Sticky fingers: *Hox* genes and cell adhesion in vertebrate limb development

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Summary

During vertebrate limb development, various genes of the *Hox* family, the products of which influence skeletal element identity, are expressed in specific spatiotemporal patterns in the limb bud mesenchyme. At the same time, the cells also exhibit 'self-organizing' behavior – interacting with each other *via* extracellular matrix and cell-cell adhesive molecules to form the arrays of mesenchymal condensations that lead to the cartilaginous skeletal primordia. A recent study by Yokouchi *et al.*[1] establishes a connection between these phenomena. They misexpressed the product of the *Hoxa-13* gene in chick limb buds and demonstrated both skeletal pattern perturbations and changes in cell-cell adhesivity in mesenchyme aberrantly expressing this protein.

The vertebrate limb: a partially self-organizing system

Why does the vertebrate limb have the structure and appearance that it does? The aspect of limb development that has held the greatest fascination for students of morphogenesis and pattern formation is the emergence of successive arrays of skeletal primordia – bars and spot-like nodules of cartilage (later to be replaced by bone in most species) – in a sequence that begins with a single element ('stylopod') attached directly to the body, followed by two parallel elements ('zeugopod') and a variable, but usually greater, number of elements constituting the wrist and hand, or ankle and foot ('autopod'). One theory for this process, which has been influential for the past two decades[2,3], holds that a system of molecular gradients arising early during limb formation imparts 'positional information' to the mesenchymal cells of the developing limb bud mesoblast. Each cell, acting essentially autonomously (according to this view), interprets its local coordinates along independent proximodistal, anteroposterior and dorsoventral axes, and makes that portion of the skeleton appropriate to its location. Over the period since this picture was first presented, a variety of molecules with particular spatial and temporal expression patterns in the developing limb bud have been described, including retinoids[4] and their receptors[5], members of the *Hox*[6] and *Msx*[7] families of DNA binding proteins, the secreted products of the *Sonic hedgehog* (*Shh*)[8] and *Wnt-7a*[9,10] genes, and fibroblast growth factor (FGF)[11] and transforming growth factor-beta (TGF-β)[12,13] family members. The wide acceptance of the positional information framework has led to suggestions that the local concentration of one or more of these molecules constitutes the 'address' that a mesenchymal cell consults as it decides to differentiate into part of a cartilaginous skeletal primordium, or to undergo programmed death[14] to open up the inter-primordium spaces. However, certain findings, some of which predate the positional information framework and some of which (including those reported in an important paper by Yokouchi *et al.*[1]) have emerged recently, suggest, rather, that the limb pattern is established by a system of cellular interactions in which the anatomical axes are not functionally separable in their developmental roles, and cellular addresses and their interpretations cannot be so easily schematized.

One early finding[15] that has gained renewed attention[16] was the observation that randomized limb mesenchymal cells repacked into an ectodermal hull are capable of developing into convincingly limb-like structures, consisting of tandemly arranged skeletal rods and nodules, usually terminating in two or more jointed, indistiguishable digits. Since this occurs without the reestablishment of anteroposterior gradients in the expression of the *Hoxd11-13* genes[16], it is probable that such gradients are involved in determining detailed differences between skeletal elements, rather than what might be considered a basic limb groundplan.

What can account for the generation of the rough skeletal pattern in the absence of axis-specific positional gradients, and the autonomous cellular interpretation thereof? It has long been known that limb bud mesenchyme exhibits 'self-

organizing' behavior: when a randomized mixture of limb precartilage cells is placed in high-density culture they undergo a process of focal cell aggregation, or condensation[17-19]. These condensations are rich in extracellular matrix (ECM) molecules such as fibronectin[18] and tenascin[20], and in cell adhesion molecules such as N-cadherin[21] and N-CAM[22]. Such condensations, which exhibit a quasi-periodic arrangement *in vitro,* also form *in vivo*[23,24], providing the mesenchymal armatures for the partly periodic arrays of cartilage elements that form during limb development.

Interestingly, non-living chemical systems that exhibit certain quantitative relationships among reaction and diffusion coefficients will spontaneously self-organize into spot- and bar-like patterns of concentration of reaction products[25,26], as predicted more than 40 years ago[27]. These chemical patterns, which emerge from a homogeneous medium as a result of simple interactions among diffusible activators and inhibitors of product formation, resemble the condensation patterns seen in limb precartilage mesenchyme *in vitro* and *in vivo*. It is therefore plausible that mesenchymal cells can generate nodules and rods of cartilage by making use of analogous simple interactions, in this case involving a balance between secreted, diffusible molecules that stimulate, and those that inhibit, the production of the ECM and adhesion molecules that mediate condensation formation[28,29]. One model for this process[28] suggests that the tendency to generate increasing numbers of parallel elements along the proximodistal axis may be automatically related to the narrowing of the distal band of unorganized mesenchyme (the 'progress zone'[30]) over time. In this context, any non-uniformly distributed transcription factors that modulate the local expression of ECM and adhesion molecules, or their activators and inhibitors, could fine-tune this self-organizing process and ensure the reliable generation of the size, shape and number of cartilage elements that form at any position.

