Limbs: a model for pattern formation within the vertebrate body plan

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Giant strides have been made in identifying the molecular basis of limb development. The four main phases are initiation of the limb bud, specification of limb pattern, differentiation of tissues and shaping of the limb, and growth of the miniature limb to the adult size. We will focus on the exciting advances that have been made in initiation and specification of limb pattern. The limb is a model system and the same sets of molecules are used at different times and places in vertebrate embryos. There is also remarkable conservation of the molecular mechanisms of limb development in insects and vertebrates.

The ideas of Lewis Wolpert have been particularly influential in viewing embryonic pattern formation, and he suggested that it can be understood as a two-step process[1]. In the first step, cells are informed of their position in a three-dimensional coordinate system, and they then interpret this information to form the appropriate structures. The limb has provided an ideal model system to explore the identity of molecules that convey and register positional information. As a result of the pattern formation process, a small bud of undifferentiated mesenchyme cells encased in ectoderm gives rise to a limb with a precise pattern of cells and tissues, skeleton, muscle and tendons. The same principles apply to specifying the body plan. The interpretation of positional signals will determine where limbs will develop and their type: either forelimb or hindlimb.

Pattern specification in the early limb bud

Signalling molecules have been identified that pattern each of the three limb axes (Fig. 1; reviewed in Ref. 2). Signalling by the apical ridge, the thickened ectodermal epithelium at the tip of the limb bud, is necessary for outgrowth and laying down the proximodistal pattern, and there is increasing evidence that this signalling is mediated by fibroblast growth factors (FGFs). *FGF8* is expressed throughout the apical ridge from very early in limb development, while transcripts of *FGF4* are detected later and are posteriorly restricted in the ridge. FGF2 protein can be detected in ectoderm and mesoderm. Removal of the apical ridge from early chick limb buds leads to truncations, but outgrowth and patterning can be maintained by stapling FGF-soaked beads in place of the ridge[3,4]. Signalling by dorsal ectoderm plays a role in dorsoventral patterning and is mediated by the *WNT7a* gene. *WNT7a* is expressed in dorsal limb bud ectoderm right up to the apical ridge, and transcripts are present before the bud forms. Direct evidence that *Wnt7a* plays a role in dorsoventral patterning comes from the limb morphology of the *Wnt7a* knockout mouse. In the absence of *Wnt7a*, the paws have a double ventral pattern[5]. Signalling of the polarizing region, a group of mesenchyme cells at the posterior margin of the limb bud, establishes anteroposterior pattern, and this signal can be mimicked by the product of *Sonic hedgehog* (*SHH*)[6]. *SHH* transcripts map to the polarizing region and expression is initiated in the early bud[6]. Signalling of the polarizing region can be demonstrated by grafting a polarizing region to the anterior margin of a second limb bud[7]. This induces a duplicate set of digits to develop in mirror-image of the normal set. When a pellet of *SHH*-expressing cells[6] or SHH protein[8] is applied to the anterior margin of a chick wing, the same mirror-image duplications result.

There appears to be mutual dependence between signalling systems (Fig. 1a, b). The expression of *SHH* in posterior mesenchyme maintains *FGF4* expression in the posterior apical ridge[9,10], and *WNT7a* expression in dorsal ectoderm together with *FGF4* maintains *SHH* expression in posterior mesenchyme[11] (Fig. 1a, b).

Retinoic acid can initiate a signalling cascade at the anterior margin of a limb bud (reviewed in Ref. 2). Retinoic acid leads to activation of *FGF4* in the anterior ridge and *SHH* in the anterior mesenchyme. *WNT7a*, which is expressed throughout the dorsal ectoderm, can help to maintain this ectopic anterior domain of *SHH* (Ref. 11). It is still controversial whether or not endogenous retinoic acid initiates the normal signalling network at the posterior limb bud margin.

A great deal of work still remains to be done to identify the intermediate steps in the signalling cascades and there are several major unresolved questions. What initiates and maintains *FGF8* expression in the ridge? What are the roles of different FGF-family members? Is there a ventral ectoderm signal in addition to a dorsal signal? Does SHH directly specify pattern across the anteroposterior axis of the limb?

