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Growing models of vertebrate limb development

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The developing limb has been a very influential system for studying pattern formation in vertebrates. In the past, classical embryological models have explained how patterned structures are generated along the two principal axes of the limb: the proximodistal (shoulder to finger) and anteroposterior (thumb to little finger) axes. Over time, the genetic and molecular attributes of these patterning models have been discovered, while the role of growth in the patterning process has been only recently highlighted. In this review, we discuss these recent findings and propose how the various models of limb patterning can be reconciled.

Introduction

The developing limb has long been a pioneering model for understanding pattern formation: the process in which the spatial organisation of differentiated cells and tissues is generated in the embryo. One aspect of limb development that has perplexed several generations of researchers is the importance of growth. This might appear to be a trivial problem because growth occurs throughout the period when pattern is laid down and so, in the broadest sense, it is obviously required for development. However, controversy surrounds whether growth is required for the actual specification of pattern.

Pattern formation can be considered as a two-step process; first cells are informed of their position and, thus, acquire a positional value (specification); cells then remember and interpret this value to form the appropriate structures (differentiation) (Wolpert, 1969). In the developing chick leg, specification cues can be experimentally overridden until quite a late stage, leading to altered differentiation and morphogenesis, thus revealing remarkable developmental plasticity (Dahn and Fallon, 2000). Three main scenarios for the role of growth in pattern formation have been suggested and can be illustrated by the classical French flag model (Wolpert, 1969; Wolpert, 1989). In one scenario, growth itself is proposed to specify positional values directly (Fig. 1A). In another, local growth generates positional values by intercalating existing disparate positional values, as seen in regenerating amphibian limbs (French et al., 1976) (Fig. 1B). In the third scenario, growth is proposed to play no direct patterning role, but simply to expand positional values that have already been specified by a different mechanism, such as a concentration gradient of a long-range morphogen (Fig. 1C).

Studies of the genetic basis of some human congenital limb defects, such as Apert syndrome (Wilkie et al., 1995) and preaxial polydactyly (PPD) (Lettice et al., 2002) have complemented experimental findings in the main model organisms, the chick and the mouse. However, in order to understand the relationship between genotype and phenotype, we need to have a better grasp of the basic patterning mechanisms that operate during limb development, knowledge that could be incorporated into our current models of embryonic pattern formation. Thus, it is encouraging that several recent papers on limb development propose patterning models in which growth features as an integral component (Towers et al., 2008; Zhu et al., 2008; Mariani et al., 2008). These findings will be the focus of this review.

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An overview of chick and mouse limb development

The three main axes of the vertebrate limb are: the proximodistal (PD), running in the human arm from shoulder to digits; the anteroposterior (AP), from thumb to the little finger; and dorsoventral (DV), from the back of the hand to the palm. Much of the classical work on vertebrate limb development has been carried out in chicken embryos because the developing wing and leg are easy to access. More recently, mice have emerged as powerful models in which to study limb patterning, owing to the ability to manipulate gene function in a spatially and temporally regulated manner in the limb (Logan et al., 2002). The main stages of chick wing and mouse forelimb development are similar, and it has been usual to extrapolate findings between these models (Martin, 1990; Fernandez-Teran et al., 2006); however, there are some differences, which are highlighted in Fig. 2.

The chick wing and the mouse forelimb skeleton have the typical vertebrate plan with three main regions along the PD axis, humerus, radius/ulna and digits together with a variable number of wrist elements (not shown). In the chick wing, there are only three digits across the AP axis, rather than five digits, as in the mouse forelimb (Fig. 2A).

The first visible signs of limb development are small bulges, called limb buds, which grow out of either side of the body wall at appropriate levels (Fig. 2B). The early bud consists of a meshwork of apparently homogeneous undifferentiated mesenchymal cells covered with ectoderm. Chick wing buds have a translucent rim due to the thickened ectoderm known as the apical ectodermal ridge (AER). This thickened AER is required for bud outgrowth, and develops about a day later in the mouse forelimb. As the bud elongates, the mouse limb forms a relatively broader hand plate than the chick wing, and cells near the body wall begin to differentiate into various specialised tissues, while cells at the bud tip remain undifferentiated. It takes 7 days after wing buds first appear (about 5 days in the mouse forelimb) for the complete skeleton to been laid down, with the humerus forming first and the digits last.

Detailed cell-marking experiments in chick wing buds have shown that, in addition to the pronounced outgrowth that occurs along the PD axis, there is also considerable expansion of the posterior region of the bud across the AP axis (Vargesson et al., 1997). Thus, the posterior-distal region of the early wing bud forms the digits, whereas the anterior-distal half contributes to more proximal structures. In the chick wing, there is also non-uniform expansion of the AER, with the posterior part expanding more than the anterior part (Vargesson et al., 1997). Fate-mapping of the mouse forelimb bud also shows that the posterior part contributes more to digit development than does the anterior part (Muneoka et al., 1989). These localised differences in chick and mouse limb bud expansion

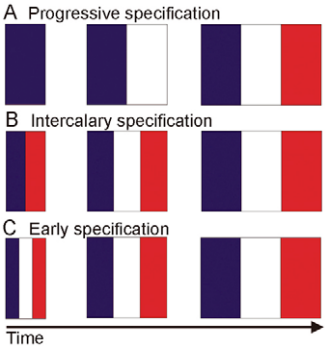

Fig. 1. French flag models illustrating potential roles of growth in embryonic patterning. The three colours of the French flag depict positional values that specify different cell fates across an embryonic field, such as the limb bud, over time (see Wolpert, 1969; Wolpert, 1989). (**A**) Progressive specification. The blue value is specified first, followed by the white and the red as growth occurs. The final positions that make up the final flag are attained by the displacement of blue, white and then red cells from the right-hand side of the field over time by growth. (**B**) Intercalary specification. The outlying blue and red positional values are specified first and the disparity between these extreme values promotes local growth that provides the white intermediate positional value. (**C**) Early specification. The coloured positional values that make up the flag are specified very early by, for example, a morphogen gradient, and further growth expands the field.

cannot readily be related to cellular behaviour because most cells are proliferating (Fernandez-Teran et al., 2006). There are, however, indications that cell cycle times may be slower in the anterior region of the chick wing than in the posterior region (Cairns, 1977), thus potentially contributing to differential expansion. Apoptosis is not thought to influence overall limb bud growth in either mouse or chick, to any large extent, and, where present, is concentrated in restricted areas. In the early chick wing bud, cell death occurs in the anterior and posterior necrotic zones (Saunders and Gasseling, 1962), and might be associated with the relatively narrow hand plate of the chick wing compared with the mouse forelimb (Fernandez-Teran et al., 2006). In mouse forelimb buds, there is also a region of cell death at the anterior margin but no posterior necrotic zone (Fernandez-Teran et al., 2006).

Models of vertebrate limb patterning

In the 1970s, experiments on chick wing buds produced two classical models of limb development (see Boxes 1 and 2). In the progress zone model, growth was suggested to have a direct role in progressively specifying PD positional values (Summerbell et al., 1973) (see Fig. 1A; Box 1), whereas in the morphogen gradient model, a morphogen gradient was proposed to specify AP values in the early bud, which are then 'remembered' throughout subsequent growth (Tickle et al., 1975) (see Fig. 1C; Box 2). DV patterning involves signals from both dorsal and ventral ectoderm (MacCabe et al., 1974), but as there appears to be relatively little growth along this axis, it will not be considered further here [for recent insights into DV patterning see Arques et al. (Arques et al., 2007), which reports an unexpected cell lineage-restricted compartment boundary that separates dorsal and ventral mesenchyme in the mouse limb bud].

