Molecular basis of limb development

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The vertebrate limb is an ideal model system in which to study the cell interactions that lead to the generation of patterns of differentiated cells and tissues during embryonic development. Over the past 40 years or so, the developing limb has been the focus of a wealth of experimental analysis. This analysis established the cell interactions that are necessary to form a limb. More recently, some of the molecules that provide the basis of these interactions have been discovered. These molecules include retinoids (the collective term for vitamin A derivatives), growth factors and homeobox-containing genes. It is intriguing that similar sets of molecules appear to operate at different times and places in vertebrate embryos. This suggests that common signalling systems are involved. Here, I will concentrate on the roles of growth factors that may provide important signals in the limb bud.

Most of the experimental work on limb development has been carried out on chick embryos [1]. This is because the developing limb bud can be manipulated in ovo and subsequent effects on pattern monitored later. The early bud consists of undifferentiated mesenchyme cells encased in ectoderm (Figure 1). During development, the bud elongates and cells in the proximal part of the limb bud (the part nearest the body wall) start to differentiate. The structures of the limb are laid down in proximo-distal sequence. For example, in the chick wing, the humerus forms first, followed by the radius and ulna and finally digits (Figure 1).

Two major interactions have been identified from experiments on chick limb buds. One set of interactions takes place at the tip of the limb bud between the thickened epithelium, the apical ectodermal ridge, and underlying mesenchyme. The interaction between apical ridge and underlying mesenchyme is necessary for outgrowth. When an additional apical ridge is grafted to the dorsal surface of a wing bud, an outgrowth is induced and an extra limb sprouts from the host limb. When the apical ridge is cut away from the bud, the limb is truncated. The apical ridge maintains at the tip of the limb bud a region of undifferentiated cells,

565

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Abbreviations used: FGF, fibroblast growth factor; TGF, transforming growth factor.
known as the progress zone [2]. The progress zone cells in turn maintain the apical ridge. As cells leave the progress zone, they lay down structures along the proximo-distal axis of the limb. A second major set of interactions is between a small group of mesenchyme cells at the posterior margin of the bud, known as the polarizing region, and cells at the limb bud tip. This mesenchyme-mesenchyme signalling is important in specifying pattern across the antero-posterior axis of the limb bud. (The antero-posterior axis runs, for example, in our hand from thumb to little finger.) The signalling of the polarizing region can be demonstrated by cutting out the polarizing region from one bud and grafting it to the anterior margin of a second bud. This results in a duplicated wing pattern with additional digits 4-3-2 developing in mirror image symmetry with the normal set of digits, 2-3-4 (reading from anterior to posterior) (Figure 2) [3].

The basis of both epithelial-mesenchymal signalling and signalling of the polarizing region is the same in wing and leg. For example, a leg ectodermal ridge can substitute for the ectodermal ridge of a wing bud and a leg polarizing region can induce additional wing digits. The signals are also conserved between chickens and mammals [1]. Very recently, growth factors have been implicated in these two pathways. A member of the fibroblast growth factor (FGF) family appears to have a central role in the epithelial-mesenchymal interaction between apical ectodermal ridge and underlying mesenchyme; a member of the transforming growth factor-β (TGF-β) family appears to participate in the polarizing region pathway.

An FGF, FGF-4, appears to be central to signalling between apical ridge and underlying mesenchyme. The first clues came from studies on Fgf-4 gene expression in mouse embryos by Niswander and Martin [4]. They showed that transcripts of Fgf-4 are found in the posterior part of the apical ridge. They then showed, using an organ culture system of mouse limb buds, that FGF-4 could promote mesenchymal cell proliferation in buds stripped of their apical ectodermal ridges [5]. However, the fate of the new cells could not be determined because the cultured mouse limb buds can only survive a short time and therefore further experimental analysis was carried out in chick embryos [6]. Removal of the apical ridge of the chick wing bud leads to a rapid inhibition of bud outgrowth. A short period (24 h) after the operation (and the implantation of a bead soaked in saline), the bud is clearly stunted (Figure 2a). When a bead soaked in FGF-4 is stapled to the bud following ridge removal, outgrowth continues and a bulbous bud is formed (Figure 3b). Removal of the apical ridge from early buds leads to truncated limbs in which only a humerus forms. However, when beads soaked in FGF-4 are stapled in appropriate positions to the bud after ridge removal, a series of structures along the proximo-distal axis develops and even digits are formed [6]. The most complete limbs were obtained when two FGF-4 beads were placed simultaneously along the bud margin at its apex and