Expression and misexpression of *HoxA* genes in the developing limb

In 1991 Yokouchi *et al.*[31] showed that members of the subfamily of the vertebrate *HoxA* gene cluster most closely related to Drosophila *Abd-B* (*Hoxa-10, Hoxa-11* and *Hoxa-13*) have unique expression domains along the proximodistal axis of the chicken limb bud (a finding that was confirmed in the mouse[32]). It was suggested that the products of these genes contribute to defining the identity of skeletal elements in their respective domains and, indeed, targeted disruption of *Hoxa-11* in the mouse led to a variety of limb defects involving abnormal fusions of small bones and widening of zeugopodal elements[33]. Even more dramatically, loss of *Hoxa-11* activity against a background null for its paralogue *Hoxd-11* led to complete or partial supression of the zeugopod in the fore limbs, but not the hind limbs, and autopodal defects that indicated a coupling between proximodistal and anteroposterior patterning[34]. These results suggested modulation of mesenchymal properties by the Hox proteins, but according to complex rules that resisted schematization in the form of a 'Hox code' for positional information[35].

In their more recent study, Yokouchi *et al.*[1] caused *Hoxa-13* to be misexpressed during chicken limb development by infecting the entire limb bud with a viral construct specifying that protein. They found, remarkably, that the cartilage primordia of the zeugopodia (radius and ulna in the fore limb; tibia and fibula in the hind limb), and these regions alone, were transformed from elongated bars to shorter, stubbier bars, some verging on the spot-like morphology normally seen only in wrist and ankle elements. Moreover, in the region between the zeugopodial cartilages in both the

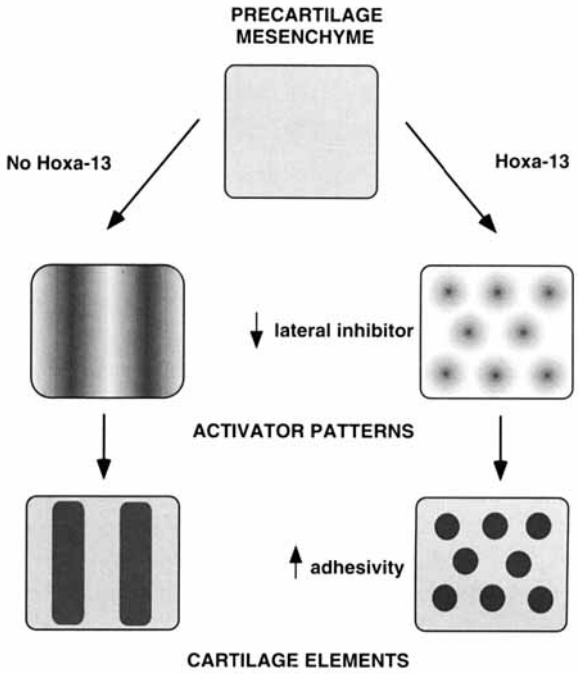

Fig. 1. Model for the role of the *Hoxa-13* gene product in pattern formation and morphogenesis of skeletal elements of the vertebrate limb. The box at the top represents unpatterned precartilage mesenchyme. The box at middle left represents the spatial pattern of an activator of precartilage condensation that would result from synthesis and diffusion of hypothetical short range activator and lateral inhibitor molecules, based on properties of the cells of the prospective zeugopod, including lack of Hoxa-13 protein. The box at bottom left represents cartilage elements that would result from this activator pattern. The box at middle right represents the activator pattern that would result from activator-inhibitor interactions with cells that contain Hoxa-13 protein, either endogenously in the prospective autopod, or by infection with a Hoxa-13 encoding retrovirus, as in the study by Yokouchi *et al.*[1], if an effect of Hoxa-13 were to decrease the production of the inhibitory molecule. The box at bottom right represents the cartilage elements that would result from this activator pattern in conjunction with the increase in adhesivity at sites of condensation associated with the presence of Hoxa-13[1]. A reduction in the extent of inhibitory fields could lead, in addition to the spot pattern shown, to a pattern of bars that are more closely spaced than those of the zeugopod, i.e. like the digits.

fore and hind limb, a prominent site of programmed cell death during normal limb development, extra cartilage elements appeared, of both the bar and spot morphologies.