Even in simple models of limb development, the relationship between patterning and growth can be complex. Thus, it will take time for a diffusible morphogen to set up a gradient, and cells will have to adjust constantly to changing morphogen concentrations.

Box 1. The development of the classical progress zone model

The classical progress zone model proposes that, as the limb bud grows out under the influence of signalling from the apical ectodermal ridge (AER), proximodistal (PD) positional values are specified progressively by the length of time cells spend in an undifferentiated region at the bud tip called the progress zone (Summerbell et al., 1973). Cells that spend a short time in the progress zone are specified to form proximal structures, whereas cells that spend longer there form more-distal structures (see Fig. 1A).

The finding that the chick wing is truncated when the AER is removed (Saunders, 1948; Summerbell et al., 1973) was the key to showing the importance of AER signalling in limb development. The extent of the truncation depends on the time at which the AER is removed: when removed early, only proximal structures develop; when removed later, more distal wing structures form. Another John Saunders study showed that when the AER from a late chick wing bud is replaced with the AER from an early wing bud and vice versa, normal limbs still develop, leading to the conclusion that AER signalling is permissive (Rubin and Saunders, 1972). Saunders also discovered that cells from the proximal region of a chick leg bud placed under the AER of a chick wing bud form toes, thus showing that proximal cells can be re-specified when placed at the bud tip (Saunders and Gasseling, 1959).

In 1973, Dennis Summerbell and colleagues reported that transplanting the undifferentiated tip of an early chick wing bud to the stump of a late wing bud, or transplanting a late bud tip to an early stump, resulted in duplications or deletions, respectively, thus showing that the limb bud tip behaves autonomously, a key finding for the progress zone model (Summerbell et al., 1973). Lewis Wolpert and colleagues then showed that killing cells in the early wing bud with high doses of X-irradiation led to loss of proximal structures, whereas distal structures remained relatively unaffected, a result that can be explained by the progress zone model (Wolpert et al., 1979).

Furthermore, the fact that the limb bud is continuously growing complicates the specification of positional values, and growth may actually play a key role in determining the size of the field over which a morphogen operates (see later). Additionally, another way of setting up a morphogen concentration gradient is by RNA or protein decay over time in a growing tissue, thus leading to short-range signals with long-range effects (Dubrulle and Pourquie, 2004). In the following sections, we consider the involvement of growth in PD and AP patterning of the developing limb.

Proximodistal patterning

The progress zone model of chick wing PD patterning

The progress zone model for patterning the PD axis emerged as a result of many embryological experiments on chick wing buds in the 1970s (Box 1; Fig. 3A). It had been known for a long time that the AER is required for limb bud outgrowth and for the accompanying sequential proximal-to-distal differentiation of skeletal elements (Fig. 3A) (Saunders, 1948). It was also known that the removal of the AER at different stages of wing development causes truncations that progressively become more distally restricted the later the operation is performed (Fig. 3A). It was, however, experiments in which transplanted tips of chick wing buds were shown to develop autonomously that led to the idea that the length of time that undifferentiated mesenchymal cells spend proliferating at the tip of the limb – in a region known as the progress zone – specifies PD positional values (Summerbell et al., 1973). It was suggested that these values are generated over time by a 'clock-like' mechanism and

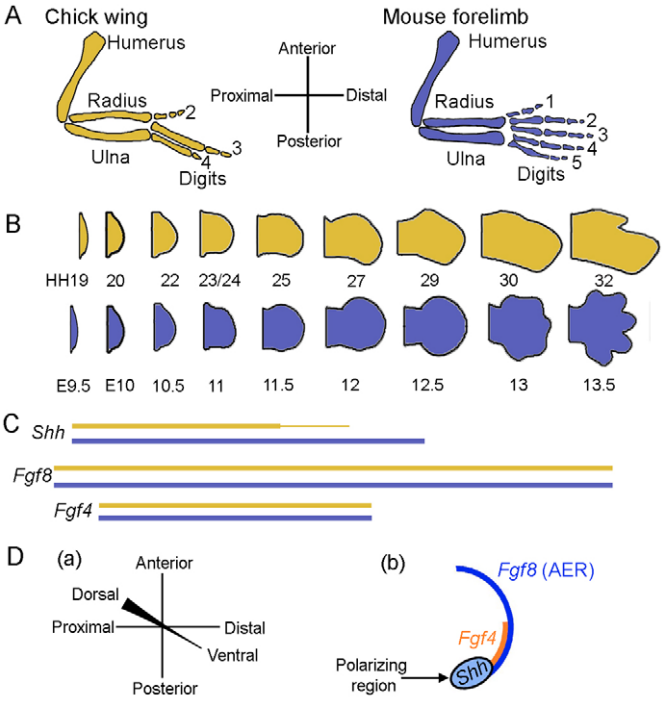

Fig. 2. Chick wing and mouse forelimb development. (**A**) A schematic of fully developed chick wing (yellow) and mouse forelimb (blue) skeletons with anteroposterior (AP) and proximodistal (PD) axes shown (as applied to all elements except the humerus). (**B**) Schematics of equivalently staged chick wing (Hamburger-Hamilton stages, HH) and mouse forelimb buds (embryonic day, E), from early stages to hand plate development. Note, mouse hindlimb development is delayed by about half a day relative to the forelimb (Martin, 1990; Fernandez-Teran et al., 2006). (**C**) Timeline of *Shh*, *Fgf4* and *Fgf8* expression in relation to embryonic stages shown in B (thin line indicates very low *Shh* expression in the chick wing). (**D**) (**a**) Orientation of AP, PD and DV axes in early stage limb buds. (**b**) A schematic of the expression of: *Shh* in the polarizing region, *Fgf8* throughout the apical ectodermal ridge (AER) and *Fgf4* in the posterior AER.

Box 2. Experimental evidence for the morphogen gradient model of anteroposterior patterning

The morphogen gradient model proposes that the polarizing region, a group of mesenchyme cells at the posterior limb bud margin (see Fig. 2D), produces a diffusible morphogen that establishes a concentration gradient across the anteroposterior (AP) axis. According to this model, cells nearest the polarizing region will be exposed to high morphogen concentrations and form posterior digits, whereas cells further away, exposed to increasingly lower concentrations, form progressively more anterior digits (Tickle et al., 1975; Wolpert, 1969).

Saunders and Gasseling discovered the polarizing region or zone of polarizing activity (ZPA) (Saunders and Gasseling, 1968). When tissue from the posterior region of a chick wing bud was grafted to the anterior margin of a second bud, a mirror-image symmetrical digit pattern resulted, with an additional set of digits arising from the anterior region of the host wing (Saunders and Gasseling, 1968). The ulna can also be duplicated if the graft is performed early in limb development, but not the humerus (Wolpert and Hornbruch, 1987), showing that the polarizing region patterns the limb distal to the elbow.

Grafts of X-irradiated polarizing regions (Smith et al., 1978), or of small numbers of polarizing region cells (Tickle, 1981), specify only additional anterior digits, showing that polarizing region signalling is dose dependent. A polarizing region must be grafted for at least 16 hours to produce an additional digit 2, and for 20 hours to produce digit 3 (Smith, 1980). An early response to a polarizing region graft was found to be increased S-phase entry in adjacent mesenchyme cells (Cooke and Summerbell, 1980). X-irradiating wing buds within 2 hours of grafting a polarizing region reduced AP growth and led to loss of anterior digits (Smith and Wolpert, 1981).

