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# Pattern formation in epithelial development: the vertebrate limb and feather bud spacing

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The ectoderm of the vertebrate limb and feather bud are epithelia that provide good models for epithelial patterning in vertebrate development. At the tip of chick and mouse limb buds is a thickening, the apical ectodermal ridge, which is essential for limb bud outgrowth. The signal from the ridge to the underlying mesoderm involves fibroblast growth factors. The non-ridge ectoderm specifies the dorsoventral pattern of the bud and Wnt7a is a dorsalizing signal. The development of the ridge involves an interaction between dorsal cells that express radical fringe and those that do not. There are striking similarities between the signals and genes involved in patterning the limb ectoderm and the epithelia of the *Drosophila* imaginal disc that gives rise to the wing. The spacing of feather buds involves signals from the epidermis to the underlying mesenchyme, which again include Wnt7a and fibroblast growth factors.

**Keywords:** epithelia; pattern; limb; feather bud

## 1. INTRODUCTION

Epithelia play a fundamental role in vertebrate development. Even at the earliest stages of mammalian development the trophoblast, which will give rise to extra-embryonic structures, forms a well-defined epithelium with polarized cells, and epithelia play a fundamental role in the development of many organs such as kidney, lungs and vascular system. However, in this paper I will only review the patterning and the role of epithelia in the development of the limb and in the development of feather bud spacing.

## 2. THE LIMB BUD

The development of the limb and the role of the ectoderm has been studied mainly in the chick and the mouse (Wolpert *et al.* 1998). The ectoderm forms an epithelial covering enclosing the mesoderm from which all the skeletal elements, such as cartilage and muscle, develop. The limb bud grows out as a small flattened bud-like structure whose patterning in three dimensions is in relation to three more-or-less independent axes: proximodistal, anteroposterior and dorsoventral (figure 1). The ectoderm plays a key role in the patterning of the limb along each of these three axes (reviewed by Zeller & Duboule (1997) and Johnson & Tabin (1997)).

At the tip of the developing limb bud is a ridge of thickened epithelium, known as the apical ectodermal ridge, which plays a key role. The ridge is essential for limb outgrowth and patterning along the proximodistal axis. Signals from the overlying apical ectodermal ridge specify the progress zone in the underlying mesenchyme, a region where cells acquire their positional information during limb outgrowth. If the apical ectodermal ridge is removed the limb is truncated, and the level of truncation depends on the time at which the apical ectodermal ridge is removed. Signals from the apical ectodermal ridge to the underlying mesenchyme involve fibroblast growth factors (FGFs), three of which are expressed in the limb ectoderm; in particular FGF4 is restricted to the posterior region of the apical ectodermal ridge, whereas FGF8 is expressed more extensively. It is possible to substitute a bead containing FGF for the apical ectodermal ridge when it is removed and to get more-or-less normal limbs (Niswander *et al.* 1993).

By labelling with DiI, a fluorescent cell marker, a fate map of the apical ectodermal ridge has been constructed (Vargesson *et al.* 1997). Cells in the posterior two-thirds of the ridge at an early stage will give rise to the entire ridge at later stages as cells in the anterior region at early stages leave the ridge as the bud grows out. It is possible to make mirror-image duplications along the anteroposterior axis of the limb by grafting a bead soaked in retinoic acid to the anterior margin. This results in the widening of the bud and also an increase in length of the apical ridge (Lee & Tickle 1985), which is probably due to cells in the anterior region not leaving the ridge. Labelling experiments also show that the apical ridge does not keep in register with the underlying mesenchyme, the ridge expanding more in the anterior direction.