In vitro analysis of *Hoxa-13* function

Taking their cue from earlier studies, which suggested that vertebrate Hox proteins can act as transcriptional regulators of the production of cell-cell adhesion and ECM proteins[36], Yokouchi *et al.*[1] sought to relate the pattern perturbations induced by ectopic expression of *Hoxa-13* to adhesion-mediated changes in cell behavior. Sorting-out of cells from a randomly mixed heterotypic population has been used to demonstrate differential adhesion of a wide variety of embryonic cell types[37,38], and this assay provided strong evidence that Hoxa-13 did indeed alter the adhesive properties of limb mesenchymal cells. The most convincing demonstration of this was an experiment in which prospective limb regions of the embryo were infected with a *Hoxa-13*-expressing construct; the 20-30% of cells that stained positively with anti-Hoxa-13 antibody in stage 20 limb buds (i.e. prior to the expression of endogenous Hoxa-13), sorted into clusters of a dozen or more cells, an indication that they were more mutually adhesive than the surrounding non-Hoxa-13-expressing cells.

A caveat that must accompany the interpretation of the sorting experiments concerns the fact that the limb bud contains both somite-derived myogenic cells and somatopleure-derived chondrogenic cells at all stages studied[39,40]. These subpopulations of the mesenchyme may themselves be differentially adhesive. In the absence of controls demonstrating the contrary, the sorting results could be interpreted as reflecting different susceptibilities of chondrogenic and myogenic mesenchymal cells to infection with the retroviral vector. This potential complication is not resolved by sorting experiments performed using naturally *Hoxa-13*-expressing and non-expressing limb mesenchymal subpopulations, since in these cases the *Hoxa-13*-expressing cells were all derived from a region of the limb bud devoid of myoblasts[41].

Assuming however, as seems reasonable, that infection with the *Hoxa-13* virus did cause limb precartilage cells to become more adhesive, the *in vivo* pattern perturbations in the limb resulting from infection with this virus, as well as the role of endogenous Hoxa-13 during limb development, begin to be interpretable. Such an interpretation cannot invoke independent positional specification along separable anteroposterior and proximodistal axes – the extra skeletal elements induced by ectopic *Hoxa-13* expression represent changes in both axes – nor can it depend on a model in which the production of any of the adhesive molecules known to be involved in condensation formation is uniquely linked to the presence of Hoxa-13. As Yokouchi *et al.* point out, neither N-cadherin nor N-CAM (nor, they may have added, fibronectin), which are all elevated at sites of precartilage condensation, have restricted expression domains similar to those of *Hoxa-13*. Moreover, contrary to the authors' original expectations (perhaps influenced by the positional information framework), uniform expression of *Hoxa-13* did not equalize all cell adhesion properties in its domain. Finally, reduced growth of cartilage primordia of *Hoxa-13*-infected limbs, while it is likely to have contributed to ultimate differences in the sizes of corresponding skeletal elements during later development, cannot account for the *pattern* differences established during the condensation phase, particularly the formation of ectopic nodules and rods of cartilage.

The simplest interpretation may be that Hoxa-13 endows the cells that express it with an enhanced capacity to produce ECM or cell-cell adhesion molecules in response to activators of condensation common to all limb precartilage cell populations. Another consequence of Hoxa-13 may be to attenuate the production of an activator-stimulated diffusible inhibitor of condensation formation. The result would be less extensive inhibitory fields around each condensation. This combination of properties would lead to more numerous, closely spaced and tighter condensations in any precartilage cell population that expressed *Hoxa-13* (Fig. 1). An analogy may be found in the morphologically distinctive precartilage condensations produced *in vitro* by fore- and hind-limb precartilage mesenchyme[42,43]. Both cell populations respond to TGF-β, an inducer of precartilage condensations, by producing additional fibronectin, but the hind limb cells, which form rounder, more compact, condensations, produce three times as much additional fibronectin as the fore limb cells[43]. Moreover, the condensing mesenchyme in hind-limb cell cultures appears to produce greater amounts of a lateral inhibitor of condensation than does the condensing mesenchyme of fore-limb cell cultures[42,43]. Thus different regions within a given limb bud, or indeed different types of limb buds, could make use of simple self-organizing properties that cause all skeletal elements to resemble each other, and fine-tune these 'generic' interactions by means of Hox proteins and other non-uniformly distributed modulators, so that distinctive skeletal elements may be formed.

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