In the 1980s, it was suggested that intercalation, involving local cell-cell interactions, could explain digit duplications produced by polarizing region grafts (Iten et al., 1981). But when two polarizing regions were grafted, the complete mirror-image duplications predicted by intercalation were not obtained. Honig showed directly that polarizing region signalling was long range; a chick wing digit 2 could be specified in cells separated from a grafted polarizing region by a 200 μm wide piece of leg tissue (Honig, 1981).

become fixed when cells are displaced from the progress zone (Fig. 3A). It was calculated using data from AER removal experiments that this timing mechanism could be linked to the cell cycle because seven cell generations are required to lay down a complete chick wing skeleton, about one cell generation for each element, if one includes the two carpal bones in the wrist and the three phalanges of digit 3 (Lewis, 1975). Thus, in the classical progress zone model, specification of PD pattern depends on growth, timing and length of exposure of a population of undifferentiated mesenchyme cells to a permissive AER signal (Fig. 3A).

The early specification model of chick wing PD patterning

Over the past few years, the progress zone model has been challenged by the early specification model (Fig. 3A), which proposes that the PD pattern is specified very early and then expands, so that structures differentiate in the observed proximal-to-distal sequence, as the limb grows out under the influence of the AER (Dudley et al., 2002). The recent data assembled in support of the early specification model can, however, be accommodated by the progress zone model (Tickle and Wolpert, 2002). Thus, for example, it is a matter of interpretation as to whether the cell death that accompanies AER removal leads to the loss of cells that have already been specified to form distal structures (indicated by crosses in Fig. 3A) or to the loss of the progress zone. Furthermore, the results of fate-mapping experiments that suggest that cell lineage-restricted compartments might exist along the PD axis, a finding used to support the early specification model, have not been confirmed (Pearse et al., 2007; Sato et al., 2007). Therefore, it is not yet clear whether the progress zone model of PD limb patterning should be abandoned for the early specification model. Indeed, a progress zone model is currently favoured to explain how somites are generated along the main body axis (Dale and Pourquie, 2000). One limitation of the early specification model is that it does not explain how the pattern is set up in the early limb bud.

In summary, embryological approaches have yielded two strikingly different models of PD patterning. In the following sections, we discuss how these models stand up in light of recent genetic and molecular advances in our understanding of AER signalling.

The molecular/genetic basis of PD patterning

A simple experiment in chick wing buds, in which an FGF-soaked bead rescued wing bud outgrowth and PD patterning in the absence of the AER, showed that AER signalling is mediated by FGFs

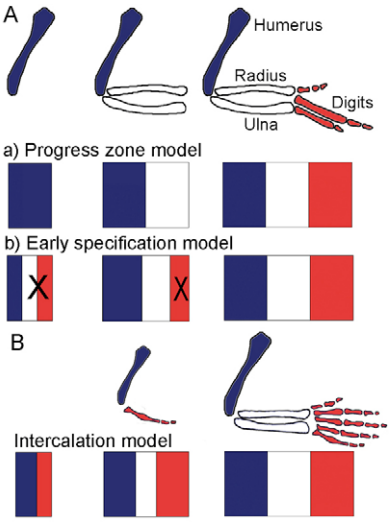

Fig. 3. Three models of proximodistal patterning in chick and mouse limbs. Schematics of how positional values (red, white and blue) are established that specify different proximodistal (PD) structures. (**A**) Apical ectodermal ridge (AER) removal in the chick wing at different stages leads to truncations, the extent of which depends on when the operation is performed (the earliest developmental stage is to the left). These results have been explained by two different models. (**a**) Progress zone model of chick wing PD patterning (Summerbell et al., 1973). Positional values are specified depending on the length of time cells remain in the distal progress zone (the right-hand side of the field in the figure). Cells that are displaced first from the progress zone form the humerus (blue), followed by forearm (white) and finally digits (red). AER removal terminates progress zone activity (shown by the loss of the white and red values). (**b**) Early specification model of chick wing PD patterning (Dudley et al., 2002). All three positional values, humerus (blue), forearm (white) and digits (red) are specified in the early chick wing bud prior to outgrowth and are expanded by growth. An alternative explanation for the limb truncations that follow AER removal is that cell death eliminates distal pre-specified positional values (crosses). Outcomes of some genetic experiments in the mouse have also supported this model (see main text for references and details). (**B**) Intercalation model of mouse limb PD patterning (Mariani et al., 2008). Reducing FGF activity in the AER of the mouse limb can lead to loss of intermediate structures, while distal and proximal structures still form (as shown in schematic of the skeleton), suggesting that positional values of the humerus (blue) and digits (red) are specified first in the early mouse limb bud with intermediate positional values (white) generated by intercalation. A loss of digits across the AP limb axis also occurs in these experiments.

(Niswander et al., 1993). It was later found that an FGF-soaked bead applied to the inter-limb region of an early chick embryo can induce the development of a complete limb (Cohn et al., 1995).

Several genes that encode FGFs are expressed in the AER at different times and with different spatial patterns, and thus the quality and quantity of FGF signalling in the limb varies over time (Martin, 1998) (Fig. 2C,D). *Fgf8* is expressed throughout the entire AP extent of the AER, from the earliest bud stages up until limb outgrowth is completed, whereas three other FGFs – *Fgf4*, *Fgf9*, *Fgf17* – are expressed for a shorter period of time and are initially more posteriorly restricted. Mesenchymal signals control these patterns of FGF gene expression in the AER (Laufer et al., 1994; Niswander et al., 1994), and this could account for the fact that, in embryological experiments, old and young AERs are interchangeable (Rubin and Saunders, 1972) (Box 1).

According to both progress zone and early specification models, FGFs secreted from the AER into the underlying mesenchyme mediate outgrowth along the PD axis and maintain the region of undifferentiated cells at the tip of the limb bud. The function of FGF signalling in mouse limb outgrowth has been tested directly by conditionally inactivating each of the FGF genes that are expressed in the AER. When *Fgf8* is functionally inactivated (Lewandoski et al., 2000; Moon and Cappechi, 2000), bud outgrowth is reduced and some digits are lost, whereas functional inactivation of the other FGFs expressed in the AER has no affect on limb development (Colvin et al., 2001; Moon et al., 2000; Sun et al., 2000; Xu et al., 2000). However, when both *Fgf4* and *Fgf8* are inactivated together at the earliest stages of bud outgrowth, limb development fails, although, when inactivated together slightly later, proximal apoptosis occurs, followed by loss of proximal structures (Sun et al., 2002). Taken together with the loss of proximal structures in mice limbs following targeted disruption of *Plzf* (Promyelocytic zinc finger) and *Gli3* (Gli/kruppel family member 3) transcription factor (Barna et al., 2005), these findings were used as support for the early specification model (Sun et al., 2002).

