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Fibroblast Growth Factors Induce Additional Limb Development from the Flank of Chick Embryos

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Summary

Fibroblast growth factors (FGFs) act as signals in the developing limb and can maintain proliferation of limb bud mesenchyme cells. Remarkably, beads soaked in FGF-1, FGF-2, or FGF-4 and placed in the presumptive flank of chick embryos induce formation of ectopic limb buds, which can develop into complete limbs. The entire flank can produce additional limbs, but generally wings are formed anteriorly and legs posteriorly. FGF application activates *Sonic hedgehog* in cells with polarizing potential to make a discrete polarizing region. *Hoxd-13* is also expressed in the ectopic bud, and an apical ectodermal ridge forms. A limb bud is thus established that can generate the appropriate signals to develop into a complete limb. The additional limbs have reversed polarity. This can be explained by the distribution of cells in the flank with potential polarizing activity. The results suggest that local production of an FGF may initiate limb development.

Introduction

Initiation and control of limb development is a fundamental issue in vertebrate development and evolution. In virtually all vertebrates, two pairs of limb buds form from lateral plate mesoderm at particular axial levels and grow out to form the limbs.

Cell interactions within the developing limb bud lead to morphogenesis and patterning, and considerable progress has been made in understanding their molecular basis. The apical ectodermal ridge (AER) rims the developing limb bud and maintains proximodistal outgrowth by keeping cells at the distal tip of the bud in a continuously proliferating, undifferentiated state (Summerbell et al., 1973). The AER signal can be substituted by application of fibroblast growth factor 4 (FGF-4) (Niswander et al., 1993) or FGF-2 (Fallon et al., 1994). The AER contains transcripts of FGF-4 posteriorly (Suzuki et al., 1992; Niswander and Martin, 1992) and FGF-8 throughout (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995). The posterior ridge, or FGF-4, also maintains another cell population at the posterior margin of the limb bud, the polarizing region (Vogel and Tickle, 1993). The polarizing region signal, in conjunction with FGF-4, establishes a progress zone at the tip of the bud and allows continued patterning of limb structures (Niswander et al., 1993). In addition, the polarizing region specifies pattern along the anteroposterior (AP) axis of the developing limb, and transplantation of the polarizing region to the anterior margin of a host wing bud leads to mirror-image duplication of digits (Saunders and Gasseling, 1968; Tickle et al., 1975).

Recently, transcripts of a vertebrate homolog of the *hedgehog* gene, *Sonic hedgehog* (*Shh*), have been found to map to the polarizing region, and grafts of *Shh*-expressing cells to the anterior limb bud induce digit duplications (Riddle et al., 1993). The ability of cells to induce digit duplications along the AP axis of the limb is known as polarizing activity. Polarizing activity is found in other regions of the embryo, such as Hensen's node (Hornbruch and Wolpert, 1986) and the floor plate of the neural tube (Wagner et al., 1990), and these tissues also express *Shh* (Riddle et al., 1993). Mesodermal cells in the flank posterior to the wing territory have polarizing potential, as they normally lie dormant and do not express *Shh*, but can induce digit duplications when grafted to the anterior margin of a host wing bud (Hornbruch and Wolpert, 1991).

Polarizing signals can regulate the expression pattern of genes of the *HOXD* complex (Izpisúa-Belmonte et al., 1991, 1992). For example, when retinoic acid, polarizing region cells, or fibroblasts expressing *Shh* are grafted anteriorly, posterior *Hoxd* genes are activated in anterior mesenchyme to give a mirror-image pattern that precedes the mirror-image duplication of the digits (Izpisúa-Belmonte et al., 1991; Nohno et al., 1991; Riddle et al., 1993).

Although the molecular networks that operate in the limb bud have received much attention, very little is known about how a limb bud is initiated. A recent clue has emerged from work on chimeric mice combining wild-type embryos and pluripotent embryonic stem (ES) cells that constitutively express FGF-4. A dramatic consequence of overexpression of FGF-4 is the development of multiple small limb bud structures from the flank of these embryos (H. A. et al., unpublished data). Based on these observations, we investigated whether FGFs could stimulate outgrowth of lateral plate mesoderm in the flank of chick embryos. The flank is the region between the two buds, and cell proliferation normally decreases here (Searls and Janners, 1971). We show that FGFs can induce additional limb buds in this region of the embryo. The established buds then independently develop into complete additional limbs, with a polarity determined by cells with potential polarizing activity that are recruited from the flank.

