2005) (reviewed in Hall and Miyake, 1995, 2000; Forgacs and Newman, 2005).

Because all the precartilage cells of the limb mesoblast are capable of producing fibronectin and CAMs but only those at sites destined to form skeletal elements do so, there clearly must be communication among the cells to divide the labor in this respect. This is mediated in part by secreted, diffusible factors of the transforming growth factor (TGF)-β family of growth factors, which promote the production of fibronectin (Leonard et al., 1991) and cadherins (Tsonis et al., 1994). Limb bud mesenchyme also shares with many other connective tissues (Van Obberghen-Schilling et al., 1988) the autoregulatory capability of producing more TGF-β upon stimulation with this factor (Miura and Shiota, 2000a).

The limb bud ectoderm is a source of fibroblast growth factors (FGFs) (Martin, 1998). Although the entire limb ectoderm produces FGFs, the particular mixture produced by the apical ectodermal ridge (AER), a narrow band of specialized ectodermal cells running in the AP direction along the tip of the growing limb bud in birds and mammals, is essential to limb outgrowth and pattern formation. FGF8 is the most important of these (Mariani and Martin, 2003). The AER affects cell survival (Dudley et al., 2002) and keeps the precondensed mesenchyme of the apical zone in a labile state (Kosher et al., 1979). Its removal leads to terminal truncations of the skeleton (Saunders, 1948).

The FGFs produced by the ectoderm affect the developing limb tissues through three distinct FGF receptors. The cells of the apical zone express FGF receptor 1 (FGFR1) (Peters et al., 1992; Szebenyi et al., 1995). Signaling through this receptor presumably mediates the suppressive effect of the AER in this region of the developing limb. As the chicken limb elongates, a second, ''active,'' zone is established at a distance of approximately 0.3 mm from the AER, where cells begin to condense. In the active zone FGFR1 is downregulated and cells that express FGFR2 appear at the sites of incipient condensation (Peters et al., 1992; Szebenyi et al., 1995; Ornitz and Marie, 2002; Moftah et al., 2002). Activation of these FGFR2-expressing cells by FGFs induces a laterally acting (that is, peripheral to the condensations), inhibitory effect which suppresses cartilage differentiation (Moftah et al., 2002). Recent work suggests that Notch signaling also plays a part in this lateral inhibitory effect (Fujimaki et al., 2006).

The roles of TGF-β, the putative lateral inhibitor of its effects, and fibronectin in mediating precarti-

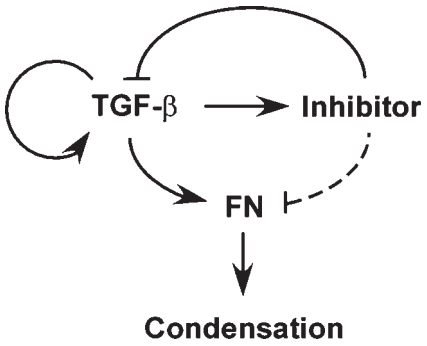

Figure 2. Core network of cell-gene product interactions leading to limb precartilage mesenchymal condensation. The molecular identity of the lateral inhibitor of condensation is unknown, but depends on interaction of ectodermal FGFs with mesenchymal FGF receptor 2 (Moftah et al., 2002) as well as the Notch signaling pathway (Fujimaki et al., 2006). This inhibitor may act at the level of TGF-β synthesis or activity (solid inhibitory vector), fibronectin synthesis (dashed inhibitory vector), or at some earlier stage. (Adapted from Kiskowski et al. (2004)).

lage condensation in the limb bud mesenchyme can be schematized in the form of a ''core'' cell-molecular-genetic network (Fig. 2). This network is characterized in terms of molecules that directly participate in cell-cell and cell-substratum interactions and thereby mediate the physical aspects of pattern formation (Newman and Bhat, in press). Transcription factors and coregulators that control changes in the levels of these multicellular ''interaction molecules,'' as well as transducers of intracellular signals to and from the nucleus, are of course essential components of any complete description of such a developmental network. In the schematic models described below, however, we treat these components implicitly, as complex response functions.

PATTERNING LIMB BUD MESENCHYME BY A ''LOCAL AUTOACTIVATION–LATERAL INHIBITION'' SYSTEM

Reaction-Diffusion and Reactor-Diffusion Systems

Reaction-diffusion systems are networks of molecular species in which positive and negative feedback in the molecules' production and consumption, and disparate diffusion rates, lead to a stable configuration in which the chemical composition is nonuniform, rather than uniform, over a spatial domain. These systems have attracted interest as biological pattern-forming mechanisms ever since Turing (1952) proposed them as the ''chemical basis of morphogenesis'' more than half a century ago.

For purely chemical systems, where Turing-type mechanisms have been unambiguously demonstrated (Castets et al., 1990; Ouyang and Swinney, 1991), chemical reactions are the sources and sinks of the molecular components, and diffusion is the classic, Brownian motion-based physical process. In the case of developing tissues, however, ''reaction'' involves production and consumption of molecules by cells, and is an elaborate, multistep processes. Transport through tissues, moreover, while capable of generating molecular gradients that attenuate with distance from the source, is typically a more complex process than simple molecular diffusion (Kruse et al., 2004; Lander, 2007). For this reason, the term ''reactor-diffusion'' has been suggested as being more appropriate than reaction-diffusion for applications of the Turing mechanism to biological development (Hentschel et al., 2004).

Reactor-diffusion models have been gaining prominence in many areas of developmental biology (reviewed in Forgacs and Newman, 2005; Maini et al., 2006), including the patterning of the pigmentation of animal skin (Yamaguchi et al., 2007), feather germs (Jiang et al., 2004), hair follicles (Sick et al., 2006), teeth (Salazar-Ciudad and Jernvall, 2002), and the limb itself (Newman and Frisch, 1979; Hentschel et al., 2004; Newman et al., 2007). The Turing scheme stipulates a slowly diffusible, positively-autoregulatory activator and a more rapidly diffusible inhibitor that is induced by the activator. For certain developmental systems, there is strong evidence for the identification of both these factors with specific molecules. In other cases, however, only one of these components, usually the activator, has been thus identified.