The range over which FGF signalling extends from the AER is unclear because although FGF8 protein can be visualised in the AER, it has not yet been detected in the mesenchyme. *Mkp3* (Map kinase phosphatase 3), a gene encoding a dual specificity phosphatase that is a transcriptional target of FGF (and which negatively regulates FGF signalling), is expressed in a gradient along the PD axis in early wing buds (Eblaghie et al., 2003). Although mRNA decay may contribute to the distribution of *Mkp3* transcripts in the limb (Pascoal et al., 2007a), the extent of *Mkp3* expression nevertheless suggests that FGF signalling from the AER can exert long-range effects on the underlying limb mesenchyme. Other genes expressed at the tip of the limb bud include the *Msx1* (muscle segment homeobox1) gene, which encodes a transcription factor that, in other contexts, including regenerating newt limbs, keeps cells in an undifferentiated state (Crews et al., 1995). It has also recently been shown that the gene *Hairy2*, which encodes a component of the somitogenesis clock, is expressed in an intriguing oscillatory fashion in cells at the tip of the chick wing bud (Pascoal et al., 2007b). The identification of further molecular clock genes would clearly support the progress zone model, although, as mentioned earlier, the periodicity of the cell cycle still provides another plausible timing mechanism.

A major question in the field concerns the identities of genes that are expressed in response to positional cues in different regions of the limb bud and then govern the development of that particular part of the pattern. Meis genes, which encode TALE-homeodomain proteins, are candidate factors for proximal limb identity that might control subsequent humerus development (Mercader et al., 2000), whereas genes that occupy 5′ positions in the *Hoxa* and *Hoxd* clusters are candidate distal identity factors that govern subsequent digit development (Zakany and Duboule, 2007). The expression patterns of these genes are established in early chick wing buds, with FGF signalling from the AER being required to maintain *Hoxa13* and *Hoxd13* expression distally, and retinoic acid (RA) signalling at the base of the bud maintaining Meis expression proximally. The overexpression of Meis genes in distal areas of chick limb buds inhibits the development of distal structures (Mercader et al., 2000), whereas knocking out *Hoxd13* and *Hoxa13* together in mice leads to loss of digits (Fromental-Ramain et al., 1996). Careful cell marking experiments in chick wing buds have indicated, however, that, as the bud grows out, some cells that express *Hoxd13* early on become displaced from the tip and cease to express *Hoxd13*

(Vargesson et al., 1997). This behaviour is difficult to reconcile with the early specification model, but also seems at odds with the idea that cells become progressively more distal in character over time. One interpretation of these data is that distal cells become progressively proximalised during limb bud outgrowth.

In addition to the gene products mentioned above that are implicated as determinants of proximal and distal limb differentiation, the product of the *Shox* (short stature homeobox containing) gene might govern intermediate limb differentiation. Mutations that affect *Shox* gene function are responsible for short stature in individuals with Turner syndrome and for the disproportionate shortening of the arm, particularly involving the radius/ulna, in individuals with Leri-Weill and Langer syndromes (Blaschke and Rappold, 2006). In chick wing buds, *Shox* is expressed in an intermediate region where it overlaps *Meis* expression proximally. This expression pattern could be explained by the fact that *Shox* is inhibited by distal FGF and by proximal RA signals (Tiecke et al., 2006).

In summary, significant progress has been made in identifying the molecules involved in PD limb patterning but this has not clarified whether this process is best explained in terms of the progress zone or early specification model. In fact, in the next section we describe how the dissection of FGF genetics in the mouse limb has led to an alternative model that, again, has classical roots.

The intercalation model of mouse PD patterning

The classical model for PD patterning in regenerating limbs involves intercalary growth (Maden, 1980) (Fig. 1B). In a recent paper, it has been suggested that intercalation might be involved in establishing PD positional values in the early mouse limb bud (Mariani et al., 2008) (Fig. 3B). Gail Martin and colleagues have used conditional gene targeting in mice to delete painstakingly FGF-encoding genes (*Fgf4*, *Fgf8*, *Fgf9*, *Fgf17*) specifically in the AER, singly and also in double and triple combinations (Mariani et al., 2008). This analysis shows that *Fgf8* can support normal forelimb development in the absence of *Fgf4*, *Fgf9* and *Fgf17*. When FGF signalling was then titrated genetically, by knocking out *Fgf8* function together with, in turn, that of *Fgf17*, *Fgf9* or *Fgf4*, and by making *Fgf4/Fgf8* conditional knockouts that were heterozygous for *Fgf9* function, mouse embryos were produced that had progressively smaller limb buds, which developed into forelimbs with correspondingly fewer skeletal elements (Mariani et al., 2008). First, digits were lost, and, in some cases, forearm elements too, although all elements of PD pattern were still represented. With further reduction in FGF function in $Fgf4^{-/-}/Fgf8^{-/-}/Fgf9^{+/-}$ conditional knockouts, forelimbs developed that had a reduced humerus and lacked a radius and ulna, but still had a digit-like structure at the distal tip. In $Fgf4^{-/-}/Fgf8^{-/-}/Fgf9^{-/-}$ conditional knockouts, no limb structures formed. This series of limb morphologies contrasts with the progressively more proximal truncations that are predicted by both early specification and progress zone models (Fig. 3A). A similar phenotype in which distal structures form in the absence of proximal structures was obtained when cell death was induced throughout chick wing buds by X irradiation (Wolpert et al., 1979). This was interpreted in terms of the progress-zone model because surviving cells would spend longer in the progress zone in order to repopulate it and thus become distalized. However, in FGF-deficient mouse limbs, this phenotype is not easy to correlate with the distribution and timing of cell death (Mariani et al., 2008). Instead, it is proposed that loss of forearm structures in $Fgf4^{-/-}/Fgf8^{-/-}/Fgf9^{+/-}$ conditional knockouts is due to loss of intermediate positional values that are normally intercalated by local growth, between proximal positional values specified by RA and distal positional values specified by FGFs. Because the role of FGFs in this model is to specify just the distal structures, the authors propose that AER signalling should be considered to be instructive rather than permissive.

As mentioned above, in the intercalation model for amphibian limb regeneration, it is the juxtaposition of cells with disparate positional values that generates local growth to restore the missing intermediate positional values (Maden, 1980). However, an intercalation model does not appear to apply to the chick limb because there is little regulation along the PD axis. Thus, for example, when the distal tip of an early wing bud is grafted to a proximal stump of an older wing bud, the intermediate part of the pattern is not produced (Summerbell et al., 1973) [but see also Kieny (Kieny, 1977)]. Some regulation can occur at very early stages of wing development when slices are cut out of the PD axis, but this regulative ability rapidly declines as the bud develops (Summerbell, 1977). The intercalation model for the mouse forelimb, however, predicts that the intermediate pattern is specified at a later stage of limb development than are the distal and proximal patterns.

In summary, there is still uncertainty as to how the PD limb pattern is specified. There could be differences in patterning mechanisms between different species that could reflect, for example, their regulative capacities, as well as overlap between seemingly opposing models. However, in the next section, we describe how such considerations based on seemingly conflicting data might yield a unified model of AP limb patterning.

Anteroposterior patterning

Classical morphogen model of chick wing AP patterning

A landmark discovery by Saunders and Gasseling was the discovery of the polarizing region, a classical organiser located at the posterior margin of a chick wing bud, which induces a new pattern of digits in mirror-image symmetry to the normal pattern when grafted to the anterior margin of another wing bud (Saunders and Gasseling, 1968) (see Box 2). The discovery of the polarizing region paved the way for a series of embryological experiments, which, over the next decade, yielded results consistent with a model in which AP positional values are specified by a gradient of a long-range morphogen (Box 2). The number and identity of the induced digits was shown to depend on both the strength (Smith et al., 1978; Tickle, 1981) and duration (Smith, 1980) of the polarizing signal (Box 2). These studies showed that only an additional digit 2 forms when the number of polarizing region cells is reduced or if the polarizing region is removed early. It should be noted that it was proposed that a morphogen gradient might act on a digit pre-pattern, which is specified by a wave-like distribution of a morphogen generated by a reaction-diffusion mechanism (Turing, 1952). One of the pieces of evidence favouring this is the formation of digits in chick limb reaggregates in which the mesenchymal cells from the limb buds were disaggregated into single cells and then placed inside a normal ectodermal jacket (Pautou, 1973) (reviewed by Wolpert, 1989). The morphogen gradient model was briefly challenged in the 1980s by the suggestion that local cell-cell interactions and intercalation might account for the digit duplications produced by polarizing region grafts (Iten et al., 1981) (Box 2).

