

Distribution of polarizing activity and potential for limb formation in mouse and chick embryos and possible relationships to polydactyly

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SUMMARY

A central feature of the tetrapod body plan is that two pairs of limbs develop at specific positions along the head-to-tail axis. However, the potential to form limbs in chick embryos is more widespread. This could have implications for understanding the basis of limb abnormalities. Here we extend the analysis to mouse embryos and examine systematically the potential of tissues in different regions outside the limbs to contribute to limb structures. We show that the ability of ectoderm to form an apical ridge in response to FGF4 in both mouse and chick embryos exists throughout the flank as does ability of mesenchyme to provide a polarizing region signal. In addition, neck tissue has weak polarizing activity. We show, in chick embryos, that polarizing activity of tissues correlates with the ability

either to express *Shh* or to induce *Shh* expression. We also show that cells from chick tail can give rise to limb structures. Taken together these observations suggest that naturally occurring polydactyly could involve recruitment of cells from regions adjacent to the limb buds. We show that cells from neck, flank and tail can migrate into limb buds in response to FGF4, which mimics extension of the apical ectodermal ridge. Furthermore, when we apply simultaneously a polarizing signal and a limb induction signal to early chick flank, this leads to limb duplications.

Key words: Polarizing activity, Apical ectodermal ridge, Chick, Mouse, Limb, *Shh*, FGF4

INTRODUCTION

In tetrapods, two pairs of limb buds develop at specific positions along the head-to-tail axis of the embryo. However, it is well-known, both from classical work in amphibians and more recent studies in chick embryos, that the potential to form limbs is much more widespread throughout the body. This may be a legacy from ancestral vertebrates from which present day vertebrates evolved. This potential could also be important in genesis of limb abnormalities. Here we explore limb-forming potential in mouse and chick embryos and suggest models for the origin of polydactyly.

Limbs develop from small buds of mesenchyme enveloped in ectoderm. Bud outgrowth is controlled by the apical ectodermal ridge, the ectodermal thickening around the limb-bud tip. The apical ridge forms from ectoderm overlying the limb-forming region in response to signals from the mesoderm and, subsequently, mediates outgrowth through production of FGFs. A second signalling centre, the polarizing region, then forms at the posterior edge of the apical ridge. Signals from the polarizing region control anteroposterior digit pattern (Saunders and Gasseling, 1968). When the polarizing region from one chick wing bud is grafted to the anterior margin of a

second bud, extra digits are induced. Signalling regions in the limb are conserved between mouse and chick. Posterior cells from a mouse limb bud, when grafted to the anterior margin of chick limb buds, lead to formation of extra digits and a rat apical ridge can support outgrowth of chick limb mesenchyme (Tickle et al., 1976; Fallon and Crosby, 1977; Wanek and Bryant, 1991; Jorquera and Pugin, 1971).

The ability of tissues to induce extra digits when placed anteriorly in chick wing buds is known as polarizing activity and, more recently, molecules with this property have been discovered. Retinoic acid was the first molecule to be identified with polarizing activity (Tickle et al., 1982) but is now thought to act via induction of expression of *Sonic hedgehog* (*Shh*; Riddle et al., 1993). *Shh* is expressed in the polarizing region in the normal limb bud and its expression corresponds to maps of polarizing activity (MacCabe et al., 1973; Honig and Summerbell, 1985). When *Shh* is ectopically expressed anteriorly, this leads to extra digits. Several non-limb tissues have been shown to have polarizing activity and this has also been found to be associated with *Shh* expression (Riddle et al., 1993).

The flank (interlimb) region of vertebrate embryos is known to have limb-forming potential. This was first demonstrated in newts in response to nasal placode grafts (Balinsky, 1965).

More recently, it was shown that a single application of FGF to the presumptive flank region of chick embryos leads to induction of an ectopic apical ridge, expressing *Fgf8*, followed by induction of a polarizing region, expressing *Shh*, and formation of an additional limb (Cohn et al., 1995; Ohuchi et al., 1995, 1997; Crossley et al., 1996; Vogel et al., 1996; Yonei-Tamura et al., 1999). It is also known from grafting experiments that cells in chick flank have polarizing activity (Hornbruch and Wolpert, 1991; Yonei et al., 1995). In chimeric mouse embryos prepared with wild-type embryos and *Fgf4*-expressing ES cells, small bud-like structures develop from the flank and these 'buds' express *Shh* and *Msx1* (Abud et al., 1996).

Here we document, in detail, the potential of cells and tissues at different positions in both mouse and chick embryos to contribute to limb structures. We find widespread potential to form both signalling regions and/or limb structures. These observations suggest that polydactyly could result from recruitment of non-limb cells into limb buds. Our analysis of mouse embryos is particularly important because there are mouse polydactylous mutants that resemble human conditions with digit abnormalities. In many polydactylous mouse mutants, *Shh* has been shown to be expressed anteriorly as well as posteriorly (Masuya et al., 1995, 1997) but the origin of these cells is not known.

Li and Muneoka (1999) have shown that FGF4, which is produced by the apical ridge, acts as chemoattractant to limb-bud cells. Therefore we implanted FGF4-soaked beads at the edges of the apical ridge in chick limb buds to imitate ridge elongation and tested whether nearby non-limb cells migrate into the buds. We also applied a ridge induction signal together with a polarizing region signal to the flank of early chick embryos and showed that this led to limb polydactyly.

MATERIALS AND METHODS

Source of animals

Mice (C57, Balb-C and CD-1; Harlan) were maintained at constant temperature ($21\pm 2^\circ\text{C}$) with 12 hours of light 6 a.m.-6 p.m. Mated females were checked for vaginal plugs three times a day (9 a.m., 2 p.m. and 5 p.m.). Fertilized White Leghorn chicken eggs were obtained from Poyndon Farm, Waltham Cross, Hertfordshire, UK.

Whole mouse embryo culture and insertion of FGF-soaked beads into the flank of E9 mouse embryos

E9 mouse embryos were cultured in rat serum with closed yolk sacs as described by Martin and Cockroft (1999). Heparin-acrylic beads (50 μm in diameter; Sigma, H5263) were soaked in 0.7 mg/ml FGF-4 or PBS as previously described (Cohn et al., 1995). After removal of Reichert's membrane, a small slit was made in the yolk sac, amnion and flank lateral plate. The FGF4- or PBS-soaked bead was implanted into the small slit in the flank and the embryonic membranes were then pinched closed. Immediately after bead implantation, embryos were placed in a warmed tube with 1 ml of warmed rat serum per embryo. The tube was then gassed with 20% O₂, 5% CO₂ and 75% N₂, sealed and placed into a roller-tube mouse incubator at 37°C. At 24 hours, the tube containing the embryos was re-gassed with 40% O₂, 5% CO₂ and 55% N₂. Embryos were harvested after 48 hours of culture and then fixed in 4% paraformaldehyde (PFA) for in situ hybridization.