Morphogen and Nonmorphogen Mediators of Condensation Spacing

Patterning of the limb skeleton is dependent on molecules of the TGF-β and FGF classes, which are demonstrably diffusible morphogens (Lander et al., 2002; Williams et al., 2004; Filion and Popel, 2004). As reviewed in the previous section (see also Hall and Miyake, 1995, 2000; Chimal-Monroy et al., 2003), TGF-β, which initiates a cascade of events that leads to precartilage condensation and chondrogenesis, is positively autoregulatory (van Obberghen-Schilling et al., 1988; Miura and Shiota, 2000a), and is therefore an excellent candidate for the activator in a reactor-diffusion scheme (Newman, 1988; Leonard et al., 1991; Miura and Shiota, 2000a). The molecular identity of the lateral inhibitor has been elusive, however. Lateral inhibition can be abrogated by blocking the function of FGF receptor 2 (FGFR2), which is localized in the incipient condensations (Moftah et al., 2002). While this can be taken to imply that the normal function of FGFR2 is to mediate the release of a diffusible lateral inhibitor of chondrogenesis (Moftah et al., 2002), it does not comport with other evidence that FGFR2s containing Apert syndrome mutations, which lead to expanded, fused skeletal elements (i.e., decreased lateral inhibition of chondrogenesis) in humans, are actually *hyperactivated* (Ibrahimi et al., 2001).

Activation of Notch signaling in vitro and in vivo is also associated with lateral inhibition of condensation and chondrogenesis. Blocking of the activated Notch state with the γ-secretase inhibitor N-S-phenyl-glycine-t-butyl ester (DAPT), for example, led to fusion of condensations (Fujimaki et al., 2006). But these results are difficult to reconcile with the known functionality of the Notch pathway, which

acts in a juxtacrine fashion, and would not be an obvious candidate for a long-range lateral inhibitor in a reactor-diffusion system.

Evidence for Activator-Inhibitor Dynamics in Limb Bud Mesenchyme

Despite the complexities mentioned, a reactor-diffusion-like mechanism remains the most compelling basis for the generic vertebrate limb skeletal pattern for the following reasons: 1) randomized limb mesenchymal cells with disrupted gradients of Hox proteins, Shh, etc., give rise to digit-like structures in vivo (Zwilling, 1964; Ros et al., 1994) and of discrete, regularly spaced cartilage nodular or stripe-like arrangements in vitro (Downie and Newman, 1994; Kiskowski et al., 2004; Christley et al., 2007); 2) the pattern of precartilage condensations in limb mesenchyme in vitro changes in a fashion consistent with a reactor-diffusion mechanism (and not with an alternative mechanochemical mechanism) when the density of the surrounding matrix is varied (Miura and Shiota, 2000b); 3) exogenous FGF perturbs the kinetics of precartilage condensation in vitro in a fashion consistent with a role for this factor in regulating the inhibitor in a reactor-diffusion-type model (Miura and Maini, 2004); 4) the "thick-thin" pattern of digits in the *Doublefoot* mouse mutant can be accounted for by the assumption that the normal pattern is governed by a reactor-diffusion process, the parameters of which are modified by the mutation (Miura et al., 2006); 5) simultaneous knockout of Shh and its inhibitory regulator Gli3 in mice yields limbs with numerous extra digits (Litingtung et al., 2002), suggesting a default propensity of the limb mesenchyme to generate regularly spaced repetitive elements of indefinite number. A reactor-diffusion-type mechanism is the most plausible basis for this.

Reaction-diffusion and allied systems exhibit scale dependence in that the number of repetitive pattern elements that form varies in proportion to the size of the reactive domain. This has sometimes been considered to count against such mechanisms for developmental processes which often produce the same pattern over a range of tissue sizes. But scale-dependence actually represents the biological reality in the developing limb. Experiments show, for example, that the number of digits is sensitive to the AP (thumb-to-little finger) breadth of the developing limb bud, and will increase (Cooke and Summerbell, 1981) or decrease (Alberch and Gale, 1983) over typical values if the limb is broadened or narrowed. Furthermore, the generally increasing number of parallel elements in the proximal to distal direction in most limbs may, in fact, be a function of dependence of the number of morphogen peaks on the changing spatial dimensions of limb bud domains during pattern formation (see below).

A broader category of pattern forming mechanism that includes reactor-diffusion systems as a special case, can be termed "local auto-activation-lateral inhibition" (LALI) systems or networks (see also Meinhardt and Gierer, 2000; Nijhout, 2003). In particular, LALI systems form patterns by exactly the same formal means as reactor-diffusion systems but need not utilize molecular diffusion or similar mechanisms to propagate activation or inhibitory functions. Living tissues are "excitable media" (Mikhailov, 1990) that can transmit signals by means other than material transport. Considering the limb mesenchyme as a LALI system, we can entertain alternatives to molecular diffusion for the spread of the inhibitor that are consistent with the molecular findings described above.

Expression of the Notch transcriptional mediator *hes1* is known to oscillate in time during somitogenesis (Palmeirim et al., 1997), and in the zebrafish there is large scale spatial coordination of these oscillations through the mechanism of synchronization (Giudicelli et al., 2007). Because synchronized oscillations of *Hes1* expression also appear in early-stage micromass cultures (Ramray Bhat and Stuart A. Newman, unpublished results), we have been considering LALI networks for limb pattern formation in which lateral inhibition of precartilage condensation is mediated by the propagation of coherent states of Notch activation peripheral to sites of initiation. This framework provides a way to understand the apparently paradoxical role of FGFR2 signaling in mediating both stimulation and inhibition of chondrogenesis. It also suggests a way to "globalize" the juxtacrine effect of Notch signaling.

In the next section we provide motivation for filling out the details of a limb-specific LALI network by showing that, in the geometric context of the growing limb bud, it can reproduce several of the important features of this developmental system. We will follow this by presenting a specific model for the oscillation-synchronization module in a LALI network for limb skeletal patterning.

