Vertebrate limb development – the early stages in chick and mouse

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More news this year about FGFs and their roles in vertebrate limb initiation; Wnt signalling is shown for the first time to be another component of the signalling cascade involved in early limb formation. Ectodermal compartments that control apical ridge formation were previously described in chick embryos and are now shown to exist in mouse embryos; *Engrailed1* is expressed in the ventral ectodermal compartment but experiments in both chick and mouse show that it is not responsible for compartment specification.

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Abbreviations
FGF fibroblast growth factor
Shh *Sonic hedgehog*

Introduction

Considerable progress has been made in understanding mechanisms that lead to limb initiation and answering general questions about these structures, such as what controls number, type and position. Classic transplantation experiments in early chick embryos showed that regions of the embryo are determined to form limbs long before there are any signs of limb development. Furthermore, these transplanted regions contain sufficient information to generate a bud that can then go on to develop autonomously into a limb. Here, we discuss recent work that identifies new components of the FGF (fibroblast growth factor) signalling cascade that initiates limb formation and controls establishment of the apical ectodermal ridge — the signalling centre that mediates bud outgrowth and patterning. We consider the role of FGF signals produced by mesenchyme and ridge in early limb buds and finally discuss work that explores how the apical ectodermal ridge is positioned with respect to the dorsoventral axis of the body so that limbs grow out from the sides. We review data from both chick and mouse embryos, illustrating how information from these two model organisms can be synthesised to arrive at general principles of vertebrate limb development.

Limb initiation and apical ridge formation

FGFs comprise a family of growth factors that play key roles at several different stages of limb development, including initiation. Application of FGF to the flank of chick embryos can trigger development of an additional limb [1]. *Fgf10* is expressed in mesenchyme of limb-forming regions and is essential for limb formation [2,3]. FGF10 induces *Fgf8* expression in overlying ectoderm, which forms the apical ectodermal ridge and FGF8 in turn maintains *Fgf10* expression in mesenchyme (Figure 1a). *Fgf8* and other *Fgfs* continue to be expressed in the ridge and beads soaked in FGFs can replace apical ridge function in limb outgrowth and patterning [4]. This year, ground-breaking work in chick embryos shows that Wnt signals are upstream of FGFs in limb initiation and also that Wnts intervene in the signalling loop between *Fgf10* and *Fgf8* [5••].

Local application of members of the Wnt family of growth factors (or cells expressing retroviruses containing activated β-catenin), like FGFs, have the spectacular property of inducing additional limbs [5••]. Two Wnt family members are reported to be expressed in limb-forming mesenchyme (*Wnt2b* in wing region and *Wnt8c* in leg). Furthermore, when β-catenin-mediated signalling in these regions is blocked by misexpressing axin, a negative regulator of the canonical Wnt pathway, limb development is impaired, thus pointing very clearly to a role for Wnt signalling in normal limb initiation. The effects of *Wnt* and *β-catenin* misexpression on expression of *Fgf10* and *Fgf8* lead the authors to propose a model whereby these respective Wnts maintain *Fgf10* expression in the limb-forming regions and thus lie upstream of FGFs in initiating limb formation.

The same paper [5••] also provides some evidence consistent with the idea that Wnt signalling (Wnt3a this time) intervenes in the signalling loop between FGF10 signalling in mesenchyme and FGF8 signalling in ridge. Other work also suggests that induction of *Fgf8* expression by FGF10 may be indirect on the basis of timing [6,7•]. Thus the picture emerging is that FGF10 induces *Wnt3a* expression in the ectoderm and then Wnt3a via β-catenin activates *Fgf8* expression, which then maintains *Fgf10* expression in a feedback loop (Figure 1b). It should be noted that *Wnt3a* transcripts cannot be detected in mouse limb ectoderm. Nevertheless the phenotype of the *Lef1/Tcf1* double knockout mouse is consistent with Wnt signalling being essential for ridge formation but presumably another Wnt is involved [8].

This unanticipated participation of Wnts in both signalling cascades leading to limb initiation and in regulation of *Fgf* expression in ectoderm underlines the synergy and intricate interactions between signalling pathways during development. This particular combination of growth factor signals has added significance because it has been reported that expression of a chick *spalt* homologue, *CSAL1*, at least in distal mesenchyme of later limb buds, is controlled by

ridge-derived FGFs and Wnts [9•]. In this case, Wnts acting through either β-catenin-dependent (Wnt3a) or β-catenin-independent (Wnt7a) pathways are effective. Very recent work on inner ear induction and early neural patterning have also demonstrated interaction of FGF and Wnt signalling pathways [10,11].