Embryo manipulations

Assaying for polarizing activity in embryonic chick and mouse limb bud and flank

In the first grafting procedure, tissues of chick or mouse embryos were dissected and placed in 1% trypsin, for 10-30 minutes at 4°C to loosen the ectoderm from the underlying mesoderm. The wedges of tissue to be grafted were then transferred to tissue-culture medium

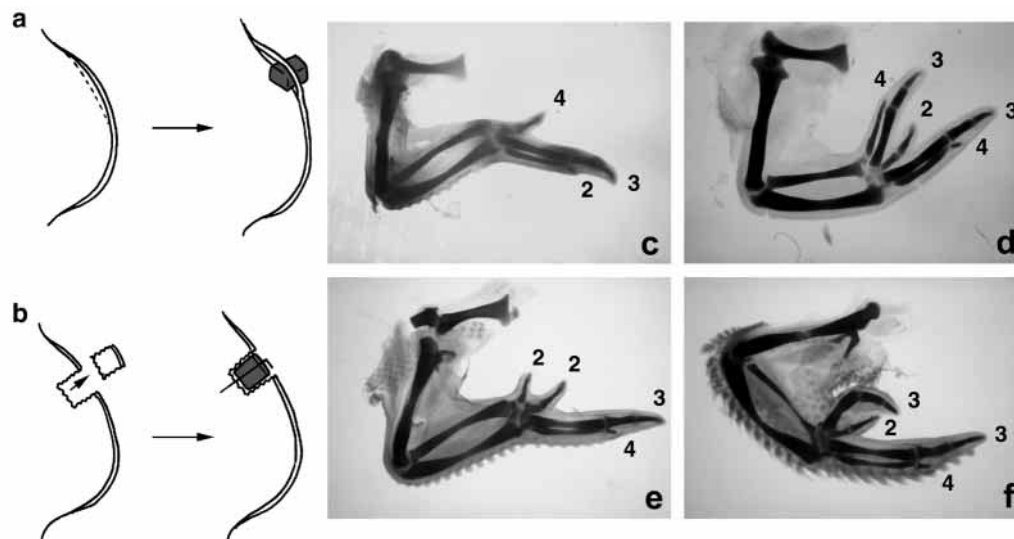


Fig. 1. (a,b) Diagram of experimental manipulations carried out with stage 20 wing buds. Grafted tissue is shaded. (a) Grafting procedure (Saunders, 1977; Tickle, 1981; Hornbruch and Wolpert, 1991) in which a cut was made along the base of the apical ectodermal ridge over the anterior part of the host bud. The apical ectodermal ridge was raised up from the underlying mesenchyme and the graft tissue (without ectoderm) was placed between ridge and mesenchyme. (b) Grafting procedure (Saunders and Gasseling, 1968; MacCabe et al., 1973) in which a piece of mesenchyme plus overlying apical ridge was removed from the anterior margin of the host limb bud and the graft tissue (with ectoderm) was placed in the hole and pinned. (c-f) Dorsal views of whole mounts of chick wings obtained after transplantation of chick graft tissue under the host anterior apical ridge. (c) Wing resulting from grafting of tissue from anterior margin of the limb bud ('A' region). Digit pattern 234. (d) Wing resulting from grafting of tissue from posterior margin of the limb bud ('H' region). Digit pattern 43234. (e) Wing resulting from grafting of tissue from central region of the limb bud ('P' region). Digit pattern 2234. (f) Wing resulting from grafting of the tissue from flank ('X' region). Digit pattern 3234.

(MEM or DMEM+10% foetal calf serum (FCS); Gibco: Biocult) at 4°C and the ectoderm removed. The graft site in the host chick wing bud was prepared by lifting the apical ectodermal ridge along the anterior margin of the wing bud of stage 19-20 host chick embryos. The graft tissue was then implanted under the ridge (Saunders, 1977; Fig. 1a).

In a second grafting procedure to assay polarizing activity, blocks of tissues (mesenchyme plus ectoderm) from chick embryos were dissected in tissue-culture medium. The tissue block was grafted to a hole cut at the anterior margin of a host chick wing bud of stage 19-20 (Hamburger and Hamilton, 1951). The graft site in the host was prepared by removal of a comparably sized piece of apical mesoderm and overlying ectodermal ridge from the anterior wing bud. The graft tissue was then secured into the host wing bud with a fine tungsten pin (Saunders and Gasseling, 1968; MacCabe et al., 1973; Fig. 1b).

In the normal chick wing, the digit pattern is **234** (reading anterior to posterior, Fig. 1c). Tissue with high polarizing activity induces the formation of an extra posterior digit, digit **4**, as in digit patterns such **43234** or **4334** (Fig. 1d; **43234**), whereas weak polarizing activity induces only an extra anterior digit, digit **2** (Fig. 1e; **2234**), and moderate polarizing activity specifies an extra digit **3** (Fig. 1f; for example **3234**).

Patterns were assigned a score (S) based on the extra digit with most-posterior identity that develops. When an extra digit **4** was produced, S was 4. When the digit with the most posterior identity was a **3** or a **2**, S was 2 or 1 respectively. When there were no anterior digits, S was 0. These scores were used to calculate the 'percentage respecification', where percentage respecification = $SS \times 100 / 4 \times n$, and n is number of wings (Tickle et al., 1985).

Insertion of heparin beads into chick embryos

Heparin-acrylic beads were loaded with FGF4 (PBS soaked as a control) as described above then inserted into slits in wing buds or flank regions or tail regions of stage 20 chick embryos. Embryos were fixed 16 hours later in 4% PFA and then subjected to in situ hybridization for *Shh*.

Insertion of tail or leg tissue into wing buds of chick embryos

Tail tissues opposite somite 32 to 35 or mesenchyme from distal central regions of leg buds of stage 20 chick or quail embryos were dissected and placed in 1% trypsin, for 10 minutes at 4°C to loosen the ectoderm and endoderm from the mesoderm, then transferred to tissue culture medium at 4°C and the ectoderm and endoderm removed. The mesoderm was then grafted to host chick wing buds. The graft site in the host chick wing bud was prepared by lifting the apical ectodermal ridge (AER) along the posterior margin of wing buds of stage 19-20 host chick embryos.

Insertion of beads into pre-limb-bud-stage chick flank

Heparin-acrylic beads were soaked in 1 mg/ml FGF2 (R&D Systems) as previously described (Cohn et al., 1995). Mouse *Shh* protein was kindly provided by Andy McMahon. Affigel CM beads were soaked in 16 mg/ml *Shh* as previously described (Yang et al., 1997). Beads were inserted into slits made in flank ectoderm and pushed into the mesoderm. Embryos were then incubated for a total of 9-10 days.

DiI labelling and application

DiI labelling of grafts

Mesenchymal tissues to be grafted were labelled by immersion in 9 µg/ml DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; 3 mg/ml stock in dimethylformamide was diluted in MEM + 10% FCS) at 37°C for 30 minutes. DiI-labelled tissues were then grafted under anterior apical ridge as described (Fig. 1a). Embryos were fixed 24 hours later in 4% PFA, and photographed under a Leica MZ FLIII microscope. Specimens were then subjected to in situ hybridization for *Shh*.

DiI application to track cells

Small deposits of DiI (3 mg/ml in dimethylformamide) were injected via microelectrodes into mesoderm, in ovo, using a picospritzer (Vargesson et al., 1997). Microelectrodes, with a tip diameter of 3 µm, were filled at their tip with a small quantity of tracer and backfilled with 1 M lithium chloride. In each embryo, the location of DiI was photographed first at 0 hours, and then later at 24 hours using a Leica MZ FLIII microscope.