**Figure 2**

_Bmp-2 expression and chick wing pattern_

(a) Application of retinoic acid leads to expression of _Bmp-2_ transcripts in anterior cells. The section shown is through the bud 24 h after application of retinoic acid. The open arrow indicates the hole left by a bead soaked in retinoic acid, the closed arrow indicates the ectopic domain of _Bmp-2_ expression (compare with the left limb). There is a normal domain of posterior expression in both right and left limb buds. (b) Limb with mirror-image pattern of digits that develops from buds such as those shown in (a) treated with retinoic acid. Digit pattern is 4-3-2-3-4. Similar duplicated limbs develop following grafts of polarizing region cells or cells expressing _hedgehog_.

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**566**

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Figure 3
Effects of FGF-4 on outgrowth and patterning of chick wing buds

(a, b) Effect of FGF-4 on wing bud outgrowth. The figures show trunks of embryos 24 h after removal of the apical ridge from the right wing bud. (a) A bead soaked in saline is applied to apex; the right bud is severely truncated, compare with the left wing bud. (b) A bead soaked in FGF-4 is applied to apex; the bud has continued to grow out and is rather bulbous. (c) A limb that developed following removal of the apical ridge from the early bud. A bead soaked in FGF-4 was stapled to the posterior margin. The limb consists of humerus, ulna and posterior digits. Note the 'bunching' of the digits. (d) Transverse section through a wing bud in which an FGF-4 bead has been placed after removal of the apical ridge. The limb section is spherical, and the bead lies in centre.

posterior. A single bead placed posteriorly after ridge removal also gives a complete set of posterior structures along the proximo-distal axis, although the radius and digit 2 do not form (Figure 3c). The importance of posteriorly applied FGF-4 is that it maintains the posterior mesenchymal signalling region, the polarizing region [6,7]. New cells generated by FGF-4 in the absence of the ridge give rise to distal structures only if they are exposed to a polarizing region signal.

FGF-4 can very effectively substitute for the ridge signal that controls bud outgrowth. However, not unexpectedly, FGF-4 cannot substitute for the mechanical role of the ridge. In the normal limb bud, the apical ridge stiffens the ectodermal covering of the bud and leads to dorso-ventral flattening. When the apical ridge is substituted by FGF-4 bead(s), the bud loses its dorso-ventral flattening (Figure 3d) and becomes spherical in cross-section. This change in bud shape could lead to the characteristic bunching of digits that occurs in FGF-4-treated limbs in the absence of the ridge [6].

Members of the TGF-β superfamily are expressed in developing limb buds and participate in the polarizing region pathway. Transcripts of bone morphogenetic proteins (BMPs) are found in both apical ridge and mesenchyme of early buds [8–10]. In the chick wing bud, transcripts that code for BMP-2 are expressed in a very restricted patch of posterior mesenchyme, which is where the polarizing region is located. As the bud grows out, the domain of Bmp-2 transcripts remains near the tip, just as polarizing activity does. The signal from the polarizing region can be mimicked by local applica-
tion of retinoic acid, a vitamin A derivative, to the anterior margin of a wing bud [11]. Endogenous retinoids are present in chick wing buds and wing bud cells are equipped with molecules that allow them to respond to retinoids [12]. However, it has been suggested that retinoic acid does not dictate cell fate directly but acts by inducing production of a second signal [13]. The pattern of gene expression of BMP-2 makes it a candidate for that signal [10]. Consistent with this hypothesis, BMP-2 does appear to act downstream of retinoic acid. When a bead soaked in retinoic acid is implanted at the anterior margin of a chick wing bud, an ectopic domain of Bmp-2 transcripts is induced in anterior cells. However, application of BMP-2 protein on a bead to the anterior margin does not lead to the formation of additional digits. This suggests that BMP-2 is expressed in response to the polarizing region signal rather than being the signal itself. Recent work has identified another signalling molecule expressed at the posterior margin of the wing bud. Transcripts of a vertebrate homologue of the Drosophila gene called hedgehog also appear to co-localize with polarizing activity and expression can be induced by retinoic acid [14]. However, unlike BMP-2, application of hedgehog protein (by grafting cells expressing hedgehog) leads to additional digits.