The width of the bud and the length of the AER is a good indicator of the number of digits that form. Several experiments on the chick wing in the 1970s and 1980s indicated that the polarizing region might directly control mesenchymal cell proliferation while the digits are being specified (Cooke and Summerbell, 1980; Smith and Wolpert, 1981) (Box 2). Enhanced mesenchymal proliferation was detected prior to the extension of the overlying AER following a

polarizing graft to the anterior margin (Cooke and Summerbell, 1980). Thus, growth and specification were considered to be controlled by the same or by different signals emanating from the polarizing region (Summerbell, 1981). This proposal gained support when it was found that instead of a fully duplicated digit pattern (432234), anterior digits were lost (4334 or 434) when AP expansion was inhibited following a polarizing region graft to the anterior margin, although the mechanism by which this occurred was unclear (Smith and Wolpert, 1981). Thus, a direct role for growth in specification of AP positional values remained speculative until the molecular basis of AP patterning began to be revealed.

Molecular basis of AP patterning

It is now clear that the sonic hedgehog (*Shh*) gene, which is expressed in the polarizing region of mouse and chick limbs (Fig. 2D), is pivotal in controlling digit number and pattern (Riddle et al., 1993). RA, however, was the first defined chemical found to have polarizing activity (Tickle et al., 1982); RA-soaked beads implanted at the anterior margins of chick wings induce full-digit duplications with the same characteristics as polarizing region grafts (Tickle et al., 1985). RA is proposed to be involved in proximal limb formation through the regulation of *Meis* gene expression (Mercader et al., 2000), and its effects on AP pattern are possibly mediated via the transcriptional activation of *Shh* (Riddle et al., 1993). Consistent with the classical morphogen model of AP patterning, Shh protein can be directly detected away from the polarizing region in mouse limb buds (Gritli-Linde et al., 2001) and is indirectly detected via patched 1 (*Ptch1*) transcripts (*Ptch1* encodes the Shh receptor), which are expressed very rapidly in response to Shh signalling in the chick wing (Marigo and Tabin, 1996). Shh signalling has been shown to specify AP positional values with the time and dose dependency of polarizing region grafts (Yang et al., 1997). Fate-mapping experiments in chick wing buds have demonstrated that the prospective digit progenitor cells can be 'promoted' to more-posterior digit fates following a longer exposure to Shh signalling (Yang et al., 1997). Bone morphogenetic protein (BMP) signalling is implicated in this promotion of AP positional values downstream of Shh signalling in the chick wing bud (Drossopoulou et al., 2000).

Again, as for the PD axis, the identity of genes that determine the development of the different digits remains a major question. Evidence suggests that 5′ Hoxd genes might play a role in patterning this axis, especially at hand plate stages (Zakany and Duboule, 2007). Other candidates include members of the Tbox (*Tbx*) family and the vertebrate Sall orthologues of the *Drosophila* Spalt genes. The overexpression of either *Tbx2* or *Tbx3*, which are expressed in stripes both anteriorly and posteriorly in chick and mouse limb buds, posteriorizes toes in chick legs (Suzuki et al., 2004). Furthermore, mutations that affect the function of *TBX3* and *SALL1* and *SALL4* genes underlie congenital digital abnormalities in humans (Sweetman and Munsterberg, 2006).

Integrated growth and specification model of chick wing AP patterning

A recent growth/morphogen model of chick wing patterning suggests that growth plays an essential role in the specification of positional values in the early bud and that both processes are controlled and integrated by Shh signalling (Towers et al., 2008) (Fig. 4A). This study showed that Shh regulates the high-level expression of several genes that encode regulators of S-phase entry, including N-myc and cyclins D1/2 in the digit-forming region of the early wing bud, both in polarizing region cells, which give rise to digit 4, and in adjacent posterior cells, which give rise to digits 2 and

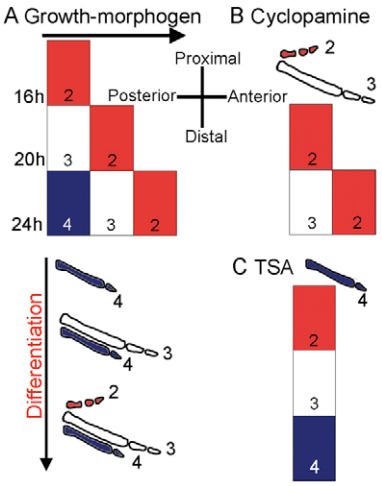

Fig. 4. Growth-morphogen model of chick wing anteroposterior patterning. [See Towers et al. (Towers et al., 2008).] In this figure, squares represent positional values and numbers indicate which digits have been specified. Timings, shown on the vertical axis, estimate the duration of Shh activity required for the specification of digits. Left is posterior, right is anterior. (**A**) Normal chick wing development. Shh protein emanating from the posterior polarizing region (left-hand side) promotes sufficient AP growth of the mesenchymal field for three digits to form (digits 2, 3 and 4). Simultaneously, the Shh specification gradient forms over the field and establishes the three AP positional values of each digit identity; low levels, digit 2 (red); intermediate levels, digit 3 (white); and high levels, digit 4 (blue). Specification involves cells being promoted through increasing AP positional values and is predicted from experimental data to take 16-24 hours (Smith, 1980; Yang et al., 1997). Positional values are remembered; digit differentiation occurs later in the sequence digit 4, 3 and 2, as shown in the skeletons below. (**B**) Reduced Shh signalling. Cyclopamine treatment blocks Shh-dependent AP expansion of the mesenchymal field to the extent that only two digits can form, as depicted in the skeleton (Scherz et al., 2007). Simultaneously, reduced Shh signalling specifies only anterior digits 2 and 3 (red and white). (**C**) Reduced proliferation and growth. TSA-treatment irreversibly inhibits AP expansion of the mesenchymal field during the interval when digits are normally specified. In the most severely affected wings, only one digit can form, as depicted in the skeleton. The duration of Shh signalling is not affected, so a single posterior digit 4 (blue) is specified.

3. It was already known that posterior digits are lost when Shh signalling is inhibited by cyclopamine (an inhibitor of smoothened, which activates the Shh signalling pathway) (Scherz et al., 2007), but this more recent study showed that loss of posterior digits was caused by a combination of reduced AP growth and specification (Fig. 4B) (Towers et al., 2008). Importantly, fate maps of cyclopamine-treated chick limbs revealed that all prospective digit progenitors contributed to the anterior elements that formed. By contrast, transient inhibition of AP growth, either by overexpressing the cyclin-dependent kinase inhibitor $p21^{cip1}$, or by applying mitotic inhibitors, including trichostatin A (TSA), resulted in loss of anterior digits (Towers et al., 2008) (Fig. 4C). In such wings, posterior specification was inhibited during growth arrest but continued for the normal duration after outgrowth recovered. However, AP expansion of the digit-forming field failed to recover following growth arrest, and fate-mapping showed this entire cell population contributed to the remaining posterior structures, often a single digit

4. These findings demonstrate that Shh normally promotes AP expansion and specification of the digit-forming field, which together determine digit number and identity in the chick wing (Fig. 4A).

