Annu. Rev. Cell Dev. Biol. 2001. 17:87–132
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# PATTERNING MECHANISMS CONTROLLING VERTEBRATE LIMB DEVELOPMENT

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**Key Words** AER, BMP, FGF, Hedgehog, limb, morphogen, pattern formation, regeneration, secreted factors, vertebrate development, WNT, ZPA

■ **Abstract** Vertebrate limb buds are embryonic structures for which much molecular and cellular data are known regarding the mechanisms that control pattern formation during development. Specialized regions of the developing limb bud, such as the zone of polarizing activity (ZPA), the apical ectodermal ridge (AER), and the non-ridge ectoderm, direct and coordinate the development of the limb bud along the anterior-posterior (AP), dorsal-ventral (DV), and proximal-distal (PD) axes, giving rise to a stereotyped pattern of elements well conserved among tetrapods. In recent years, specific gene functions have been shown to mediate the organizing and patterning activities of the ZPA, the AER, and the non-ridge ectoderm. The analysis of these gene functions has revealed the existence of complex interactions between signaling pathways operated by secreted factors of the HH, TGF-$\beta$/BMP, WNT, and FGF superfamilies, which interact with many other genetic networks to control limb positioning, outgrowth, and patterning. The study of limb development has helped to establish paradigms for the analysis of pattern formation in many other embryonic structures and organs.

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## INTRODUCTION

Embryonic development could be defined as the orderly, stereotyped process that adds complexity to the initial relative simplicity of a fertilized egg. Complexity not only in terms of the increase in the number of total cells and the number of different cell types that comprise the embryo, but also in the number of recognizable organs or structures that accessorize the basic body plan, and in the number of functions that these organs and structures perform. Thus as development proceeds, a tight spatial and temporal control of gene expression and cell behavior sculpts the developing embryo by adding specific morphological and functional features, which will actually determine the lifestyle and functionality of the adult animal.

During the development of many structures and organs there is an initial stage in which a primordium (or precursor structure) is induced in a specific location in the embryo in response to pre-existent combinatorial positional cues. The primordium is composed of a selected group of embryonic cells, which may or may not belong to the same type of embryonic layer (endoderm, mesoderm, or ectoderm). In the primordium, very often it is the establishment of cell-cell interactions between its mesenchymal and epidermal components that results in coordinated growth and patterning of structures derived from both layers. Interestingly, it appears that an evolutionarily conserved set of specific molecular interactions and cellular events operates in the primordia of many structures and organs, including some as diverse as the limbs, teeth, facial structures, hair follicles, kidneys, lungs, gut, and pancreas. For example, members of the Hedgehog (HH), WNT, transforming growth factor-$\beta$ (TGF-$\beta$)/bone morphogenetic proteins (BMPs) and fibroblast growth factor (FGF) families of secreted factors, along with their transducers and modulators, appear to constitute a sort of ancient "genetic toolbox" that is used time and again in the embryo to build limbs, teeth, lungs, or kidneys. The final result (what the structure or organ actually looks like) depends on the activity of particular downstream (or "realizator" genes) that are expressed (or active) in one organ or structure but not in

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another, so that a limb ends up looking very different from a tooth, even though the underlying molecular and cellular mechanisms that have sculpted both structures are remarkably similar.

Among the structures and organs mentioned above, the vertebrate limb bud is clearly an excellent experimental model to study the cellular and molecular mechanisms that regulate pattern formation during embryogenesis. Vertebrate limbs are appendages that perform crucial roles, being involved in locomotion, feeding, copulation, communication, and other complex tasks. Over the years, classical embryological studies in avian and amphibian embryos, combined with essays of ectopic expression in the chick and gene knockouts in the mouse, have greatly contributed to our understanding of how growth and patterning are integrated in the limb bud (reviewed by Tickle & Eichele 1994, Johnson & Tabin 1997, Schwabe et al. 1998). From these and other studies, it is clear that many mechanisms that regulate growth and patterning in the limb bud are also used in many other embryonic structures and organs throughout embryonic development. A synthesis of embryological, cellular, molecular, and evolutionary approaches to the study of limb development in different organisms has provided useful insights into a variety of patterning mechanisms that appear to be conserved during evolution.

In the case of the vertebrate limb, a primordium (the limb bud) appears at specific locations in the developing embryo (Figure 1*A*), positioned by combinations of factors that provide positional cues. Later on, the limb bud, which is made up of mesenchymal cells covered by ectoderm, starts growing out of the body wall, and specific epithelial-mesenchymal interactions are established that coordinate growth and patterning through the activities of specialized regions of the limb bud that act as organizers (Figure 1*B*). This basic mechanism, which operates (with some variations) in most tetrapods (vertebrates with four limbs), results in the development of a variety of adult vertebrate limbs that, despite their varied morphologies and functionalities, share a common morphologic plan (Figure 1*C*). Here we review what is currently known about the patterning mechanisms that control the early development of vertebrate limbs. For simplicity, we mostly refer to what is known about mouse and chick limbs because they have been extensively studied using a variety of experimental techniques; we refer to peculiarities of other vertebrate limbs when necessary. We start with the allocation of the limb primordia in the embryo, followed by the mechanisms of limb induction, outgrowth, and patterning, also mentioning what is known about some aspects of cell differentiation. Finally, we discuss some open questions and future directions regarding patterning mechanisms in the vertebrate limb bud.

## ALLOCATING THE LIMB FIELDS

The first step in the development of a vertebrate limb is the determination of a group of embryonic cells that will give rise to the limb primordium (or limb bud). These so-called limb fields are initially composed of cells within the lateral plate

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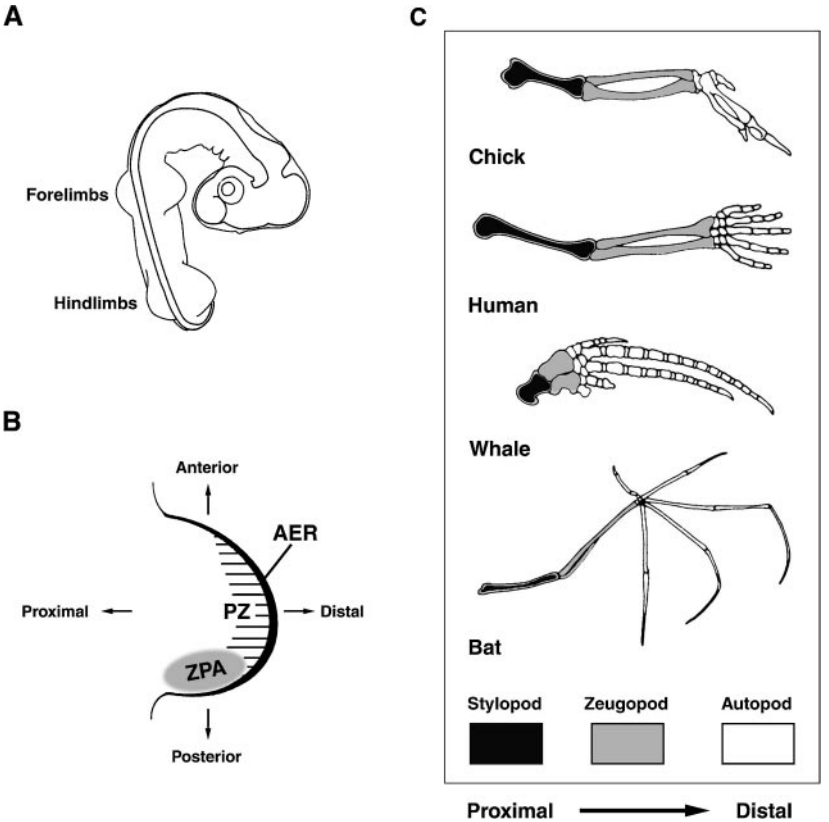


**Figure 1** The basic structures of the limb bud and the adult limb are conserved among vertebrates. (*A*) Vertebrate limbs originate as two pairs of primordia (limb buds) that appear at specific levels of the embryonic flank (forelimb and hindlimb buds are indicated in a chick embryo). (*B*) Dorsal view of the limb primordium (limb bud), which is composed of mesenchymal cells encased in an ectodermal jacket and contains specific regions that pattern the bud along the anterior-posterior (AP), dorsal-ventral (DV) and proximal-distal (PD) axes. The ZPA (zone of polarizing activity) patterns the AP axis, and the AER (apical ectodermal ridge) maintains outgrowth of the limb bud, keeping underlying mesenchymal cells in the PZ (progress zone) in an undifferentiated state. Not shown, the dorsal and ventral ectoderm determine the DV polarity of the distal part of the limb. In fish and amphibians, the region corresponding to the AER is broader and is called apical epidermal cap. (*C*) Schematic representations of the skeletal structure of forelimbs from several vertebrates, as indicated. The basic skeletal structure of the vertebrate limb is remarkably conserved among amniote tetrapods; it consists of a proximal part (stylopod, in black) with a single skeletal element, a medial part (zeugopod, in gray) with two elements, and a distal part (autopod, in white) composed of carpus or tarsus and a variable number of radiating digits. Despite this general conservation, there is great morphological and functional diversity, most likely derived from variations in the molecular mechanisms that sculpt the limb, some of which are already known (see text).

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mesoderm (LPM) that are located in specific positions in the flank of the embryo. In this sense, development of the vertebrate limb bud is no different from that of any other embryonic structure or organ, where the first step is always the selection of a group of cells that are competent to form an anlage or primordium.

Before considering how limb fields are allocated in the vertebrate embryo, it is perhaps worthwhile to point out some evolutionary considerations about limb number. Invertebrate chordates, such as Amphioxus (the closest invertebrate relative of vertebrates), are limbless. Among vertebrates, the first paired appendages (which were paired fins) appeared in jawless fish (agnathans), and tetrapods like frogs, mice, or humans, have two sets of paired appendages (or limbs) (Figure 1*A*). Our forelimbs and hindlimbs are evolutionarily derived, respectively, from the pectoral and pelvic fins of primitive jawed vertebrates (gnathostomes) (reviewed by Carroll 1988, Coates 1994). Most living vertebrates are tetrapods, but not all vertebrates have limbs. For example, several vertebrate taxa display reduction (and even absence) of limbs, including animals as diverse as snakes and whales (Carroll 1988, Cohn & Tickle 1999, Greene & Cundall 2000). For the purpose of this review, however, we focus on the basic tetrapod body plan, where two pairs of appendages are present. Thus the primordia of the two pairs of limbs or fins (limb or fin buds) originate in four specific areas of the flanks of the early embryo, where groups of cells in the LPM form small buds of mesenchymal cells encased in an ectodermal jacket (Searls & Janners 1971) (Figure 1*B*). But how are these four areas of the early embryo selected in the first place?

## The *Hox* Code and Other Elements of the Pre-Pattern

*Hox* genes, first identified in *Drosophila melanogaster*, encode homeodomain transcription factors shown to provide spatial cues during the development of many embryonic structures in vertebrates and invertebrates (reviewed by Krumlauf 1994, Deschamps et al. 1999), including those that allocate the limb fields in a variety of organisms. Both in *Drosophila* and vertebrates such as mouse and chick, specific combinations of *Hox* genes are expressed at different levels of the embryonic trunk, thus conferring positional identity along the AP embryonic axis. A detailed analysis of the expression profiles, evolutionary significance, and roles of *Hox* genes in the vertebrate embryonic axis is beyond the scope of this review (but see Duboule 1998, Deschamps et al. 1999, Valentine et al. 1999), and thus we refer only to experimental results relevant to the problem of limb positioning.

Several lines of evidence support the involvement of a combinatorial *Hox* code in positioning the vertebrate limb fields. First, in a variety of vertebrates the anterior expression boundaries of *Hox* genes such as *Hoxc6*, *Hoxc8*, and *Hoxb5* in the LPM occur exactly at the forelimb (or pectoral fin) level (Oliver et al. 1990, Rancourt et al. 1995, Nelson et al. 1996), suggesting a role for these genes in the specification of this particular axial level of the embryo. Mice lacking the *Hoxb5* gene have the shoulder girdle slightly shifted (Rancourt et al. 1995), which confirms a role for *Hoxb5* in allocating the forelimb field. Additionally, it has been shown that axial

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shifts in the position of the forelimb correspond with shifts in *Hox* expression domains when comparing chick and mouse wild-type embryos (Burke et al. 1995, Gaunt 2000). Second, a thorough study of the embryonic expression of several *Hox* genes in the chick embryo has revealed that specific combinations of *Hox* gene expression in the embryonic trunk and LPM associate well, both with the levels at which the limbs are going to develop, and with the type of limb that develops (Cohn et al. 1997). Third, the absence of forelimbs in some snakes correlates well with specific changes in *Hox* gene expression domains in both paraxial mesoderm and LPM (Cohn & Tickle 1999). Taken together, these results suggest that the limb fields are induced in the embryonic flank at specific positions that contain a certain combination of *Hox* gene expression.

But how are the overlapping domains of *Hox* gene expression established in the embryonic trunk? In the embryonic axis, *Hox* gene expression is controlled by a variety of factors that include at least three types of transcriptional regulators, retinoic acid receptors (RARs), the *Krox20* gene, members of the *Pbx/Exd* family of cofactors, the *Hox* genes themselves, and also secreted factors of the FGF and TGF-$\beta$ superfamilies. Here we briefly mention only a few of these regulators and how they relate to this process.

Retinoic acid (RA) is involved in controlling *Hox* gene expression in the LPM at the time at which the limb fields are determined (Marshall et al. 1996). Inhibition of RA activity in the embryonic flank of the chick downregulates expression of the *Hoxb8* gene (Lu et al. 1997), which has some involvement in the initial AP polarity of the limb bud, and retinoid-deficient quail embryos have limb buds with abnormal AP and DV patterning (Stratford et al. 1999). Conversely, an excess of RA administered during embryogenesis can alter the pattern of the axial skeleton, probably due to rostral shifts in *Hox* gene expression (Iulianella & Lohnes 1997). Also, it has been demonstrated that the *Hoxb8* gene has regulatory elements that bind Cdx proteins (Charité et al. 1998). These proteins are homologs of *Drosophila* Caudal, a protein involved in AP patterning in the fly embryo. Other *Hox* genes are also regulated by Cdx proteins, and Charité and collaborators have proposed an ancestral role for Cdx/Caudal proteins in specifying AP axial patterning in a variety of organisms through the control of *Hox* gene expression boundaries.

Recently, it was shown that GDF11, a TGF-$\beta$ factor, plays a role in the AP patterning of the axial skeleton. *Gdf11*-deficient mice show anteriorly directed homeotic transformations throughout the axial skeleton and posterior displacement of the hindlimbs (McPherron et al. 1999). These defects are correlated with alterations in patterns of *Hox* gene expression, which suggests that GDF11 acts upstream of the *Hox* genes to specify positional identity along the AP axis. Moreover, the promyelocytic leukemia zinc finger (PLZF) protein may also act as a regulator of *Hox* gene expression im the embryonic axis and limb buds (Barna et al. 2000).

Thus the mechanisms controlling *Hox* expression in the embryonic trunk are not entirely known, but it appears that the interaction of the *Hox* code with a variety

of regulators establishes a sort of pre-pattern in the embryonic axis that contributes to allocate the limb fields in a variety of vertebrates.

## INDUCTION OF LIMB BUDDING

After the forelimb and hindlimb fields have been specified at precise locations along the embryonic flank, the corresponding cells in the LPM engage in active cell division, whereas cells in the non-limb flank LPM divide more slowly (Searls & Janners 1971). This differential cell proliferation results in the development of a noticeable limb primordium (or limb bud) consisting of a mass of mesenchymal cells encased in an ectodermal jacket (Figure 1*B*). The series of events that culminate in the initiation of limb budding in the embryonic flank is called limb induction, and it involves a directional transfer of positional information in the embryonic axis and flank between several key tissues and structures.

### The Role of the Intermediate Mesoderm and Other Axial Structures

It is important to point out that the exact mechanism of limb induction is still a matter of controversy (reviewed by Martin 1998). As indicated above, a variety of factors set up a pre-pattern that specifies the levels at which limb buds are going to develop in the flank. Subsequently, this positional information needs to be interpreted by several key tissues that play important roles during the actual induction of limb budding. It is known that limb induction in the chick embryo is inhibited when a barrier is placed between the LPM and the intermediate mesoderm (IM). The IM (precursor of the kidney) lies between the somites and the LPM (Figure 2), and its extirpation results in limb reduction (Stephens & McNulty 1981, Strecker & Stephens 1983, Geduspan & Solursh 1992). These observations first suggested that the IM may be the source of a diffusible limb inducer, which would operate on the LPM. However, because the IM develops along the entire length of the embryo and limb budding is restricted to very specific positions along the flank, the putative inducer must necessarily display a restricted pattern of expression and/or activity in the IM.