In addition to this signal from the apical ectodermal ridge, there are signals from the dorsal and ventral ectoderm of the limb. These pattern the underlying mesoderm and therefore determine dorsoventral patterning of the mesoderm that gives rise to the muscles, tendons and cartilaginous elements (figure 2). Reversal of the ectoderm 180° about its dorsoventral axis results in an inversion of the dorsoventral pattern of the underlying mesoderm (MacCabe *et al.* 1974; Akita 1996). Three genes are known now to be differentially expressed along the dorsoventral axis and to be involved in dorsoventral patterning, two of them in the ectoderm and one in the mesoderm. In the dorsal ectoderm, *Wnt7a*, which codes for a secreted factor,

 

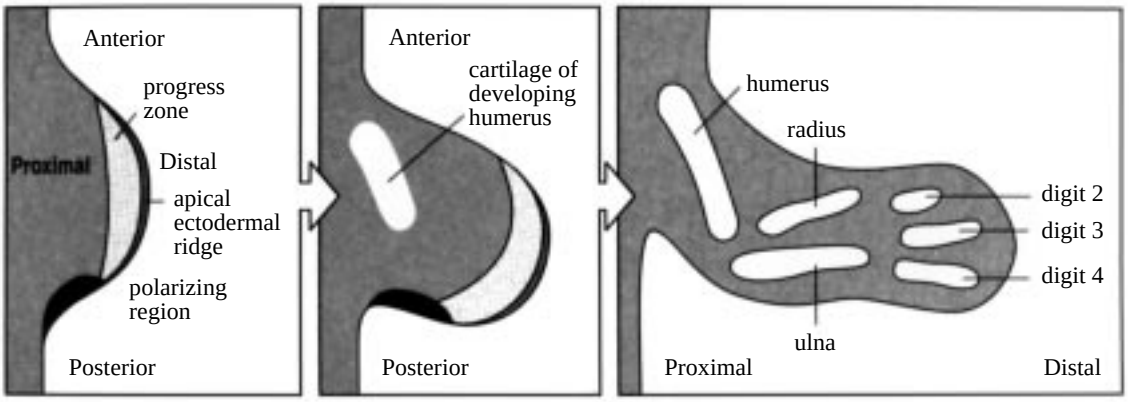


Figure 1. The apical ectodermal ridge specifies the progress zone in the underlying mesenchyme. The limb is laid down in a proximodistal sequence and when the proliferating cells in the progress zone leave the zone they may begin to differentiate as cartilaginous elements. The polarizing specifies positional information along the anteroposterior axis.

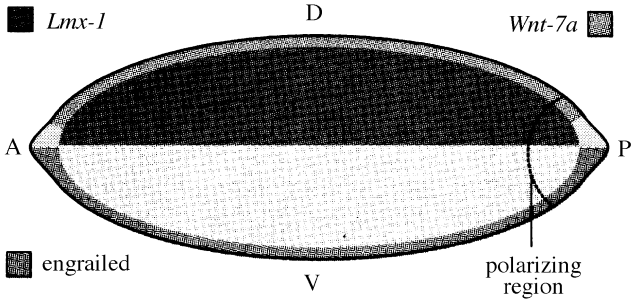


Figure 2. Transverse section through progress zone of a limb bud. The ectoderm controls the dorsoventral pattern in the developing limb. The gene *Wnt-7a* is expressed in the dorsal ectoderm and induces *Lmx-1* in the dorsal mesoderm, and *engrailed* is expressed in the ventral ectoderm. Anterior (A) is to the left.

is expressed (Dealy *et al.* 1993), whereas in the ventral ectoderm, *engrailed1*, a nuclear factor, is expressed (Loomis *et al.* 1996). The function of each of these factors has been partly understood by ectopic expression in the chick limb bud or by targeted mutations in mice. *Wnt7a* is involved in dorsalizing the limb; knockouts result in double ventral mirror-image limbs (Parr & McMahon 1995). Similarly the loss of *engrailed1* in mice results in dorsalization of the ventral ectoderm and in double dorsal limbs (Loomis *et al.* 1996). *Engrailed1* is a ventral regulator to repress the expression of *Wnt7a* in ventral ectoderm. In the dorsal mesoderm *Lmx1* is expressed and this is due to the action of *Wnt7a* (Vogel *et al.* 1995). Moreover, in the limb buds of the limbless chick embryo that lacks an apical ridge, *Wnt7a* is expressed throughout the ectoderm and *engrailed* is not expressed in the limb (Normaly *et al.* 1996).