A "BARE-BONES" SYSTEM FOR LIMB SKELETAL PATTERNING

Modeling Mesenchymal Patterning In Vitro and In Vivo

A LALI mechanism based on the core mechanism for precartilage condensation formation is fully capable, under realistic assumptions, of generating authentic patterns of condensations on a 2D plane (Kiskowski et al., 2004; Christley et al., 2007). This simulation design is a good representation of the micromass culture system, for which a great deal of experimental data is available (Mello and Tuan, 1999; DeLise et al., 2000; Newman et al., 2007). In the most sophisticated of these models the stipulated conditions included values for cell dimensions, average distances traveled, initial cell density, and diffusion coefficient of the activator. The simulations produced condensation patterns (both "spots" and "stripes") with average width and spacing statistics that were virtually indistinguishable from experimental values in

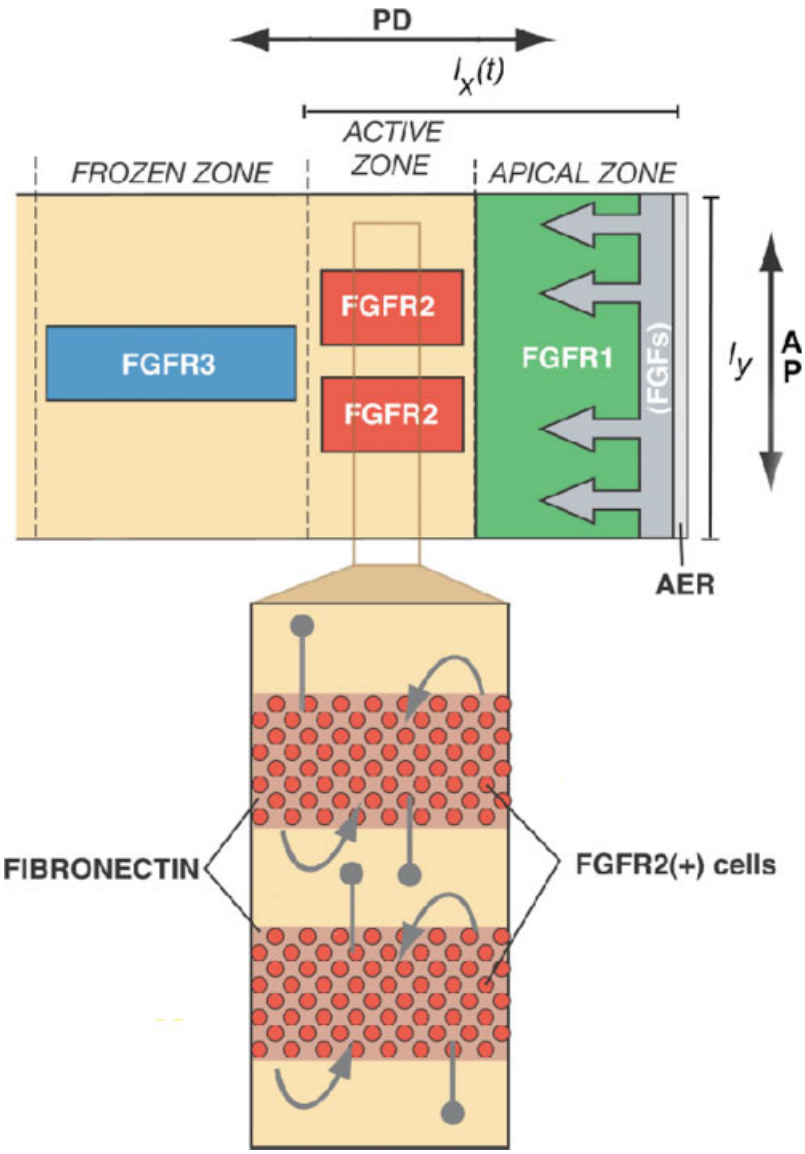

Figure 3. "Bare-bones" mechanism for vertebrate limb development. The interactions of the core mechanism are superimposed on a 2D schematic limb bud organized into zones defined by experimentally-determined expression patterns of FGF receptors 1, 2, and 3. In the "apical zone" fibronectin synthesis and precartilage condensation is suppressed by the FGFs emanating from the AER. In the "LALI zone," comprising the apical zone and an "active zone" just proximal to it, a local autoregulatory-lateral inhibitory system sets up peaks of an activator of fibronectin synthesis and precartilage condensation (i.e., TGF-β). The length of the LALI zone, l_x, is a function of time. The active zone (a detailed view of which is shown below) is the region in which cells are sufficiently far from the AER to respond to the activator and undergo cell condensation. 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, constituting a "frozen zone." The length of the AP axis, l_y, is constant during chicken wing development in which three digits are generated, but expands during limb development in mouse and human, where five digits form. The dorsoventral axis is collapsed to zero in this simplified model. PD, proximodistal; AP, anteroposterior. In lower panel, curved arrows: positively autoregulatory activator; lines ending in circles: lateral inhibitory effect. (Adapted from Hentschel et al. (2004) and Forgacs and Newman (2005)).

the in vitro system (Kiskowski et al., 2004; Christley et al., 2007).

Behavior of the simulated system under attenuated inhibitor conditions indicated that lateral inhibition was critical for realistic patterns (Kiskowski et al., 2004). And although, as mentioned, no direct evidence exists for a diffusible mediator of lateral inhibition, the putative inhibitor would have to spread about four times as fast as the activator for authentic patterns to form, given other constraints (Christley et al., 2007). This is both physically and biologically reasonable, whatever the actual process mediating lateral inhibition may turn out to be. It would be useful to determine the additional assumptions needed for this substantiated in vitro pattern-forming mechanism to mediate the generation of skeletal elements in the appropriate number and order in a 3D developing limb bud.

This is an exceedingly difficult problem, both mathematically and computationally, because formal representation and simulation of 3D multicomponent dynamical systems, of systems with moving boundaries, and of systems with nonstandard shapes, are all formidable. Based on information reviewed in earlier sections we have modeled the developing limb using several different idealizations. One such representation is a growing parallelepiped housing a continuous-variable ("continuum") set of reactor-diffusion equations describing the spatiotemporal evolution of AER-derived FGF, activating and inhibitory morphogens, fibronectin, and different cell types (defined by expression of different FGF receptors) (Hentschel et al., 2004). Here the distribution of cell types was represented by a density, an approximation to independently acting, physically discrete cells. Though inherently a 3D model, we have simulated its behavior in two dimensions, i.e., assuming a negligible thickness in the dorsoventral dimension (see Figs. 1 and 3).

We have also simulated the developing limb in 3D, using much

simplified (and therefore less biologically founded) equations for morphogen dynamics, but more realistic discrete, stochastic cell dynamics (Chaturvedi et al., 2005; Cickovski et al., 2005). In particular, the cells in these 3D representations were modeled as distinct, independently motile, deformable, biosynthetically active entities. These model cells move randomly, consistent with a requirement to minimize their energy of interaction with the patterns of fibronectin induced by the activator (TGF-β-like) morphogen. The theoretical framework that permits the simulation of cell rearrangement by differential adhesion or, as in this case, haptotaxis, is the Cellular Potts model of Glazier and Graner (1993).