An excellent candidate to mediate limb induction from the IM is the product of the *Fgf-8* gene, which encodes a member of the FGF superfamily of secreted factors. The *Fgf-8* gene is expressed transiently and dynamically in the IM at the forelimb and hindlimb levels before and during limb induction (Figure 2, shown in black), and the FGF-8 protein can maintain cells in a proliferative state at the flank positions that correspond to the limb fields (Crossley et al. 1996, Vogel et al. 1996). Moreover, the FGF-8 protein (and other proteins of the same family) are capable of directing initiation and normal development of an ectopic limb bud from the embryonic flank (Cohn et al. 1995, Mahmood et al. 1995, Ohuchi et al. 1995, Crossley et al. 1996, Vogel et al. 1996, Yonei-Tamura et al. 1999). This indicates that

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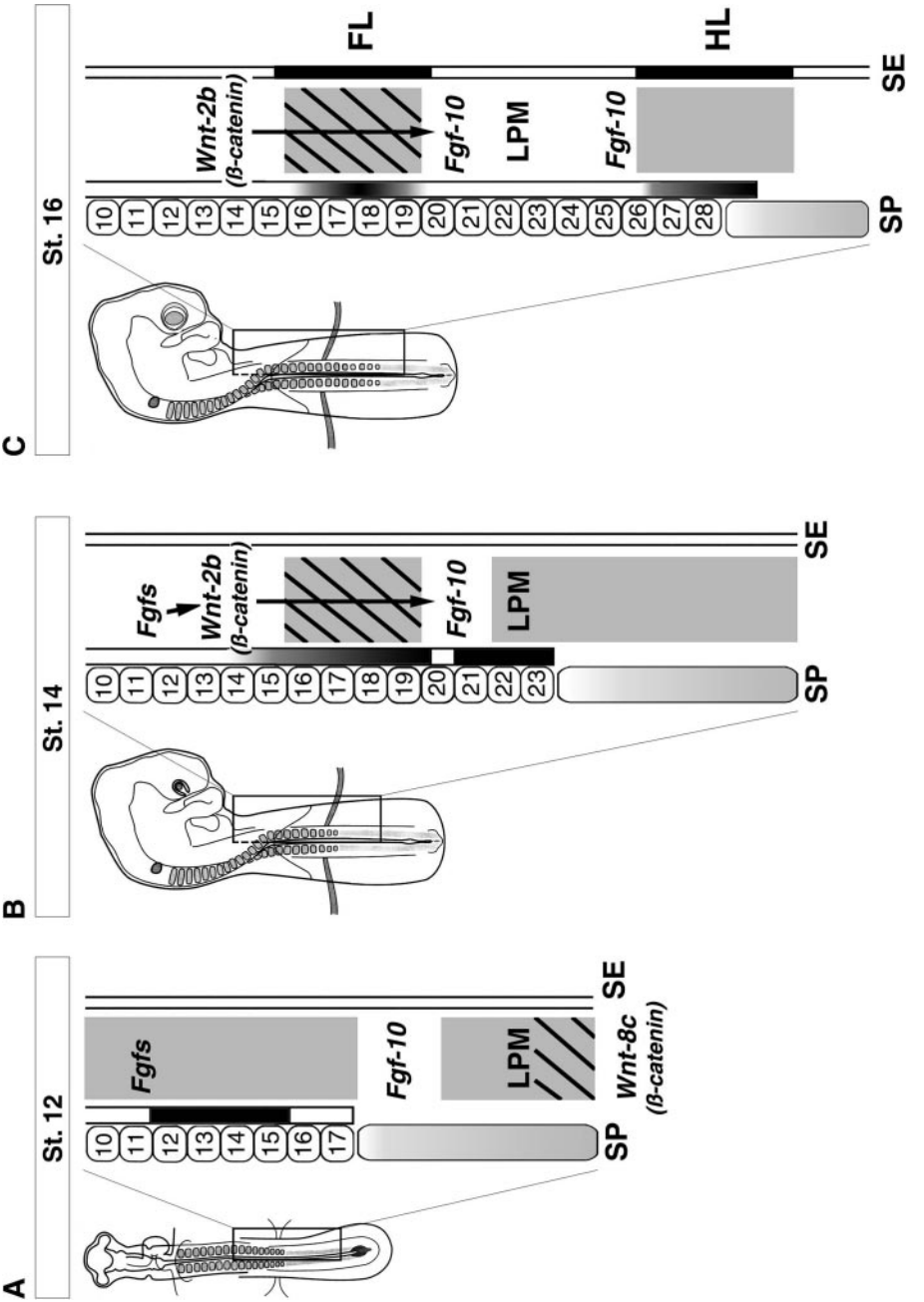

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the whole embryonic flank is, in principle, competent to form a limb and suggests that the sole purpose of the pre-pattern could be to localize expression of *Fgf* genes to specific positions in the axial structures (such as the IM) that normally induce limb formation. Based on these and other results, it has been proposed that FGF-8 expression in the mesonephros (one of the two components of the IM) induces limb initiation (Crossley et al. 1996). However, a recent study has challenged the notion that the mesonephros is required for limb initiation (Fernández-Terán et al. 1997). The elucidation of the exact role of FGF-8 in limb induction will probably require tissue-specific ablation of *Fgf-8* activity in the mesonephros at stages prior to limb induction, along with the analysis of other *Fgf* genes that could display partially redundant activities.

The IM is not the only tissue involved in limb induction. In the chick embryo, experiments involving grafting of the prospective forelimb region to an ectopic site have shown that limb induction occurs between stages 13–15 (although the limb bud itself is not morphologically recognizable until stage 17; stages according to Hamburger & Hamilton 1951). However, at early stages (8–9), grafts of prospective forelimb region can also develop a limb, but only if the embryonic organizer (Hensen's node), somites, and IM are all included in the graft. As development proceeds, fewer tissues are required to induce a limb, so that at stages 12–14, only the IM is required for limb induction. This indicates that several axial tissues medial to the LPM may indeed be involved in limb induction, including somites

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**Figure 2** Interpretation of the pre-pattern and allocation of the limb fields in the embryonic flank by FGFs and WNTs. In the early chick embryo, a pre-pattern of combinatorial gene expression (*Hox* genes, RA, *Gdfs*, etc, not shown here for simplicity) is translated into restricted patterns of gene expression in the key tissues involved in limb induction. In this way, the limb fields are allocated to specific levels of the flank LPM (indicated to the right as FL, forelimb, and HL, hindlimb). The tissues involved in the allocation of the limb fields and limb induction are somites, indicated as circles with their respective number; SP, segmental plate; IM, intermediate mesoderm, indicated as a bar right next to the somites; LPM, lateral plate mesoderm; SE, surface ectoderm. (*A*) At stage 12, the *Fgf-10* gene (gray) is still widely expressed in the SP and the LPM, whereas the *Fgf-8* gene (black) is restricted to a specific level of the developing IM, adjacent to somites 12–16. Caudally, expression of the *Wnt-8c* gene in the LPM (black stripes) partially overlaps with *Fgf-10*. (*B*) At stage 14, a restricted anterior domain of *Fgf-10* expression is clearly visible, probably activated by another *Wnt* gene, *Wnt-2b* (black stripes), which is controlled by FGFs produced by the adjacent IM. Caudally, a second restricted domain of *Fgf-10* expression begins to resolve. (*C*) At stage 16, the two restricted domains of *Fgf-10*, which correspond to the prospective forelimb (FL) and hindlimb (HL) areas, are clearly defined at specific levels of the LPM. FGFs are still present in adjacent segments of the IM, and *Wnt-2b* is still present in the LPM of the presumptive FL area. In the SE, expression of *Fgf-8* (black) is activated by FGF-10 coming from the LPM.

and IM, both tissues where FGFs and other secreted factors are present. Again, the elucidation of the individual roles of FGFs and other factors expressed in these tissues may require tissue-specific gene ablation techniques in mouse embryos.

## The FGF-8/FGF-10 Loop and Its Interaction with WNT Signals

The limb initiation model indicated above states that localized sources of FGF-8 (and probably other FGF proteins also present in the IM and other axial tissues) signal to the adjacent LPM cells to induce limb formation. Interestingly, another member of the same gene family, *Fgf-10*, seems to be involved in mediating the inductive effect of FGF-8 on the LPM cells (Ohuchi et al. 1997, Xu et al. 1998, Yonei-Tamura et al. 1999). The *Fgf-10* gene is initially widely expressed in the segmental plate (SP) and the LPM (Figure 2*A*; pattern of expression shown in gray) (Ohuchi et al. 1997), but around stage 14 in the chick embryo, it becomes restricted to the LPM cells of the prospective forelimb area (Figure 2*B*). Recent results indicate that FGF-8 (and probably other FGFs and even other secreted factors expressed in the LPM) controls expression of the *Wnt-2b* gene in the LPM of the prospective forelimb area, and that *Wnt-2b* acts as an upstream regulator of *Fgf-10* in the LPM, thus mediating the induction of *Fgf-10* by *Fgf-8*. Indeed, *Wnt-2b* is expressed in the somites, the IM, and the LPM of the prospective forelimb area, and its ectopic expression alone can induce development of an ectopic limb in the flank (Kawakami et al. 2001). Expression of *Wnt-2b* is shown as black stripes in the forelimb area in Figure 2*B–C*. Expression of another *Wnt* gene, *Wnt-8c* (black stripes in Figure 2*A*), may be involved in restricting *Fgf-10* expression to the prospective hindlimb area (Figure 2*A–C*). Both WNT-2B and WNT-8C proteins signal through $\beta$-catenin and, moreover, the canonical WNT/$\beta$-catenin pathway appears to be both necessary and sufficient for induction of both forelimbs and hindlimbs through the control of *Fgf-10* (Kawakami et al. 2001). Thus WNT/$\beta$-catenin pathways mediate the FGF-8/FGF-10 loop that controls limb initiation, and localized expression of *Fgf-10* in the LPM appears to be the key factor for limb induction.

Consistent with this model of limb initiation, mostly derived from studies in the chick, targeted mutation of the *Fgf-10* gene in mice results in the absence of limbs, most likely owing to the interruption of limb budding and the inability to induce an apical ectodermal ridge (AER) (Min et al. 1998, Sekine et al. 1999) (see below).

## Determining the Identity of Wings and Legs

The series of events explained above culminates in the initiation of limb budding in four specific locations of the embryonic flank, giving rise to a pair of forelimbs and a pair of hindlimbs. Despite the fact that forelimb and hindlimb buds look very similar at the earliest stages of development, morphological differences soon begin to appear, and in most tetrapods adult forelimbs and hindlimbs look very

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different and usually perform different functions. Recent discoveries have shed light on the molecular mechanisms that determine morphological and functional differences between forelimbs and hindlimbs.

During the evolution of vertebrates, forelimbs and hindlimbs appeared at around the same time as pectoral and pelvic fins in jawless fish (agnathans) (Coates 1994, Ruvinsky & Gibson-Brown 2000). Despite some controversies on the exact path of vertebrate limb evolution, forelimbs and hindlimbs are generally considered serially homologous structures, which implies that the molecular mechanisms used to build these two pairs of very similar (but not identical) structures at different locations in the embryo are basically the same (Shubin et al. 1997, Ruvinsky & Gibson-Brown 2000). In fact, most genes display the same expression pattern in forelimbs and hindlimbs, which of course results in the generation of similar patterns of bone structure and other morphological features in forelimbs and hindlimbs. Still, some specific molecular differences must exist that account for the morphological and functional differences observed betwen these two kinds of appendages.

The decision to become either a forelimb or a hindlimb appears to be made at the earliest stages of limb initiation, prior to limb budding, as shown by transplantation experiments performed in the chick embryo. For instance, when pre-bud LPM cells belonging to the forelimb field are transplanted into an ectopic location, the limb always develops as a forelimb, indicating that the identity of the limb resides in the mesoderm (and not the ectoderm) and is determined even before limb budding begins (Zwilling 1955). Indeed, when beads soaked in FGF protein are implanted in the interlimb region (resulting in induction of an ectopic limb), ectopic expression of fore- and hindlimb-specific genes such as *Tbx-5* or *Tbx-4* (see below) is induced very rapidly (i.e., 1 h after) (Isaac et al. 2000), which further suggests that the selection of limb type occurs very early.

In recent years, several genes have been shown to be expressed exclusively in forelimbs or hindlimbs in mouse, chick, zebrafish, and other organisms, including two members of the T-box gene family, *Tbx-4* and *Tbx-5*. Both genes are detected in the LPM prior to limb budding, *Tbx-5* in the presumptive forelimb area and *Tbx-4* in the presumptive hindlimb area (Bollag et al. 1994, Simon et al. 1997, Gibson-Brown et al. 1998, Isaac et al. 1998, Logan et al. 1998, Ohuchi et al. 1998, Tamura et al. 1999, Begemann & Ingham 2000, Ruvinsky et al. 2000, Takabatake et al. 2000). Other fore- or hindlimb-specific genes include members of the *Hox* gene family (*Hoxc4* and *Hoxc5*, restricted to the forelimb) (Nelson et al. 1996) and another transcription factor, *Pitx-1*, which encodes a member of the Otx-related subclass of paired-type homeodomain proteins (Lamonerie et al. 1996, Szeto et al. 1996) and is exclusively expressed in the hindlimb (Shang et al. 1997, Logan et al. 1998). Experiments involving loss of gene function in mice and ectopic expression in chick embryos have recently demonstrated the role of *Tbx-5* as a forelimb determinant (Rodríguez-Esteban et al. 1999, Takeuchi et al. 1999) and of *Tbx-4* and *Pitx-1* (most likely acting in concert) as hindlimb determinants (Lanctot et al. 1999, Logan & Tabin 1999, Rodríguez-Esteban et al. 1999, Szeto et al. 1999, Takeuchi

et al. 1999, reviewed by Weatherbee & Carroll 1999, Ruvinsky & Gibson-Brown 2000). Interestingly, expression of *Pitx* genes in the posterior mesendoderm of the developing embryo appears to be a conserved feature among all chordates (Yasui et al. 2000). This has led researchers to propose that posterior expression of *Pitx* genes predated the event of limb duplication, so that after the establishment of limb outgrowth program in the *Pitx* domain, *Pitx* and *Tbx* genes could co-evolve and cooperate in the establishment of hindlimb identity (Ruvinsky & Gibson-Brown 2000).

Despite all these recent advances, it is evident that we are far from a clear understanding of how limb identity is determined. The identification of additional genes displaying forelimb- or hindlimb-specific expression, along with the isolation of targets of *Tbx-4*, *Tbx-5*, and *Pitx-1*, will be necessary if we are to have a more complete picture of the mechanisms that control this process.

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## AER INDUCTION AND LIMB OUTGROWTH

Up until now, we have been calling the induction of *Fgf-10* (in the LPM) by FGF-8 (coming from the IM) a "loop". The reason for this is that the next step in the process of limb induction involves the activation of *Fgf-8* expression (in the ectoderm) by FGF-10 (produced in the forelimb and hindlimb areas). After expression of *Fgf-10* in the prospective forelimb and hindlimb areas has been consolidated in the LPM (Figure 2*C*), the FGF-10 protein signals to the overlying ectoderm (or surface ectoderm; SE) to initiate a program of gene expression that includes activation of *Fgf-8* transcription even before a limb bud is recognizable. These events are absolutely required for limb outgrowth because they culminate in the induction of the AER.

In many tetrapods and concomitantly with the initial stages of limb budding, inductive signals from LPM cells of the prospective limb bud area induce the overlying ectoderm to form a specialized structure (the AER), an ectodermal thickening that runs along the AP axis of the limb bud, separating the dorsal side of the limb from the ventral side (Figure 1*B*). In the chick embryo, where its properties have been extensively studied, the AER is morphologically detectable at stage 18, and its integrity is essential to keep the limb cells proliferating after the initiation of limb budding (Saunders 1948, Todt & Fallon 1984). When the AER is surgically removed, proliferation of the mesenchymal limb bud cells is affected and the limb is truncated (distal structures are missing). Truncations are more severe when the AER is removed early in development, which indicates that there is a differential temporal requirement for the AER.

AER activity is thought to apply to most tetrapods. However, not all tetrapod limbs have an AER. Most likely, the AER was already present in the common ancestor of anurans and amniotes, but it was later lost in several species that are direct developers, including several species of frogs, whose limb buds have a thickened apical ectoderm but no AER (Richardson et al. 1998). In other vertebrates, such as slowworms and other reptilians, the AER degenerates, and the adult is

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limbless. The molecular basis of this phenomenon is not well known, although it has been shown that it may be related to specific changes in *Hox* expression in the trunk. Moreover, FGF application can partially rescue limb bud outgrowth in embryos of slow worms and python (Raynaud et al. 1995, Cohn & Tickle 1999). In fish (such as the zebrafish, *Danio rerio*), the apical fin bud ectoderm does not form an AER, rather it transforms into a protruding fold (or apical epidermal cap) that encloses the dermal rays, thus terminating proliferation of the mesenchyme of the bud (Geraudie 1978). As a result, there is a proximal-distal subdivision of the mesenchyme, which forms four elements (called radials), and several peripheral foci form other distal radials. Thus different kinds of apical ectodermal structures control mesenchymal proliferation and patterning in specific ways that explain variations in morphology and function between limb and fin buds. In this section, we focus on the chick AER because it has been studied extensively to illustrate pattern mechanisms known to exist in several higher vertebrates.

## Allocating the AER

How is the AER precisely positioned within the ectodermal field, right at the interface between the presumptive dorsal and ventral cells of the limb ectoderm? Experiments involving fate mapping of the presumptive dorsal and ventral ectoderm of the limb have shown that ectodermal cells covering the LPM prior to limb induction are already committed to form the AER (marked as pre-AER cells in Figure 3*A*, *B*, black). The ectoderm that will give rise to the dorsal ectoderm of the limb overlies the somites (Figure 3*A*, light gray), and ectodermal cells located above the lateral somatopleural mesoderm will give rise to the ventral ectoderm of the limb (Figure 3*A*, *B*, dark gray) (Altabef et al. 1997, Michaud et al. 1997). As the limb bud grows out, the ectodermal cells migrate laterally (thin arrows, Figure 3*B*) to cover the mesenchyme. Recent results have shown that two distinct lineage boundaries exist in the mouse limb ectoderm prior to limb budding: one corresponding to the DV midline of the AER and the second to the dorsal AER margin (Kimmel et al. 2000) (thick arrows, Figure 3*B*). The molecular basis of the generation of ectodermal DV compartments prior to limb budding is still unknown, and there may be some differences among vertebrates.