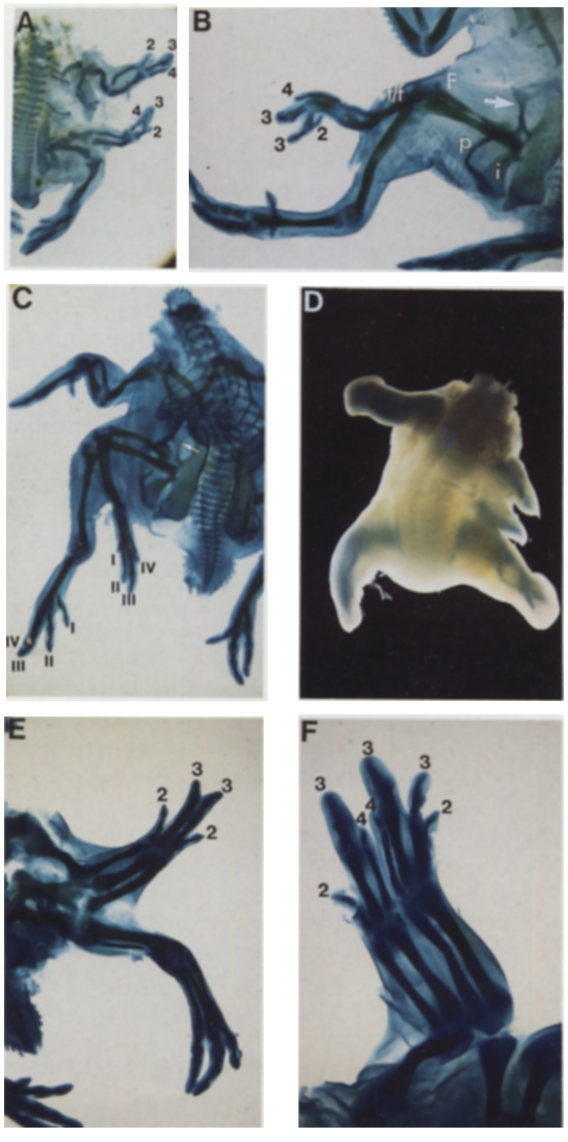

Figure 1. Additional Limbs Produced by Application of FGF-2 Beads to the Flank, as Seen in Whole-Mount Chick Embryos Stained with Alcian Green to Reveal Skeletal Patterns

(B), (C), (E), and (F) are at 10 days of development. (A) is at 9 days, and (D) is at 6 days. Anterior is at top of page, except in (F).

(A) Additional wing that developed between normal wing and leg after implantation of a bead opposite somite 24 at stage 17. Note the reversed polarity of the digits, with a pattern of 4-3-2 compared with the normal wing pattern of 2-3-4. Dorsal view.

(B) Additional limb that developed after implantation of an FGF-2 bead opposite somite 20, at stage 14. The limb consists of proximal leg structures (femur [F], tibia and fibula [t/f]) with wing digits. Note reversed digit pattern, 4-3-3-2, with duplicated digit 3. Additional femur is anterior and articulates between the pubis (p) and an additional ischium (arrow). Normal ischium (i). Ventral view.

(C) Additional leg that developed following bead implantation to the midflank, opposite somite 23 at stage 15, with digit pattern in reversed polarity (IV-III-II-I). Digit IV is not complete. Note additional ischium (arrow), as in (B). Ventral view.

(D) Embryo stained but not cleared 4 days after bead implantation opposite somite 21. The wing on the treated (right) side is truncated and shifted posteriorly along the body axis. The additional limb is also truncated. Dorsal view.

(E) Fusion of additional limb and normal wing following bead implantation opposite somite 26 at stage 13. The forearm of the fused limb consists of radius, ulna, and radius, and the digit pattern is 2-3-3-2. Note absence of digit 4 and single ulna. Dorsal view.

(F) Fusion of additional wing and normal wing following bead implantation opposite somite 23 at stage 14. The normal wing, with digit pattern of 2-3-4, is connected by soft tissue to the additional wing, which has a reversed sequence of digits in a pattern of 4-3-3-2. Proximally, there is a double set of skeletal elements. Anterior is to the left; dorsal view.

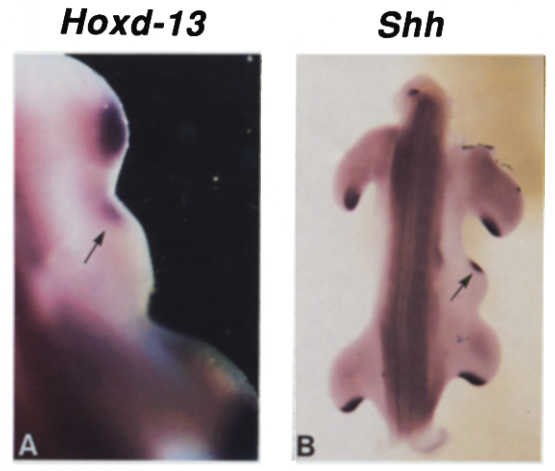

Figure 2. Distribution of *Hoxd-13* and *Shh* Transcripts in Whole-Mount Preparations of Embryos with Additional Limb Buds 48 hr after FGF-2 Bead Implantation

Regions to which the probes have hybridized are stained purple. Expression of *Hoxd-13* (A) and *Shh* (B) is restricted to the posterior margin of the normal wing and leg buds, but is found anteriorly (arrows) in the additional limb buds.