Our ultimate aim, of course, is to incorporate realistic morphogen and cell dynamics into a single geometrically realistic framework (Newman et al., 2007), but at present this is beyond the limits of available methods. The family of models described (beginning with the 3D model of Newman and Frisch (1979)), however, contains a shared set of assumptions concerning the zonal organization of the developing limb. Moreover, despite the different idealizations employed, they reproduce a common set of its key features. We will focus on the properties of the continuum model of Hentschel et al. (2004) in the remainder of this section.

Zonal Organization of the Developing Limb

Reiterating the description in an earlier section, we take the limb mesoblast to be divided into an apical zone, under the condensation-suppressive effects of the AER, and an active zone consisting of cells that, based on distance from the apical source of FGF8, are susceptible to patterning interactions. The model also includes a frozen zone proximal to the active zone where differentiation of the condensed cells into chondrocytes is stipulated to be irreversible and no additional pattern changes are possible.

This organization of the limb bud mesoblast into tandemly arranged zones of: 1) unresponsive but unpatterned; 2) responsive to patterning signals; and 3) irreversibly patterned, which was carried over from an early simpler version of this model (Newman and Frisch, 1979), also has similarities to current models of somitogenesis (Pourquié, 2003; Giudicelli et al., 2007). The similarity extends also to the molecule constituting the suppressive gradient emanating from the terminal region, which is FGF8 in both cases. In the somitogenesis models the patterning signal operating in the equivalent of the active zone is a synchronized biochemical oscillation, while in the limb model it is a reactor-diffusion, or more generally, LALI system. We will revisit the possible relationships between these patterning signals below.

For representational and computational expediency the following geometric idealization was employed (Newman and Frisch, 1979; Hentschel et al., 2004): the limb bud is considered to have time-dependent PD length, $L(t)$, taken along the x-axis, and fixed lengths, l_y and l_z, along the AP (y-axis) and dorsoventral (z-axis) directions (Fig. 3). Another variable, $l_x(t)$, represents the length along the PD axis of the domain of the limb bud where activator-inhibitor interactions take place.

We refer to the model for limb development in this growing domain as "bare-bones," because while it incorporates the core mesenchymal cell-morphogen-ECM network summarized in Fig. 2, it omits spatiotemporally distributed modulatory factors such as Hox protein gradients, Shh, and so on, that cause the various skeletal elements (e.g., the radius and ulna, the different digits) to appear different from one another.

As mentioned, the division of the distal portion of the limb into an apical and active zone reflects the activity of the AER in suppressing differentiation of the mesenchyme subjacent to it (Kosher et al., 1979). The spatial relationship between the apical and active zones results from the graded distribution of FGFs, the presumed AER-produced suppressive factors. The active zone, therefore, is where the mesenchyme cells no longer experience high levels of FGFs and therefore become responsive to the activator, TGF-β, and the factors that mediate lateral inhibition. The dynamic interactions of cells and morphogens in the unpatterned portion of the limb bud give rise to spatial patterns of condensations in the active zone.

Factors Controlling the Number of Elements

LALI systems are sensitive to spatial scale. In general, the designated interactions set a characteristic "chemical wavelength," but do not determine how many peaks will form overall. The latter number is a function of the dimensions of the "LALI domain" in which the reaction-diffusion or allied process takes place. In embryonic tissues this domain is the tissue region in which inductive patterns of morphogens are established, independently of whether the cells are prepared to respond to these signals by differentiating.

There are no experimental data that directly bear on the boundaries of the LALI domain in the developing limb bud; in different versions of the bare-bones model the domain has been considered to comprise the apical and active zones together (Newman and Frisch, 1979), or only the active zone (Hentschel et al., 2004; Chaturvedi et al., 2005; Cickovski et al., 2005). As will be seen below, assuming that the LALI domain extends fully to the end of the limb bud (i.e., comprises both the active and apical zones) brings the model into conformity with predictions of some classic experiments.

In all versions of the model, the greater the value of the AP (AP) length, l_z, the more parallel peaks will form. Thus, whether three digits (as in the chicken forelimb), four digits (as in the chicken hindlimb), or five digits (as in mouse or human limbs), arise, is pre-

dicted to be positively correlated with this distance, and this is borne out in the actual systems. What is somewhat counterintuitive is the model's determination of a negative correlation between the PD length of the LALI domain, $l_x(t)$, and the number of parallel elements generated. This is a straightforward mathematical consequence of the behavior of the LALI system under the asymmetric conditions imposed by the AER-dependent gradient (Newman and Frisch, 1979; Hentschel et al., 2004; Chaturvedi et al., 2005; Cickovski et al., 2005). Since it appears in a number of different mathematical embodiments of the model, it is probably a robust feature.

In the developing chicken wing, where l_z remains constant during the period of pattern formation, the length of the unpatterned distal region (encompassing, in our terminology, both the apical and active zones) declines progressively over the course of development (Summerbell, 1976). The model's stipulation that $l_x(t)$ acts as a "control parameter" influencing the number of parallel elements is thus consistent with (though not compelled by) existing evidence.

Cell proliferation enters into this scheme in the following fashion: cells are recruited into the active zone from the proximal end of the apical zone, as dividing cells move away from the influence of the AER. (This is similar to the role of the caudal FGF gradient in somitogenesis) (Dubrulle et al., 2001). The active zone loses cells, in turn, to the proximal frozen zone, the region where cartilage differentiation has occurred and a portion of the definitive pattern has become set. The overall effect of this growth and zonal reassignment is a time-dependent reduction of l_x. The growth of the domains implies the presence of a local velocity field and thus cell rearrangement by convection. However, estimates of the Péclet number, defined by the mathematical relation LV/D, where L is the characteristic length scale of the limb, V the characteristic velocity of the flow, and D the morphogen diffusion coefficient, is relatively small, meaning that diffusion is more important than convection, at least for the purposes of our basic model (Newman et al., 2007).

Four main types of mesenchymal cells are involved in chick limb skeletal pattern formation. These are represented in the continuum model by their spatially and temporally varying densities. The cell types are characterized by their expression of one of the three FGF receptors found in the developing limb. The cells expressing FGFR1, FGFR2 (and cells), and FGFR3 are denoted, respectively, by R_1, $R_2 + R_2'$, and R_3. The apical zone consists of R_1 cells, and those of the frozen zone, R_3 cells (reviewed in Ornitz and Marie, 2002). The active zone contains R_2 cells and the direct products of their differentiation, R_2' cells. These latter cells secrete elevated levels of fibronectin. The R_1, R_2, and R_2' cells are mobile, while the R_3 (cartilage) cells are immobile.