Growth factors clearly provide important signals in the limb. A number of target genes that could be regulated by these limb signals have been identified. Genes whose expression in the progress zone is regulated by apical ridge signals include Evx-1 (a vertebrate homologue of the Drosophila gene called even-skipped) [15] and Msx-1 [16]. Genes whose expression is regulated by polarizing region signals include genes of the Hox-D complex [17], which are expressed in overlapping domains across the antero-posterior axis of the limb bud [17]. When wing buds are manipulated by grafting a polarizing region or cells expressing hedgehog, or by application of retinoic acid, a mirror-image pattern of Hox-D gene expression is induced that presages development of a mirror-image pattern of digits. The anterior cells now express 5' (posterior) Hox-D genes. Grafs of polarizing region or application of retinoic acid also induce ectopic domains of Bmp-2 transcripts. In the future, it should be possible to elucidate these pathways in more detail, find out which targets are direct and which indirect and identify the sequence of genes in the signal and response cascades. A major challenge will then be to understand how molecular responses to signals are translated at the cellular and tissue levels to give particular structures. How does expression of, say, a particular combination of Hox genes lead to the development of a particular digit?

Finally, the two signalling systems must act in concert. The limb pattern is established in a co-ordinated fashion along both the proximo-distal and the antero-posterior axis as the bud grows out. A link between signalling of the apical ridge and signalling of the polarizing region is seen in activation of genes of the Hox-D complex [18]. When a polarizing signal (retinoic acid) is applied to the anterior margin of the limb bud, 'posterior' Hox-D genes are not activated in anterior cells if the apical ridge is removed. A second link between growth and patterning is also mediated by the apical ridge. The number of structures across the antero-posterior axis, in particular the number of digits, is related to the length of the apical ridge. When additional digits are specified by either grafting a polarizing region or applying retinoic acid, the apical ridge is maintained over the anterior part of the bud and the bud becomes broader [19]. It is possible that BMP-2 could act as a signal between mesenchyme and ridge to regulate ridge length.

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3 Saunders, J. W. and Gasseling, M. T. (1968) in Epithelial–Mesenchymal Interactions (Fleischmajer, R. and Billingham, R. E., eds.), pp. 78–97, Williams & Wilkins, Baltimore, MD
8 Lyons, K. M., Pelton, R. W. and Hogan, B. L. M.
Sonic hedgehog: a key mediator of anterior-posterior patterning of the limb and dorso-ventral patterning of axial embryonic structures

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Introduction
The physical complexity of higher organisms arises during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic signalling. Instructive cell-cell interactions are crucial in vertebrate development from the earliest establishment of the body plan to the patterning of the organ systems to the generation of diverse cell types during tissue differentiation (reviewed in [1-3]). During the past century of experimental embryology, the spatial origin of many important embryonic inductive signals has been defined by various ablation and transplantation experiments. However, for many of these inductive interactions, the molecules mediating the signals have not been identified. For example, the organization of neural and mesodermal structures along the body axis is known to be regulated by a complex series of signals originating from the ventral midline (Figure 1a). Signals from the notochord direct the differentiation of the floor plate, a specialized group of ventral midline neural tube cells [4-8]. Subsequently, both the notochord and the floor plate produce instructive signals which determine the identity of ventral neuronal cells such as motor neurons [8-14]. In addition, signals from the floor plate are responsible for the orientation and direction of commisural neuron outgrowth [15]. Besides patterning the neural tube, the notochord and floor plate are responsible for producing signals which control patterning of the somites by promoting ventral somite differentiation [16,17].

Another important signalling centre exists in posterior mesenchyme of developing limb buds, called the zone of polarizing activity (ZPA) (Figure 1b). Grafting of mesenchymal tissue from the ZPA to the anterior margin of a second limb bud results in the development of a wing with duplicated structures along the anterior-posterior axis with mirror-image symmetry [18]. It is believed that this assay reflects an endogenous function of the ZPA in specification of structures along the anterior-posterior limb axis. The posterior limb bud mesenchyme also plays critical roles in the maintenance of specialized limb bud ectoderm [18] and in the regulation of programmed cell death [19-21].

The molecular architects of these processes are beginning to be revealed. Recently, we [22] and others [23-25] have identified a potentially secreted protein, Sonic hedgehog, which appears to be a key mediator of the activity of the ZPA. When ectopically expressed early in limb development, Sonic profoundly affects the pattern of skeletal structures of the limb [22]. More specifically, when introduced on the anterior side of the limb bud, ectopic Sonic leads to mirror-symmetric duplications of digits much like those induced by the ZPA. In addition to its dramatic effect on limb development, Sonic can

Abbreviations used: CNS, central nervous system; ZPA, zone of polarizing activity.

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