Immunohistochemistry

7 days after transplantation of quail tail tissue into chick wing buds, embryos were fixed in 4% PFA in PBS, embedded in O.C.T. compound (Miles), frozen and then serially sectioned at 10 µm. Sections were blocked for 1 hour in 5% heat-inactivated goat serum + 5 mg/ml bovine serum albumin (Sigma) in PBS, incubated for 1 hour in QCPN anti-quail monoclonal antibody (Developmental Studies Hybridoma Bank, University of Iowa), washed five times for 20 minutes in PBS, incubated for 1 hour in 1:400 horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma), washed as before with second antibody, and then developed in 0.25 mg/ml diaminobenzidine in PBS with synchronous addition of 0.03% H₂O₂. The reaction was stopped by addition of excess PBS.

In situ hybridization

The *Tbx4* probe was transcribed from a *HindIII* fragment by T3 polymerase (Isaac et al., 1998) and the *Shh* probe was transcribed from a *XhoI* fragment by T3 polymerase (Riddle et al., 1993). *Hoxc9* cDNA-containing plasmid was generously supplied by Cliff Tabin and probe was transcribed from a *EcoRI* fragment by T3 polymerase (Nelson et al., 1996). In situ hybridization was performed as previously described (Wilkinson, 1992).

Alcian blue staining

Host chick embryos were incubated until day 9-10. The embryos were then fixed in 5% TCA, stained in 0.1% Alcian blue in 70% acid alcohol, dehydrated in ethanol and cleared in methyl salicylate.

RESULTS

The future flank of mouse embryos can form an apical ectodermal ridge

To test whether mouse flank is competent to respond to FGF and form limb buds, we implanted FGF4-soaked beads into the flank lateral plate mesoderm of 14-somite-stage mouse embryos (embryonic day 9, E9, before limb buds have developed). Manipulated mouse embryos were then cultured in rat serum with special gas mixtures for 48 hours. After this length of time in culture, most of the 13 embryos that had survived, had developed both forelimb and hindlimb buds but some embryos had forelimb buds only. Implantation of an FGF4-soaked bead to the flank led either to enlargement of the forelimb bud towards the flank on the manipulated side (10 out of 13 embryos) or to ectopic limb-bud formation in the flank (3 out of 13 embryos).

In order to examine apical ridge formation in treated embryos, we examined the pattern of transcripts of *Fgf8*, a gene that is expressed in the apical ectodermal ridge of developing limb buds. In bead-treated embryos where a morphological limb bud extends posteriorly down the embryonic flank, we saw punctuated linear expression of *Fgf8* throughout flank and hindlimb regions (Fig. 2a,b). Thus the ectoderm all the way along the flank of mouse embryos is competent to form an apical ridge. When the FGF bead triggered outgrowth of an

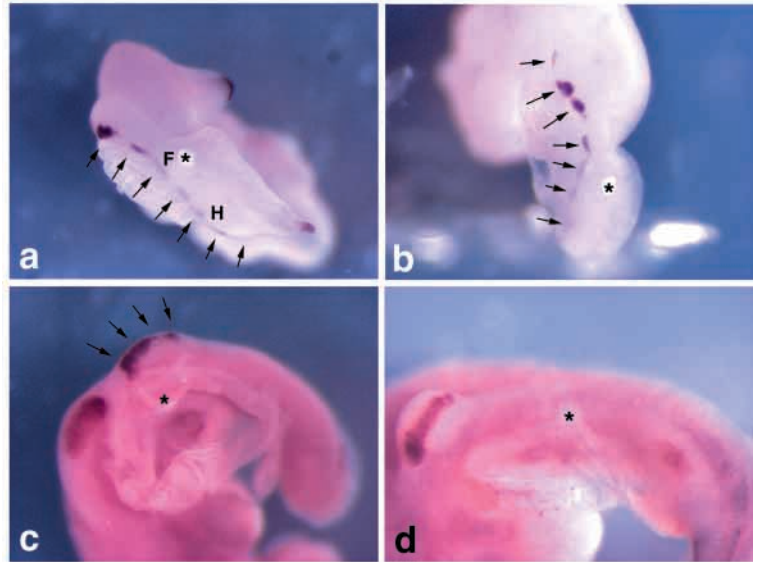


Fig. 2. Expression of *Fgf8* in cultured mouse embryos 48 hours after FGF4- or PBS-soaked bead implantation. (a,b) Expression of *Fgf8* is observed continuously from apical ridge of forelimb bud to hindlimb bud throughout the flank (arrows) after implantation of an FGF4-soaked bead (asterisk). F, flank; H, hindlimb. (c) Ectopic expression of *Fgf8* is observed in the apical ridge of an additional limb bud (arrows) that developed in the flank after implantation of a FGF4-soaked bead (asterisk). (d) No ectopic *Fgf8* expression was observed after the implantation of a PBS-soaked bead (asterisk).

additional limb bud, ectopic expression of *Fgf8* was confined to the apical ridge of the new limb bud (Fig. 2c). Implantation of PBS-soaked beads never induced extended apical ridge formation or outgrowth of ectopic limb buds (0 out of 8; Fig. 2d).

Polarizing activity can be detected in limb bud and flank of mouse embryos

Polarizing activity of lateral plate mesoderm of mouse embryos at various stages and from various locations was measured by grafting the mesenchyme under the apical

ectodermal ridge at the anterior margin of a stage 20 chick wing bud (Fig. 1a). Limb-bud and flank tissues to be used as grafts were excised from mouse embryos as shown in Fig. 3.

In 14-somite-stage (E9) mouse embryos, we observed weak polarizing activity in presumptive forelimb ('A' and 'B' region) and most anterior flank region ('C' region; Fig. 3a,d). Grafts of mesenchyme from these regions induced extra digit 2s. However, grafts of mesenchyme from posterior flank ('D' region) and presumptive hind limb ('E' and 'F' region) showed no polarizing activity.