Prior to AER induction by the underlying mesenchymal cells, the expression of several genes in the ectoderm covering the limb bud already reveals a DV heterogeneity, which again stresses the notion that DV polarity information originates in the embryonic trunk (somitic mesoderm and presumptive limb bud). For instance, the product of the gene *Radical fringe* (*Rfng*) is expressed in the dorsal ectoderm of the chick limb bud prior to AER induction (Laufer et al. 1997, Rodríguez-Esteban et al. 1997), and the homeobox-containing transcription factor EN-1 is expressed in the ventral ectoderm (Davis & Joyner 1988). Both *Rfng* and *En-1* genes are also expressed later in the AER (Figure 3*C*). The AER forms right at the interface between the cells that express *Rfng* (dorsal ectoderm) and the cells that do not (ventral ectoderm). The *En-1* gene acts by preventing *Rfng* from being expressed in the

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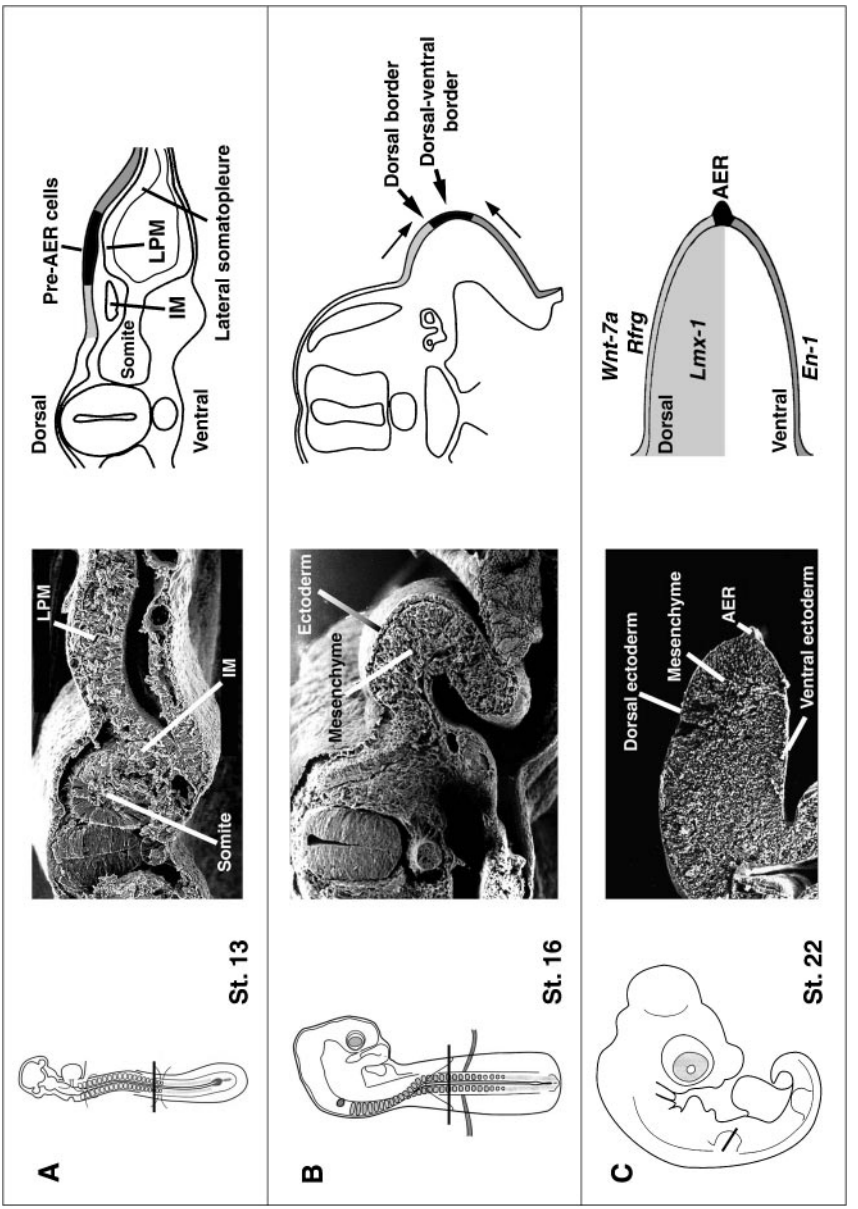

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ventral ectoderm, thus ensuring that a sharp boundary between *Rfng* expressing and non-expressing cells is maintained. When *En-1* is ectopically expressed in the dorsal ectoderm by using a retroviral vector (Laufer et al. 1997, Rodríguez-Esteban et al. 1997), *Rfng* is repressed in some cells and ectopic AERs appear, giving rise to outgrowths. Similar outgrowths and AER induction can be induced by transplanting *En-1*-overexpressing ectoderm (Tanaka et al. 1998). When *Rfng* is ectopically expressed in the ventral ectoderm, *Rfng* positive and negative cells juxtapose, giving rise to ectopic AERs and outgrowths. Although *Rfng* is expressed in the mouse limb bud in a pattern comparable to the chick, mutant mice that lack *Rfng* are normal, which indicates that *Rfng* function is not required for limb development in the mouse. This could result from functional overlapping with some other *fringe*-related gene expressed in the limb (Moran et al. 1999, Zhang & Gridley 1999).

## AER Induction

The specific requirements for AER induction in the limb ectoderm are only partially known, but the secreted factor encoded by the gene *Wnt-3a* appears to play an important role in this process. *Wnt-3a* is involved in the control of *Rfng* expression (and therefore, in the positioning of the AER) but also in the actual induction of the AER. Expression of *Wnt-3a* in the limb bud ectoderm is detected around the time at which *Fgf-8* also appears in the ectoderm, in response to FGF-10 emanating from the LPM (Figure 4*A*). In fact, recent data suggest that, in the surface ectoderm, *Wnt-3a* mediates the induction of *Fgf-8* by FGF-10. It is important to point out that the initial expression of *Wnt-3a* (and also *Rfng* or *Fgf-8*) occurs in a wide ectodermal domain, which is subsequently refined and restricted to the cells that form the AER. The mechanisms involved in this refinement, which actually control

←

**Figure 3** Origin of DV information and interactions that allocate the AER in the vertebrate limb bud. (*A*, *B*, *C*) Each panel first shows a scheme of a whole chick embryo at stages 13 (*A*), 16 (*B*), and 22 (*C*), followed by scanning electron micrographs (SEMs) of transverse sections (made at the levels indicated by black lines) and schemes that depict the fates of ectodermal cells at each stage. Light gray indicates cells fated to become dorsal limb ectoderm. Black indicates cells fated to become AER. Dark gray indicates cells fated to become ventral limb ectoderm. In the last scheme in (*C*), however, different levels of gray represent domains of gene expression, as indicated (see text). Thin arrows in (*B*) indicate ectodermal cells migrating to cover the developing mesenchyme, and thick arrows indicate the two DV compartment borders that exist in the developing limb ectoderm (Kimmel et al 2000). Some of the tissues referred to in the text are indicated. IM, intermediate mesoderm; LPM, lateral plate mesoderm; AER, apical ectoderm ridge. SEMs (that only show part of each embryo for simplicity) were generously provided by G Schoenwolf and J Hurlé. Some schemes were adapted from Michaud et al (1997).

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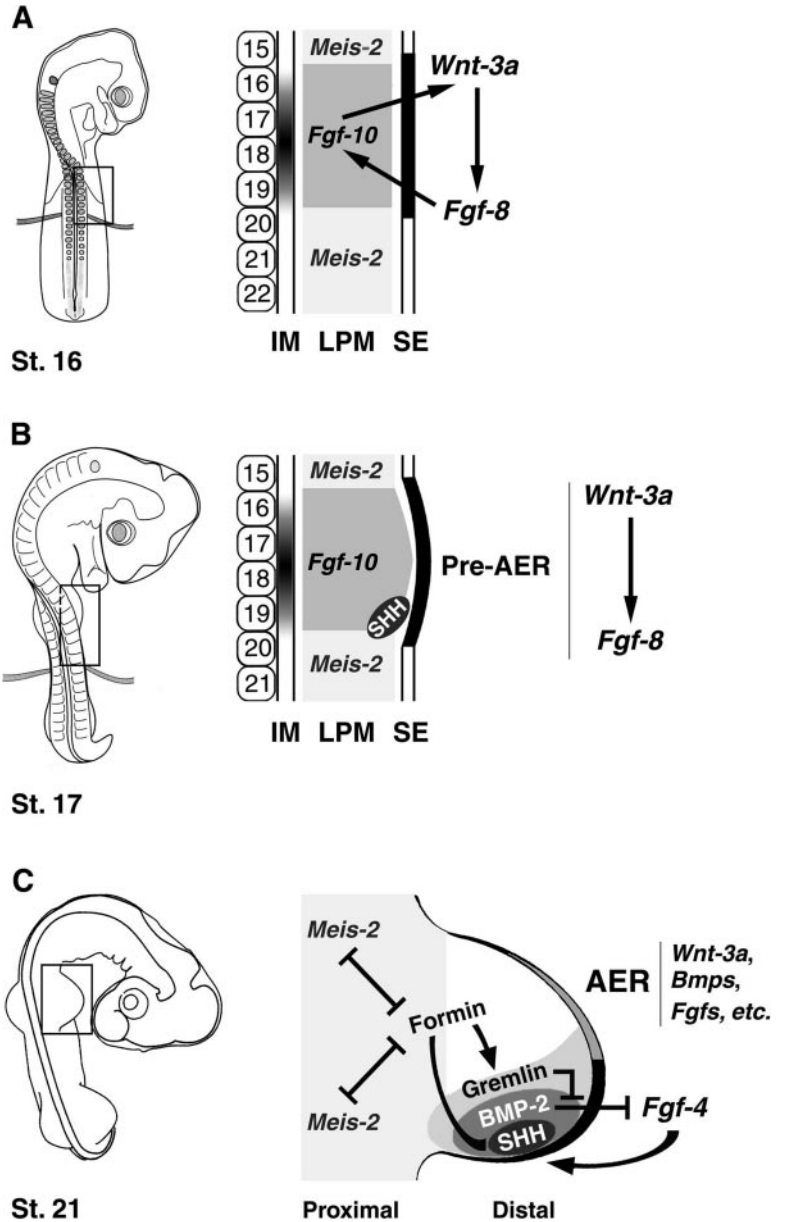

the width of the AER, are unknown, but they most likely read and interpret DV information already present in the ectoderm prior to limb budding.

Several lines of evidence indicate that AER induction by the WNT-3A protein involves the intracellular mediators $\beta$-catenin and LEF-1/TCF. For instance, in mice double mutant for *Lef-1* and *Tcf-1* (two genes encoding related, partially redundant mediators of the WNT/$\beta$-catenin pathway), *Fgf-8* expression is absent from the limb ectoderm, which does not form an AER (Galcerán et al. 1999). This is consistent with the proposed roles of WNT-3A and Lef-1 as regulators of AER formation, derived from ectopic expression studies in the chick (Kengaku et al. 1998). It is important to point out that ectopic expression of *Wnt-3a* (but not of *Fgf-8*) is capable of inducing ectopic AER formation and that experiments of ectopic induction of limb buds in the flank show that *Wnt-3a* appears in the limb before *Fgf-8*. Moreover, ectopic *Wnt-3a* can induce *Fgf-8* expression, but ectopic *Fgf-8* is unable to induce *Wnt-3a*. These and other data (Kengaku et al. 1998, Kawakami et al. 2001) suggest that FGF-10 activates *Wnt-3a* in the overlying ectoderm and that WNT-3A then signals through $\beta$-catenin to activate *Fgf-8* expression (Figure 4*A*).

## Dorsal-Ventral Patterning

A topic intimately related to the positioning of the AER is the generation of DV polarity in the limb bud itself (reviewed by Chen & Johnson 1999). Marked

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**Figure 4** Control of limb budding and coordinated development of AP and PD axes. In the schemes, only the forelimb area is shown, for simplicity. (*A*) At stage 16 in a chick embryo, FGF-10 (domain of gene expression is shown in dark gray), signals from the LPM to the SE, where it induces expression of *Wnt-3a*, which, in turn, triggers a signaling pathway mediated by $\beta$-catenin that activates *Fgf-8*. FGF-8 then signals back to the LPM to maintain *Fgf-10* expression. At this stage, *Meis* genes (light gray) are expressed in the whole LPM, including the *Fgf-10* domain (*Meis-2* is indicated here, but *Meis-1* has the same pattern). (*B*) At stage 17, budding is already evident, and the ectoderm cells expressing *Wnt-3a* and *Fgf-8* are considered as pre-AER. The AER will be morphologically defined slightly later, at stage 18. A small domain of *Shh* expression appears at the posterior margin of the budding limb mesenchyme. *Meis* genes are still expressed in the whole LPM, including the *Fgf-10* domain, but they begin to be restricted to the more proximal part. (*C*) From stage 17 on, *Meis* genes become restricted to the proximal part of the limb bud, and distally, the SHH protein controls expression of target genes such as *Bmp-2* and *Gremlin*, in a pathway mediated by Formin proteins (stage 21 is shown here). The Gremlin protein, a BMP antagonist, is postulated to antagonize a negative effect of BMPs on the AER, so that the AER can be maintained and *Fgf-4* can be expressed in the posterior half of the AER. FGF-4 and other FGFs signal back to the limb bud mesenchyme to maintain expression of *Shh* and other genes (not shown). There is mutual antagonism between *Meis* (proximal) and the SHH/BMP/Gremlin/FGF distal pathway.

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differences along the DV axis are evident in many vertebrate appendages. For example, in the case of the human hand, the back of the hand is dorsal and the palm is ventral, and muscles, tendons, and other structures show an orderly disposition along this axis. Surgical manipulations involving rotation of the chick limb bud ectoderm had previously suggested that signaling from the non-ridge ectoderm was responsible for the specification of cell fates along the DV axis, at least of the distal part of the experimental limb (MacCabe et al. 1974, Pautou 1977). These experiments indicate that, from around embryonic stage 15 on, the limb ectoderm provides DV positional information to the distal part of the limb bud.

The secreted factor encoded by the gene *Wnt-7a*, which is expressed in the dorsal ectoderm (Dealy et al. 1993, Parr et al. 1993) (Figure 3*C*), is a good candidate to convey a dorsal signal. WNT-7A controls the dorsal mesenchymal expression of the LIM-homeodomain factor LMX-1. Combined data from experiments involving ectopic expression in chick embryos and targeted gene disruption in mice have demonstrated that WNT-7A/Lmx-1 are involved in the specification of dorsal identities in the limb (Parr & McMahon 1995, Riddle et al. 1995, Vogel et al. 1995, Chen et al. 1998). Moreover, the expression of *En-1* in the ventral ectoderm appears to be required for the specification of ventral fates because limbs of *En-1*$^{-/-}$ mice display a double-dorsal phenotype (Loomis et al. 1996). In this scenario, WNT-7A acts as a dorsalizing factor expressed in the dorsal ectoderm, controlling the expression of *Lmx-1* in the underlying dorsal mesenchyme, and *En-1* acts as a ventralizing factor expressed in the ventral ectoderm, where it prevents *Wnt-7a* from being expressed (Cygan et al. 1997, Logan et al. 1997, Loomis et al. 1998). Interestingly, distinct mechanisms seem to control *Lmx-1* expression in the proximal and distal regions of the limb. Distal DV pattern seems to be controlled by the limb ectoderm and proximal DV pattern by the mesoderm. The identification of regulatory sequences that direct *Lmx-1* expression in the limb should provide valuable insights into the regionalized control of DV limb patterning. The mechanism by which the expression of *Lmx-1* in the dorsal mesenchyme determines dorsal fates is still unknown. Ectoderm-mesoderm interactions clearly continue to be important during the initial stages of development of tendons and muscles, which display DV polarity and develop directly underneath the dorsal and ventral ectoderm (Blagden & Hughes 1999, Büscher & Izpisüa Belmonte 1999). Development of muscles and tendons most likely involves interactions with signals such as BMPs, Noggin, and Indian hedgehog (IHH), which are expressed in the underlying cartilage elements (Blagden & Hughes 1999).

RA also seems to be involved in this particular patterning process. Retinoid-deficient quail embryos have limb buds with abnormal AP and DV patterning, the latter characterized by ectopic expression of *Wnt-7a* in the ventral ectoderm, corresponding expansion of *Lmx-1* into the ventral mesoderm, and absence of *En-1* from the ventral ectoderm (Stratford et al. 1999), all similar to what is observed in the chick *limbless* (Fallon et al. 1983, Grieshammer et al. 1996, Noramly et al. 1996, Ros et al. 1996) and mouse *legless* (Bell et al. 1998) mutants. Although these

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mutants display alterations of both DV polarity and AER formation, this does not imply that these two processes are necessarily co-dependent. For example, dramatic alterations of DV polarity that do not affect the AER at all are observed in a number of mutants and experimental situations, which indicates that DV polarity and AER formation are processes initiated by a common mechanism and later become independent.

Although many details are already known about AER positioning and DV patterning of the limb bud, the problem of how and when DV polarity actually originates in the early embryo is still a matter of controversy (reviewed by Chen & Johnson 1999). As indicated above, some experiments involving surgical manipulations of the chick limb bud and mesoderm and ectoderm recombinations suggest that the trunk mesoderm already has DV polarity at stage 12 and that the information is transferred to the ectoderm around stage 15 (Geduspan & MacCabe 1989). However, recent results stress the importance of inductive signals coming from the somites (Michaud et al. 1997), although it is not yet clear whether the somitic mesoderm influences primarily the mesoderm, the ectoderm, or both.