In the next sections, we discuss how models of AP patterning derived in the chick wing stand up in the light of results derived from genetic studies in the mouse.

Genetics of mouse limb AP patterning

Many of the fundamental concepts of mouse digit AP patterning originate in embryological studies undertaken in the chick wing. For example, grafts of tissue from the posterior of the mouse limb to the anterior of chick wing buds can induce a full set of chick wing digits (Tickle et al., 1976). The simplest model that accounts for these results is that a gradient of polarizing activity patterns mouse digits, as it does in the chick wing. However, it is becoming evident that mouse AP limb patterning is much more complicated than chick AP wing patterning, not least because the mouse has five digits rather than three. The complete inactivation of *Shh* in the mouse results in the loss of all digits in the forelimb and of all but the most anterior digit (digit 1) in the hindlimb, which is therefore considered to be independent of Shh (Chiang et al., 1996). The same pattern of digit loss is also seen in the chick mutant, *oligozeugodactyly*, which lacks Shh function in the wing and leg (Ros et al., 2003). The inactivation of *Gli3* alone and of *Shh* and *Gli3* together causes many unpatterned digits to form (Litingtung et al., 2002; te Welscher et al., 2002). Gli3 is one of the transcriptional effectors of Shh signalling, and Shh signalling prevents its activator form (Gli3A) from being processed into its repressor form (Gli3R). This shows that the function of Shh in controlling digit number and identity is achieved principally by repressing Gli3R activity in the posterior part of the limb bud that forms the digits. The precise balance of Gli3A and Gli3R may provide the basis for the graded response to Shh signalling. The generation of many unpatterned digits is also consistent with the proposed digit pre-pattern (see earlier).

The above findings, although contributing highly important molecular insights, have not revealed the mechanism by which the AP axis of the mouse limb is patterned. However, recent conditional gene-targeting approaches have yielded two strikingly different models of AP patterning of the mouse digits, which we discuss below.

Temporal expansion model of mouse limb AP patterning

Recent conditional gene-targeting approaches in mice have been designed to follow the descendants of polarizing region cells and to manipulate the duration, dose and range of Shh signalling in the mouse limb. In one study (Harfe et al., 2004), which invokes a new timing mechanism for the patterning of the most posterior mouse digits (Fig. 5A), the lineages of *Shh*-expressing cells were traced using an inducible *lacZ* reporter line (*ShhGFPCre/+,R26R/+*), revealing that these cells progressively contribute to part of digit 3 and to the two most-posterior digits (4 and 5). This contrasts with the chick wing, in which polarizing region descendants contribute only to digit 4 (Towers et al., 2008). Furthermore, the formation of posterior mouse digits does not appear to depend on Shh diffusion because only digit 2 was lost when long-range Shh signalling was severely reduced following the inactivation of the dispatched 1 (*Disp1*) gene (Disp1 is responsible for transporting cholesterol-modified Shh) (Harfe et al., 2004) (Fig. 5B). This suggests that the development of the three most-posterior digits in the mouse limb occurs by a mechanism that is related to the proliferation of the polarizing region cell lineage, rather than being specified by the highest levels of Shh signalling. Thus, proliferation could provide a timing mechanism by which polarizing cells become committed to different posterior digit fates. Although these fate-mapping studies do not actually reveal when digit identities are specified, the fact that three digits derive from the same population of cells suggests a requirement for an extended period of proliferation (Fig. 5A). *Shh* is expressed for around 60 h in the mouse limb and could underpin such a mechanism (Fig. 2C). This is considerably longer than the 24 h exposure to Shh signalling required to specify the full set of AP values in the chick wing (Yang et al., 1997) (Fig. 4A). It has also recently been shown that posterior digits still form in limbs of *Shhgfpcre/Shh^{c}* mice, in which levels of long-range Shh signalling are reduced but in which *Shh* is expressed for the normal length of time, although in such limbs, digit 2 is absent (Scherz et al., 2007).

Together, these data strongly suggest that, in the mouse limb, a specification gradient of Shh patterns anterior digits and that the length of time that proliferating polarizing region cells are exposed to direct Shh signalling patterns the posterior digits. It remains to be seen whether Shh signalling from the polarizing region also controls the growth of the adjacent digit-forming field in the mouse limb as in the chick wing (see earlier) and thus whether the growth-morphogen model outlined above for the chick wing applies to mouse anterior digits.

Biphasic model of mouse AP limb patterning

The temporal requirements of Shh signalling for mouse digit patterning have now been tested in further careful experiments in which Shh function has been rapidly inactivated at different stages using an inducible *Hoxb6CreER* line (Zhu et al., 2008). The outcome of these experiments forms the basis of a new biphasic model, in which Shh specifies digits at the very earliest stages of limb development (possibly by a concentration gradient) and then is required as a mitogen for the progressive formation of individual digits (Fig. 5C). This study reported that only two digits form following a 3-hour pulse of *Shh* transcription in the hindlimb (which provides 9 hours of Shh activity, as assessed by *Ptch1* expression) and following 9 hours in the forelimb (15 hours Shh activity). In such limbs, the digits that formed were digit 1 and, unexpectedly, digit 4. A longer period of Shh signalling permitted other digits to form in the alternating sequence, digit 2, digit 5 and then digit 3 (Fig. 5C). Strikingly, using *Sox9* and *Noggin* knock-in alleles to drive *lacZ* expression, the authors observed that the cartilage condensation of each digit differentiates in the same order, i.e., digit 4, digit 2, digit 5 and finally digit 3. The exception is the condensation of digit 1, which appears last, but forms after a short pulse of *Shh* expression in the forelimb. It should be noted, however, that other accounts of mouse limb development suggest that digit 5 forms after digit 3 (Martin, 1990).

This interdigitating pattern of digit condensation does not fit with the expected anterior-to-posterior sequence of specification, which is pivotal to all other models of AP patterning. Instead, the authors suggest that digit morphogenesis relies on Shh-dependent proliferation allowing sufficient numbers of specified cells to survive to form a condensation. This could explain why cells that require the shortest duration of Shh-dependent proliferation form the cartilage condensation of digit 4, the digit that differentiates first (Fig. 5C). Thus, in this biphasic model, the authors suggest that the control of digit identity and number are temporally uncoupled and that Shh signalling acts first as a morphogen and then second as a growth-promoting factor.