Given the importance of the role of ectoderm in patterning the vertebrate limb, a central question is how the pattern of gene expression and the form of the ectoderm is specified during development. The mesoderm is involved in the initiation of limb bud formation. There is evidence that fibroblast growth factors are involved in limb bud initiation and the specification of the apical ectodermal ridge. When beads soaked in FGF2, or other members of the fibroblast growth factor family, are placed in the mesoderm between presumptive fore and hind limbs, a new ectopic limb bud can form from the lateral plate mesoderm; these form an apical ectodermal ridge at their tip and develop into quite normal limbs but with reversed anteroposterior polarity (Cohn *et al.* 1995).

The apical ectodermal ridge develops as a columnar epithelium (Todt & Fallon 1995) and is quite a narrow band of cells located between the dorsal and ventral limb ectoderm. At early pre-bud stages the lateral plate mesoderm is a flat layer of cells covered by the ectoderm. As the body wall tucks round to begin to close up ventrally, a thickened strip of ectoderm that gives rise to the ridge begins to develop over the mesoderm and eventually forms the group of tightly packed columnar cells. Using cell fate tracers it has been shown that the dorsal and ventral ectoderm are quite separate compartments, i.e. once a cell is in the dorsal region or the ventral region it never moves across the boundary represented by the apical ectodermal ridge (Altabef *et al.* 1997). This is important as it is one of the first examples in vertebrates of the presence of a compartment in non-neural ectoderm. The underlying mesoderm, unlike the ectoderm, does not contain two separate dorsal and ventral cell lineages and cells can cross the dorsoventral boundary. Surprisingly, the cells that form the apical ectodermal ridge are initially scattered in a wide region of early ectoderm both dorsally and ventrally (in addition, see Michaud *et al.* (1997)). These scattered cells contribute to the apical ectodermal ridge where they intermingle to some extent within it. The limb ectoderm arises from an area approximately 500 μm wide that lies lateral to the medial edge of the somites. The ectodermal compartmentalization may be a very early process directly related to dorsoventral compartmentalization of the body.

As the apical ridge is a narrow strip of cells in the ectoderm, how is it specified? The genes *radical fringe* and *engrailed1* play an important role in positioning the apical ectodermal ridge. *Radical fringe* is expressed in the dorsal ectoderm, and the region where cells expressing *radical fringe* and cells not expressing *radical fringe* meet defines where the apical ectodermal ridge forms (Laufer *et al.* 1997; Rodriguez-Esteban *et al.* 1997). Ectopic expression of *radical fringe* can result in new apical ectodermal ridges forming at the boundaries where there are cells expressing *radical fringe* and cells not expressing *radical fringe*. *Engrailed1*, which is expressed in ventral ectoderm, plays

an important role in restricting *radical fringe* expression to the dorsal ectoderm and thus ensuring that a sharp ventral boundary is made.

The differentiation of the apical ectodermal ridge is altered by mutations in the gene *limb deformity*, which encodes a nuclear protein. In *limb deformity* mutants (Zeller *et al.* 1989) the apical ectodermal ridge remains as a flattened and patchy epithelium expressing FGF8 and other early apical ridge markers; however, FGF4 is not expressed and expression of sonic hedgehog in the underlying mesenchyme, which acts as a positional signal along the anteroposterior axis, is not maintained. The *limb deformity* gene is expressed in both the mesoderm and the apical ridge. The mesoderm is the tissue affected by the mutation as a combination of *limb deformity* ectoderm from mouse limb buds with wild-type avian mesoderm results in the *limb deformity* ectoderm forming an apical ridge. The site of limb deformity action is in the mesoderm and it probably is involved in the relay of a signal from *sonic hedgehog* to the ectoderm, which may also involve bone morphogenetic proteins (BMPs). There is evidence that sonic hedgehog protein can specify an apical ridge as localized application in anterior regions leads to expression of FGF4 and ridge extension.