The role of SHH signalling is an important issue. It has been proposed that the signal from the polarizing region is long range and acts in a graded fashion[1]. According to this view, cells at different distances from the polarizing region would be exposed to different concentrations of morphogen. Cells next to the polarizing region would be exposed to high morphogen concentrations and form posterior digits, and cells further away would be exposed to lower concentrations of morphogen, thus forming anterior digits. If SHH acts as a long-range signal, then it should be distributed in a gradient across the limb bud and signalling should be dose dependent. High concentrations of SHH should give posterior digits whereas lower concentrations of SHH should give anterior digits. Another possibility is that SHH acts indirectly. SHH activates *BMP2* in limb bud mesenchyme[10], and *BMP2* could mediate the effects of SHH. This appears to be a widely used pathway in vertebrates[12]. Also, in *Drosophila*, *decapentaplegic*, the homologue of *BMP2*, is activated as a result of *hedgehog* signalling in the wing imaginal disc[13]. *BMP2* transcripts map to the polarizing region and are present in the apical ridge[14]. Anterior application of BMP2 to chick wing buds does not, however, lead to digit duplications, which suggests another role for BMP2, perhaps in signalling between the mesenchyme and the ridge[14]. BMP4 and BMP7 are expressed in the mesenchyme and the ridge and could also participate[14].

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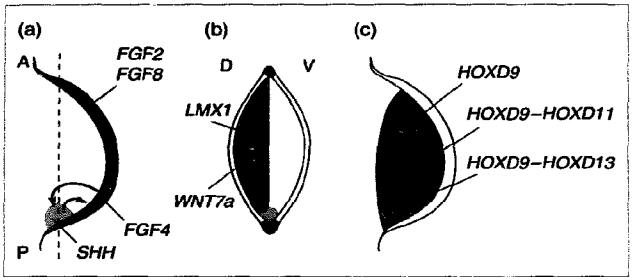

FIGURE 1. Molecules involved in pattern specification in early chick wing bud. (a) Dorsal view of the early chick wing bud showing localization of mRNAs encoding secreted molecules in mesenchyme and apical ectodermal ridge. *FGF8* is expressed throughout the apical ridge (light purple), *FGF4* in the posterior ridge (dark purple) and *SHH* in the posterior mesenchyme (light green). FGF2 can be detected throughout the ridge and in underlying mesenchyme. Interactions between *FGF4* and *SHH* are indicated by arrows, but this does not mean that interactions are direct. (b) Transverse section through the wing bud. Plane of section is indicated by dashed line through bud shown in (a). *LMX1* (dark green) is restricted to dorsal mesenchyme and *WNT7a* (yellow) is restricted to dorsal ectoderm. Arrows indicate induction of *LMX1* in dorsal mesenchyme and maintenance of *SHH* in polarizing region by *WNT7a*. (c) Dorsal view of the early chick wing bud, showing localization of transcripts of *HOXD* genes activated in response to signalling. *HOXD9–13* are expressed in nested domains in limb mesenchyme; the domains of only three genes are shown for clarity. Abbreviations: A, anterior; P, posterior; D, dorsal; V, ventral.

Signalling in the limb bud activates genes that encode transcription factors (Fig. 1b, c). *LMX1* is expressed in dorsal mesoderm in response to *WNT7a* signalling[15,16] (Fig. 1b). *LMX1* is a relative of the *Drosophila* gene *apterous* and expression of this gene is activated very early in lateral plate mesoderm, even before a limb bud is formed. It is striking that *apterous* is also expressed in the dorsal compartment of the *Drosophila* wing disc (reviewed in Ref. 17). Infection of chick wing bud ectoderm with a retrovirus containing *Wnt7a* results in ectopic activation of *LMX1* in ventral mesoderm, and when presumptive limb is infected with a retrovirus containing *LMX1*, parts of the ventral limb are dorsalized[15,16].

Genes situated 5′ in the *Hoxa* and *Hoxd* clusters are activated in response to SHH and FGF signals[9,10] and initially form a series of overlapping domains centred on distal and posterior-distal regions of the limb, respectively (reviewed in Ref. 18; Fig. 1c). These genes are activated during outgrowth of the limb bud in the region of undifferentiated proliferating mesenchyme cells beneath the apical ectodermal ridge known as the progress zone[19]. *MSX1* and *MSX2*, two related homeobox genes, are expressed in the progress zone, and *MSX1* can be maintained by FGF. When a polarizing signal is applied next to the apical ridge at the anterior margin of a chick wing, a mirror-image pattern of *HOXD* gene expression is induced, which correlates with the mirror-image pattern of digits that develops[20]. Overexpression of one of the most 5′ *HOXD* genes produces local pattern alterations and an anterior digit in the chick leg appears to be posteriorized[21].