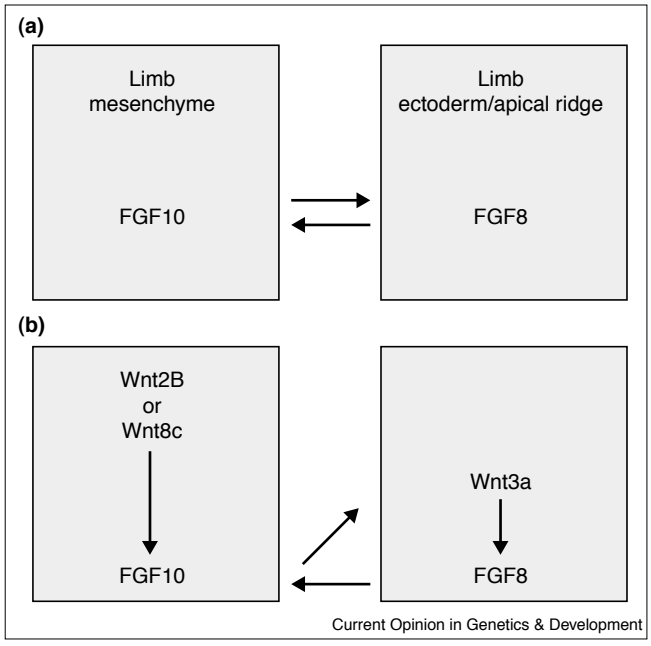
Signalling in early limb bud

The importance of FGF signalling for early limb development has been further tested in the past year by mouse knockouts of FGF ligands and their receptors. A dramatic phenotype was reported a few years ago in *Fgf10* knockouts. Both forelimbs and hindlimbs are almost completely absent [2,3] and, depending on genetic background, no buds form at all or limb buds are initiated but do not grow out. However, importantly, in both cases, the apical ectodermal ridge does not form morphologically or molecularly (*Fgf8* is never expressed). A strikingly similar limb phenotype was observed more recently in the knockout of *FGF receptor 2-IIIb* [12•], for which FGF10 had previously been suggested to be the major ligand [13]. Mice deficient for this particular receptor isoform show agenesis of limbs but, surprisingly, *Fgf8* expression is still induced, indicating that FGF10 may act through a different receptor or receptor isoform; but even though both *Fgf10* and *Fgf8* are expressed, *Shh* (*Sonic hedgehog*), which also plays an essential role in maintaining outgrowth, is not.

In the past year, *Fgf8* has been knocked-out in the apical ridge of mouse embryos with somewhat surprising results. As *Fgf8* null mice die before limbs develop, conditional knockouts had to be made and this was done by two different groups using the Cre-*LoxP* system but with different ridge promoters driving Cre [14••,15••]. Forelimbs are affected in one study because the *RARβ2* promoter used to drive Cre is only active in forelimb whereas hindlimbs are more affected in the other study because the *Msx2* promoter driving Cre is active at much earlier stages in hindlimb compared to forelimb. Conditional ablation of *Fgf8* in apical ridge of developing forelimbs [15••] or hindlimbs [14••] has substantial effects on development. In both studies, morphological changes are observed in skeletal structures along the entire proximo-distal axis including upper part of forelimb/hindlimb (humerus/femur), lower part (radius and ulna/tibia and fibula) and digit region (fingers/toes). The upper part of the limb is most severely affected and humerus/femur is either very reduced or even absent. In the lower part of the limb and digital region, anterior structures (but not posterior) structures are missing. Thus, functional inactivation of *Fgf8* in the mouse ridge seems to have almost the opposite effect to removing chick ridge, which leads to truncated limbs lacking distal structures.