It is striking that the ectoderm plays such an important role in patterning the vertebrate limb and it is also striking that the signals and genes involved are very similar to those involved in organizing appendage development in *Drosophila*, which occurs in the epithelial sheet of the imaginal disc (Wolpert *et al.* 1998). For example, genes such as *fringe, engrailed* and *wnt* are all involved in insect development in a rather similar way. The boundary at the junction of dorsal and ventral surfaces of the *Drosophila* imaginal wing disc gives rise to the wing margin and acts as a signalling centre for further patterning of the wing (Laufer *et al.* 1997). In insect wing development, *fringe* acts by activating genes in the *Notch* signalling pathway, and vertebrate homologues of this pathway, *serrate* and *Notch*, are expressed in the ridge. Moreover, a key gene involved in the proximodistal axis of the insect limb, and which is expressed at its tip—*Distal-less*—is also expressed in the apical ectodermal ridge (Ferrari *et al.* 1995). The mechanisms for patterning appendages thus show remarkable conservation during evolution. A further example of this conservation is shown in the patterning of feather buds.

## 3. FEATHER BUDS

Some of the simplest patterns observed in development are spacing patterns, which involve the maintenance of a regular distance between neighbouring elements (Sengel 1976). Examples of spacing patterns include teeth, skin, glands and scales. The development of feather buds in the chick skin provides a very good model system as the pattern will develop in cultured embryonic skin. It is striking that once again, many of the genes patterning the epithelia of vertebrate and insect limbs are involved in spacing the feather buds.

Feather primordia begin their development in the chick at stage 29 to 35, which is between six and nine days of incubation (Davidson 1983; figure 3). They first appear as dense regions about 0.24 mm in diameter on the chick skin surface, and develop in well-defined lines and well-defined