In older mouse embryos at early limb-bud stage (E10.5), we

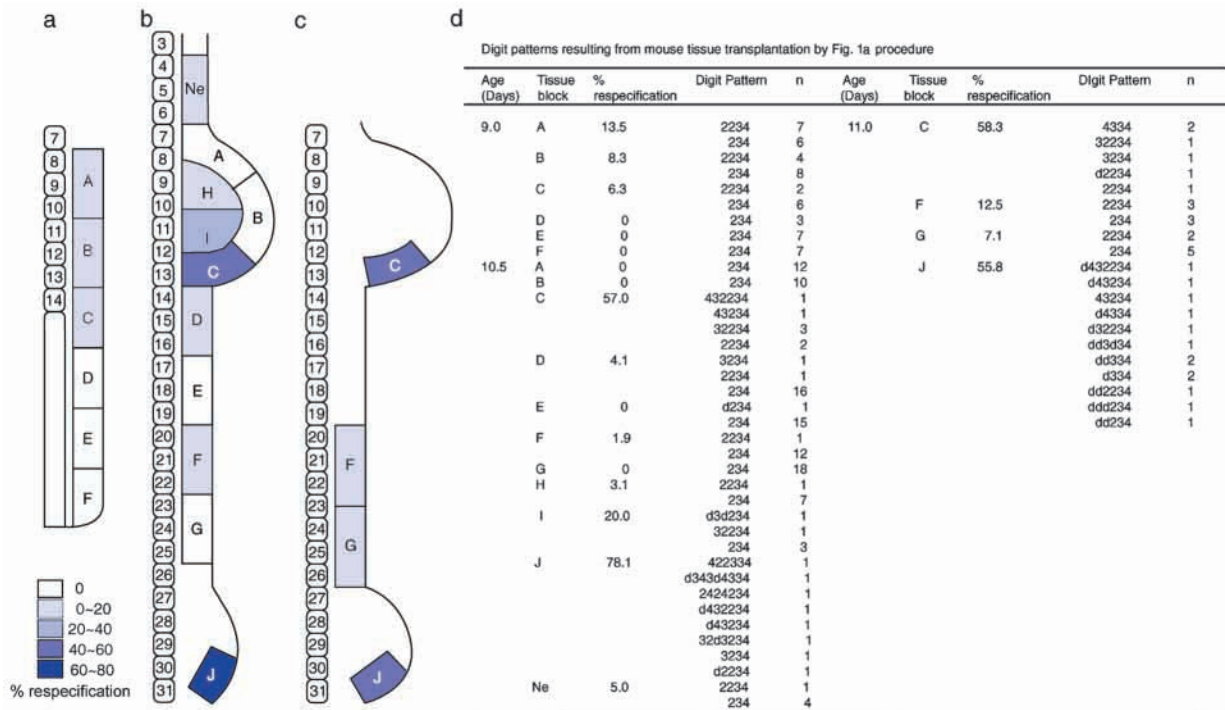


Fig. 3. Diagram of (a) E9, (b) E10.5 and (c) E11.0 mouse embryos. Pieces of mesenchyme were cut as shown in diagrams and assayed for polarizing activity by grafting to the anterior margin of a host chick wing bud as in Fig. 1a. (d) Digit patterns and percentage respecification values resulting from mouse tissue transplants. n, number of cases.

found that polarizing activity was now more widespread and could be detected in anterior to mid-flank ('D' and 'F' region), although the most posterior region of the flank still did not show any polarizing activity ('G' region; Fig. 3b,d). Very weak polarizing activity could also be detected in mesenchyme from the posterior neck region ('Ne' region). Grafts from the posterior margin of both forelimb bud and hindlimb bud where the polarizing region is known to be located ('C' and 'J' region) had very strong polarizing activity. More unexpectedly, mesenchyme from proximal forelimb bud showed strong polarizing activity posteriorly ('I' region) and weak activity anteriorly ('H' region).

In still older mouse embryos (E11.0), polarizing activity could now be detected in the most posterior part of the flank just anterior to the hindlimb bud ('G' region; Fig. 3c,d). The posterior margin of the forelimb bud and hindlimb bud also still had strong polarizing activity ('C' and 'J' region).

Distribution of polarizing activity in chick embryos is similar to that in mouse embryos

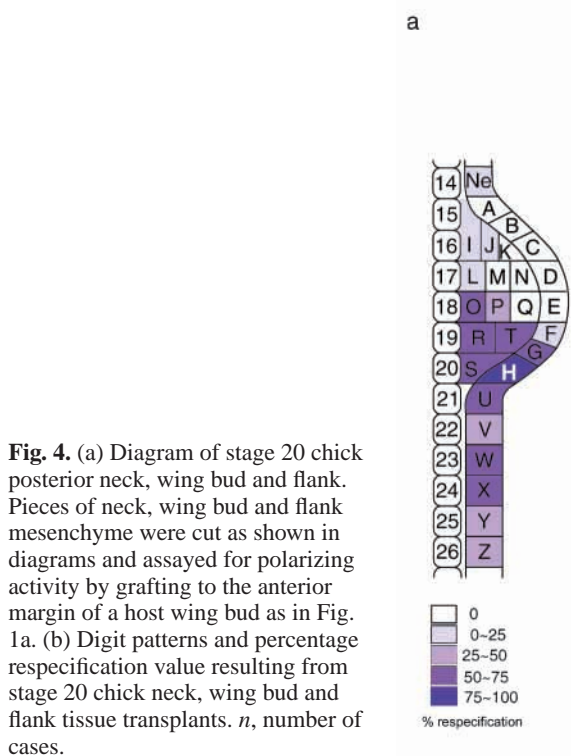
In parallel with our map of polarizing activity in mouse embryos, we also prepared a more detailed map of polarizing activity in chick embryos (Fig. 4a,b). In general, the two maps are similar, although there are some important differences. As in MacCabe's map of the chick wing bud (MacCabe et al., 1973), mesenchyme from the posterior 'H' region, which is now known to express *Shh* (Riddle et al., 1993), had very high polarizing activity and always specified an extra digit 4. However, in contrast to MacCabe's map, strong polarizing activity was also detected in the proximal central region of the chick limb bud. For example, grafts from this region of the developing bud ('O' region) generally induced formation of an extra digit 4. While proximal posterior tissue ('S' region) has

similar activity, proximal tissue more anteriorly (for example 'I' region) has weaker activity. None of these proximal regions in the normal chick limb expresses *Shh*.

There are some discrepancies between our maps of polarizing activity in the chick limb bud and those of MacCabe et al. (1973). We therefore repeated MacCabe's mapping procedures in which mesenchyme plus ectoderm was grafted to a hole at the anterior margin of a host chick wing bud (Fig. 1b). Using this approach, we too found that while posterior margin grafts from a stage 20 chick wing buds ('H' region) showed very strong polarizing activity (5/5 wings with extra digit 4), grafts from the proximal center of the limb bud ('O' region) had hardly any polarizing activity (1/7 wing had an unidentified element, 6/7 wings were normal). To test whether this difference between our results and those of MacCabe was simply due to presence or absence of overlying ectoderm, we implanted tissue from 'O' region without removal of the ectoderm under an intact apical ridge, but these grafts again showed strong polarizing activity (4/4 wings with extra digit 4). These data suggest that our assay may detect additional sources of polarizing activity.

Using our grafting assay, we also found that when we dissected all the regions in the limb bud with polarizing activity into dorsal and ventral halves, the dorsal half always had stronger polarizing activity than the ventral half. Thus for example, dorsal tissue from the proximal posterior region (S-Dorsal) often induced digit 4, while ventral tissue (S-Ventral) never specified additional digits (or ectopic cartilage).

Our maps do reveal one area of the embryo with dramatically different polarizing activities between chick and mouse. We found that posterior flank of early limb-bud-stage mouse embryos (E10.5), has no activity (Fig. 3d) and yet our grafts revealed that the entire flank of a chick embryo at an



b Digit patterns resulting from tissue transplantation.