## FGFs Mediate AER Function

We have discussed how, in the chick embryo, *Fgf-10* is already restricted to the prospective forelimb and hindlimb areas of the LPM by stage 16, before *Wnt-3a* and *Fgf-8* appear in the presumptive limb ectoderm, and before the AER is morphologically detectable. Moreover, implants of *Fgf10*-expressing cells in the interlimb region are capable of inducing ectopic *Wnt-3a* and *Fgf-8* expression in the overlying ectoderm, all of which suggest that FGF-10 normally induces *Fgf-8* in the overlying ectoderm. Consistent with this, *Fgf-10*-deficient mice lack AER formation and *Fgf-8* expression in the limb bud ectoderm. Thus a regulatory loop that spans at least three different tissues (IM, LPM, and surface ectoderm) exists between *Fgf-8* and *Fgf-10* because the *Fgf-10* gene has been shown to be induced in the LPM by FGF-8 (emanating from the IM), and FGF-10 has been shown to signal to the surface ectoderm to induce *Fgf-8* and other AER markers (Ohuchi et al. 1997, Yonei-Tamura et al. 1999). As indicated above, the induction of *Fgf-8* by FGF-10 in the limb ectoderm is mediated by WNT-3A, and thus three Wnt genes that signal through $\beta$-catenin mediate the FGF-8/FGF-10 loop.

Interestingly, among all the factors known to be expressed in the AER (which are not discussed in detail here), only FGFs (including FGF-2, -4, and -8) are capable of substituting for the AER after its surgical removal (reviewed by Martin 1998). Thus these FGFs, which are unable to induce the AER, are nonetheless capable of performing its morphogenetic function. Owing to partial functional redundance, it is difficult to determine the individual importance of each particular FGF expressed in the AER. However, expression analyses and the generation of mice deficient in FGF receptors and in individual FGF factors have provided valuable information (Xu et al. 1998, Lizarraga et al. 1999, reviewed by Xu et al. 1999). From all these data, a model has been proposed where the spatial restriction of FGF ligands and

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receptors and the ligand-receptor specificity control an orderly transfer of signals between tissues involved in limb induction and outgrowth. Thus FGF-10 and FGF-8 appear to signal through the FGFR2b and FGFR2c alternative splice receptor isoforms, respectively. FGF-10 (expressed by the LPM) signals via ectodermally restricted FGFR2b to regulate *Fgf-8* expression in the overlying ectoderm; in turn, FGF-8 (from the AER) signals via mesodermally restricted FGFR2c to maintain FGF-10 expression in the LPM. This is precisely the last leg of the FGF-8/FGF-10 loop, which culminates in the maintenance of *Fgf-10* expression in the limb bud mesenchyme (Ohuchi et al. 1997), which appears to be required in turn to maintain the proliferation of these mesenchymal cells.

In the following we see that the main roles of the FGFs produced in the AER are to stimulate cell proliferation in the underlying mesenchyme (the progress zone) and to maintain *Sonic hedgehog* (*Shh*) expression; however, FGFs may also play additional roles, for example acting as chemoattractive agents that regulate patterns of mesenchymal cell migration during limb outgrowth (Li & Muneoka 1999). In general, the details of the cellular and molecular mechanisms involved in the transfer of signals from the AER to the mesenchyme are not well understood. For example, because the basal lamina that separates the AER from mesenchymal cells in the limb prevents passage of molecules as large as FGFs (Kelley & Fallon 1976, Wilcox & Kelley 1993), it is unclear how FGFs affect mesenchymal cells in the limb bud. The binding of FGFs to their receptors is a multistep process that requires interactions with additional factors. For example, the association of FGFs with cell-surface heparan sulfate proteoglycans (HSPGs) is a required step for high-affinity FGF receptor activation. Thus FGF activity may be regulated by a number of extracellular modulators. Recently, a specific CD44 splice variant has been shown to be involved in a crucial step of the epithelial-mesenchymal interaction that controls growth and patterning in the limb (Sherman et al. 1998). The CD44 variant has been shown to function in the AER by presenting FGFs to the underlying mesenchymal cells, thus revealing a novel growth factor presentation mechanism that could be involved in other physiological and pathological situations. AER-derived signals may also be passed on through gap junctions in the mesenchyme. Interestingly, FGF-4 has been recently shown to increase mesenchymal gap junctional communication (Makarenkova et al. 1997, Makarenkova & Patel 1999). The integrity of the basal lamina itself is also required for normal limb development. The basal surface of the epithelial cells of the limb bud is coated by a laminin $\alpha$5-rich basal lamina, which separates ectoderm from mesenchyme. Mice lacking the $\alpha$5 chain of laminin, a major glycoprotein of all basal laminae, display failure of digit septation (syndactyly), which could be due to disruption of the integrity of the surface ectoderm, locally affecting the AER (Miner et al. 1998). The AER is also disrupted by mutations in the *p63* gene, encoding a homolog of the tumor-suppressor protein p53. p63 is highly expressed in the basal or progenitor layers of many epithelial tissues, including limb ectoderm (Mills et al. 1999, Yang et al. 1999). Mutations in the human *p63* gene have been found in individuals suffering from SHFM (split-hand/split-foot malformation) or EEC

(ectrodactyly, ectodermal dysplasia, and facial cleft) syndromes, which display limb malformations consistent with defects in the maintenance of the AER (Celli et al. 1999, Ianakiev et al. 2000).

## ZPA, SHH, AND THE LINK BETWEEN ANTERIOR-POSTERIOR AND PROXIMAL-DISTAL PATTERNING

Differences along the AP axis are observed in most vertebrate limbs, the different lengths, shapes, and functions of the digits (and other skeletal elements) being only an obvious example with clear implications for the functionality of the appendage. Growth and patterning along the AP axis appear to be tightly coordinated with the development of the PD axis, in a process mediated by specific interactions between the AP organizer (the zone of polarizing activity; ZPA) and the controller of limb outgrowth (the AER).

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### ZPA, Retinoic Acid, Sonic Hedgehog, and the Organizer of AP Patterning

A group of cells located in the posterior mesenchyme of the limb bud, the ZPA (Figure 1*B*), acts as the organizer of the AP polarity of the limb bud (Saunders & Gasseling 1968). When the ZPA from one limb bud is grafted into the anterior margin of a host limb, mirror-image duplications of the digits along the AP axis are produced. The organizing activity of the ZPA was initially interpreted in terms of a morphogen gradient that diffuses across the limb bud to determine pattern in a concentration-dependent manner (reviewed by Tickle 1999). In 1993, it was shown that the polarizing activity of the ZPA is mediated by *Sonic hedgehog* (*Shh*) (Riddle et al. 1993, Chang et al. 1994, López-Martínez et al. 1995), a gene that encodes a secreted factor homologous to the product of the *Drosophila* segment polarity gene *hedgehog* (*hh*), involved in many patterning processes in the embryo and imaginal discs (reviewed by Ingham 1998). As first shown in the chick embryo, *Shh* expression is detected at stage 17 in the posterior margin shortly after the limb bud is induced, co-localizing with the ZPA (Figure 4*B*). A similar pattern is observed in mouse, zebrafish, and other vertebrates (Echelard et al. 1993, Krauss et al. 1993, Roelink et al. 1994). RA, which was previously known to be capable of inducing duplications similar to the ZPA when ectopically applied to the anterior margin of the limb (Tickle et al. 1982), appears to do so by inducing *Shh* transcription. Here, we discuss only the aspects of SHH function directly related to the vertebrate limb because the biochemical aspects of SHH signaling have been recently reviewed (Villavicencio et al. 2000).

SHH displays potent organizing activities in assays of ectopic application, but it is important to point out that the *Shh* gene is required neither for the initiation of limb development nor for the establishment of initial AP polarity of the limb because even in its absence there is some AP polarity in the limb bud (Noramly

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et al. 1996, Ros et al. 1996). Moreover, *Shh* is not involved in patterning the most proximal limb structures, but *Shh* activity is absolutely required for the maintenance of growth and patterning of intermediate and distal limb structures. Mice that are null for *Shh* (Chiang et al. 1996, Kraus et al. 2001) have limbs, but they are reduced and the skeletal pattern is severely perturbed. Intermediate structures are severely truncated and fused, and the autopod is almost completely absent, although in some cases a single, digit-like bone element is present, most likely the result of residual expression of *Indian hedgehog* (*Ihh*, another member of the *hh* gene family with similar biochemical properties). *Shh* is also required for the outgrowth of the limb ectoderm.

SHH is the only factor capable of mediating the polarizing activity of the ZPA known to date, and thus it is of particular importance to understand the mechanisms that control its expression and modulate its signaling activities. Several *Hox* genes appear to be important in delimiting the region of the limb bud mesenchyme where the *Shh* gene is going to be transcribed. In particular, the distribution of *Hoxb8* transcripts in the chick flank and early forelimb mirrors the distribution of polarizing activity (Lu et al. 1997, Stratford et al. 1997), which suggests that *Hoxb8* could be an upstream regulator of *Shh*. Indeed, *Hoxb8* was proposed to be required for the initiation of *Shh* expression in the posterior mesenchyme of the forelimb bud, although it would not be required for its maintenance (Charité et al. 1994). Besides, ectopic *Hoxb8* in the anterior margin of the mouse limb bud is able to induce ectopic *Shh*, which results in pattern duplications. However, *Hoxb8* cannot be the only regulator of *Shh* expression because *Shh* is only activated in the most distal cells that express *Hoxb8*. In fact, *Hoxb8* is not required for establishing AP polarity in the mouse limb bud (Stratford et al. 1997, van den Akker et al. 1999), and thus its activities are most likely dependent on its interaction with other *Hox* genes. Other *Hox* genes, such as *Hoxd11* and *Hoxd12*, are also involved in the control of *Shh* in the limb bud (Mackem & Knezevic 1999, see also Sordino et al. 1995). The basic helix-loop-helix (HLH) transcription factor dHAND also appears to control *Shh* expression, and *dHAND*-deficient mice (which die around E10.5), have smaller limbs with no detectable expression of *Shh* (Charité et al. 2000, Fernández-Terán et al. 2000).

Inhibition of RA signaling via the retinoid receptors (Helms et al. 1996, Lu et al. 1997) or inhibition of its synthesis (Stratford et al. 1996) prevents the establishment of the ZPA, the appearance of *Shh* expression, and the outgrowth of the limb bud. The effect of RA on *Shh* can be explained, in principle, by the fact that RA induces *Hoxb8* and other *Hox* genes involved in *Shh* control (Stratford et al. 1997), although there is also the apparently contradictory result that in limb buds of retinoid-deficient quail embryos, *Hoxb8* is ectopically expressed, whereas *Shh* is downregulated (Stratford et al. 1999). The dependence of *Shh* expression on AER signals (Laufer et al. 1994, Niswander et al. 1994) also restricts *Shh* to the more distal region of the posterior mesenchyme of the limb bud. The secreted factor WNT-7A, produced by the dorsal ectoderm, may also play a role in regulating *Shh* expression (Parr & McMahon 1995, Yang & Niswander 1995).

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*Shh* expression is also under negative transcriptional regulation. Analysis of a number of polydactylous mutants reveals that *Shh* transcription is negatively regulated in the anterior margin of the limb bud by several genes expressed in the anterior mesenchyme. These include the *aristalless*-like gene *Alx4*, which is mutated in the *Strong's luxoid* mouse (Qu et al. 1997, Takahashi et al. 1998) and interacts with the related gene *Cart1* (Qu et al. 1999), and the zinc finger-encoding gene *Gli-3* (Büscher et al. 1997, Masuya et al. 1997, Mo et al. 1997), mutated in the *extra toes* mouse (Xt) (Büscher et al. 1997, Schimmang et al. 1992, Hui & Joyner 1993). Both mutants display ectopic expression of *Shh* in restricted areas of the anterior margin of the limb bud (Chan et al. 1995; Büscher et al. 1997; Qu et al. 1997,1998), which results in duplications of pattern elements. Other mouse mutants also display anterior ectopic expression of *Shh* (*Rim4*, *Hx*, *Sasquatch*) (Chan et al. 1995, Masuya et al. 1995, Sharpe et al. 1999) or *Ihh* (*Doublefoot*) (Yang et al. 1998). Interestingly, the *Patched1* (*Ptc1*) gene, which encodes the SHH receptor, also appears to repress *Shh* expression in the anterior margin of the mouse limb bud (Milenkovic et al. 1999). Thus a complicated network of genetic interactions allocates and restricts *Shh* expression to the posterior distal margin of the limb bud. Also, a number of extracellular modulators (including the Ptc1 protein) and post-translational modifications regulate the extracellular availability and range of action of the SHH protein (reviewed by Capdevila & Izpisúa Belmonte 1999).

Although SHH is able to mimic the ZPA activity, it seems unlikely that the SHH protein itself gives positional information to all the cells in the limb bud. It does not seem to diffuse a long distance in vivo (Martí et al. 1995), and a membrane-tethered form is still able to elicit a dose-dependent patterning response, which suggests that at least part of the organizing activities of SHH are mediated by secondary signals (Yang et al. 1997).

## The Shh/Gremlin/FGF Loop and the Maintenance of the AER

As indicated above, SHH is not involved in AER induction, but it is certainly involved in AER maintenance. At the same time, maintenance of *Shh* expression in the posterior margin of the limb bud requires the integrity of the AER, which again illustrates the importance of epithelial-mesenchymal interactions during limb bud development.

The mesenchymal cells in the distal part of the limb bud constitute the so-called progress zone (PZ) (Figure 1*B*), which is kept in a proliferating, undifferentiated state by the AER (Summerbell et al. 1973). Cells in the PZ give rise to most of the mesenchymal elements in the limb. As the limb grows, mesenchymal cells exit the PZ, moving proximally and acquiring positional information to give rise to the mature appendages, which display a reproducible pattern of anatomical elements such as bones, muscles, and nerves. The AER is also required for maintaining *Shh* expression in the posterior margin of the limb bud and, reciprocally, the maintenance of the AER is also dependent on *Shh* expression. Thus SHH seems to act in

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a regulatory loop with FGF proteins expressed in the AER to maintain cell growth and proliferation in the mesenchyme, and to maintain the integrity of the AER (Laufer et al. 1994, Niswander et al. 1994). This regulatory loop is usually referred to as the *Shh/Fgf-4* loop because specific interactions between these two genes have been demonstrated (Figure 4*C*). For instance, ectodermal FGF signaling is initiated normally in limb buds of $Shh^{-/-}$ mouse embryos (Zuniga et al. 1999, Sun et al. 2000), but eventually they lose expression of both *Fgf-4* (in its normal domain in the posterior part of the AER) (Zuniga et al. 1999), and *Fgf-8* (in most of the AER, only remaining a small dot of posterior expression in some hindlimbs) (Kraus et al. 2001). On the other hand, the exact FGF or combination of FGFs that mediates the activities of the AER in vivo is not known, and this also applies to the control of *Shh* expression. For instance, elimination of *Fgf-4* expression from the AER of the mouse limb bud by means of a Cre/loxP binary transgenic system has no effect on *Shh* expression or limb development (Moon et al. 2000, Sun et al. 2000). This clearly indicates that there is redundancy among FGFs, further illustrated by similar experiments that eliminate *Fgf-8* expression in the AER (Lewandoski et al. 2000, Moon & Capecchi 2000). Although the results indicate that *Fgf-8* expression in the AER is necessary for normal limb development (including maintenance of *Shh*), other *Fgfs* seem to be able to compensate for the lack of *Fgf-8* in this experimental situation, and thus the analysis of other *Fgf* mutant situations or mutant combinations will presumably be required in order to assign specific functions to each FGF.

If AER-derived signals are required for continued growth of the limb, signals from the mesenchyme are required for maintaining the AER, and we are beginning to understand the mechanisms involved in the exchange of information between the mesenchyme and AER. For example, the *formin* gene, which encodes several protein isoforms thought to function in cytokinesis and/or cell polarization, is required to establish the *Shh/Fgf-4* feedback loop (Zeller et al. 1999) (Figure 4*C*). The *formin* gene is disrupted in the mouse *limb deformity* (*ld*) mutation. Homozygous *ld* mutants display shortened and malformed limbs, and their AERs are poorly organized. Although expression of *Fgf-8* is maintained in the AER of *ld* mutants, *Fgf-4* is not expressed. *ld* embryos also show a decrease in the expression of *Shh* in the limb mesenchyme. Thus the limb defect of *ld* mutants could be due to the absence of the proliferative function of *Fgf-4* combined with the reduction in *Shh* expression.

Recent results have also implicated BMPs in the negative regulation of the AER (Gañán et al. 1998, Dahn & Fallon 1999, Pizette & Niswander 1999, Capdevila et al. 1999, Zuniga et al. 1999). BMP beads implanted under the AER cause a precocious disruption of the AER owing to cell death, without affecting the rest of the ectoderm. Ectopic expression of the BMP antagonist Noggin in the limb bud has the opposite effect, maintaining the AER and reinforcing expression of AER markers, including *Fgf-8* and *Fgf-4* (which is anteriorly expanded without concomitant expansion of *Shh*). Recently, it has been shown that another BMP antagonist expressed in the limb bud, Gremlin, is required to

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antagonize the repressive effect of BMPs on the AER and to maintain *Fgf-4* expression in the posterior AER (Capdevila et al. 1999, Merino et al. 1999, Zuniga et al. 1999). The *formin* gene appears to interact with *Gremlin* in the regulation of the *Shh/Fgf-4* feedback loop. Thus Gremlin could be the factor produced by the distal mesenchyme that maintains the AER. The existence of an AER maintenance factor (AERMF) was proposed based on classic embryological studies in the chick embryo (Zwilling 1956, Saunders & Gasseling 1963). Since *Fgf-10*-expressing cells can induce both *Fgf-8* expression and thickening of non-ridge ectoderm, it has also been proposed that FGF-10 might be the AERMF (Ohuchi et al. 1997).