Transitions and associations between the different cell types are regulated in the model by the gene products of the core mechanism (Fig. 2). The model contains terms representing, respectively, the spatially and temporally varying concentrations of FGFs (produced by the AER), TGF-β (produced throughout the mesenchyme), a diffusible inhibitor of chondrogenesis produced by R_2 cells, and fibronectin, produced by R_2' cells. The model thus comprises eight independent variables, with an equation for the behavior of each of them. These eight variables correspond to the core set of interactions necessary to describe the development of a basic, bare-bones skeletal pattern.

Simulations using the eight-equation system are computationally unfeasible. In Hentschel et al. (2004) a biologically motivated separation of time scales was employed to reduce the system to four equations. This involved averaging the values of variables that evolve very rapidly relative to the activating and inhibitory morphogens and holding constant those that evolve more slowly.

The mathematical technique of linear stability analysis was then used to identify solutions of the four-equation system on a 2D plane (i.e., under the simplifying assumption that the dorsoventral width is very small) for LALI domains of progressively decreasing width (Fig. 4). 3D simulations of the growing limb bud, based on the model of Hentschel et al. (2004), but using different simplifying assumptions for the activator-inhibitor dynamics, and a "multimodel" computational framework that permitted representation of cells as autonomous, discrete entities (Cickovski et al., 2007), also accurately portrayed the PD order of appearance and increase in the number of skeletal elements (Fig. 5) (Chaturvedi et al., 2005; Cickovski et al., 2005).

Additional Predictions of the Model

Several emergent properties of the bare-bones model add to its plausibility as an accurate account of the cell-molecular basis of limb pattern formation. It has long been known, for example, that experimental removal of the AER before full elaboration of the pattern leads to terminal deletions, which are less extensive the later the procedure is performed (Saunders, 1948). If the distal source of FGF is removed as the model limb "develops," the entire distal region is immediately unsuppressed. Under the assumption that the LALI domain extends to the end of the limb (one of the possibilities mentioned above), the full domain would become "active," and the activator pattern currently under generation would become the final, albeit terminally incomplete, one. (The alternative assumption, that the LALI domain is coextensive with the active zone, would predict tandem duplications of proximal elements from AER removal, contrary to experimental findings.)

Another unanticipated explanatory feature of the bare-bones model concerns predicted devia-

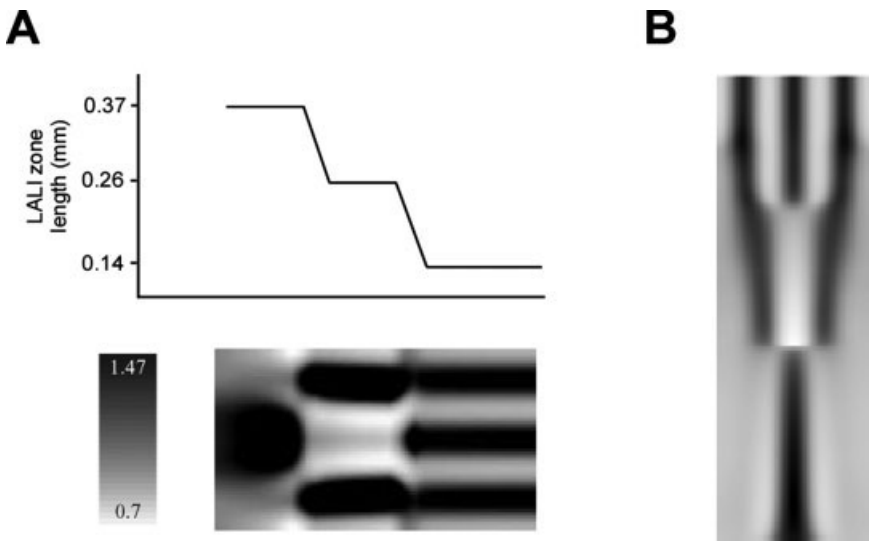

Figure 4. Relationship of size of LALI zone and number of activator morphogen peaks formed. **A:** Top, graph of change in size of the unpatterned distal portion of the embryonic chicken wing between 3.5 and five days of development, based on measurements by Summerbell (1976); bottom, computational results of activator distribution as a function of the proximodistal length, $l_x(t)$, of the LALI zone, which is identified with the unpatterned tissue in above graph. The scale represents the concentrations as a fraction of the concentration (= 1.0) at the uniform stationary state. **B:** A typical developed ''limb skeleton'' arising from the bare-bones model represented in Figure 3 and the calculations in (A), above, allowing for growth. Specifically, after the cellular condensations form based on patterns of activator peaks, growth is assumed to occur at a constant rate. Consequently, earlier forming cartilage elements are subject to more growth than later ones. All panels adapted from Hentschel et al. (2004).