Tissue block	% respecification	Digit Pattern	n	Tissue block	% respecification	Digit Pattern	n
A	0	234	7	S	65.0	432234	1
B	0	234	5			43234	2
C	0	234	6			4334	3
D	0	234	5			2234	2
E	0	d234	1			234	2
		234	6	T	65.0	432234	1
F	15.6	43234	1			43234	1
		2234	1			4334	1
		234	6			d2234	1
G	70.6	432234	1			234	1
		43234	1	U	62.5	43234	1
		4334	5			4334	1
		234	3			d3234	1
H	100	43234	4			234	1
		4334	2	V	25.0	43234	2
I	8.3	2234	2			2234	4
		234	4			234	6
J	4.2	2234	1	W	50.0	4334	2
		234	5			d2234	1
K	0	234	5			2234	1
L	10.7	32234	1			234	1
		2234	1	X	55.6	432234	1
		d234	1			43234	1
		234	4			4334	2
M	0	234	6			3234	2
N	0	234	8			234	3
O	62.5	432234	2	Y	28.6	43234	1
		4334	1			4334	1
		32234	1			234	5
		d2234	1	Z	31.3	43234	1
		234	1			4334	1
P	25.0	43234	1			2234	2
		2234	3			234	4
		234	3	Ne	8.3	d3d234	1
Q	0	234	5			2234	1
R	62.5	432234	1			234	7
		43234	1				
		4334	1				
		d3234	1				
		2234	1				
		234	1				

Fig. 4. (a) Diagram of stage 20 chick posterior neck, wing bud and flank. Pieces of neck, wing bud and flank mesenchyme were cut as shown in diagrams and assayed for polarizing activity by grafting to the anterior margin of a host wing bud as in Fig. 1a. (b) Digit patterns and percentage respecification value resulting from stage 20 chick neck, wing bud and flank tissue transplants. *n*, number of cases.

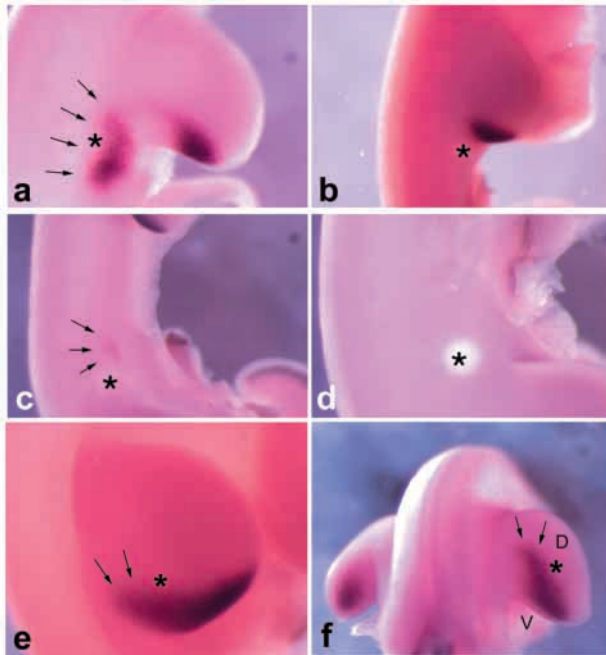


Fig. 5. *Shh* expression in flank region (a-d) and wing buds (e,f) 16 hours after insertion of an FGF4- (a,c,e,f) or PBS- (b,d) soaked heparin bead indicated by an asterisk. (a,b) Bead inserted in anterior flank ('U' region). (c,d) Bead inserted in posterior flank ('Z' region). (e,f) Bead inserted in proximal posterior region ('S' region). Arrows show ectopic *Shh* expression. D, dorsal; V, ventral.

equivalent stage has very high activity with the capacity to induce digit 4 (Fig. 4b), as previously described (Yonei et al., 1995).

Relationship between polarizing activity and expression of *Shh*

Without exception all mouse and chick tissues from regions where *Shh* is expressed have polarizing activity. However, the converse is not true. *Shh* is not expressed in flank, nor in proximal posterior and central regions of the limb, all of which have polarizing activity. It is already known that proximal posterior wing (Yang and Niswander, 1995) and pre-limb-bud-stage flank (Cohn et al., 1995) of chick embryos can express *Shh* when FGF4 is applied. Therefore, we tested whether application of FGF4 to the additional regions that we found to have polarizing activity resulted in induction of *Shh* (Fig. 5). We found that strong expression of *Shh* could be induced in the anterior flank ('U' region; Fig. 5a) while weaker expression was induced in posterior flank (Fig. 5c). Control beads did not result in ectopic expression of *Shh* in either zone (Fig. 5b,d). We also showed that FGF4 beads in proximal-posterior mesenchyme ('S' region) led to induction of ectopic *Shh* expression dorsally but not ventrally (Fig. 5e,f; see also Yang and Niswander, 1995). In contrast, FGF4 beads grafted into proximal-central mesoderm ('O' region) did not induce *Shh*, although we had found that these regions have strong polarizing activity.

In order to determine whether the polarizing activity of proximal central limb-bud tissue is via its capacity to induce *Shh* expression in host limb buds, we labelled this tissue with

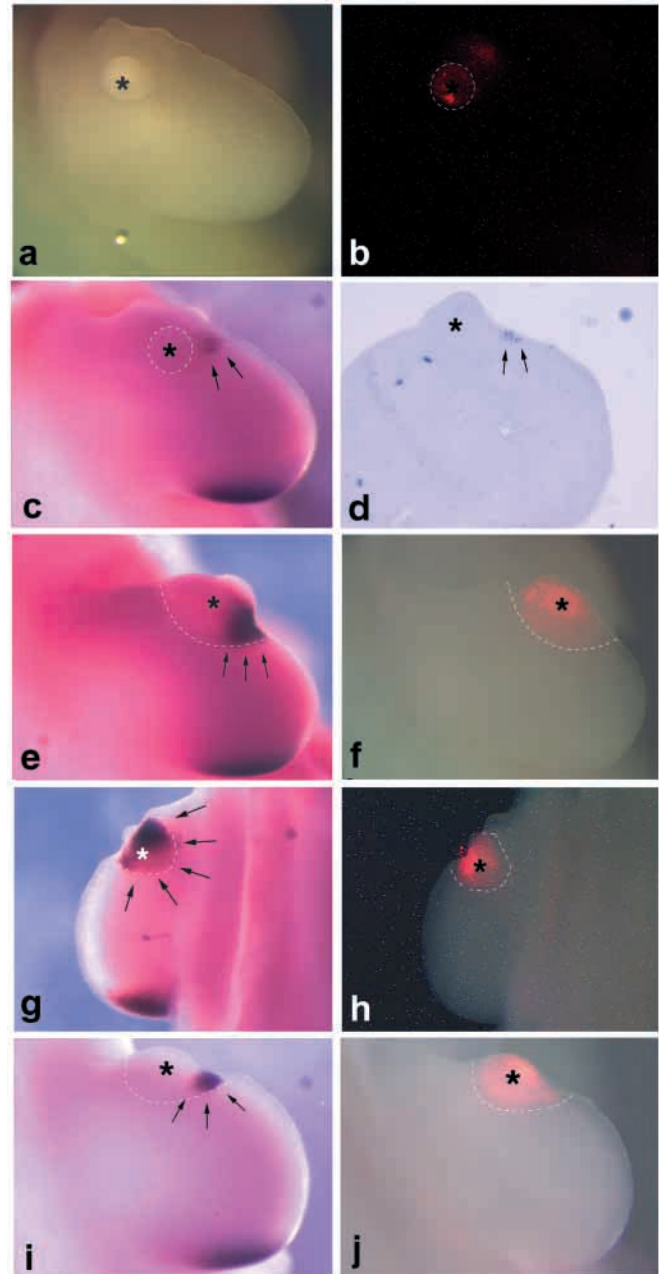


Fig. 6. Wing buds following grafting of a DiI labeled tissue (asterisk) as shown in Fig. 1a. (a-c) Dorsal view of wing bud following grafting of 'O' region of chick wing bud. (a) Bright field; (b) fluorescence confirming the position of the DiI-labelled graft. *Shh* expression (c) in same wing bud; (d) in section. (e,f) Dorsal view of wing buds following grafting of 'H' region of chick wing bud. (g,h) Ventral view of wing buds following grafting of 'S' region of the chick wing bud. (i,j) Dorsal view of wing buds following grafting of 'U' region of the chick flank. *Shh* expression (e,g,i) bright field; (f,h,j) fluorescence. Arrows show ectopic *Shh* expression. Dotted lines show boundaries between grafted mesoderm and host.