Remarkably, the activities of the factors involved in maintaining the *Shh/Fgf-4* feedback loop may be coordinated by the regulation of protein degradation. Recently, it has been shown that a novel member of the *F-box/WD40* gene family, encoding the Dactylin protein, is disrupted in the mouse *dactylaplasia* (*Dac*) mutant (Sidow et al. 1999), which resembles the human autosomal dominant split hand/foot malformation (SHFM) diseases. *Dac* homozygotes lack hands and feet, except for rudimentary single digit structures, and this phenotype is due to disruptions of AER maintenance linked to a lack of cell proliferation in the mutant AER (Crackower et al. 1998). The *F-box/WD40* gene family encodes adapter molecules that target several proteins for destruction by the ubiquitination machinery (Patton et al. 1998), including transducers of the NF-$\kappa$B, WNT/Wingless, and HH signaling pathways (Maniatis 1999). Several members of the *F-box/WD40* gene family have been shown to play important roles in the development of different organisms, including limb patterning in *Drosophila*. Sidow and collaborators propose that the function of Dactylin is to mediate degradation of a suppressor of AER proliferation. In *Dac* mutants, the suppressor would not be degraded and cell proliferation would be diminished, thus shifting the balance between proliferation and cell death in the AER toward increased cell death, which results in premature elimination of the AER. Identification of targets for Dactylin and the molecular cloning of *Mdac*, a known suppressor of *Dac*, are expected to provide additional clues on this process.

From the Formin, BMP, and Dac results outlined above, it seems clear that there is an AER maintenance activity distinct from activities involved in AER induction and differentiation. The combined data also suggest that AER maintenance may be achieved by promoting cell proliferation in the AER, by inhibiting PCD, or both. More studies are needed in order to determine how the activities of Formin, BMPs, and Dactylin interact in the mechanism that maintains the AER.

## *Meis* Genes and Proximal Patterning

Shh is not required for the development of the more proximal structures of the vertebrate limb (Chiang et al. 1996, Kraus et al. 2001). This, along with the fact that these structures are also unaffected even when the AER is removed very early, clearly

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indicates that the whole distal program of gene expression (the Shh/Gremlin/FGF loop) is not required for proximal development. Thus the question remains as to which specific molecular mechanisms control growth and patterning of the proximal limb bud. In principle, *Hox* genes are good candidates, as they display dynamic, combinatorial patterns of gene expression in this and other regions of the limb bud (Nelson et al. 1996). However, the analysis of the individual contribution of each *Hox* gene may be complicated by the high level of partial redundancy and reciprocal regulation that exists among these genes. Despite these difficulties, recent results have shed light on the mechanisms that control proximal limb development.

An increasing amount of evidence indicates that HOX proteins perform their roles of specifying regional identities by interacting with a number of cofactors. For instance, the homeodomain protein Pbx1 has been shown to modulate HOX function by binding to several HOX proteins that contain tryptophan dimerization motifs. Pbx1 is the vertebrate homolog of *Drosophila* extradenticle (Exd), and numerous experiments in *Drosophila* (reviewed by Morata & Sánchez-Herrero 1999) have shown that Exd is only active (and required) in the domain where it is nuclear and that this nuclear localization of Exd (and Pbx1) is promoted by interaction with Hth proteins (Meis in vertebrates). An added twist is that in *Drosophila* the subcellular localization of Exd is regulated by the *Bithorax* complex (*BX-C*) homeotic (or *Hox*) genes, and each *BX-C* gene can prevent or reduce nuclear translocation of Exd to varying extents. Interestingly, this Meis/Pbx/HOX interaction also operates in the vertebrate limb and appears to control development of the more proximal structures. *Pbx1* transcripts and protein are present throughout the limb bud, although, similar to *Drosophila*, Pbxl proteins are localized to the nuclei of mesenchymal cells in the proximal limb bud, whereas they are cytoplasmic in the distal part of the limb (González-Crespo et al. 1998). Consistently, transcription of two vertebrate *Meis* genes, *Meis-1* and *Meis-2*, is restricted to the proximal limb bud (Capdevila et al. 1999; Mercader et al. 1999, 2000; Saleh et al. 2000) (Figure 4*C*). *Pbx1*-deficient mice display numerous patterning defects, including malformation of the more proximal skeletal elements (stylopod) of the limb, although distal elements are completely normal (L. Selleri, personal communication). Thus as expected, phenotypes are observed only in domains that express HOX proteins known to contain the Pbx1 dimerization motif (the proximal limb), whereas the distal limb, which is thought to be specified by HOX proteins that lack Pbx dimerization motifs, is spared. Complementing these results, experiments involving ectopic expression of *Meis* genes in the distal limb bud of the chick embryo have shown that ectopic *Meis* activity is capable of repressing the distal program of gene expression, and vice versa, which indicates that the vertebrate limb bud is clearly divided into well-defined (and antagonistic) proximal and distal territories, similar to *Drosophila* appendages (Capdevila et al. 1999, Mercader et al. 1999), controlled, respectively, by Meis/Pbx/HOX and by the Shh/Gremlin/FGF loop (reviewed by Vogt & Duboule 1999).

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## CELL DIFFERENTIATION

The patterning mechanisms that operate in the vertebrate limb bud result in the generation of a stereotyped array of elements easily recognizable in the adult limb, such as bones, muscles, nerves, and blood vessels, and epithelial appendages such as hair, scales, or feathers. For some of these elements, relatively little is known about the mechanisms that control terminal cell differentiation. In this section we briefly comment on some molecular mechanisms that operate during cartilage formation, determination of regions of programmed cell death (PCD), and digit specification, since they are representative of the mechanisms that sculpt the vertebrate limb.

### Control of Cartilage Formation and Programmed Cell Death

In terms of skeletal elements, all vertebrate limbs consist of a proximal part with a single element (stylopod: humerus or femur), a medial part with two elements (zeugopod: radius and ulna or tibia and fibula), and a distal part (autopod) composed of carpus or tarsus and a variable number of radiating digits (Figure 1*C*). In the vertebrate limb, skeletal structures develop in a characteristic proximal-to-distal sequence. In all vertebrate limbs, the more proximal skeletal structures are determined first and are formed by the mesenchymal cells that leave the PZ first, whereas mesenchymal cells that leave the PZ later form more distal structures (Summerbell et al. 1973). After limb initiation and AER induction, mesenchymal cells that exit the PZ (and presumably get out of reach of the factors produced by the AER) aggregate to form precartilaginous condensations that will give rise to the skeletal components (Figure 5*A*). Concomitantly, some cells located around or between the primordia of the skeletal elements undergo programmed cell death (PCD), which contributes to shape the limb, for example, by freeing the digits. In some vertebrates, however, interdigital PCD is either reduced or absent, and thus the resulting limbs have webbed or lobulated digits (Gañán et al. 1998). Later stages of skeletal development include replacement of cartilage by bone through endochondral ossification, the growth of long bones through proliferation and differentiation of chondrocytes in growth plates, and bone formation through differentiation of osteoblasts from mesenchymal cells in areas of intramembranous ossification.

Although the exact mechanisms that direct the differentiation of the cell types present in the limb bud (including chondrogenic cell types) are still unknown, a model has recently emerged that explains how cells that exit the PZ and become autopod decide between two opposite fates: forming skeletal elements or becoming interdigital tissue that will eventually be removed by PCD. General aspects of skeletal development (Hall & Miyake 1995, Mundlos & Olsen 1997, Ferguson et al. 1998, Olsen et al. 2000) and the role of PCD in controlling limb shape and defining the digits (Chen & Zhao 1998) have been recently reviewed, thus we focus here on the specific problem of deciding between forming skeletal elements or dying by PCD.

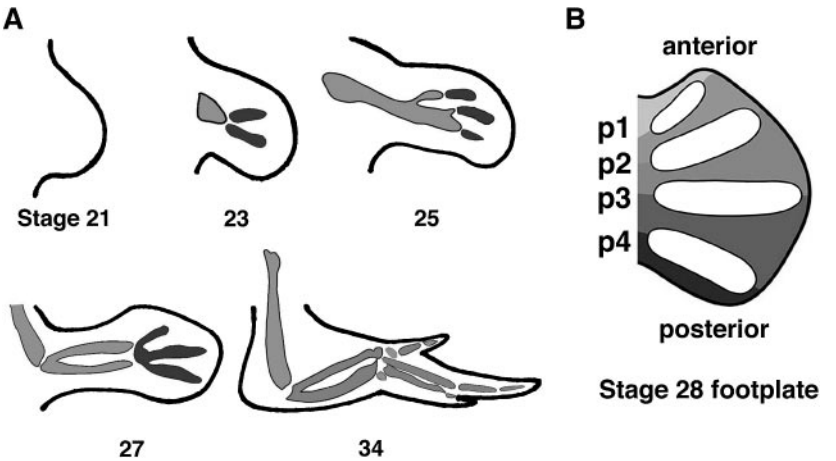


**Figure 5** PD patterning and digit identity. (*A*) Schemes of a chick forelimb bud at different stages showing appearance of cartilage elements in a stereotypical proximal-to-distal sequence. Dark gray indicates the last elements to be formed. (*B*) Scheme showing a proposed model of determination of digit identity in the autopod, where the identity of the digital primordia (p1 to p4) is determined by the adjacent interdigital mesenchyme (see text). There is a sort of posterior prevalence (probably linked to a BMP-related activity, shown as levels of gray), so that each digit primordium acquires its identity from the more posterior interdigital mesenchyme. Scheme adapted from Dahn & Fallon (2000).

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Several lines of evidence indicate that members of the TGF-$\beta$ superfamily of secreted factors, along with their receptors, extracellular modulators, and intracellular transducers, control the choice between digital and interdigital fates in the autopod. BMPs, for example, appear to have dual roles as inducers of PCD and promoters of cartilage growth. The *Bmp-2*, *-4*, and *-7* genes are expressed in the interdigital regions of the limb bud that undergo PCD, and when beads soaked in BMP protein are implanted in the interdigital regions of the limb bud, cell death is prematurely induced. Interestingly, when the beads are implanted close to the digits, they seem to increase the size of the cartilage, which indicates that BMPs also promote cartilage growth. Experiments involving constitutive activation of BMP receptors (Zou et al. 1997) and blocking of BMP activity by dominant-negative BMP receptors (Kawakami et al. 1996, Yokouchi et al. 1996, Zou & Niswander 1996) or extracellular antagonists such as Noggin (Capdevila & Johnson 1998, Pizette & Niswander 1999) also support this dual role of BMPs. It has been proposed that BMP receptor-1a (encoded by *BmpR-1a*, expressed in the distal mesenchyme) mediates PCD induction and that BMP receptor-1b (encoded by *BmpR-1b*, expressed in the prechondrogenic condensations) primarily mediates cartilage growth, although it can also mediate PCD induction at other stages of development (Zou et al. 1997). The effect of RA on PCD has been recently shown to be mediated by BMP signaling (Rodríguez-León et al. 1999).

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Interestingly, a different member of the TGF-$\beta$ superfamily, TGF-$\beta$2, seems to fulfill the requirements for an endogenous inducer of digit formation in the autopod. The *tgf-β2* gene is expressed in the prechondrogenic condensations of the presumptive digit regions of the limb (Millan et al. 1991, Merino et al. 1998), and a bead soaked in TGF-$\beta$2 protein can induce the formation of extra digits when implanted in the interdigital spaces (Gañán et al. 1996). Digit induction is preceded by induction of *BmpR-1b*. Other TGF-$\beta$s are also able to induce digits in this assay, and this functional redundancy could presumably explain why *tgf-β2*-deficient mice do not display the digit alteration phenotype expected (Sanford et al. 1997). Hurlé and collaborators have proposed that *tgf-β2* (expressed in the presumptive digital areas) acts on the cells leaving the PZ by sensitizing those cells to the chondrogenic influence of BMPs (reviewed by Macías et al. 1999). This is accomplished by the induction of *BmpR-1b* by TGF-$\beta$2. In the interdigital spaces, where *tgf-β2* expression is absent, BMPs would promote PCD. *Noggin* is expressed in the presumptive digital areas, where it could conceivably modulate the amount of BMPs available to bind the BMPR-1B.

FGFs also play an important role in this process, since cells in the PZ must be protected from both chondrogenic and apoptotic stimuli if limb outgrowth is to be maintained. Thus it has been shown that FGFs oppose both BMPs and TGF-$\beta$s, blocking BMP-induced interdigital cell death (Macías et al. 1996) and TGF-$\beta$2-induced chondrogenesis (Gañán et al. 1996). This is consistent with the observed role of FGFs as mediators of AER function (Martin 1998).

Besides TGF-$\beta$s, BMPs, FGFs, and the components of their transduction pathways, other molecules are also involved in different stages of skeletal development. For example, the HMG-domain transcription factor Sox9 plays an important role in cartilage formation. The *Sox9* gene is expressed in mesenchymal condensations throughout the embryo before and during the deposition of cartilage (Wright et al. 1995). In mouse chimeras, *Sox9*$^{-/-}$ mesenchymal cells are excluded from all cartilage and lack expression of chondrocyte-specific markers (Bi et al. 1999). Misexpression of *Sox9* in limbs results in ectopic cartilage formation, and *Sox9* is able to change the aggregation properties of limb mesenchymal cells in vitro, suggesting that *Sox9* functions at the level of mesenchymal cell condensation, probably acting downstream of BMPs (Healy et al. 1999). In humans, *Sox9* haploinsufficiency results in a skeletal malformation syndrome, campomelic dysplasia (CD), which is characterized by bowing of the long bones and is often associated with male-to-female sex reversal (Foster et al. 1994, Wagner et al. 1994). Other *Sox* genes are coexpressed with *Sox9* in mesenchymal condensations and cooperate in the control of genes of the chondrocytic program (Lefebvre et al. 1998), which is consistent with the fact that the absence of *Sox9* in the human syndrome fails to cause total disruption of cartilage formation.

Thus a model arises where interactions between different TGF-$\beta$s and their extracellular modulators regulate the digital and interdigital fates of the autopodial limb mesoderm (reviewed by Macías et al. 1999). Conceivably, high levels of BMP expression in the interdigital mesenchyme induce PCD in a process probably mediated by the activity of *Msx* genes (Gañán et al. 1998). *tgf-β2* (expressed in the

digital areas) induces *BmpR-1b* so that cells in the presumptive digit areas may respond to BMPs by initiating cartilage formation. The presumptive digit areas also express the BMP antagonist Noggin, which antagonizes BMPs by binding to them and preventing interaction with their receptors (Zimmerman et al. 1996). Coexpression of Noggin and BMPR-1B in the digit-forming areas could be a mechanism to fine-tune the levels of BMP protein that effectively interact with their receptors (Capdevila & Johnson 1998). Other BMP antagonists expressed in the limb could also be involved in the process, so partial redundancy might be expected. In *noggin*$^{-/-}$ embryos, for example, excess of BMP activity enhances the recruitment of cells into cartilage, resulting in hyperplasia of cartilage condensations and failure of joints to develop (Brunet et al. 1998).

It is important to point out that the induction of skeletal elements seems to be under the control of different signals along the PD axis of the limb. For example, TGF-$\beta$2 is not expressed in the skeletal precursors of the stylopod and zeugopod (Merino et al. 1998), so clearly another factor or factors must regulate skeletal patterning in the proximal region of the limb.

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## Digit Identity

Most vertebrate limbs display a complement of digits (as part of the autopod) that shows clear differences along the AP axis, each digit being recognizable by its characteristic shape, length, and number of phalanges (Figure 1*C*). Until recently it was thought that the AP patterning of the autopod (and hence digit identity) was specified and fixed at early limb bud stages by the ZPA, perhaps mediated by a SHH morphogen gradient that would control both the number and identity of the digits. This initial positional information would subsequently be interpreted and refined by other factors (such as *Hox* code) that would then influence the size and number of digits (in a *Hox*-dose-dependent fashion involving *Hoxd* and *Hoxa* genes) (Zákány et al. 1997, Zákány & Duboule 1999).

Recently, experiments involving surgical manipulation of the digits and interdigital regions of the chick foot have broadened our understanding of the problem of digit identity. These experiments have shown that digital identity in the chick limb remains labile even after the digital primordia form, so that the identity of digits is actually specified by the interdigital mesoderm at relatively late stages of limb bud development (Dahn & Fallon 2000) (Figure 5*B*). BMP proteins, present in the interdigital mesoderm, appear to be required for the correct assignment of digit identities since inhibition of BMP signaling in the interdigital mesoderm results in transformations of digit identity. Interestingly, more posterior interdigital regions specify more posterior digital identities, and each digital primordium develops in accordance with the most posterior cues received (a sort of "posterior prevalence"). It is still not clear whether there are actual differences in the level of activation of the BMP pathways among different interdigital areas; thus the postulated existence of thresholds of BMP signaling throughout the developing autopod remains speculative. In any event, these and other results (Drossopoulou

et al. 2000) illustrate the complexity of interactions involved in the specification of digit identity in the vertebrate limb by SHH, BMPs, and other factors (Newman 2000, Hagmann 2000).

## OPEN QUESTIONS AND FUTURE DIRECTIONS

Many important questions regarding the molecular and cellular mechanisms that control limb development remain to be answered. In this final section we briefly mention only three, which illustrate how the multidisciplinary study of vertebrate limb development may deepen our understanding of a variety of issues that have a bearing on important aspects of both basic and applied sciences.

### The Nature of the Organizing Activity of SHH

Because the SHH protein is the only known factor capable of mediating all the patterning activities of the ZPA, there is, of course, considerable interest in characterizing its activities in detail. What does SHH actually do to its target cells in the limb bud? SHH has already been shown to act as a survival factor for several cell types in the embryo, including sclerotome, neural tube cells, and cultured fetal rat ventral spinal interneurons and ventral dopaminergic and GABAergic mesencephalic neurons (Miao et al. 1997, Borycki et al. 1999), but it is also involved in inducing PCD of ventral neuronal precursors and floor-plate cells (Oppenheim et al. 1999). The possible role of SHH as a trophic factor for limb cells remains practically unexplored. The problem is relevant because the relationship between growth and pattern specification during development of the limb remains elusive. Some molecules known to function as growth factors are also potent agents of pattern formation, and recent studies involving manipulation of the cell cycle in chick limb buds have raised the interesting possibility that growth factors such as SHH or BMPs operate through the control of the length of the cell cycle (Ohsugi et al. 1997).