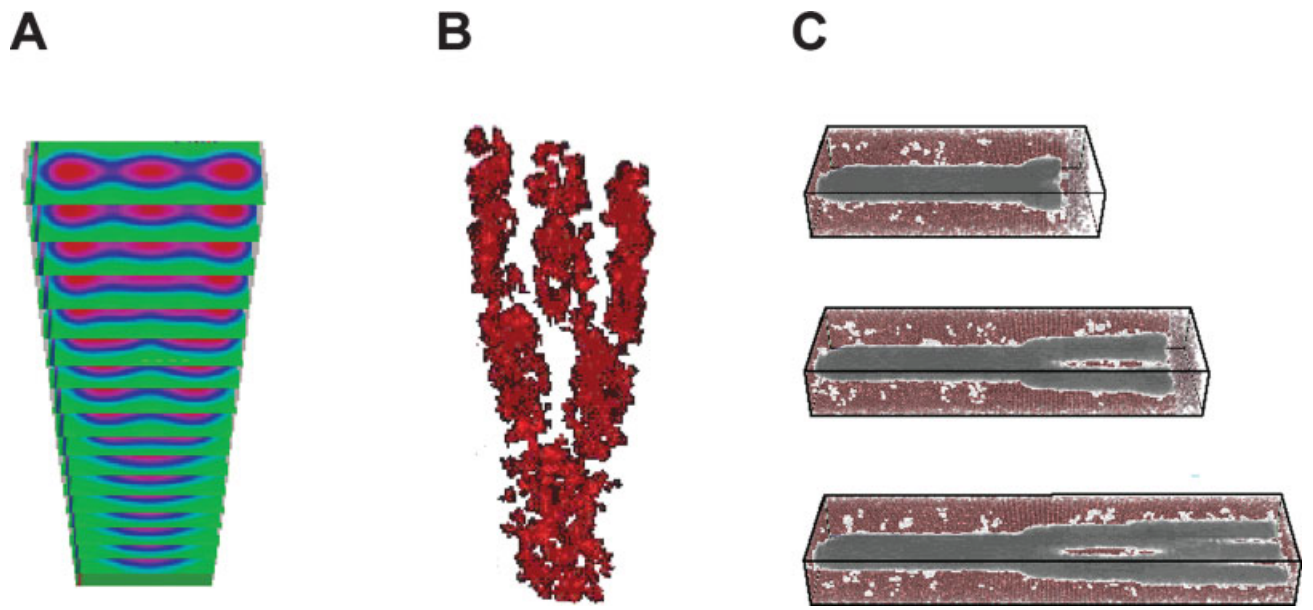

Figure 5. 3D simulations of limb development. **A:** Simulation of distribution of TGF-β (activator) over progressive developmental stages (time increasing from bottom to top) in a 3D computational model based on the bare-bones mechanism represented in Figure 3 and a simplified version of the core-mechanism equations in Hentschel et al. (2004), using a ''multimodel'' representation of autonomous cells (Cickovski et al., 2007). **B:** Simulation of cell distributions after completion of development into humerus (lower), ulna and radius (middle), and digits (top). **C:** Simulation of successive stages (top to bottom) of proximodistal development of limb skeleton using the multimodel described in Cickovski et al. (2007) and a different simplification of the core-mechanism equations than in (A) and (B). (A) and (B) are from Chaturvedi et al. (2005); (C) is from Forgacs and Newman (2005), based on Cickovski et al. (2005), courtesy of Trevor Cickovski.

tions from the PD appearance of limb skeletal elements. Unlike amniotes (reptiles, birds, and mammals), for which this developmental progression universally pertains, for urodele amphibians some distal elements may appear before some proximal ones (Franssen et al., 2005). The developing limbs of amniotes and urodeles also differ in that whereas the former produce FGF8 only in the apical

ectoderm, the latter produce it in the mesenchyme as well (Han et al., 2001). When we performed simulations using a modified version of the bare-bones model in which both the limb bud tip and the mesoblast produced suppressive FGFs, we obtained two active zones and patterns that developed out of strict PD order (Tilmann Glimm and Stuart A. Newman, unpublished results).

OSCILLATION-SYNCHRONIZATION AS A MECHANISM FOR LATERAL INHIBITION

In a study of limb bud mesenchymal cells in culture we examined the response to perturbation by exogenous TGF-β of several developmental variables (Leonard et al., 1991). These variables included production of fibronectin mRNA, the extent and rate of appearance of condensations, and levels of production of the cartilage-specific sulfated proteoglycan, aggrecan. We found, surprisingly, that there was a temporal periodicity in the dynamics of all these factors. For example, peaks of fibronectin mRNA or aggrecan production appeared at increasing durations of TGF-β exposure, but exposure durations interdigitated between the ones that elicited peaks produced, instead, response minima (Leonard et al., 1991).

It had been known for some time that processes controlled by an oscillatory mechanism can respond to continuously varying external perturbations by changing in alternating directions, producing so-called type 0 resetting curves (Winfree, 1980). Such indirect data, though diagnostic of an underlying oscillator, give no hint of what is actually oscillating. We concluded from our experiments, however, that the generation of precartilage condensations or their molecular determinants may involve one or more periodic processes (Leonard et al., 1991). Since then, evidence has been presented for periodic expression of *hes2* in the chicken limb (Pascoal et al., 2007) and, as mentioned above, *hes1* has been found to be expressed in a synchronized, periodic fashion during the condensation-forming period in micromass cultures of limb bud mesenchyme (our unpublished results).

The synchronization of oscillations provides a way of propagating a signal that may begin as a juxtacrine interaction and convert it to a long-range effect (see for example, Garcia-Ojalvo et al., 2004). This has led us to consider a mechanism for lateral inhibition of precartilage condensation that is an alternative to one based on a diffusible morphogen. We suggest, instead, that this effect results from Notch-Delta juxtacrine signaling followed by synchronization of a Notch-activated oscillatory state. As will be seen, this proposed solution to the elusive inhibitor problem can potentially reconcile a set of puzzling cell-molecular findings relating to the spacing of condensations discussed earlier. Like the reactor-diffusion mechanism described earlier, in which both the activator and inhibitor are diffusible morphogens, the oscillation-synchronization-dependent system is a LALI mechanism and, as such, can play the same role in the bare-bones model for limb development as the reactor-diffusion process considered in earlier versions of this model (Chaturvedi et al., 2005; Cickovski et al., 2005; Hentschel et al., 2004).

The precartilage mesenchymal condensation pattern seen in micromass cultures shows a spatial regularity that depends on a zone of inhibition forming around incipient condensations. These zones of inhibition consist of cells which have failed to enter the condensation and thus do not undergo differentiation into cartilage. In preliminary studies using quantitative real-time PCR, we have found that the expression of *hes1*, a Notch pathway target gene, enters into synchronous oscillations in a significant portion of the cells within the first day of culture, just as the spatial pattern of condensations is becoming established (Ramray Bhat and Stuart A. Newman, unpublished; shown schematically in Fig. 6A).

Notch is transiently activated in precartilage mesenchymal cells as they first enter condensations and pharmacological abrogation of Notch activation leads to expansion and fusion of the condensations (Fujimaki et al., 2006). Since the Notch-activated state is transduced by Hes1, the oscillatory nature of *hes1* expression (the ''Hes1 clock'') implies that the synchronized cells periodically cycle through Notch-activated and Notch-inhibited phases. We assume that when *hes1* expression is minimal, there is a ''phase window'' during which the cells become permissive to respond to the activator signal (by our earlier assumption, TGF-β) and produce more activator and fibronectin.

If the window for activator propagation in the Hes1 clock is short, in a field of cells with random phases of the Hes1 clock there will be a low but finite probability of propagation of the condensation state from any point. Therefore, in the absence of synchrony, although the default state for activator propagation is ''mostly inhibited,'' i.e., refractory to activator (indicated by there being more light blue cells than dark blue cells in the upper row of panel B in Fig. 6), activator/TGF-β expression will eventually percolate through the entire field, leading all the cells to condense. However, in reality, the cells become largely synchronized in *hes1* expression. This is shown schematically in the upper row of Fig. 6C, where the ''high Hes1'' phase of the clock, nonpermissive to propagation of activation, is represented by the preponderance of light blue cells.