DiI prior to grafting it to the anterior margin of stage 20 host wing bud. 24 hours after grafting, we observed ectopic *Shh* expressed in the host wing bud adjacent to the grafted tissue, but not in the grafted tissue itself (Fig. 6a-d). This contrasts with grafts taken from the posterior margin of the wing bud

('H' region), the proximal posterior region of the wing ('S' region) and the anterior flank region ('U' region), in which it is clear, from comparing the locations of DiI-labelled tissue with the *Shh* expression patterns, that *Shh* is expressed in the graft itself (compare Fig. 6e and f; g and h; i and j, respectively).

Tail cells can give rise to toes

Our previous studies have shown that flank tissues between the normal limb-forming regions have the potential to form limb structures. We were therefore interested to find out whether other tissues adjacent to the limbs might also have this capacity. We tested whether tail tissue has the ability to respond to limb signals by implanting chick tail tissue under the apical ridge at the posterior edge of a stage 20 chick wing bud. In several of the wings that developed, we could see digits with extra phalanges that resembled toes (3/10; Fig. 7d). Similar toe-like digits are routinely obtained when tissue from the tip of a leg bud is implanted into wing (4/4; Fig. 7c) as already shown by Saunders et al. (1959).

To determine whether the tail tissue had indeed formed the toe-like digit(s) in this experiment, we implanted quail tail tissue under the apical ridge in the posterior edge of stage 20 chick wing bud. 6/10 of the chick wings with quail grafts had an elongated digit 4 with additional phalanges. When these wings were sectioned and stained with the quail-specific monoclonal antibody (QCPN) to distinguish between host (chick) cells and graft (quail) cells, we found that the elongated digit 4 was composed of quail cells (arrows; Fig. 7e). We also observed the expression pattern of *Tbx4* in tail tissue grafts, since this gene is a marker of hindlimbs and not wing. Tail tissue does not express *Tbx4* at the time of grafting but 24 hours after implantation into the wing bud, however, we now see strong expression of *Tbx4* in the graft (arrows; Fig. 7f). We also monitored *Hoxc9* expression in tail grafts. *Hoxc9* is expressed in flank, proximal anterior part of leg bud and tail but not in posterior leg bud or in wing bud at stage 20. 2 hours after implantation of tail tissue into wing bud, *Hoxc9* expression persisted but had disappeared in the grafted tail tissue by 24h (5/5 cases). These patterns of gene expression in the tail graft are the same as those normally found in the posterior part of the leg.

In order to test whether activation of *Tbx4* expression in the tail tissue grafted into the wing bud is a consequence of exposure to FGFs, we placed FGF4-

soaked beads in the tail bud. When the FGF4 bead was placed in the tail just posterior to the leg bud, *Tbx4* expression expanded from the leg bud into the anterior part of the tail. However, when the FGF4 bead was placed mid-way down the tail, no ectopic *Tbx4* expression was induced.

Cells from neck, flank and tail can migrate into the limb bud in response to FGF4

Since tissues surrounding the limb buds have the potential to participate in limb development, this raises the possibility that preaxial and postaxial polydactyly in limbs could arise due to incorporation of cells from neck, flank and tail in response to an extended apical ridge. To test this possibility, we labelled neck, flank and tail cells of stage 20 chick embryos with DiI and simultaneously implanted an FGF4-soaked bead at the end of apical ridge of the nearby limb bud. As controls, PBS beads were implanted. We then examined the embryos 24 hours later.

This series of experiments showed that cells both anterior and posterior to limb buds can migrate into the adjacent bud when an FGF4 bead is implanted. Thus, DiI-labelled neck cells adjacent to somite 14 (Fig. 8a) migrated into the wing bud towards an FGF4-soaked bead implanted at the anterior edge of the apical ridge (Fig. 8b; 3/3 cases), but were not attracted towards a control PBS-soaked bead (Fig. 8c; 1 case). Similarly, DiI-labelled anterior flank cells and posterior flank cells migrated into nearby limb buds towards an FGF4-soaked bead placed at the edge of the apical ridge (Fig. 8e and h respectively; 4/4 cases). Finally, when tail tissue opposite somite 32 was labelled with DiI, many dispersed labelled cells were found in the posterior part of the leg bud (Fig. 8j; 1 case).

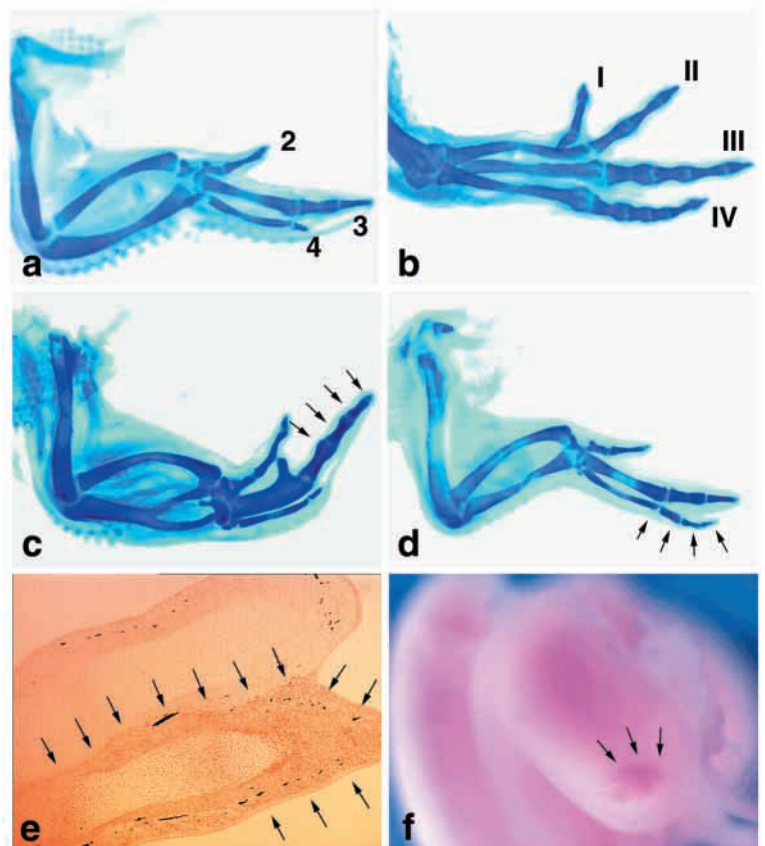


Fig. 7. Dorsal views of whole mounts of chick wings and leg. (a) Normal wing; digit pattern 234. (b) Normal leg; digit pattern I III III IV. (c) Wing resulting after grafting tissue from the central distal region of stage 20 chick leg bud under the posterior apical ectodermal ridge of a chick wing bud. Arrows indicate toe. (d) Wing resulting after grafting tail tissue (opposite somite 32-35 level) under the posterior part of the apical ectodermal ridge of chick wing bud. Arrows indicate a digit that has more phalanges than a normal wing digit and thus distally resembles a toe. (e) Immunohistochemistry using quail-specific antibody QCPN to show position of grafted quail tail tissue. Quail cells were observed in the posterior digit (arrows). (f) Expression of *Tbx4* (arrows) in a graft of tail tissue, 24 hours after the implantation of the graft.