Three other FGFs are also expressed in the apical ridge (*Fgf4*, *Fgf9*, *Fgf19*; Figure 2) and it is possible that some or all of these FGFs could compensate for absence of *Fgf8*. Indeed, limb development in *Fgf4* conditional mutants is normal, probably for this very reason [16,17]. These other FGFs are expressed somewhat later than *Fgf8* and in a more posteriorly restricted domain (Figure 2). This difference in extent of expression along the antero-posterior axis could go some way to explaining why posterior structures such as ulna and posterior digits are 'rescued' in conditional mutants whereas anterior structures are not. Similarly, the fact that these other Fgfs are expressed at a later stage in development could account for the 'rescue' of distal structures but not proximal structures.

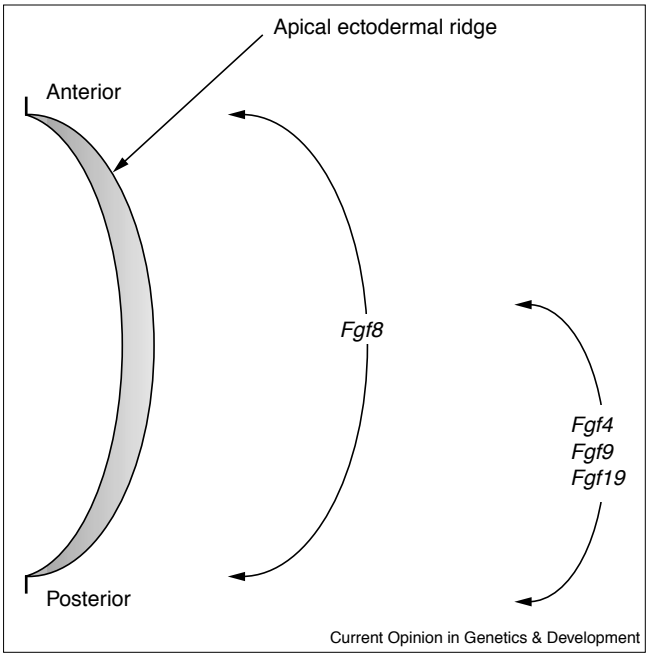
Figure 1



Regulatory interactions during limb initiation. **(a)** FGF10 derived from limb mesenchyme activates expression of *Fgf8* in ectodermal cells of the apical ridge. FGF8 maintains *Fgf10* gene expression in a positive feedback loop. **(b)** Model incorporating recent work. Wnt2b and Wnt8c, acting via β-catenin, induce *Fgf10* expression in limb mesenchyme. FGF10 activates *Wnt3a* in apical ridge, which in β-catenin dependent manner regulates *Fgf8* expression. FGF8 maintains *Fgf10* gene expression in a positive feedback loop.

The phenotype of these conditional *Fgf8* knockout mice is stimulating a new debate about the mechanism of patterning along the proximo-distal axis. At present, the widely accepted model is the progress zone model which proposes that the length of time that cells spend in the region of undifferentiated mesenchyme at the tip of the limb bud determines whether they form proximal or distal structures. Thus, cells become progressively 'distalized' as limb buds grow out under the influence of the apical ridge [18]. The authors of both papers suggest that the progress zone model should be revisited because removal of the ridge outgrowth signal, FGF8, in these mice does not preferentially affect distal structures. Interestingly, however, the phenotype of these *Fgf8* mutant mice in which proximal structures are most affected is reminiscent of that of chick limb buds which have

Figure 2



Expression of FGF family members in the apical ectodermal ridge. *Fgf8* is the first FGF to be expressed; its expression extends throughout the apical ectodermal ridge. *Fgf4, Fgf9* and *Fgf19* are expressed slightly later and are restricted to more posterior regions in normal embryos. Conditional ablation of *Fgf8* in apical ridge of fore- and hindlimbs results in an anterior expansion of *Fgf4* gene expression. The presence of other FGFs during limb development may explain the phenotype in these mice [14••,15••].

been irradiated and mesenchyme cells killed [19]. The explanation advanced in this old work is that, in irradiated wing buds, cells spend longer at the tip replacing the cells that were killed and therefore, according to the progress zone model, would form distal rather than proximal structures. In *Fgf8* conditional mutants, there will be a lag period before the other FGFs are expressed, in which there is no FGF signalling to sustain mesenchyme cells in the early limb bud and indeed transient cell death was noted in one study [15••]. Therefore, we wonder if the progress zone model should be discounted just yet on the basis of this evidence alone. We will have to wait and see what happens when several of the Fgfs expressed in the ridge are knocked-out together.