Mesenchyme cells in different regions of the limb bud express different combinations of *HOX* genes, and cells in the dorsal mesenchyme express *LMX1*. This approximates crudely to a three-dimensional molecular representation of cell position. An attractive possibility is that this system could then control local cell behaviour, differentiation, proliferation, adhesion and so on, so that structures develop in appropriate positions. The idea that the sole function of *HOX* gene expression domains is to encode position might, however, be an oversimplification. Patterns of *HOX* gene expression are dynamic and do not remain fixed. *Hoxd13*, for example, which is restricted posteriorly in the early bud, later comes to be expressed throughout the mesenchyme at the distal tip of the limb bud, where digits will develop[18]. When *Hoxd13* is functionally inactivated, digit development is delayed and there are only small changes in digit morphology[22]. It is possible that *Hoxa13* could partially compensate for the lack of *Hoxd13*, and in double knockouts of *Hoxa11* and *Hoxd11*, which are expressed more proximally and anteriorly than the *Hoxa13* and *Hoxd13* genes, the forearm is almost completely absent[23].

It is important to note that the same signals convey positional information in wing and leg buds, and that the same transcription factors are activated in response. The major challenges will be to understand how this information is interpreted, and to identify downstream targets of the *HOX* genes and *LMX1*.

Analysis of mutants

Recent molecular analysis of the signal and response network in chick and mouse limb mutants has complemented experimental analysis of chick limb development. In the chicken *TALPID**3* polydactylous mutant, an increased number of morphologically identical digits is correlated with uniform expression of genes that are activated in response to SHH, instead of the distinct posteriorly restricted patterns described above[24]. Thus, although *SHH* expression is normal and posteriorly restricted, transcripts of the *BMP* and *HOXD* genes are expressed all across the anteroposterior axis in the mesenchyme, and *FGF4* transcripts are found throughout the apical ridge[24,25]. In *Drosophila*, uniform expression of *dpp* leads to morphologically uniform wings[26]. A non-polarized pattern of digit-like structures also develops when mesenchyme cells from limb buds are disaggregated, mixed together and replaced in an ectodermal jacket[27,28]. These reaggregates have some similarities with *TALPID**3* limb buds, in that expression of the *BMP* and *HOXD* genes occurs throughout the distal mesenchyme. In reaggregates of anterior leg mesenchyme, *BMP* and *HOX* genes appear to be activated in the absence of *SHH*, and this suggests that uniform expression of these genes could be the default state[28]. It has been suggested that inhibitory signals produced anteriorly in the normal limb could play a

role in restricting expression of posterior genes[29]. There is very little evidence for the existence of such signals, but the idea is attractive because it suggests an analogy with neural tube patterning, in which an SHH signal emanating ventrally is antagonized by a BMP-related signal emanating dorsally[30,31]. In the chick wing bud, although *BMP2* and *BMP7* transcripts are localized posteriorly, *BMP4* is expressed predominantly anteriorly[24], and in *Bmp7* knockout mice, additional digits develop anteriorly[32,33]. It is possible that different BMP family members and/or heterodimers could have distinct roles in limb development and this needs to be investigated.

Gene expression has also been analysed in four different polydactylous mouse mutants, which, unlike *TALPID*3*, have partial digit duplications, with one or two additional digits developing anteriorly[34,35]. In these mutants, a new anterior signalling centre can be detected, with ectopic expression of *Shh* in anterior mesenchyme and of *Fgf4* in anterior ridge, and this is associated with ectopic expression of *Hoxd* genes. One of the mutants studied was *extra toes*, a mutation that affects the mouse *Gli3* gene[34]. Interestingly, the human Greig cephalopolysyndactyly syndrome is caused by loss of *GLI3*, and results in limb and craniofacial malformations. In another mouse mutant, *limb deformity* (*ld*), digits are reduced and fused[36,37]. In *ld*, *Shh* expression is not maintained in posterior mesenchyme as the limb bud grows out, *Fgf4* expression cannot be detected in the posterior ridge, and later phases of *Hoxd* expression are delayed. The data for polydactylous mutants and *ld* confirm the close relationship between *Shh* and *Fgf4* and provision of a polarizing signal. In *ld* mutants, *Fgf8* is expressed posteriorly in the ridge, but *Shh* is not maintained[36,37]. Thus, it is possible that *Fgf4* plays a special role in maintaining the polarizing region. There have been conflicting reports, however, about whether *Fgf8* can maintain *Shh* expression in vertebrate limb buds after the apical ridge has been removed[38,39].