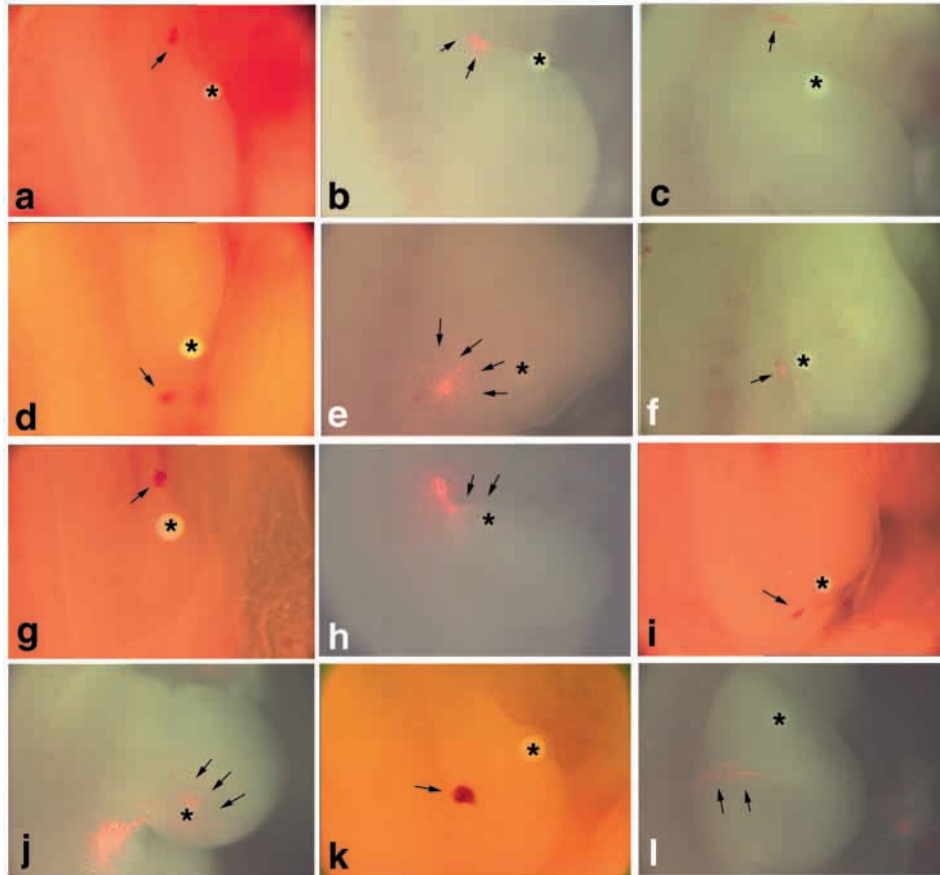


Fig. 8. Cells from neck, flank and tail migrate into limb bud in response to FGF4. (a) Neck cells opposite somite 14 were labelled with DiI and FGF4-soaked bead implanted at anterior end of wing apical ridge at time zero. (b) 24 hours later. (c) Control in which neck cells were labelled with DiI and a PBS-soaked bead was implanted at anterior end of wing apical ridge. (d) Anterior flank cells opposite somite 22 were labelled with DiI and an FGF4-soaked bead was implanted at the posterior end of wing apical ridge. (e) 24 hours later. (f) Control in which anterior flank cells were labelled with DiI and a PBS-soaked bead was implanted at posterior end of wing apical ridge. (g) Posterior flank cells opposite somite 26 were labelled with DiI and an FGF4-soaked bead was implanted at the anterior end of leg apical ridge. (h) 24 hours later. (i) Tail cells opposite somite 32 were labelled with DiI and FGF4-soaked bead was implanted at posterior end of leg apical ridge. (j) 24 hours later. (k) Proximal central wing bud cells were labelled with DiI and FGF4-soaked bead was implanted at the anterior end of wing apical ridge. (l) 24 hours later. Implanted beads indicated by asterisks. DiI-labelled cells were indicated by arrows. (a,d,g,i,k) Limb buds of stage 20 chick embryos bright field; (b,c,e,f,h,j,l) Composite picture with fluorescence picture using FITC/rhodamine filter of limb buds.

In contrast, DiI-labelled proximal central limb cells showed no tendency to migrate towards an anteriorly grafted FGF bead (Fig. 8l; 0/7 cases).

Extra digits versus extra limbs

Our experiments in mouse and chick embryos show that tissues in the flank can form new limb signalling regions. Moreover, in both chick and mouse embryos extra limb buds can be induced from the flank when FGF beads are added at early stages. In chick embryos, these FGF-induced buds develop into limbs with reversed anteroposterior polarity (Cohn et al., 1995). We therefore tested whether grafting of an Shh-soaked bead lead into the flank alongside the limb-inducing FGF bead might alter the anteroposterior pattern of the extra limb. These experiments revealed that Shh appears to restrict apical ridge formation in the flank and trigger polydactyly rather than formation of extra limbs.

As previously reported, when an FGF bead was implanted opposite somite level 23-25 in the posterior flank of stage 14-

17 chick embryos, an ectopic apical ridge developed and extra limbs were produced from the flank anterior to the bead (Cohn et al., 1997; Fig. 9a). In contrast, when an Shh bead was implanted opposite somite level 23-25, no additional limbs were produced (0/11 cases) and very occasionally legs with extra digits (2/11 cases; Fig. 9a). Unexpectedly, when an Shh bead was implanted just anterior to an FGF bead in posterior flank, then, in most embryos (4/5 cases), we now observed legs with extra digits resembling polydactyly (Fig. 9a,b).

DISCUSSION

Widespread potential for limb formation

We have shown, in both mouse and chick embryos, that flank ectoderm can form an apical ridge. In addition, both mouse and chick flank mesoderm has substantial polarizing activity, while neck has weak activity. Cells from the tail of chick embryos can form toes when transplanted to the wing bud. Thus the potential

a

Effects of SHH and FGF2 beads applied to posterior flank mesoderm of stage 14-17 chick embryos

Protein	Bead position (somite)	Additional limb	Duplicated leg
SHH	23-25	0/11	2/11
FGF2	23-25	36/48	0/48
FGF2 + SHH	FGF 24-25 + SHH 23-24	1/5	4/5

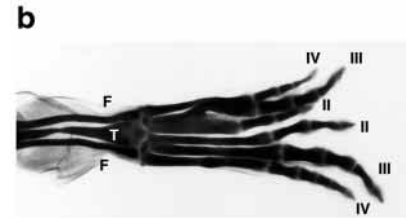


Fig. 9. (a) Effects of SHH and FGF2 beads applied to flank of stage 14-17 chick embryos. (b) Simultaneous application of an FGF bead opposite somite 25 and a SHH bead opposite somite 24 at stage 15 resulted in leg with extra digits (*IV III II II III IV*). T, tibia; F, fibula.

of cells to form limb structures and limb-signalling regions is widespread and not confined just to the limb-forming regions.