Also, although the evidence seems to point to the existence of secondary signals that mediate the patterning activities of SHH (Yang et al. 1997), so far no other factor has been shown to display the organizing capabilities that SHH demonstrates in assays of ectopic expression. BMPs, which are targets of SHH and belong to a superfamily of factors known to have multiple organizing capabilities in *Drosophila* and other organisms, fail to induce pattern duplications when applied to the anterior margin of the limb bud (Francis et al. 1994). Since BMPs act as heterodimers, and several BMPs are expressed in the limb, the possibility remains that a particular heterodimer may actually have some polarizing activity, but evidence for this is still lacking. The molecular characterization of the genes mutated in chick *talpid* mutants (Izpisúa Belmonte et al. 1992, Francis-West et al. 1995, Rodríguez et al. 1996, Caruccio et al. 1999, Lewis et al. 1999) has the potential to reveal new elements of the SHH pathway, because in these mutants *Shh* expression is not altered,

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but *Shh* targets are ectopically expressed, and the anterior margin of the mutant limbs have polarizing activity. The problem of the putative secondary signal or signals that mediate SHH patterning activities could be addressed by experiments involving constitutive activation of the SHH pathway, analyzing the genes induced and the phenotypes caused by this ligand-independent activation, as it is used in *Drosophila* to analyze the organizing properties of several signaling molecules, including HH. Another twist to the mechanisms used by signaling molecules to affect neighboring cells has been added by the recent discovery of novel cellular structures (cytonemes) that could be involved in long-distance transport of patterning signals (Ramírez-Weber & Kornberg 1999, reviewed by Morata & Basler 1999). The possible involvement of these novel structures in limb development remains unexplored.

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## Molecular Mechanisms Involved in the Evolution of Vertebrate Limbs

The tetrapod limb, as an evolutionary novelty capable of performing an astonishing variety of functions, has played a key role in the appearance of terrestrial vertebrates. In recent years, a new synthesis of developmental and evolutionary biology (reviewed by Wagner 2000), supported mostly by the reinterpretation of the fossil record and by new discoveries from developmental genetics, has aimed to provide detailed and plausible molecular explanations for the developmental changes that culminated in the adoption of the basic tetrapod limb plan (reviewed by Capdevila & Izpisúa Belmonte 2000, Ruvinsky & Gibson-Brown 2000). For instance, it is generally accepted that the genetic network that controls appendage initiation, growth, and patterning in a variety of organisms (a sort of "appendage toolbox") was most likely already present before appendages and other outgrowths appeared (Panganiban et al. 1997), and it was later co-opted to initiate and control limb development. Indeed, genes and gene families that play key roles in limb initiation and patterning, such as *Shh*, *Bmps*, *Fgfs*, *Wnts*, *Hox*, and many others, are involved in many embryonic patterning processes in vertebrates (including several taxa with reduced or absent limbs) and even invertebrate chordates such as Amphioxus (Shimeld 1999, Wada et al. 1999, Schubert et al. 2000). For instance, it has been proposed that the *Hox* code originally evolved to control rostral-caudal patterning of the digestive tract and was ng co-opted later to control limb positioning and other aspects of limb patterning (Coates & Cohn 1998, 1999).

Molecular studies have produced the interesting suggestion that patterning genes such as *Wnt-11*, expressed in the tailbud and the tail fin in Amphioxus (and probably lower vertebrates also), could have been co-opted for controlling development of lateral appendages (Schubert et al. 2000). This remains as hypothesis, however, because (among other complications) the regulatory sequences of this gene and many other genes that may play key roles in limb positioning and limb initiation remain unknown. For instance, the regulatory mechanisms that restrict expression of *Fgf-8* (and other *Fgf* genes) to specific portions of the IM are also

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unknown, as are the specific sequences that direct expression of *Shh* in the limb bud. The identification of these and other regulatory sequences would be key if we are to unveil the genetic changes that led to the appearance of paired appendages and the establishment of the tetrapod limb plan.

In terms of our knowledge of the regulatory mechanisms that control expression of limb-patterning genes, *Hox* genes are especially interesting, since expression studies and sophisticated genetic techniques have provided valuable information (reviewed by Zákány & Duboule 1999), and thus we comment on it only briefly. Aside from playing a key role in the pre-pattern that allocates the limb fields in the flank, *Hox* genes are also expressed in the limb bud, where their proper regulation by Meis/Pbx in the proximal part is essential for normal patterning. Moreover, the activity of *Hoxa* and *Hoxd* gene complexes is absolutely required for the proper development of digits. Some authors have even proposed that the appearance of digits correlates with the adoption of novel spatial and temporal patterns of expression of *Hox* genes in the distal part of the limb bud (reviewed by Shubin et al. 1997, Capdevila & Izpisúa Belmonte 2000). Additionally, digit size and number appear to be determined (at least partially) by the dose of HOX proteins (Zákány et al. 1997, Zákány & Duboule 1999). It is already known that a complex set of enhancers within the regulatory regions of each *Hoxd* gene is used during the phase of nested domains of expression in the posterior margin of the limb bud (Beckers et al. 1996, van der Hoeven et al. 1996). On the contrary, the late phase, characterized by antero-distal expansion and polarity reversal of *Hoxd* expression, is regulated by a single enhancer, or global enhancer sequence that is also used to control *Hoxd* expression in the gut (Gérard et al. 1993, van der Hoeven et al. 1996). Recently, a silencer regulatory element has been discovered that is absolutely required to restrict expression of the *Hoxd13* gene, at the same time allowing the activity of the global enhancer (Kmita et al. 2000). Thus these and future discoveries will allow us to complete the picture of the regulatory mechanisms that control the activity of *Hox* genes.

## Adult Limb Regeneration and Its Applications in Tissue Engineering

Remarkably, urodele amphibians such as newts and salamanders are capable of regenerating their adult limbs after amputation (Stocum 1995, Brockes 1997, Gardiner et al. 1999). Teleost fish such as zebrafish are also capable of regenerating their adult fins after partial amputation (Santamaría & Becerra 1991, Wagner & Misof 1992). In contrast, adult limb regeneration in mammals is very modest, being limited to digit tip regeneration in some rodents and primates, including humans (Borgens 1982, Muller et al. 1999). Understandably, the molecular bases of adult limb regeneration have attracted considerable attention owing to their potential applications in the reconstruction of structures such as cartilage, bone, nerves, or blood vessels in patients suffering degenerative or traumatic processes. Notably, bone has considerable regenerative powers, and advances in tissue engineering

(partially inspired by the endogenous processes of limb development and limb regeneration) have made possible partial restoration of altered skeletal functions by localized induction of bone (Reddi 1998, 2000).

Adult limb regeneration recapitulates normal limb development, with some minor differences (Gardiner & Bryant 1996), and many of the molecules involved in this process in urodele amphibians or teleost fish are the same as described in chick or mouse limbs (Geraudie & Ferretti 1998). Thus SHH (Imokawa & Yoshizato 1998, Laforest et al. 1998, Poss et al. 2000a), FGFs (Mullen et al. 1996, Poss et al. 2000b, Endo et al. 2000), and HOX proteins (Gardiner & Bryant 1996) are expressed in patterns comparable to those in chick or mouse and appear to play similar patterning roles, although some peculiarities exist. For example, nerves are needed as a mitotic stimulant for adult limb regeneration in urodele amphibians (reviewed in Brockes 1984), but a patterning requirement for nerve function during normal development of chick or mouse limbs has not been described so far.

Limb regeneration has been more extensively studied in urodele amphibians. In these animals, amputation of an adult limb results in formation of a "regeneration blastema" composed of dedifferentiated mesenchymal progenitor cells that subsequently re-enter the cell cycle and proliferate to form a complete limb with normal pattern elements. This blastema results from a massive breakdown of extracellular matrix at the site of the amputation, which requires the activity of a variety of proteases and releases a number of cell types that subsequently dedifferentiate. The capability of urodele cells (especially fibroblasts) to proliferate, dedifferentiate, and contribute to regenerate a limb appears to be the result of their specific ability to re-enter the cell cycle after injury (reviewed in Stocum 1999). This is in contrast with mammalian fibroblasts, which form scar tissue instead of contributing to regeneration. Thus studies on the molecular nature of the response of urodele fibroblasts to injury may provide suggestions on how to confer regenerative potential to mammalian fibroblasts and other cell types.

In summary, the study of basic mechanisms of growth control and pattern formation has benefited enormously from a new synthesis that integrates methods and visions derived from classic embryology, molecular and cellular biology, and evolutionary biology. In this context, it is clear that the study of the cellular and molecular interactions that shape the vertebrate limb, and their evolutionary implications, will continue to provide valuable insights into the epithelial-mesenchymal interactions and other morphogenetic mechanisms that sculpt the vertebrate embryo.

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## ACKNOWLEDGMENTS

We apologize to authors for the impossibility to cite all the references relevant to this review. We thank Gary Schoenwolf, Juan Hurlé, and Concepción Rodríguez Esteban for kindly providing pictures. We also thank Lorraine Hooks for helping with the manuscript. This work is supported by grants from the G Harold and Leila Y Mathers Foundation, and the National Institutes of Health to JCIB, who is a Pew Scholar.

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## LITERATURE CITED


Altabef M, Clarke JD, Tickle C. 1997. Dorsoventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb. *Development* 124:4547–56

Barna M, Hawe N, Niswander L, Pandolfi PP. 2000. Plzf regulates limb and axial skeletal patterning. *Nat. Gen.* 25:166–72

Beckers J, Gérard M, Duboule D. 1996. Transgenic analysis of a potential Hoxd-11 limb regulatory element present in tetrapods and fish. *Dev. Biol.* 180:543–53

Begemann G, Ingham PW. 2000. Developmental regulation of Tbx5 in zebrafish embryogenesis. *Mech. Dev.* 90:299–304

Bell SM, Schreiner CM, Scott WJ. 1998. The loss of ventral ectoderm identity correlates with the inability to form an AER in the legless hindlimb bud. *Mech. Dev.* 74:41–50

Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B. 1999. Sox9 is required for cartilage formation. *Nat. Gen.* 22:85–89

Blagden CS, Hughes SM. 1999. Extrinsic influences on limb muscle organisation. *Cell Tissue Res.* 296:141–50

Bollag R, Siegfried Z, Cebra-Thomas JA, Garvey N, Davison EM, Silver LM. 1994. An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T locus. *Nat.* 7:383–89

Borgens RB. 1982. Mice regrow the tips of their foretoes. *Science* 217:747–50

Borycki AG, Brunk B, Tajbakhsh S, Buckingham M, Chiang C, Emerson CP Jr. 1999. Sonic hedgehog controls epaxial muscle determination through Myf5 activation. *Development* 126:4053–63

Brockes JP. 1984. Mitogenic growth factors and nerve dependence of limb regeneration. *Science* 225:1280–87

Brockes JP. 1997. Amphibian limb regeneration: rebuilding a complex structure. *Science* 276:81–87

Brunet LJ, McMahon JA, McMahon AP, Harland RM. 1998. Noggin, cartilage morphogenesis, and joint formation in the mammalian skeleton. *Science* 280:1455–57

Burke AC, Nelson CE, Morgan BA, Tabin C. 1995. Hox genes and the evolution of vertebrate axial morphology. *Development* 121: 333–46

Büscher D, Bosse B, Heymer J, Rüther U. 1997. Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development. *Mech. Dev.* 62:175–82

Büscher D, Izpisúa Belmonte JC. 1999. Muscle development during vertebrate limb outgrowth. *Cell Tissue Res.* 296:131–39

Capdevila J, Izpisúa Belmonte JC. 1999. Extracellular modulation of the Hedgehog, Wnt and TGF-beta signalling pathways during embryonic development. *Curr. Opin. Genet. Dev.* 9:427–33

Capdevila J, Izpisúa Belmonte JC. 2000. Perspectives on the evolutionary origin of tetrapod limbs. *J. Exp. Zool.* 288:287–303

Capdevila J, Johnson RL. 1998. Endogenous and ectopic expression of noggin suggests a conserved mechanism for regulation of BMP function during limb and somite patterning. *Dev. Biol.* 197:205–17

Capdevila J, Tsukui T, Rodríguez Esteban C, Zappavigna V, Izpisúa Belmonte JC. 1999. Control of vertebrate limb outgrowth by the proximal factor Meis2 and distal antagonism of BMPs by Gremlin. *Mol. Cell* 4:839–49

Carroll RL. 1988. *Vertebrate Paleontology*. San Francisco: Freeman

Caruccio NC, Martínez-López A, Harris M, Dvorak L, Bitgood J, et al. 1999. Constitutive activation of sonic hedgehog signaling in the chicken mutant talpid(2): Shh-independent outgrowth and polarizing activity. *Dev. Biol.* 212:137–49

Celli J, Duijf P, Hamel BC, Bamshad M, Kramer B, et al. 1999. Heterozygous germline

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
by CAPES on 09/28/05. For personal use only.

mutations in the p53 homolog p63 are the cause of EEC syndrome. *Cell* 99:143–53

Chan DC, Laufer E, Tabin C, Leder P. 1995. Polydactylous limbs in Strong's luxoid mice result from ectopic polarizing activity. *Development* 121:1971–78

Chang DT, López A, von Kessler DP, Chiang C, Simandl BK, et al. 1994. Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* 120:3339–53

Charité J, de Graaff W, Consten D, Reijnen MJ, Korving J, Deschamps J. 1998. Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. *Development* 125:4349–58

Charité J, de Graaff W, Shen S, Deschamps J. 1994. Ectopic expression of Hoxb-8 causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures. *Cell* 78:589–601

Charité J, McFadden DG, Olson EN. 2000. The bHLH transcription factor dHAND controls Sonic hedgehog expression and establishment of the zone of polarizing activity during limb development. *Development* 127:2461–70

Chen H, Johnson RL. 1999. Dorsoventral patterning of the vertebrate limb: a process governed by multiple events. *Cell Tissue Res.* 296:67–73

Chen H, Lun Y, Ovchinnikov D, Kokubo H, Oberg KC, et al. 1998. Limb and kidney defects in Lmx1b mutant mice suggest an involvement of LMX1B in human nail patella syndrome. *Nat. Genet.* 19:51–55

Chen Y, Zhao X. 1998. Shaping limbs by apoptosis. *J. Exp. Zool.* 282:691–702

Chiang C, Litingtung Y, Lee E, Young KE, Corden JL, et al. 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 383:407–13

Coates MI. 1994. The origin of vertebrate limbs. *Dev. Suppl.*, 169–80

Coates MI, Cohn MJ. 1998. Fins, limbs, and tails: outgrowths and axial patterning in vertebrate evolution. *BioEssays* 20:371–81

Coates MI, Cohn MJ. 1999. Vertebrate axial and appendicular patterning:the early development of paired appendages. *Am. Zool.* 39:676–85

Cohn MJ, Izpisúa-Belmonte J-C, Abud H, Heath JK, Tickle C. 1995. Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* 80: 739–46

Cohn MJ, Patel K, Krumlauf R, Wilkinson DG, Clarke JD, Tickle C. 1997. Hox9 genes and vertebrate limb specification. *Nature* 387:97–101

Cohn MJ, Tickle C. 1999. Developmental basis of limblessness and axial patterning in snakes. *Nature* 399:474–79

Crackower MA, Motoyama J, Tsui LC. 1998. Defect in the maintenance of the apical ectodermal ridge in the Dactylaplasia mouse. *Dev. Biol.* 201:78–89

Crossley PH, Minowada G, MacArthur CA, Martin GR. 1996. Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* 84:127–36

Cygan JA, Johnson RL, McMahon AP. 1997. Novel regulatory interactions revealed by studies of murine limb pattern in Wnt-7a and En-1 mutants. *Development* 124:5021–32

Dahn RD, Fallon JF. 1999. Limiting outgrowth: BMPs as negative regulators in limb development. *BioEssays* 21:721–25

Dahn RD, Fallon JF. 2000. Interdigital regulation of digit identity and homeotic transformation by modulated BMP signaling. *Science* 289:438–41

Davis CA, Joyner AL. 1988. Expression patterns of the homeo box-containing genes En-1 and En-2 and the proto-oncogene int-1 diverge during mouse development. *Genes Dev.* 2:1736–44

Dealy CN, Roth A, Ferrari D, Brown AM, Kosher RA. 1993. Wnt-5a and Wnt-7a are expressed in the developing chick limb bud in a manner suggesting roles in pattern formation along the proximodistal and dorsoventral axes. *Mech. Dev.* 43:175–86

Dschamps J, van den Akker E, Forlani S, De

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
by CAPES on 09/28/05. For personal use only.