Ectopic anterior expression of *Shh* and *Fgf4* is also seen in forelimbs of a transgenic mouse that has mirror-image duplicated patterns of forelimb digits[40]. In this mouse, the anterior limit of *Hoxb8*, which is normally up to the posterior edge of the forelimb, is shifted anteriorly along the body axis under the control of the promoter of the gene encoding the retinoic acid receptor β2. The effects on gene expression and limb pattern suggest that *Hoxb8* might control position of the polarizing region in the forelimb. Expression of another *Hox* gene would presumably control development of the polarizing region of the hindlimb. As a consequence of expression of these genes, cells at the posterior margin of forelimbs and hindlimbs would become competent to activate *Shh*, for example, and become a polarizing region (Fig. 2). Thus, different genes would act initially in hindlimb and forelimb, but then the same signalling network would operate to establish the limb pattern. In the mouse polydactylous mutant, *Strong's Luxoid*, in which fore- and hindlimbs are both affected, *Hoxb8* expression is not altered in the forelimb[35], suggesting that the mutation acts downstream of *Hoxb8* and in the part of the pathway common to upper and lower limbs.

An intriguing feature of the *Hoxb8* transgenic mouse is that, although the transgene is initially expressed throughout the forelimb region, *Shh* is only expressed at limb margins. This suggests that a boundary of FGF expression, such as occurs at the ends of the ridge, might be important for activation of *Shh* expression in competent cells[41]. In normal chick limb buds, the competence of cells to express *SHH* in response to FGF is posteriorly restricted[11] and anterior cells do not activate *SHH* when only FGF is applied[9]. According to this view, anterior extension of the competence to express *Shh* in the limb buds could be the basis for ectopic domains of *Shh* in polydactylous mutants (Fig. 2).

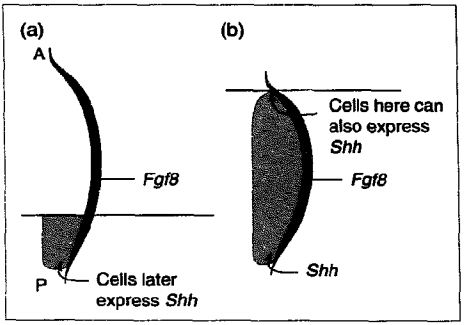

FIGURE 2. Interactions between mesenchyme cells competent to express *Shh* and the boundary of *Fgf* expression in overlying apical ridge govern the position in which a polarizing region develops. (a) In presumptive normal limbs, competence to express *Shh* is posteriorly restricted (light green). *Shh* is then activated in mesenchyme adjacent to the posterior end of the apical ridge, which expresses *Fgf8* (light purple). (b) In the presumptive polydactylous limbs of mouse mutants, competence to express *Shh* now extends throughout the limb-forming region (light green), and is present at the anterior end of the *Fgf8*-expressing apical ridge. An ectopic domain of *Shh* is induced anteriorly in the bud. *Shh*-expressing cells will locally activate *Fgf4* expression in the adjacent ridge, so that *Shh*-expressing cells are associated with ridge expressing both *Fgf4* and *Fgf8*.

Initiating limbs

Development of a limb at each corner of the body is a hallmark of the higher vertebrate body plan. Limb formation can be initiated by FGFs. A single application of FGF1, -2, -4, or -8 to the presumptive flank region of chick embryos, between the wing and leg, leads to the development of complete additional limbs[39,42,43]. Anterior flank can be induced to form additional wings and posterior flank can be induced to form additional legs (Fig. 3a). The earliest effect of FGF application on gene expression so far detected is activation of *FGF8* in the body wall ectoderm[39], which is soon followed by activation of *SHH* in flank cells with polarizing potential, converting them to a polarizing region[42] (Fig. 3b). Recent work shows that during normal limb initiation, before expression in presumptive limb ectoderm, *FGF8* transcripts are present in intermediate mesoderm near the presumptive limb[39], but it is not clear whether expression in intermediate mesoderm is part of the endogenous signalling system that initiates limb development.