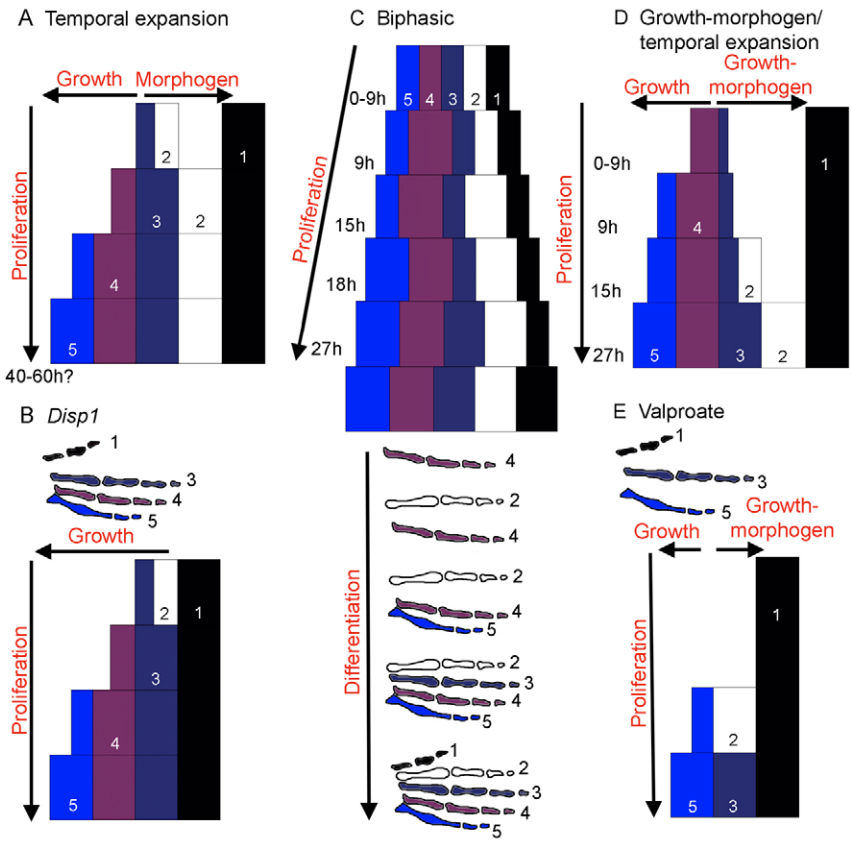

Fig. 5. Extended flag models of mouse limb anteroposterior patterning. In this figure, the squares represent positional values and numbers indicate which digits have been specified. Timings, shown on the vertical axis, estimate the duration of Shh activity required for the specification of hindlimb digits. Left is posterior, right is anterior. Digit 1 is considered to form independently of Shh signalling in the hindlimb (black). (**A**) Temporal expansion model (Harfe et al., 2004). Positional values of the digits 3 (dark blue) and digit 2 (white) are specified by a morphogen gradient mechanism, involving Shh, although the involvement of mesenchymal expansion is unclear. Posterior digits 4 (purple) and 5 (light blue) are specified according to the length of time cells remain in the polarizing region, which exclusively contributes to these two digits. This could require the full duration of Shh activity (~60 hours) but does not require a concentration gradient of Shh protein. Digit 3 (dark blue) can be specified by a combination of the above processes, as depicted. (**B**) Temporal expansion model in dispatched 1 (*Disp1*) mutant limbs (Harfe et al., 2004). Top, schematic of mouse *Disp1* mutant limb skeleton. Bottom, restricted long-range Shh movement prevents the positional value of digit 2 (white) from being established. Posterior digits are still specified by a timing mechanism linked to the proliferation of *Shh*-expressing polarizing region cells. (**C**) Biphasic model (Zhu et al., 2008). Positional identities of all five digits require less than 9 hours of Shh activity to be specified by an unknown mechanism that could involve a concentration gradient. A longer duration of Shh activity allows the survival of specified digit progenitor cells (shown by progressive enlargement of boxes representing different digits); digits differentiate in the order shown in the skeletons underneath. (**D**) Alternating temporal expansion/growth morphogen model. Positional values of digits 2 (white) and 3 (dark blue) are specified in the same way as digits 2-4 of the chick wing by a growth-morphogen mechanism (see Fig. 4A). At the same time, digits 4 (purple) and 5 (light blue) begin to be specified by temporal expansion (see Fig. 5A), resulting in an alternating sequence of specification that recapitulates the sequence in which digits develop with increasing lengths of exposure to Shh signalling (see Fig. 5C). (**E**) Alternating temporal expansion/growth morphogen model in valproate-treated limbs. Top, schematic of valproate-treated mouse limb skeleton (Faiella et al., 2000). Valproate treatment could irreversibly restrict AP expansion of the mouse limb, without affecting the timing of posterior digit specification or the Shh concentration gradient. This results in all descendant cells of the polarizing region being specified as digit 5 (light blue) and the concentration gradient of Shh establishing the positional values of digit 3 (dark blue) (compare with Fig. 4C).

Early or late specification of posterior digits in the mouse limb?

The temporal and biphasic models of AP mouse limb patterning are clearly at odds with each other; the major issue concerns the duration of *Shh* expression required to specify the posterior digits. The identification of digit 4, which underpins the biphasic model, and the use of *Tbx3* expression to identify posterior digits have been recently challenged (Tabin and McMahon, 2008), highlighting the problems of not being able to identify unequivocally mouse digits. There is clearly an urgent need to identify molecular markers for individual digits (if they exist) to aid the interpretation of patterning defects, and to provide insights into digit evolution.

In the biphasic model, if the digit with the shortest requirement for Shh signalling turns out to be a more-anterior digit than digit 4, then this would agree with the more conventional models of AP patterning. Unfortunately, other independent studies also fail to clarify this issue. For example, three digits form in the *Prx1Cre;Shh*$^{c/c}$ mouse mutant, in which *Shh* expression is attenuated early. This phenotype is interpreted as representing the loss of digits 4 and 5 by the authors (Scherz et al., 2007), but as the loss of digits 3 and 5 by others (Zhu et al., 2008). The first interpretation fits with the temporal model because a much longer time would be required to specify digit 4; the second with the biphasic model, because digit 4 would have been specified earlier.

The temporal and biphasic models of mouse digit patterning are not readily reconciled with the growth-morphogen model of chick wing digit patterning. For example, there is no evidence that the posterior digits of the chick wing are specified by a temporal expansion mechanism controlled only by the duration of polarizing region signalling because a morphogen gradient still best explains the specification of digit 4, the posterior-most digit (see Box 2). Additionally, there appears to be no evidence that a correlation exists between the duration of Shh signalling required to specify a digit and the order in which cartilage condensations appear, as predicted by the biphasic model. For example, in chick limbs, digit 4 requires the longest exposure to Shh signalling to be specified (Yang et al., 1997; Scherz et al., 2007), and yet its cartilage condensations appear first (Hinchliffe, 1977). Furthermore, in lizards such as *Ambystoma mexicanum*, digit 4 requires the longest exposure to Shh to be specified, and yet its cartilage condensations appear last (Stopper and Wagner, 2007).

Alternating specification model of mouse limb AP patterning

One common theme underpinning the biphasic and temporal models of mouse digit patterning is the fundamental concept of the anterior-to-posterior sequence of digit specification that is inherited from chick limb studies. For the biphasic model, it is assumed that because a digit 4 can form after only 2-3 hours of *Shh* transcription, more-anterior positional values have already been specified, but are not then realised. Likewise, in the temporal model, as digit 4 is derived from *Shh*-expressing cells, it is also assumed that more anterior digits have been specified much earlier. It is possible, however, that two separate processes – a growth-morphogen mechanism for digits 2-3, like that proposed for the chick wing, and a temporal expansion mechanism for digits 4-5 – occur simultaneously, leading to an alternating sequence of mouse digit specification (Fig. 5D). Thus, in the mouse hindlimb, early descendants of *Shh*-expressing cells might be specified as a digit 4 at around the same time that the Shh morphogen concentration specifies a positional value for digit 2. Later descendants of *Shh*-expressing cells might be specified as a digit 5 at around the same time that the Shh morphogen concentration specifies a positional value of digit 3 (Fig. 5D). This interpretation fits with the results that led to the biphasic model, while avoiding the assumption that all the digits are specified very early. In addition, the alternating specification model agrees well with the suggestion that the two digits that are missing in forelimbs of the *Prx1Cre;Shh*$^{c/c}$ mice are digits 3 and 5 (see earlier).