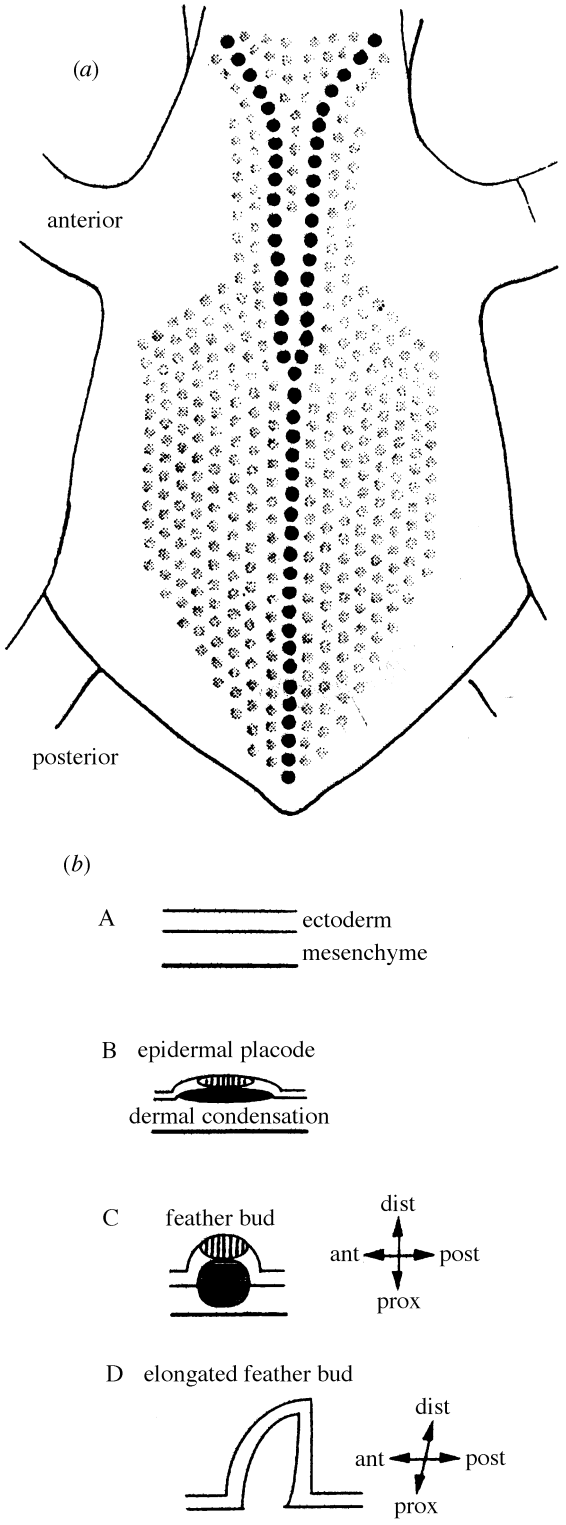


Figure 3. (*a*) The pattern of dorsal skin feather buds. The initial buds are in black. (*b*) Process of feather formation. The first indication of bud formation is the thickening of the epidermal placode, which is followed by condensation of the dermal cells.

temporal order. Initially the feather-forming region is a columnar epidermis overlying a loose mesenchyme. The first sign of feather formation is a thickening in the ectoderm to form a placode. This is followed by the underlying mesenchyme forming a dense condensation. Cells in the placode elongate until they are about twice the height of the cells in the adjacent ectoderm.

Interactions between the mesenchyme and the overlying ectoderm play a key role in feather morphogenesis. A wide variety of epithelia that would never normally form feathers does so in combination with feather-forming dermis. For example, chorionic ectoderm and amniotic epithelium form feathers when mesoderm from feather-forming regions is placed beneath them (Sengel 1971).

There is a well-defined pattern of gene expression during feather development in back skin before any morphological signs of feather bud formation. The molecules involved in spacing the buds seem to be similar to those patterning the ectoderm and mesoderm of the limb bud. A stripe of sonic hedgehog transcripts is expressed along the midline of the back within the epidermis, which then breaks up into patches that form the primary row of feather buds (Nohno *et al.* 1995; Jung *et al.* 1998). At a slightly later stage, *Cek8*, a member of the eph-receptor tyrosine kinase family, is present in the epidermal placodes. FGF4 transcripts are also present in the epidermis of the feather-forming region as a single stripe (Chuong *et al.* 1996; Jung *et al.* 1998) and expression then becomes localized to the epidermal placodes. In contrast with these expressions within the ectoderm, follistatin and BMP2 are expressed in the dermal condensations beneath the epidermal placodes. Wnt7a transcripts are expressed in epidermal placodes, but in a wider domain than sonic hedgehog expression. As the feather bud grows, Wnt7a always remains in the epithelium at the most lateral edges of the bud.

Sonic hedgehog, fibroblast growth factor 2, follistatin and BMP2 are all secreted molecules and in order to investigate their role as signalling molecules, beads soaked in these molecules were added back to the skin, which was then cultured for a further 48 h. Beads soaked in sonic hedgehog protein and placed in the midline before feather bud formation resulted in an increase in the number of feathers that formed in the region around the bead (Ting Berreth & Chuong 1996). Similarly, application of fibroblast growth factors and follistatin (Widelitz *et al.* 1996; Jung *et al.* 1998) resulted in an increased number of feather buds. In contrast, beads soaked in BMP2 resulted in the inhibition of the development of feather buds around the beads and there was an absence of feather buds up to a distance of *ca.* 750 μm.

Feather buds can also be formed from dispersed and then reaggregated mesenchymal cells provided they are combined with an intact epithelium (H.-S. Jung, personal communication). They then develop in about the same time rather than sequentially as in normal cultured skin. Although the buds form a somewhat irregular spacing, the size of most of the feather buds is normal and sonic hedgehog transcripts are localized at the epithelium of individual feather buds as in normal buds. This result shows that the normal pattern of feather bud formation is determined by the underlying mesenchyme rather than the epithelium.

These results suggest a model for the spacing of feather buds in which sonic hedgehog and fibroblast growth factors expressed in the epithelium initiate dermal condensations in the mesoderm beneath. The dermal condensations express BMPs that inhibit bud formation in adjacent areas, but they also express follistatin, which blocks the inhibitory action of BMP and so allows the bud to continue its own development. These processes are linked to a wave of competence to express sonic hedgehog and fibroblast growth factors, which spreads over the skin and so generates the spacing pattern.

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