FIGURE 3. Additional limb development following application of an FGF2 bead to the flank of chick embryos. (a) 10-day-old chick embryo stained with Alcian Green to reveal skeletal pattern. Arrow indicates additional leg that developed following application of an FGF2 bead opposite somite 24. (b) *SHH* expression at the anterior margin of additional limb (arrow) 48 h after application of an FGF2 bead to the flank.

The fact that only two limbs form on either side of the embryo raises interesting questions about the control of limb number. A similar question is presented by mammary gland development (Fig. 4). In pigs, for example, the number of mammary glands can range from one pair in the babirusa to six pairs in the domestic pig[44]. Figure 4 shows a plan of the mouse body illustrating the positions at which limbs will appear. This will occur at two particular levels along the main anteroposterior axis of the body and at the same distance from the midline on either side. It seems likely that *Hox* gene expression determines the anteroposterior position of limbs by specifying where initiation signals are produced. Consistent with this idea is the mouse *Hoxb5* mutant, in which the level of the forelimb is shifted[45]. In addition, the pattern of Hox gene expression could also determine limb type; either forelimb or hindlimb. This information will be used to interpret the pattern specified in the limb bud and translate it into appropriate forelimb or hindlimb structures.

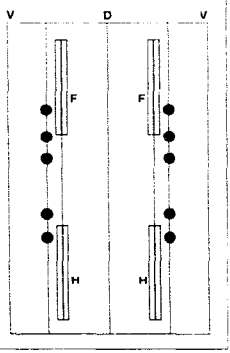

FIGURE 4. Plan of the mouse body flattened out to show the positions at which limbs and mammary glands develop. Black spots represent the mammary glands. The continuous lines between limbs and between mammary glands indicate the dorsoventral level at which additional limbs or mammary glands can develop. Abbreviations: D, dorsal midline; V, ventral midline; F, forelimb; H, hindlimb.

The position at which limbs and mammary glands develop must be specified also with respect to the dorsoventral axis of the body. Interestingly, mammary glands develop along a so-called milk line, and although different numbers of mammary glands develop in different mammals, they are always positioned along the line. In chick embryos, FGF-induced ectopic limbs arise at the same dorsoventral level as normal limbs (M.J. Cohn, unpublished) and *FGF8* is initially expressed in a narrow stripe at a particular dorsoventral level[39]. It will be important to identify the molecules that specify dorsoventral limb positioning.

A remarkable feature of the additional limbs induced by FGF is that they have reversed polarity[42]. The cells of the flank have been shown to possess the potential to become a polarizing region, and this property is graded in the flank with highest potential anteriorly[16]. Exposure of these cells to FGF either by implanting an FGF bead into the flank[42], or by grafting flank cells beneath the anterior apical ridge[47] (which express *FGF8*), activates *SHH* expression (Fig. 3b). Cells recruited into ectopic limb buds from the anterior flank have the highest polarizing potential, so the polarizing region of the ectopic bud arises anteriorly and, thus, the polarity of the developing limb is reversed[42].

Concluding remarks

It is apparent that FGFs are central signalling molecules in limb development. Not only can FGFs trigger initiation of the limb, FGFs later mediate outgrowth and continued expression of *Shh* and, thus, are pivotal to pattern specification. FGFs also play important roles in shaping and growth during later phases of limb development. Recently, mutations in fibroblast growth factor receptors have been identified in human genetic syndromes. A specific mutation leads to a syndrome characterized by digit fusions and craniosynostosis, and a different mutation in another FGF receptor is now known to be the basis of achondroplasia, the most common form of dwarfism (reviewed in Ref. 48).

Another general theme that is beginning to emerge is the similarity of the molecular basis of pattern specification in different regions of vertebrate embryos. There are common components in the molecular networks of limb, gut[49], lung[50], face[51], central nervous system[51], hair[52] and feather[53], tooth[54], mammary gland[12] and eye[32,33]. Even more remarkable is the conservation of molecules involved in patterning insect wings and vertebrate limbs. Signalling molecules common to vertebrate and *Drosophila* limbs include *Shh* (*hh*), *Wnt7a* (*wg*) and *Bmp2* (*dpp*). The recent finding that chick *LMX1* and the related *apterous* gene in *Drosophila* are expressed dorsally in wing buds and imaginal discs is striking. Signalling by FGFs and retinoic acid might be confined to vertebrates, although receptors for FGFs and retinoids have been identified in *Drosophila*[55,56]. It will be very interesting to see how far these analogies can be pushed and whether or not other molecules implicated in development of the insect wing also participate in vertebrate limb development.

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