Graaff W, Oosterveen T, et al. 1999. Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. *Int. J. Dev. Biol.* 43:635–50

Drossopoulou G, Lewis KE, Sanz-Ezquerro JJ, Nikbakht N, McMahon AP, et al. 2000. A model for anteroposterior patterning of the vertebrate limb based on sequential long- and short-range Shh signalling and Bmp signalling. *Development* 127:1337–48

Duboule D. 1998. Vertebrate hox gene regulation: clustering and/or colinearity? *Curr. Opin. Genet. Dev.* 8:514–18

Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, et al. 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75:1417–30

Endo T, Tamura K, Ide H. 2000. Analysis of gene expressions during *Xenopus* forelimb regeneration. *Dev. Biol.* 220:296–306

Fallon JF, Frederick JM, Carrington JL, Lanser ME, Simandl BK. 1983. Studies on a limbless mutant in the chick embryo. *Prog. Clin. Biol. Res.* 110:33–43

Ferguson CM, Miclau T, Hu D, Alpern E, Helms JA. 1998. Common molecular pathways in skeletal morphogenesis and repair. *Ann. NY Acad. Sci.* 857:33–42

Fernández-Terán M, Piedra ME, Kathiriya IS, Srivastava D, Rodriguez-Rey JC, Ros MA. 2000. Role of dHAND in the anterior-posterior polarization of the limb bud: implications for the Sonic hedgehog pathway. *Development* 127:2133–42

Fernández-Terán M, Piedra ME, Simandl BK. Fallon JF, Ros MA. 1997. Limb initiation and development is normal in the absence of the mesonephros. *Dev. Biol.* 189:246–55

Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature* 372:525–30

Francis PH, Richardson MK, Brickell PM, Tickle C. 1994. Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb. *Development* 120:209–18

Francis-West PH, Robertson KE, Ede DA, Rodriguez C, Izpisúa-Belmonte JC, et al. 1995. Expression of genes encoding bone morphogenetic proteins and sonic hedgehog in talpid (ta3) limb buds: their relationships in the signalling cascade involved in limb patterning. *Dev. Dyn.* 203:187–97

Galcerán J, Farinas I, Depew MJ, Clevers H, Grosschedl R. 1999. Wnt3a$^{-/-}$-like phenotype and limb deficiency in Lef1($^{-/-}$) Tcf1($^{-/-}$) mice. *Genes Dev.* 13:709–17

Gañán Y, Macías D, Basco RD, Merino R, Hurlé JM. 1998. Morphological diversity of the avian foot is related with the pattern of msx gene expression in the developing autopod. *Dev. Biol.* 196:33–41

Gañán Y, Macías D, Duterque-Coquillaud M, Ros MA, Hurlé M. 1996. Role of TGF betas and BMPs as signals controlling the position of the digits and the areas of interdigital cell death in the developing chick limb autopod. *Development* 122:2349–57

Gardiner DM, Bryant SV. 1996. Molecular mechanisms in the control of limb regeneration: the role of homeobox genes. *Int. J. Dev. Biol.* 40:797–805

Gardiner DM, Carlson MR, Roy S. 1999. Towards a functional analysis of limb regeneration. *Semin. Cell Dev. Biol.* 10:385–93

Gaunt SJ. 2000. Evolutionary shifts of vertebrate structures and Hox expression up and down the axial series of segments: a consideration of possible mechanisms. *Int. J. Dev. Biol.* 44:109–17

Geduspan JS, MacCabe JA. 1989. Transfer of dorsoventral information from mesoderm to ectoderm at the onset of limb development. *Anat. Rec.* 224:79–87

Geduspan JS, Solursh M. 1992. A growth-promoting influence from the mesonephros during limb outgrowth. *Dev. Biol.* 151:212–50

Gérard M, Duboule D, Zákány J. 1993. Cooperation of regulatory elements involved in the activation of the Hoxd-11 gene. *CR Acad. Sci. III* 316:985–94

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
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Geraudie J. 1978. Scanning electron microscope study of the developing trout pelvic fin bud. *Anat. Rec.* 191:391–94

Geraudie J, Ferretti P. 1998. Gene expression during amphibian limb regeneration. *Int. Rev. Cytol.* 180:1–50

Gibson-Brown JJ, Agulnik SI, Silver LM, Niswander L, Papaioannou VE. 1998. Involvement of T-box genes Tbx2–Tbx5 in vertebrate limb specification and development. *Development* 125:2499–509

González-Crespo S, Abu-Shaar M, Torres M, Martínez-A C, Mann RS, Morata G. 1998. Antagonism between extradenticle function and Hedgehog signalling in the developing limb. *Nature* 394:196–200

Greene HW, Cundall D. 2000. Limbless tetrapods and snakes with legs. *Science* 287: 1939–41

Grieshammer U, Minowada G, Pisenti JM, Abbott UK, Martin GR. 1996. The chick limbless mutation causes abnormalities in limb bud dorsal-ventral patterning: implications for the mechanism of apical ridge formation. *Development* 122:3851–61

Hagmann M. 2000. Why chicks aren't all thumbs. *Science* 289:372–73

Hall BK, Miyake T. 1995. Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int. J. Dev. Biol.* 39:881–93

Hamburger V, Hamilton HL. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88:49–92

Healy C, Uwanogho D, Sharpe PT. 1999. Regulation and role of Sox9 in cartilage formation. *Dev. Dyn.* 215:69–78

Helms JA, Kim CH, Eichele G, Thaller C. 1996. Retinoic acid signaling is required during early chick limb development. *Development* 122:1385–94

Hui CC, Joyner AL. 1993. A mouse model of greig cephalopolysyndactyly syndrome: the extra-toesJ mutation contains an intragenic deletion of the Gli3 gene. *Nat. Gen.* 3:241–46

Ianakiev P, Kilpatrick MW, Toudjarska I, Basel D, Beighton P, Tsipouras P. 2000. Split-hand/split-foot malformation is caused by mutations in the p63 gene on 3q27. *Am. J. Hum. Genet.* 67:59–66

Imokawa Y, Yoshizato K. 1998. Expression of sonic hedgehog gene in regenerating newt limbs. *Wound Repair Regen.* 6:366–70

Ingham PW. 1998. Transducing Hedgehog: the story so far. *EMBO J.* 17:3505–11

Isaac A, Rodríguez-Esteban C, Ryan A, Altabef M, Tsukui T et al. 1998. Tbx genes and limb identity in chick embryo development. *Development* 125:1867–75

Isaac A, Cohn MJ, Ashby P, Ataliotis P, Spicer DB, et al. 2000. FGF and genes encoding transcription factors in early limb specification. *Mech. Dev.* 93:41–48

Iulianella A, Lohnes D. 1997. Contribution of retinoic acid receptor gamma to retinoid-induced craniofacial and axial defects. *Dev. Dyn.* 209:92–104

Izpisúa Belmonte JC, Ede DA, Tickle C, Duboule D. 1992. The mis-expression of posterior Hox-4 genes in talpid (ta3) mutant wings correlates with the absence of anteroposterior polarity. *Development* 114:959–63

Johnson R, Tabin C. 1997. Molecular models for vertebrate limb development. *Cell* 90:979–90

Kawakami Y, Ishikawa T, Shimabara M, Tanda N, Enomoto-Iwamoto M, et al. 1996. BMP signaling during bone pattern determination in the developing limb. *Development* 122:3557–66

Kawakami Y, Capdevila J, Büscher D, Itoh T, Esteban CR, Belmonte JC. 2001. WNT signals control FGF-dependent limb initiation and AER induction in the chick embryo. *Cell* 104:891–900

Kelley RO, Fallon JF. 1976. Ultrastructural analysis of the apical ectodermal ridge during vertebrate limb morphogenesis. I. The human forelimb with special reference to gap junctions. *Dev. Biol.* 51:241–56

Kengaku M, Capdevila J, Rodríguez-Esteban C, De La Pena J, Johnson RL, et al. 1998. Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science* 280:1274–77

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
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Kimmel RA, Turnbull DH, Blanquet V, Wurst W, Loomis CA, Joyner AL. 2000. Two lineage boundaries coordinate vertebrate apical ectodermal ridge formation. *Genes Dev.* 14:1377–89

Kmita M, Kondo T, Duboule D. 2000. Targeted inversion of a polar silencer within the HoxD complex re-allocates domains of enhancer sharing. *Nat. Gen.* 26:451–54

Kraus P, Fraidenraich D, Loomis CA. 2001. Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech. Dev.* 100:45–58

Krauss S, Concordet JP, Ingham PW. 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 75:1431–44

Krumlauf R. 1994. Hox genes in vertebrate development. *Cell* 78:191–201

Laforest L, Brown CW, Poleo G, Geraudie J, Tada M, et al. 1998. Involvement of the sonic hedgehog, patched 1 and bmp2 genes in patterning of the zebrafish dermal fin rays. *Development* 125:4175–84

Lamonerie T, Tremblay JJ, Lanctot C, Therrien M, Gauthier Y, Drouin J. 1996. Ptx1, a bicoid-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene. *Genes Dev.* 10:1284–95

Lanctot C, Moreau A, Chamberland M, Tremblay ML, Drouin J. 1999. Hindlimb patterning and mandible development require the Ptx1 gene. *Development* 126:1805–10

Laufer E, Dahn R, Orozco OE, Yeo CY, Pisenti J, et al. 1997. Expression of Radical fringe in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* 386:366–73

Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C. 1994. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* 79:993–1003

Lefebvre V, Li P, de Crombrugghe B. 1998. A new long form of Sox5 (L-Sox5), Sox6 and Sox9 are coexpressed in chondrogenesis and cooperatively activate the type II collagen gene. *EMBO J.* 17:5718–33

Lewandoski M, Sun X, Martin GR. 2000. Fgf8 signalling from the AER is essential for normal limb development *Nat. Gen.* 26:460–63

Lewis KE, Drossopoulou G, Paton IR, Morrice DR, Robertson KE, et al. 1999. Expression of ptc and gli genes in talpid3 suggests bifurcation in Shh pathway. *Development* 126:2397–407

Li S, Muneoka K. 1999. Cell migration and chick limb development: chemotactic action of FGF-4 and the AER. *Dev. Biol.* 211:335–47

Lizarraga G, Ferrari D, Kalinowski M, Ohuchi H, Noji S, et al. 1999. FGFR2 signaling in normal and limbless chick limb buds. *Dev. Genet.* 25:331–38

Logan C, Hornbruch A, Campbell I, Lumsden A. 1997. The role of Engrailed in establishing the dorsoventral axis of the chick limb. *Development* 124:2317–24

Logan M, Simon H-G, Tabin C. 1998. Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. *Development* 125:2825–35

Logan M, Tabin CJ. 1999. Role of Pitx1 upstream of Tbx4 in specification of hindlimb identity. *Science* 283:1736–39

Loomis CA, Harris E, Michaud J, Wurst W, Hanks M, Joyner AL. 1996. The mouse Engrailed-1 gene and ventral limb patterning. *Nature* 382:360–63

Loomis CA, Kimmel RA, Tong CX, Michaud J, Joyner AL. 1998. Analysis of the genetic pathway leading to formation of ectopic apical ectodermal ridges in mouse Engrailed-1 mutant limbs. *Development* 125:1137–48

López-Martínez A, Chang DT, Chiang C, Porter JA, Ros MA, et al. 1995. Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Curr. Biol.* 5:791–96

Lu HC, Revelli JP, Goering L, Thaller C, Eichele G. 1997. Retinoid signaling is required for the establishment of a ZPA and

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
by CAPES on 09/28/05. For personal use only.

for the expression of Hoxb-8, a mediator of ZPA formation. *Development* 124:1643–51

MacCabe JA, Errick J, Saunders JW Jr. 1974. Ectodermal control of the dorsoventral axis in the leg bud of the chick embryo. *Dev. Biol.* 39:69–82

Macías D, Gañán Y, Rodríguez-León J, Merino R, Hurlé JM. 1999. Regulation by members of the transforming growth factor beta superfamily of the digital and interdigital fates of the autopodial limb mesoderm. *Cell Tissue Res.* 296:95–102

Macías D, Gañán Y, Ros MA, Hurlé JM. 1996. In vivo inhibition of programmed cell death by local administration of FGF-2 and FGF-4 in the interdigital areas of the embryonic chick leg bud. *Anat. Embryol.* 193:533–41

Mackem S, Knezevic V. 1999. Do 5'Hoxd genes play a role in initiating or maintaining A-P polarizing signals in the limb? *Cell Tissue Res.* 296:27–31

Mahmood R, Bresnick J, Hornbruch A, Mahony C, Morton N, et al. 1995. A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol.* 5:797–806

Makarenkova H, Becker DL, Tickle C, Warner AE. 1997. Fibroblast growth factor 4 directs gap junction expression in the mesenchyme of the vertebrate limb bud. *J. Cell. Biol.* 138:1125–37

Makarenkova H, Patel K. 1999. Gap junction signalling mediated through Connexin-43 is required for chick limb development. *Dev. Biol.* 207:380–92

Maniatis T. 1999. A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes Dev.* 13:505–10

Marshall H, Morrison A, Studer M, Popperl H, Krumlauf R. 1996. Retinoids and Hox genes. *FASEB J.* 10:969–78

Martí E, Takada R, Bumcrot DA, Sasaki H, McMahon AP. 1995. Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* 121:2537–47

Martin GR. 1998. The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* 12:1571–86

Masuya H, Sagai T, Moriwaki K, Shiroishi T. 1997. Multigenic control of the localization of the zone of polarizing activity in limb morphogenesis in the mouse. *Dev. Biol.* 182:42–51

Masuya H, Sagai T, Wakana S, Moriwaki K, Shiroishi T. 1995. A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* 9:1645–53

McPherron AC, Lawler AM, Lee SJ. 1999. Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. *Nat. Genet.* 22:260–64

Mercader N, Leonardo E, Azpiazu N, Serrano A, Morata G, et al. 1999. Conserved regulation of proximodistal limb axis development by Meis1/Hth. *Nature* 402:425–29

Mercader N, Leonardo E, Piedra ME, Martínez-AC, Ros MA, Torres M. 2000. Opposing RA and FGF signals control proximodistal vertebrate limb development through regulation of meis genes *Development* 127:3961–70

Merino R, Gañán Y, Macías D, Economides AN, Sampath KT, Hurlé JM. 1998. Morphogenesis of digits in the avian limb is controlled by FGFs, TGFbetas, and noggin through BMP signaling. *Dev. Biol.* 200:35–45

Merino R, Rodríguez-Leon J, Macías D, Gañán Y, Economides AN, Hurlé JM. 1999. The BMP antagonist Gremlin regulates outgrowth, chondrogenesis and programmed cell death in the developing limb. *Development* 126:5515–22

Miao N, Wang M, Ott JA, D'Alessandro JS, Woolf TM, et al. 1997. Sonic hedgehog promotes the survival of specific CNS neuron populations and protects these cells from toxic insult in vitro. *J. Neurosci.* 17:5891–99

Michaud JL, Lapointe F, Le Douarin NM. 1997. The dorsoventral polarity of the presumptive limb is determined by signals produced by the somites and by the lateral somatopleure. *Development* 124:1453–63

Milenkovic L, Goodrich LV, Higgins KM,

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
by CAPES on 09/28/05. For personal use only.

Scott MP. 1999. Mouse patched1 controls body size determination and limb patterning. *Development* 126:4431–40

Millan FA, Denhez F, Kondaiah P, Akhurst RJ. 1991. Embryonic gene expression patterns of TGF beta 1, beta 2 and beta 3 suggest different developmental functions in vivo. *Development* 111:131–43

Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* 398:708–13

Min H, Danilenko DM, Scully SA, Bolon B, Ring BD, et al. 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless. *Genes Dev.* 12:3156–61

Miner JH, Cunningham J, Sanes JR. 1998. Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. *J. Cell. Biol.* 143:1713–23

Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, et al. 1997. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. *Development* 124:113–23

Moon AM, Boulet AM, Capecchi MR. 2000. Normal limb development in conditional mutants of Fgf4. *Development* 127:989–96

Moon AM, Capecchi MR. 2000. Fgf8 is required for outgrowth and patterning of the limbs. *Nat. Gen.* 26:455–59

Moran JL, Levorse JM, Vogt TF. 1999. Limbs move beyond the radical fringe. *Nature* 399:742–43

Morata G, Basler K. 1999. Cells in search of a signal. *Nat. Cell Biol.* 1:E60–61

Morata G, Sánchez-Herrero E. 1999. Patterning mechanisms in the body trunk and the appendages of Drosophila. *Development* 126:2823–28

Mullen LM, Bryant SV, Torok MA, Blumberg B, Gardiner DM. 1996. Nerve dependency of regeneration: the role of Distal-less and FGF signaling in amphibian limb regeneration. *Development* 122:3487–97

Muller TL, Ngo-Muller V, Reginelli A, Taylor G, Anderson R, Muneoka K. 1999. Regeneration in higher vertebrates: limb buds and digit tips. *Semin. Cell Dev. Biol.* 10:405–13

Mundlos S, Olsen BR. 1997. Heritable diseases of the skeleton. Part I: molecular insights into skeletal development-transcription factors and signaling pathways. *FASEB J.* 11:125–32

Nelson CE, Morgan BA, Burke AC, Laufer E, DiMambro E, et al. 1996. Analysis of Hox gene expression in the chick limb bud. *Development* 122:1449–66

Newman SA. 2000. Fingering digit identity. *Science* 290:275–77

Niswander L, Jeffrey S, Martin GR, Tickle C. 1994. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 371:609–12

Noramly S, Pisenti J, Abbott U, Morgan B. 1996. Gene expression in the limbless mutant: polarized gene expression in the absence of Shh and an AER. *Dev. Biol.* 179:339–46

Ohsugi K, Gardiner DM, Bryant SV. 1997. Cell cycle length affects gene expression and pattern formation in limbs. *Dev. Biol.* 189:13–21

Ohuchi H, Nakagawa T, Yamamoto A, Araga A, Ohata T, et al. 1997. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* 124:2235–44

Ohuchi H, Nakagawa T, Yamauchi M, Ohata T, Yoshioka H, et al. 1995. An additional limb can be induced from the flank of the chick embryo by FGF4. *Biochem. Biophys. Res. Commun.* 209:809–16

Ohuchi H, Takeuchi J, Yoshioka H, Ishimaru Y, Ogura K, et al. 1998. Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. *Development* 125:51–60

Oliver G, De Robertis EM, Wolpert L, Tickle C. 1990. Expression of a homeobox gene in the chick wing bud following application of retinoic acid and grafts of polarizing region tissue. *EMBO J.* 9:3093–99

Olsen BR, Reginato AM, Wang W. 2000.