One test of the idea that two separate interdigitating processes, both involving growth, specify AP pattern would be to challenge developing mouse limbs with cell cycle inhibitors, such as TSA. In fact, the anticonvulsant drug, valproic acid (valproate), which is a deacetylase inhibitor like TSA (Phiel et al., 2001) and which causes the loss of anterior digits in the chick wing (Whitsel et al., 2002), can cause the loss of an anterior digit, that could either be digit 2 or 3, but also the loss of a posterior digit, digit 4 in the mouse forelimb (Faiella et al., 2000). The reduced AP expansion of both the digit field and the polarizing region at an early stage could result in these two unexpanded cell populations giving rise to the highest possible posterior values if the normal duration of specification is maintained (digit 3 and digit 5, respectively) (compare Fig. 5E with TSA chick wing model in Fig. 4C). Interestingly, inactivation of the *N-myc* gene (*Mycn*) in mouse limbs reduces AP expansion and leads to digit fusions (Ota et al., 2007). It is possible that, in such limbs, cell proliferation was not reduced sufficiently to cause loss of digits. Additionally, the most common clinical affect on the limb following loss of N-myc function in individuals with Feingolds's syndrome (van Bokhoven et al., 2005) is fusion of the second and third, and also fourth and fifth toes (Brunner and Winter, 1991). This again suggests that two independent growth processes are affected: one operating in the anterior part of the limb bud, the other in the posterior part. Individuals with Feingolds's syndrome sometimes also lose the thumb (Alessandri et al., 2000), consistent with the effects of valproate on the hands of babies whose mothers were exposed to this drug during pregnancy and also with the effects of valproate and TSA on chick wings (as discussed earlier).

Integrating patterning along the proximodistal and anteroposterior axes

Although we have considered the specification and growth of each axis as independent processes, it has long been known that they are integrated, and the molecular basis of this integration has recently been identified. Early evidence that specification along the AP and PD axes is integrated came from the finding that the duplicating effects that polarizing region grafts have on AP patterning become more distally restricted the later the operation is performed (Summerbell, 1974). The finding that the polarizing region has to be grafted in contact with the AER to induce the formation of additional digits provided further evidence that the patterning of the AP and PD axes is integrated. In turn, the polarizing region maintains the AER, via the production of a maintenance factor (Zwilling and Hansborough, 1956). It is now known that FGF signalling in the posterior AER, in particular by FGF4, transcriptionally regulates *Shh* expression in the polarizing region and that Shh signalling maintains *Fgf4* expression in the AER, thus forming a positive-feedback loop (Niswander et al., 1994; Laufer et al., 1994). However, it should be noted that genetic experiments in mice show that *Shh* is still expressed in the absence of *Fgf4*, *Fgf9* and *Fgf17* when *Fgf8* is present. A landmark finding was that the AER maintenance factor is a BMP antagonist, encoded by the *Gremlin* gene (Zuniga et al., 1999). Shh signalling by the polarizing region maintains mesenchymal *Gremlin* expression and activity, which in turn prevents BMPs from repressing *Fgf4* expression in the posterior AER.

It remains unclear how the AER is involved in promoting the AP expansion of the posterior part of the bud. In the chick wing, recent experiments show that Shh can induce the expression of cell cycle genes in the anterior mesenchyme in the absence of the AER (Towers et al., 2008). Indeed, recent work in the mouse limb shows that loss of individual digits results when the function of posteriorly expressed FGFs is progressively deleted in combination with *Fgf8* function before any PD structures are completely lost (Mariani et al., 2008). Previous cell labelling experiments in the chick wing have shown that groups of proliferating mesenchymal cells extend towards an FGF4-soaked bead (Li and Muneoka, 1999). Therefore, one possibility is that FGF4 signalling (and that of other FGFs) in the posterior AER, as well as being involved in limb outgrowth and maintaining the progress zone, also acts an external cue that informs mesenchymal cells about the direction in which they should proliferate. The ability of cells to proliferate towards sources of signals might be under the control of the planar cell polarity (PCP) pathway, which has not been investigated in limb development. Interestingly, *Wnt5a* and genes that encode other components of the PCP pathway are expressed in mesenchyme cells at the tip of the limb bud (Yamaguchi et al., 1999).

A recent focus of attention has been how *Shh* expression is terminated in the developing limb. This is particularly important in light of recent models of AP digit patterning in the mouse, which suggest that posterior digits are specified by the length of time that cells express *Shh*. The first hint that growth itself may fulfil an

important role in regulating the duration of *Shh* transcription came from the observation that considerable tissue expansion occurs in the posterior region of chick wing buds where the FGF4-Shh feedback loop operates. It was suggested that descendants of the polarizing region are unable to express *Gremlin* in response to Shh signalling (Scherz et al., 2004). Thus, it was proposed that expansion of the posterior part of the chick wing bud displaces the *Gremlin*-expressing domain too far away from the polarizing region to be maintained by Shh signalling, thus leading to the de-repression of BMP signalling (Scherz et al., 2004). As a result, *Fgf4* expression would not be maintained in the posterior AER, and this, in turn, would switch off *Shh* transcription, thus terminating the feedback loop. It is not clear, however, whether descendants of the polarizing region in the chick wing make a sufficiently significant contribution to AP expansion for this mechanism to operate, because the polarizing region only contributes to digit 4 (Towers et al., 2008). In *Fgf4* overexpressing mouse limbs, the duration of *Shh* expression is extended and the limbs exhibit postaxial polydactyly (extra posterior digits) (Lu et al., 2006). This finding can be explained in terms of the alternating specification model of mouse AP patterning, because if posterior proliferation is extended, extra digits would be predicted to develop. Another parallel extrinsic mechanism has recently been proposed to account for the termination of the *Shh* expression loop in both chick and mice limbs, in which *Gremlin* transcription is repressed by the progressive accumulation of FGF signalling during development (Verheyden and Sun, 2008).

Recent work in the chick wing suggests that intrinsic mechanisms might control the duration of Shh signalling in the polarizing region (Towers et al., 2008) because in TSA-treated wings, which fail to expand significantly across the AP axis, *Shh* transcription was terminated after the normal duration of about 38 hours in proliferating cells (Towers et al., 2008). This strongly suggests that in the absence of AP growth, an intrinsic mechanism operates that counts elapsed polarizing region cell generations, as outgrowth continues proximodistally. It should be noted that the termination of *Shh* expression occurs at the early hand plate stage and that *Fgf8* expression throughout the AER continues and is responsible for the outgrowth of digits. Therefore, termination of *Fgf8* transcription, and thus termination of limb outgrowth, occurs independently of FGF4-Shh signalling (Sanz-Ezquerro and Tickle, 2003).

Conclusions

In this review, we have outlined how models of limb patterning have grown over time. It is important that the results of older embryological investigations and of newer molecular studies are unified. Towards this aim, we have highlighted the pivotal role of growth, a component of many classical models that is being re-evaluated in the light of recent molecular advances and models of limb development. However, although many inroads have been made towards understanding how the limb is patterned, our knowledge of this process is still quite fragmentary, and many of the proposed models remain controversial. It is likely that the principles that govern the patterning of the limb axes are shared among species, but that subtle differences also exist, reflecting evolutionary changes in skeletal organisation, particularly in digit number and identity.

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