Compartmentalisation and ridge formation

One of the more surprising mechanisms for positioning vertebrate limbs was discovered in chick embryos and is based on compartmentalisation of ectoderm in both limb and inter-limb regions with respect to the dorso-ventral body axis [20,21]. This year, cell-lineage-restricted boundaries have been reported for the first time, in mouse embryos, again in ectoderm along both sides of the body where the thickened apical ridges of limb buds will form [22••]. Furthermore an 'invisible' boundary along the sides of mouse embryos is also revealed by applying FGF beads which induce a stripe of *Fgf8* expression [23]. The fact that the apical ridge signalling centre arises at a compartment boundary in mouse and chicks confirms an unexpected parallel between vertebrate limb development and insect development.

In chick embryos, ectoderm compartmentalisation is revealed either by making chick–quail chimeras or by labelling small groups of cells with DiI [20,21]. In the work this year with mice, two different methods are used: a sophisticated Cre–*LoxP* system to mark permanently with LacZ those cells expressing at that time *Engrailed 1*, a gene expressed in the ventral compartment; and injection of replication-incompetent retroviruses expressing LacZ into the amniotic cavity of mouse embryos *in utero* using ultrasound [22••].

Several consistent findings have emerged. All the studies in both chick and mouse agree that the boundary of the ventral compartment lies at the mid-point of the apical ridge (at least at early stages in ridge formation) and that this ventral compartment corresponds precisely with the domain of expression of the transcription factor *Engrailed 1* (Figure 3). In contrast, the reported extent of the dorsal compartment differs. In chick–quail chimeras, the dorsal compartment boundary abuts the ventral compartment boundary at ridge midpoint whereas in chick DiI labelling studies, dorsal cells are found throughout the ridge (Figure 3). In mice, a dorso-ventral compartment boundary in mid-ridge is transient and disappears at later stages. There also appears to be a second cell-lineage restriction at the interface of dorsal ectoderm and ridge which has not been detected in chick and is present when the border in the middle of the apical ridge is deleted (at E8.5). Later, there is a third cell lineage restriction at the ventral apical ridge boundary [22••]. In chick embryos, the expression domain of *radical fringe* encompasses the dorsal ectoderm and entire ridge [24,25]. This year, a rash of papers reported that fringe is a glycosyltransferase [26–28] which modifies notch. Interestingly, notch–delta signalling is known to be important in ridge development [29].

The correspondence between the ventral cell lineage restricted compartment and domain of *Engrailed 1* expression has attracted attention with experiments in both mouse and chick embryos to test its significance [22••,30•]. It is already established that in the *Engrailed* knock-out mouse the ridge is broad and flat instead of thickened [31]. Furthermore, in chick embryos, ectopic expression of *Engrailed 1* had been found either to induce additional ridges or to block ridge formation leading to the suggestion that an *Engrailed*-negative/*Engrailed*-positive boundary is necessary for proper ridge formation [24,32]. One possibility, therefore, is that *Engrailed* specifies the ventral compartment. To test this hypothesis in mice, *Engrailed 1* expression was driven throughout the ridge using the *Msx2* promoter, thus producing ectopic

Engrailed 1 with exquisite precision in dorsal ridge. When the distribution of ventral cells was followed using the indelible LacZ marker, however, the compartment appears undisturbed [22••]. The same result was obtained in chick embryos when *Engrailed 1* was misexpressed in ectoderm by a retroviral method followed by DiI labelling to monitor the extent of both dorsal and ventral compartments [30•]. Even though the ridge is absent and *Engrailed 1* is expressed ectopically, cell-lineage restrictions appear to remain intact. All of this taken together suggests that *Engrailed 1* does not specify the ventral compartment in either chick or mouse. The molecular mechanism that defines these ectodermal compartments, therefore, remains to be identified.