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
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Bone development. *Annu. Rev. Cell Dev. Biol.* 16:191–220

Oppenheim RW, Homma S, Martí E, Prevette D, Wang S, et al. 1999. Modulation of early but not later stages of programmed cell death in embryonic avian spinal cord by sonic hedgehog. *Mol. Cell Neurosci.* 13:348–61

Panganiban G, Irvine SM, Lowe C, Roehl H, Corley LS, et al. 1997. The origin and evolution of animal appendages. *Proc. Natl. Acad. Sci. USA* 94:5162–66

Parr BA, McMahon AP. 1995. Dorsalizing signal Wnt-7a required for normal polarity of D-V and A-P axes of mouse limb. *Nature* 374:350–53

Parr BA, Shea MJ, Vassileva G, McMahon AP. 1993. Mouse Wnt genes exhibit discrete domains of expression in the early embryonic CNS and limb buds. *Development* 119:247–61

Patton EE, Willems AR, Tyers M. 1998. Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends Genet.* 14:236–43

Pautou MP. 1977. Dorso-ventral axis determination of chick limb bud development. In *Vertebrate Limb and Somite Morphogenesis*, ed. DA Ede, JR Hinchliffe, M Balls, pp. 257–66. Cambridge, UK: Cambridge Univ. Press

Pizette S, Niswander L. 1999. BMPs negatively regulate structure and function of the limb apical ectodermal ridge. *Development* 126:883–94

Poss KD, Shen J, Keating MT. 2000a. Induction of lef1 during zebrafish fin regeneration. *Dev. Dyn.* 219:282–86

Poss KD, Shen J, Nechiporuk A, McMahon G, Thisse B, et al. 2000b. Roles for Fgf signaling during zebrafish fin regeneration. *Dev. Biol.* 222:347–58

Qu S, Niswender KD, Ji Q, van der Meer R, Keeney D, et al. 1997. Polydactyly and ectopic ZPA formation in Alx-4 mutant mice. *Development* 124:3999–4008

Qu S, Tucker SC, Ehrlich JS, Levorse JM, Flaherty LA, et al. 1998. Mutations in mouse Aristaless-like4 cause Strong's luxoid polydactyly. *Development* 125:2711–21

Qu S, Tucker SC, Zhao Q, de Crombrugghe B, Wisdom R. 1999. Physical and genetic interactions between Alx4 and Cart1. *Development* 126:359–69

Ramírez-Weber FA, Kornberg TB. 1999. Cytonemes: cellular processes that project to the principal signaling center in Drosophila imaginal discs. *Cell* 97:599–607

Rancourt DE, Tsuzuki T, Capecchi MR. 1995. Genetic interaction between hoxb-5 and hoxb-6 is revealed by nonallelic noncomplementation. *Genes Dev.* 9:108–22

Raynaud A, Kan P, Bouche G, Duprat AM. 1995. Fibroblast growth factors (FGF-2) and delayed involution of the posterior limbs of the slow-worm embryo. *CR Acad. Sci. III* 318:573–78

Reddi AH. 1998. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat. Biotechnol.* 16:247–52

Reddi AH. 2000. Morphogenesis and tissue engineering of bone and cartilage: inductive signals, stem cells, and biomimetic biomaterials. *Tissue Eng.* 6:351–59

Richardson MK, Carl TF, Hanken J, Elinson RP, Cope C, Bagley P. 1998. Limb development and evolution: a frog embryo with no apical ectodermal ridge (AER). *J. Anat.* 192:379–90

Riddle RD, Ensini M, Nelson C, Tsuchida T, Jessell TM, Tabin C. 1995. Induction of the LIM homeobox gene Lmx1 by WNT7a establishes dorsoventral pattern in the vertebrate limb. *Cell* 83:631–40

Riddle RD, Johnson RL, Laufer E, Tabin C. 1993. Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75:1401–16

Rodríguez C, Kos R, Macías D, Abbott UK, Izpisúa Belmonte JC. 1996. Shh, HoxD, Bmp-2, and Fgf-4 gene expression during development of the polydactylous talpid2, diplopodia1, and diplopodia4 mutant chick limb buds. *Dev. Genet.* 19:26–32

Rodríguez-Esteban C, Schwabe JW, De La Peña J, Foys B, Eshelman B, Belmonte JC. 1997. Radical fringe positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* 386:360–66

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
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Rodríguez-Esteban C, Tsukui T, Yonei S, Magallon J, Tamura K, Izpisúa-Belmonte JC. 1999. The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. *Nature* 398:814–18

Rodríguez-León J, Merino R, Macías D, Gañán Y, Santesteban E, Hurlé JM. 1999. Retinoic acid regulates programmed cell death through BMP signalling. *Nat. Cell Biol.* 1:125–26

Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, et al. 1994. Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* 76:761–75

Ros MA, Lopez-Martinez A, Simandl BK, Rodriguez C, Izpisua Belmonte JC, et al. 1996. The limb field mesoderm determines initial limb bud anteroposterior asymmetry and budding independent of sonic hedgehog or apical ectodermal gene expressions. *Development* 122:2319–30

Ruvinsky I, Gibson-Brown JJ. 2000. Genetic and developmental bases of serial homology in vertebrate limb evolution. *Development* 127:5233–44

Ruvinsky I, Oates AC, Silver LM, Ho RK. 2000. The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Dev. Genes Evol.* 210:82–91

Saleh M, Huang H, Green NC, Featherstone MS. 2000. A conformational change in PBX1A is necessary for its nuclear localization. *Exp. Cell Res.* 260:105–15

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, et al. 1997. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 124:2659–70

Santamaría JA, Becerra J. 1991. Tail fin regeneration in teleosts: cell-extracellular matrix interaction in blastemal differentiation. *J Anat.* 176:9–21

Saunders JW Jr. 1948. The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool.* 108:363–403

Saunders JW, Gasseling MT. 1963. *Trans*-filter propagation of apical ectoderm maintenance factor in the chick embryo wing bud. *Dev. Biol.* 7:64–78

Saunders JW Jr, Gasseling MT. 1968. Ectoderm-mesenchymal interaction in the origins of wing symmetry. In *Epithelial-Mesenchymal Interactions*, ed. R Fleischmajer, RE Billingham, pp. 289–314. Baltimore: Williams & Wilkins

Schimmang T, Lemaistre M, Vortkamp A, Ruther U. 1992. Expression of the zinc finger gene Gli3 is affected in the morphogenetic mouse mutant extra-toes (Xt). *Development* 116:799–804

Schubert M, Holland LZ, Holland ND. 2000. Characterization of an amphioxus wnt gene, AmphiWnt11, with possible roles in myogenesis and tail outgrowth. *Genesis* 27:1–5

Schwabe JW, Rodríguez-Esteban C, Izpisúa Belmonte JC. 1998. Limbs are moving: where are they going? *Trends Genet.* 14:229–35

Searls RL, Janners MY. 1971. The initiation of limb bud outgrowth in the embryonic chick. *Dev. Biol.* 24:198–213

Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, et al. 1999. Fgf10 is essential for limb and lung formation. *Nat. Genet.* 21:138–41

Shang J, Luo Y, Clayton DA. 1997. Backfoot is a novel homeobox gene expressed in the mesenchyme of developing hind limb. *Dev. Dyn.* 209:242–53

Sharpe J, Lettice L, Hecksher-Sorensen J, Fox M, Hill R, Krumlauf R. 1999. Identification of sonic hedgehog as a candidate gene responsible for the polydactylous mouse mutant Sasquatch. *Curr. Biol.* 9:97–100

Sherman L, Wainwright D, Ponta H, Herrlich P. 1998. A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev.* 12:1058–71

Shimeld SM. 1999. The evolution of the hedgehog gene family in chordates: insights

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
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from amphioxus hedgehog. *Dev. Genes Evol.* 209:40–47

Shubin N, Tabin C, Carroll S. 1997. Fossils, genes and the evolution of animal limbs. *Nature* 388:639–48

Sidow A, Bulotsky MS, Kerrebrock AW, Birren BW, Altshuler D, et al. 1999. A novel member of the F-box/WD40 gene family, encoding dactylin, is disrupted in the mouse dactylaplasia mutant. *Nat. Genet.* 23:104–7

Simon H-G, Kittappa R, Khan PA, Tsilfidis C, Liversage RA, Oppenheimer S. 1997. A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate genes involved in vertebrate forelimb/hindlimb patterning. *Development* 124:1355–66

Sordino P, van der Hoeven F, Duboule D. 1995. Hox gene expression in teleost fins and the origin of vertebrate digits. *Nature* 375:678–81

Stephens TD, McNulty TR. 1981. Evidence for a metameric pattern in the development of the chick humerus. *J. Embryol. Exp. Morphol.* 61:191–205

Stocum DL. 1995. *Wound Repair, Regeneration and Artificial Tissues*. Austin: Landes

Stocum DL. 1999. Limb regeneration: reentering the cell cycle. *Curr. Biol.* 9:R644–46

Stratford T, Horton C, Maden M. 1996. Retinoic acid is required for the initiation of outgrowth in the chick limb bud. *Curr. Biol.* 6:1124–33

Stratford TH, Kostakopoulou K, Maden M. 1997. Hoxb-8 has a role in establishing early anterior-posterior polarity in chick forelimb but not hindlimb. *Development* 124:4225–34

Stratford T, Logan C, Zile M, Maden M. 1999. Abnormal anteroposterior and dorsoventral patterning of the limb bud in the absence of retinoids. *Mech. Dev.* 81:115–25

Strecker TR, Stephens TD. 1983. Peripheral nerves do not play a trophic role in limb skeletal morphogenesis. *Teratology* 27:159–67

Summerbell D, Lewis JH, Wolpert L. 1973. Positional information in chick limb morphogenesis. *Nature* 244:492–96

Sun X, Lewandoski M, Meyers EN, Liu YH, Maxson RE, Martin GR. 2000. Conditional inactivation of Fgf4 reveals complexity of signalling during limb bud development. *Nat. Genet.* 25:83–86

Szeto DP, Rodriguez-Esteban C, Ryan AK, O'Connell SM, Liu F, et al. 1999. Role of the Bicoid-related homeodomain factor Pitx1 in specifying hindlimb morphogenesis and pituitary development. *Genes Dev.* 13:484–94

Szeto DP, Ryan AK, O'Connell SM, Rosenfeld MG. 1996. P-OTX: a PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development. *Proc, Natl. Acad. Sci. USA* 93:7706–10

Takabatake Y, Takabatake T, Takeshima K. 2000. Conserved and divergent expression of T-box genes Tbx2-Tbx5 in Xenopus. *Mech. Dev.* 91:433–37

Takahashi M, Tamura K, Büscher D, Masuya H, Yonei-Tamura S, et al. 1998. The role of Alx-4 in the establishment of anteroposterior polarity during vertebrate limb development. *Development* 125:4417–25

Takeuchi JK, Koshiba-Takeuchi K, Matsumoto K, Vogel-Hopker A, Naitoh-Matsuo M, et al. 1999. Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. *Nature* 398:810–14

Tamura K, Yonei-Tamura S, Belmonte JC. 1999. Differential expression of Tbx4 and Tbx5 in Zebrafish fin buds. *Mech. Dev.* 87:181–84

Tanaka M, Shigetani Y, Sugiyama S, Tamura K, Nakamura H, Ide H. 1998. Apical ectodermal ridge induction by the transplantation of En-1-overexpressing ectoderm in chick limb bud. *Dev. Growth Differ.* 40:423–29

Tickle C. 1999. Morphogen gradients in vertebrate limb development. *Semin. Cell Dev. Biol.* 10:345–51

Tickle C, Alberts B, Wolpert L, Lee J. 1982. Local application of retinoic acid to the limb bond mimics the action of the polarizing region. *Nature* 296:564–66

Tickle C, Eichele G. 1994. Vertebrate limb development. *Annu. Rev. Cell Biol.* 10:121–52

Annu. Rev. Cell. Dev. Biol. 2001.17:87-132. Downloaded from arjournals.annualreviews.org
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Todt WL, Fallon JF. 1984. Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. Exp. Morphol.* 80:21–41

Valentine JW, Jablonski D, Erwin DH. 1999. Fossils, molecules and embryos: new perspectives on the Cambrian explosion. *Development* 126:851–59

van den Akker E, Reijnen M, Korving J, Brouwer A, Meijlink F, Deschamps J. 1999. Targeted inactivation of Hoxb8 affects survival of a spinal ganglion and causes aberrant limb reflexes. *Mech. Dev.* 89:103–14

van der Hoeven F, Zákány J, Duboule D. 1996. Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. *Cell* 85:1025–35

Villavicencio EH, Walterhouse DO, Iannaccone PM. 2000. The sonic hedgehog-patched-gli pathway in human development and disease. *Am. J. Hum. Genet.* 67:1047–54

Vogel A, Rodriguez C, Izpisúa-Belmonte JC. 1996. Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* 122:1737–50

Vogel A, Rodriguez C, Warnken W, Izpisúa Belmonte JC. 1995. Dorsal cell fate specified by chick Lmx1 during vertebrate limb development. *Nature* 378:716–20

Vogt TF, Duboule D. 1999. Antagonists go out on a limb. *Cell* 99:563–66

Wada H, García-Fernández J, Holland PW. 1999. Colinear and segmental expression of amphioxus Hox genes. *Dev. Biol.* 213:131–41

Wagner GP, Misof BY. 1992. Evolutionary modification of regenerative capability in vertebrates: a comparative study on teleost pectoral fin regeneration. *J. Exp. Zool.* 261: 62–78

Wagner GP. 2000. What is the promise of developmental evolution? Part I: why is developmental biology necessary to explain evolutionary innovations? *J. Exp. Zool.* 288:95–98

Wagner T, Wirth J, Meyer J, Zabel B, Held M, et al. 1994. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. *Cell* 79:1111–20

Weatherbee SD, Carroll SB. 1999. Selector genes and limb identity in arthropods and vertebrates. *Cell* 97:283–86

Wilcox MJ, Kelley RO. 1993. Cell of the apical ectodermal ridge in quail limb buds are coupled to one another but are isolated from subjacent mesoderm. *Prog. Clin. Biol. Res.* 383A:339–49

Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, et al. 1995. The Sry-related gene Sox9 is expressed during chondrogenesis in mouse embryos. *Nat. Genet.* 9:15–20

Xu X, Weinstein M, Li C, Deng C. 1999. Fibroblast growth factor receptors (FGFRs) and their roles in limb development. *Cell Tissue Res.* 296:33–43

Xu X, Weinstein M, Li C, Naski M, Cohen RI, et al. 1998. Fibroblast growth factor receptor 2 (FGFR2)-mediated reciprocal regulation loop between FGF8 and FGF10 is essential for limb induction. *Development* 125:753–65

Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, et al. 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 398:714–18

Yang Y, Drossopoulou G, Chuang PT, Duprez D, Marti E, et al. 1997. Relationship between dose, distance and time in Sonic Hedgehog-mediated regulation of anteroposterior polarity in the chick limb. *Development* 124:4393–404

Yang Y, Guillot P, Boyd Y, Lyon MF, McMahon AP. 1998. Evidence that preaxial polydactyly in the Doublefoot mutant is due to ectopic Indian Hedgehog signaling. *Development* 125:3123–32

Yang Y, Niswander L. 1995. Interaction between the signaling molecules WNT7a and SHH during vertebrate limb development: dorsal signals regulate anteroposterior patterning. *Cell* 80:939–47

Yasui K, Zhang S, Uemura M, Saiga H. 2000. Left-right asymmetric expression of BbPtx, a Ptx-related gene, in a lancelet species and the developmental left-sidedness in deuterostomes. *Development* 127:187–95

Yokouchi Y, Sakiyama J, Kameda T, Iba H, Suzuki A, et al. 1996. BMP-2/-4 mediate programmed cell death in chicken limb buds. *Development* 122:3725–34

Yonei-Tamura S, Endo T, Yajima H, Ohuchi H, Ide H, Tamura K. 1999. FGF7 and FGF10 directly induce the apical ectodermal ridge in chick embryos. *Dev. Biol.* 211:133–43

Zákány J, Duboule D. 1999. Hox genes in digit development and evolution. *Cell Tissue. Res.* 296:19–25

Zákány J, Fromental-Ramain C, Warot X, Duboule D. 1997. Regulation of number and size of digits by posterior Hox genes: a dose dependent mechanism with potential evolutionary implications. *Proc. Natl. Acad. Sci. USA* 94:13695–700

Zeller R, Haramis AG, Zuniga A, McGuigan C, Dono R, et al. 1999. Formin defines a large family of morphoregulatory genes and functions in establishment of the polarising region. *Cell Tissue Res.* 296:85–93

Zhang N, Gridley T. 1999. Limbs move beyond the Radical fringe. *Nature* 399:743

Zimmerman LB, De Jesús-Escobar JM, Harland RM. 1996. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* 86:599–606

Zou H, Niswander L. 1996. Requirement for BMP signaling in interdigital apoptosis and scale formation. *Science* 272:738–41

Zou H, Wieser R, Massagué J, Niswander L. 1997. Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. *Genes Dev.* 11:2191–203

Zuniga A, Haramis AP McMahon AP, Zeller R. 1999. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 401:598–602

Zwilling E. 1955. Ectoderm-mesoderm relationship in the development of the chick embryo limb bud. *J. Exp. Zool.* 128:423–41

Zwilling E. 1956. Interaction between limb bud ectoderm and mesoderm in the chick embryo. IV. Experiments with a wingless mutant. *J. Exp. Zool.* 132:241–54

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*Annual Review of Cell and Developmental Biology*
*Volume 17, 2001*

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