The precartilage mesenchymal cells, derived from the apical zone of the limb bud, express FGFR1 (Peters et al., 1992; Szebenyi et al., 1995; see discussion in A CORE GENETIC NETWORK FOR PRECARTILAGE CONDENSATION, above), a condition that may be conducive to the *hes1* oscillatory state. Once they are removed from the influence of the AER in the developing limb, or are placed in culture, however, some cells

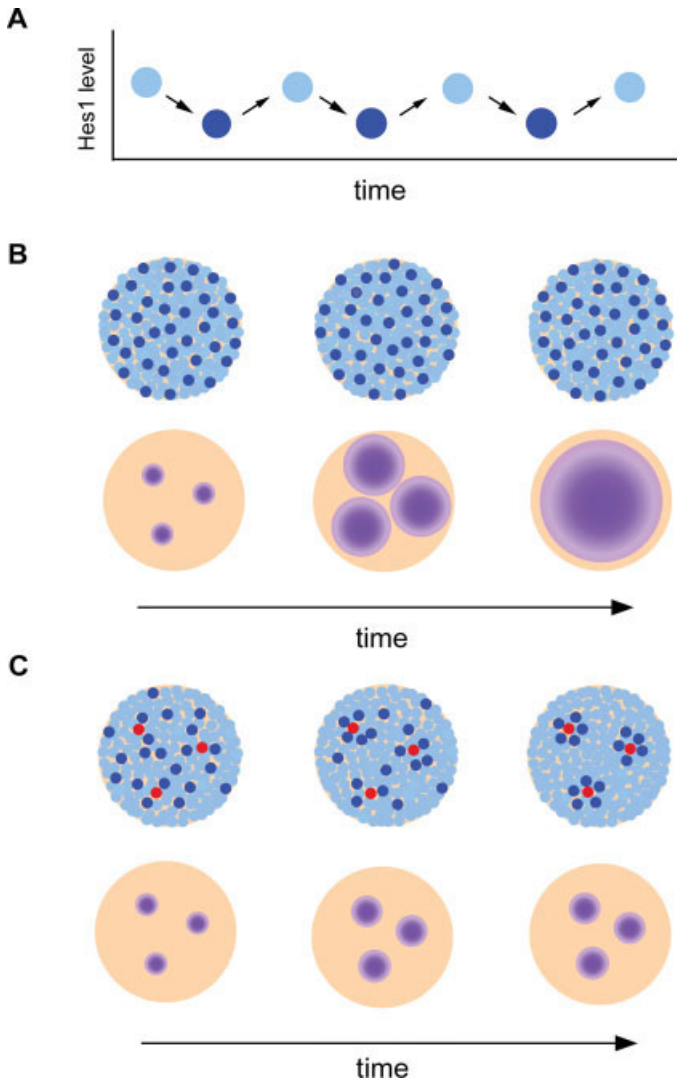

Figure 6. Oscillator-synchronization mechanism for lateral inhibition of condensation formation. **A:** Schematic representation of periodic changes in *hes1* (and presumably Hes1 protein) expression over time during the period of pattern formation. Light blue represents the "high Hes1," TGF-β-refractory state; dark blue represents the "low Hes1," TGF-β-permissive state. **B:** Upper row, schematic representation of precartilage mesenchymal cells oscillating in *hes1* expression over time, with random phases. Lower row, randomly initiated centers of activator production (purple) expand with time, because there is always a pathway for activator propagation in the phase-randomized field of cells. **C:** Upper row, cells expressing FGFR2 (red) appear, possibly induced by local elevation of activator. The Hes1 clock is halted or slowed, causing local desynchrony (scattered light and dark blue cells). Self-organized synchronization continues peripheral to the condensations (mainly light blue cells). Since, by our assumption, most of the Hes1 cycle is inhibitory to propagation of the activator, the latter fails to spread (lower panel) and pattern formation ensues.

become FGFR2-positive (red cells in upper row of Fig. 6C), and these are located at sites of incipient condensation (Szebenyi et al., 1995; Moftah et al., 2002). Since TGF-β can induce the functional activation of FGFR2 in some cell types (Kanda et al., 2003), we suggest that this factor, in conjunction with FGF itself, acts on the precartilage cells to stop or slow down the Hes1 clock.

This implies that there will be an antagonism between the induced cessation or slowing of the Hes1 clock at the condensation sites and the self-organizing (probably Notch-dependent; Giudicelli et al., 2007) synchronization of the clock at a characteristic distance from the condensation centers. (See Riedel-Kruse et al., 2007 for a description of how competition between synchrony and desynchrony sets an analogous "defect range" in perturbed segmental plates).

Synchronization thus converts local juxtacrine signaling into broad-range lateral inhibition, but without the need for a diffusible inhibitor. FGFR2-positive sites can continue to be initiated within these synchronized domains only when the synchronized Hes1 oscillation is at its trough, but the small phase window permissive to propagation of the activator ensures that two such cell clusters have a low probability of being next to each other. Unlike the case with initially asynchronous field that is mostly inhibited all of the time, but across which the activator signal will thus always have a pathway to propagate (Fig. 6B), in the synchronized situation the domains surrounding the initiation sites will be totally inhibited most of the time (Fig. 6C, upper row). The condensations can expand periodically but will be limited by the duration and decay of the activator signal, and the outcome will be a LALI-mediated pattern (Fig. 6C, lower row).

The synchrony-based pattern forming mechanism described above, while sufficient to generate patterns of the regularity seen in limb bud mesenchymal cultures, is missing a key element required to generate the entirely regular patterns seen in vivo by a LALI mechanism: feedback between the control of the initiation sites and the zones of inhibition. This feedback circuitry is specified in several previously proposed reactor-diffusion-type LALI mechanisms for limb

mesenchymal pattern formation (Hentschel et al., 2004; Christley et al., 2007; Alber et al., 2007), but so far these activator-inhibitor interactions are hypothetical. Some evidence suggests that the amounts and types of ectodermal FGFs to which the mesenchyme is exposed influences the regularity of the condensation pattern (Moftah et al., 2002). This would be well-regulated by the surrounding ectoderm in the in vitro context and may mediate the connection between initiation and inhibitory effects.