Even though *Engrailed 1* does not specify the ventral compartment, it clearly plays an important role in ridge development although some features are still puzzling. Recent work on mice shows that ectopic ridges form when *Engrailed 1* is misexpressed throughout the ridge at low levels, however, no ridge forms when *Engrailed 1* is misexpressed at high levels [22••]. Thus it appears that it is only when a boundary of *Engrailed 1* expression falls within the presumptive ridge that ectopic ridges can be induced. One possible explanation is that the ability of ectoderm cells to form a ridge becomes restricted to cells already in the ridge and that other non-ridge ectoderm cells are inhibited from forming a ridge. Recently, there has been the intriguing report in chick embryos that the transcription factor, Cux1, which is expressed in non-ridge ectoderm next to the ridge (and next to induced ectopic ridges) may do just this and prevent non-ridge ectodermal cells from forming ridge [33•]. This idea of ridges inhibiting formation of other ridges could also explain why ectopic ridges, which form when *Engrailed-1*-expressing ectoderm is grafted to dorsal limb bud, seem to be composed of *Engrailed*-expressing cells [34].

Conclusions

We have concentrated on specific issues about limb initiation but it will be important to work out how these fit into the wider picture. For example, we have discussed signals that initiate limb development but a critical question is how these signals are produced at the correct time and place in vertebrate embryos. In addition, we have not discussed the relationship of these signals with genes that encode 'limbness' such as *Tbx* genes that appear to be responsible for determining forelimb versus hindlimb [35–37]. Other transcription factors, such as Snail, are also expressed in limb-forming regions. *Snail* expression is induced rapidly in response to FGF [7•], although its function needs to be elucidated. Last, as with all repeated structures, we also need to understand spacing and mechanisms that inhibit limb formation in flank.

Acknowledgements

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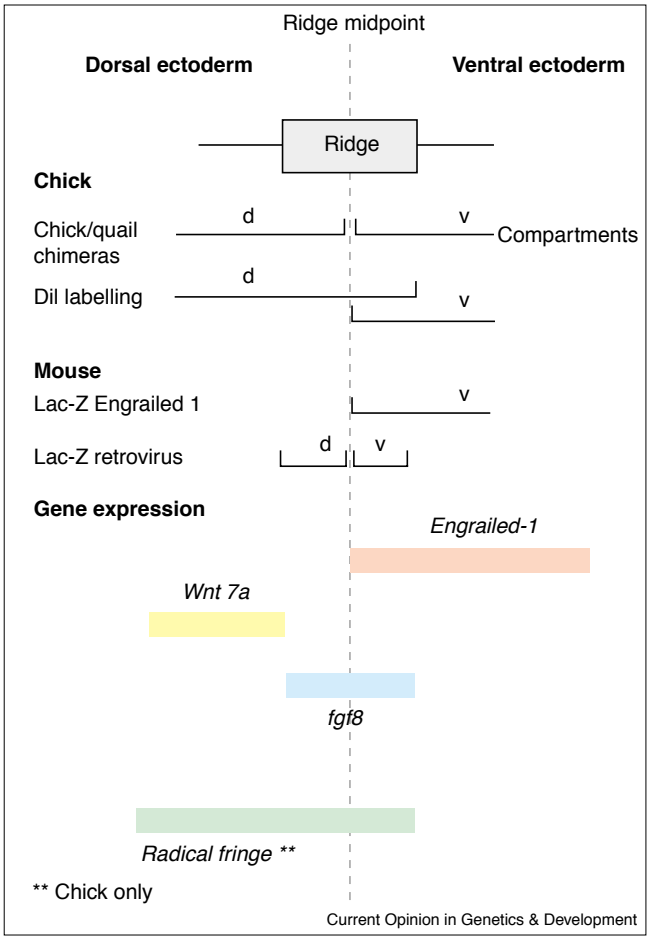
Figure 3



Compartment boundaries and gene expression within the apical ectodermal ridge. Experiments in chick and mouse have defined the extent of cell lineage restricted compartments. The dorsal–ventral midpoint of the apical ridge is represented as a dashed line. In chick–quail chimæras, the midpoint of the ridge is the compartment boundary whereas DiI labelling studies in chick suggest that the dorsal compartment extends throughout the ridge. In mouse, ventral cells, which express *Engrailed 1*, permanently marked with Lac-Z respect a mid-ridge boundary (at least during early stages). Cells labelled with a Lac-Z retrovirus in mouse show cell-lineage restricted boundaries at mid-ridge and at the dorsal and ventral edges of the ridge. The expression of *Engrailed 1* correlates with the extent of the ventral compartment, *Fgf8* is expressed throughout the ridge, *Wnt7a* is expressed in dorsal ectoderm only whereas *radical fringe* is expressed in dorsal ectoderm and throughout the ridge. d, dorsal; v, ventral.

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