The existence of such widespread limb potential may be a legacy from an ancestral vertebrate. It could be related to the way in which mesodermal patterns of expression of *Hox* and *Tbx* genes, which are thought to encode positional information about limb position and identity, are established along the head-to-tail axis of the embryo. For example, *Tbx4* is expressed throughout the posterior end of the early embryo and later becomes restricted to the leg bud. We have shown that, when cells from the anterior part of the tail are transplanted to a limb bud, they can re-express *Tbx4* and form toes. *Hoxc9* is expressed in tail bud and anterior leg bud but not in wing bud. We have shown that, when anterior tail cells are transplanted to a wing bud, *Hoxc9* expression in the graft disappears. This loss of *Hoxc9* expression suggests that the grafted tail cells have lost tail identity. We have found that FGF-4 beads can induce *Tbx4* expression in anterior tail bud and therefore it seems likely that the changes in gene expression seen in the grafted tail tissue are due, at least in part, to FGF signals in the wing bud.

Our data show that polarizing potential throughout the flank is not confined to the polarizing regions of the limb buds. Polarizing potential based on the ability to express *Shh* extends into the flank. The ability of tissues to express *Shh* in forelimb bud and flank has been linked to *Hoxb8* expression in lateral plate mesoderm (Charite et al., 1994; Stratford et al., 1997) and thus to anteroposterior patterning of the main axis of the body. Polarizing activity can also be detected in proximal forelimb bud, which appears to be unable to express *Shh*. In this case, polarizing activity is correlated with the ability of this tissue to induce *Shh* expression in anterior mesenchyme. Induction of *Shh* could be via the production of retinoic acid since retinoids and transcripts of a retinoic-acid responsive gene *Strat6* have been detected in this area of mouse limb buds (Ang et al., 1996; Rossant et al., 1991; Bouillet et al., 1995, 1997; Chazaud et al., 1996).

Finally, we have shown that the entire flank ectoderm, in addition to the ectoderm in the limb regions, in mouse and chick embryos has the potential to form an apical ridge (see also the work of Yonei-Tamura et al., 1999 in chick embryos). The region of the body where an apical ectodermal ridge can form appears to correspond precisely to the region where EN is expressed in the ventral ectoderm (Davis et al., 1991). The

basis of this regional expression of EN in the ectoderm is unknown but is probably controlled by the underlying mesenchyme.

Models for polydactyly

We have shown that non-limb cells adjacent to normal limb-forming regions have the potential both to form signalling

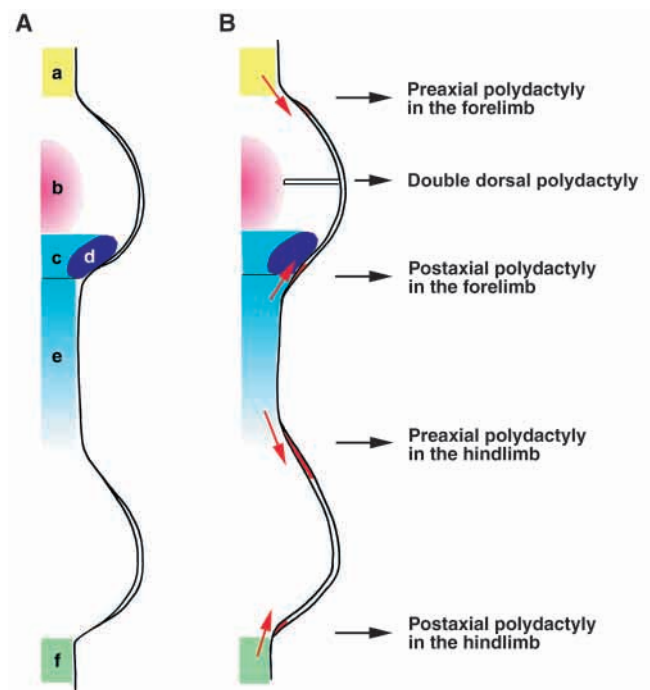


Fig. 10. Models for the polydactyly. (A) Characteristics of different regions of normal mouse and chick embryos with respect to distribution of polarizing activity and potential for limb formation. (a) Posterior neck with weak polarizing activity. (b) Proximal central region of the limb bud with polarizing activity and ability to induce *Shh*. (c) Proximal posterior of the limb bud with polarizing activity. (d) Polarizing region. (e) Flank with ability to form apical ridge and polarizing activity. (f) Anterior part of tail with potential to form digits. (B) Types of polydactyly that could arise due to distribution of polarizing activity and potential for limb formation indicated in A. Cells could be recruited into limb buds as indicated by red arrows due to extension of the apical ridge of nearby limb bud indicated in red.

regions and extra limb structures. Furthermore we have also shown that cells from surrounding regions, neck, flank and tail, can migrate into limb buds toward FGF4 bead mimicking extension of the apical ridge. These two findings taken together suggest that tissue adjacent to the limbs could contribute to both preaxial polydactyly, where extra digits arise at the anterior margin and postaxial polydactyly, where these arise posteriorly (Fig. 10).

In most of the mouse mutants with preaxial polydactyly, (*Extra toes (Xt)*, *Recombination induced mutant4 (Rim4)*, *Strong's luxoid (Ist)*, *luxate (lx)*, *X-linked polydactyly (Xpl)*, *Sasquatch (Ssq)*), *Shh* is expressed ectopically at the anterior of the developing limb bud (Masuya et al., 1995, 1997; Sharpe et al., 1999). Since we have shown that posterior neck and posterior flank tissue have polarizing activity in both chick and mouse, one possibility is that these non-limb-bud cells are recruited into the limb bud and make an anterior polarizing region. Neck cells exhibit weaker polarizing activity than flank in both mouse and chick and this could explain the lower frequency of preaxial polydactyly in forelimbs as compared with hindlimbs in these mouse mutants. The fact that we observe polarizing activity from posterior neck and flank tissue only after E10.5 in mouse also fits with this explanation since, in mouse preaxial polydactyly mutants, expression of ectopic anterior *Shh* lags behind the normal *Shh* expression at the posterior margin (Masuya et al., 1995, 1997).

We have also shown that, when *Shh* beads are applied to the posterior flank together with FGF beads, this leads to polydactyly in the leg and not ectopic limb formation. We interpret this as being due to *Shh* acting to restrict apical ridge formation to the region of flank between the beads and the nearby leg bud. This locally induced apical ridge in the flank then fuses with the ridge of the nearby leg bud.

Our experiments suggest that extra digits in postaxial polydactyly could be derived from anterior flank in upper limbs and anterior tail tissue in lower limbs since both flank and tail cells can form digits. Flank ectoderm has the ability to form apical ridge and therefore elongation of ridge into the anterior flank ectoderm could attract anterior flank tissue into the posterior part of the forelimb. The higher frequency of postaxial polydactyly in forelimbs as compared with hindlimbs could be related to the special ability of the flank to form an apical ridge.

This demonstration that limb-forming potential is widespread throughout the trunk of vertebrate embryos suggests some new ideas about how polydactyly could arise. Our data on limb-forming potential in normal mouse embryos provide a foundation for future work, in which limb-forming potential could be assayed directly in mutant and knock-out mice.

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