This oscillation-synchronization mechanism of lateral inhibition is consistent with existing experimental data and helps explain some earlier puzzles. TGF-β, for example, is much more effective in inducing fibronectin expression and condensation formation a day after cultures are established than within the first day of culture (Leonard et al., 1991). This later period is when FGFR2-positive cells first appear. It is significant in this regard that in osteogenic cells FGFR2 synergizes with TGF-β in inducing the production of fibronectin (Tang et al., 2007).

The role of juxtacrine Notch signaling in mediating long-range lateral inhibition (Fujimaki et al., 2006) becomes understandable when synchronization is taken into account. Finally, the paradoxical role of FGFR2 mentioned above can be explained as follows: FGFR2 promotes condensation formation, and thus if it is present in a hyperactivated form (as it is in Apert syndrome in humans) (Ibrahimi et al., 2001) condensations will expand. Nonetheless, without FGFR2 present, the mesenchyme will not initiate centers from which Hes1 synchrony can spread. In the absence of broad inhibitory zones, TGF-β will eventually induce all cells to produce more TGF-β and also fibronectin. Thus if FGFR2 is knocked down, ectopic condensations are predicted to form, as reported (Moftah et al., 2002).

DISCUSSION

We have presented a combined physical and molecular-genetic approach to limb skeletal pattern formation that accounts for a number of major features of this process. These include: 1) the generation of discrete, regularly spaced precartilage condensations, 2) the PD emergence of increasing numbers of parallel elements over time, 3) the dependence of successive patterning steps on the presence of a graded signal from the AER, 4) the deviation from strict PD development, as in urodele amphibians, when an AER type signal is produced by the mesoblast, as it is in urodeles, and 5) roles consistent with experiment of the following factors: FGF8, FGFR2, TGF-β, fibronectin, and Notch.

The general perspective of our analysis is based on the pattern forming capabilities of LALI systems (Meinhardt and Gierer, 2000). Such systems, based on diffusible activators and inhibitors, have been shown to quantitatively reproduce the pattern characteristics of limb bud precartilage mesenchyme in vitro (Kiskowski et al., 2004; Christley et al., 2007). But while there is strong evidence (reviewed above under A CORE GENETIC NETWORK FOR PRECARTILAGE CONDENSATION) that the diffusible, positively autoregulatory morphogen TGF-β plays the requisite role of activator in a limb mesenchymal LALI system (Leonard et al., 1991; Miura and Shiota, 2000a), the identity of the lateral inhibitor has been more elusive. Moreover, the dependence of long-range lateral inhibition of precartilage condensation on FGFR2 (Moftah et al., 2002), which is expressed at sites of condensation and is a positive mediator of chondrogenesis (Ornitz and Marie, 2002), and on the Notch pathway (Fujimaki et al., 2006), which acts in a juxtacrine fashion, suggests that a mechanism other than diffusion may mediate this process.

Drawing on indications from an earlier study that patterning of condensations may involve an underlying oscillatory process (Leonard et al., 1991), recent findings that Notch transcriptional mediators of the Hes family show oscillatory dynamics in vivo (Pascoal et al., 2007) and in vitro (our unpublished results), and evidence that Notch-based juxtacrine signaling can mediate the synchronization of Hes oscillations and thus convert short-range into long-range signals (Giudicelli et al., 2007), we have here proposed a novel mechanism for the laterally inhibitory component of the LALI system of limb precartilage mesenchyme.

The reformulated LALI system (with appropriate activator-inhibitor feedback; see above) can play the same role in the bare-bones model for skeletal development as the earlier-proposed reactor-diffusion system (Hentschel et al., 2004; Alber et al., 2007). Indeed, the robustness of the morphogenetic outcomes of this model to changes in the molecular details of one of its functional modules, the LALI mechanism, is a general characteristic of developmental systems. These can often change dramatically at the molecular level over the course of evolution while leaving anatomical structures relatively unaltered (Müller and Newman, 1999; Félix and Wagner, 2006). Further experimentation will be required to determine whether one or another version of a reactor-diffusion system (Newman et al., 2007; Alber et al., 2007), the oscillator-synchronization LALI system proposed here, or a different category of mechanism, best accounts for chondrogenic pattern formation in vivo and in vitro.

The spatially quasi-periodic nature of the vertebrate limb skeleton, however, is an invariant property of all limb types in all tetrapod species.

One set of issues not addressed in the foregoing is the bases of dif-

ferences in size and shape among the limb's individual skeletal elements. These include fairly dramatic differences between corresponding fore- and hindlimb elements, differences along the PD axis such as between the humerus and the ulna, more subtle ones along the AP axis such as between the radius and ulna, or among the various digits, and even finer ones along the dorsoventral axis such as between the dorsal and ventral aspects of the femur. The processes regulating such differences has been a major concern of research in limb developmental biology over the last 35 years, and has led to information concerning the influence of Hox and Tbx transcription factors, Sonic hedgehog and other molecular determinants on the identities of the skeletal elements. These effects, although they typically vary for a given molecule between limb types and different species, are clearly important, since the limb skeleton is not a tandem arrangement of indistinguishable elements, as in our bare-bones model, but a finely honed result of evolutionary forces. It seems likely that modulatory factors, acting at a variety of regulatory levels, could fine-tune the morphology of the developing skeletal elements by altering relative amounts of common tissue components (Downie and Newman, 1994, 1995). The spatially quasi-periodic nature of the vertebrate limb skeleton, however, is an invariant property of all limb types in all tetrapod species. Indeed, its very ubiquity may have led to its relative neglect as a scientific problem. It is this aspect of the vertebrate limb, which is central to developmental, evolutionary, and clinical concerns, that the approach described here is directed towards understanding.

Conceptualizing a developmental process such as limb skeletal patterning as an interplay between molecular-genetic and physical and physicochemical mechanisms can provide new insight into the genesis of birth defects.

Finally, we note that conceptualizing a developmental process such as limb skeletal patterning as an interplay between molecular-genetic and physical and physicochemical mechanisms (Newman and Comper, 1990) can provide new insight into the genesis of birth defects. Because genes and their products generate biological forms only as participants in dynamical systems, the properties of such systems, specifically their nonlinear behaviors and sensitivity to physical (including mechanical) and chemical externalities, need to be taken into account in understanding their typical and variant morphological outcomes. Recognizing that developing embryos are not genetically programmed machines, but rather complex, excitable systems in interaction with the physical world, can help decipher the often puzzling relationships between familial and genetic background and environmental exposure to teratogens and other agents in the production of congenital anomalies and disease (Dournon et al., 1998; Ozanne and Constancia, 2007; Godfrey et al., 2007).

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