Results

FGF Beads Induce Additional Limbs

Beads soaked in FGF-1, FGF-2, or FGF-4 and implanted in presumptive flank lead to the development of additional limbs (Figures 1A–1F). A major set of experiments was carried out with FGF-2, in which FGF-2 beads were placed at different levels along the primary body axis in the lateral plate mesoderm of chick embryos between stages 13 and 17. Stage 13 occurs well before there is any sign of limb development, and at stage 17, a slight thickening in the lateral plate mesoderm marks the place where buds will form. When beads are placed in lateral plate mesoderm opposite somites 20–26 (the presumptive flank lies between somites 21 and 25), additional limbs developed in 25 out of 29 embryos (Table 1). Both complete wings (Figure 1A) and complete legs (Figure 1C) could develop from the flank (summarized in Table 1 and Figure 4). When beads soaked in FGF-2 were placed anterior to somite 15 in the neck region ($n = 4$), or in the tail bud ($n = 3$), no additional limbs were produced.

The potential for additional limb development in the flank appears to be higher from stage 13 onward. Embryos treated from stages 10 to 12 ($n = 6$ cases) did not develop additional limbs, but the wing and leg were sometimes

Table 1. Effects of Beads Soaked in FGF-2 and Implanted in the Lateral Plate Mesenchyme of Chick Embryos between Stages 13 and 17

		Additional Limb Development				Additional Limb Morphology	
Bead Position (Somite level)	Total n	Normal (No Additional Limbs) n	Additional Wing Structures n (n)[a]	Additional Leg Structures n (n)[a]	Additional Limb Structures n (n)[a]	Reversed Digit Pattern n	Duplicated Digit Pattern n
20	3	0	1 (0)	1 (1)	1 (1)	1[b]	1
21	4	0[c]	3 (2)	0	0	2	1
22	4	0	4 (4)	0	0	4	3
23	6	0	3 (2)	2 (2)	1 (0)	3	1
24	6	1	3 (1)	2 (2)	0	3	0
25	4	1	0	3 (2)	0	2	0
26	2	1	0	0	1 (1)	1	0
Totals	29	3	14 (9)	8 (7)	3 (2)	16	6

[a] (n) = number of additional limbs with digits.
[b] Polarity could not be determined in one specimen.
[c] One specimen developed an extra coracoid with no additional limb structures.

shifted along the body axis and appeared to be drawn together. Malformations in limbs, vertebrae, and ribs were also observed in these embryos. Ability of the flank to produce complete additional limbs lasts at least through stage 17, when wing and leg buds are present.

FGF-1 and FGF-4 beads also induced additional limbs, but the limbs were less complete than those induced by FGF-2, in that they rarely developed digits. Beads soaked in FGF-7 (also known as keratinocyte growth factor or KGF) did not lead to the development of additional limbs (n = 10). Nine of ten embryos receiving FGF-7 beads developed normally; however, a single embryo developed with a duplicated pattern in the leg (duplicated fibula and digit pattern x^a-II-I-I-II-III-IV; x^a = unidentifiable digit). Beads soaked in phosphate-buffered saline (PBS) and implanted at flank levels had no detectable effects on embryo development (n = 4).

Pattern of the Additional Limbs

The additional limbs were often remarkably complete. A full range of skeletal elements from girdles to digits was produced in 18 of 25 cases receiving FGF-2 beads (Table 1; Figures 1A–1F). Additional pelvic or shoulder girdle structures were almost always present. One embryo developed an isolated additional coracoid but failed to form any other limb structures. Of the 22 clearly identifiable wings and legs, the additional legs were almost always complete, but slightly retarded in development (7 of 8 cases). Additional wings could lack distal structures (5 of 14 cases), and this was sometimes accompanied by truncation of the original wing.

The nature of the limb that developed was related to the position at which the bead was placed along the body axis (see Figure 4). When beads were placed in the anterior part of the flank (opposite somites 21 and 22), all of the limbs that developed were additional wings (seven cases). Beads implanted to the midflank (opposite somite 23; n = 6) resulted in the development of either wings (three cases) or legs (two cases; the remaining limb was very rudimentary). Beads placed slightly more posteriorly (opposite somite 24) also resulted in the formation of both wings (three cases) and legs (five cases). However, beads placed still more posteriorly in the flank (opposite somite 25) induced only legs (three cases).

A few implants were made at levels where the wing and leg would normally form. Two of these resulted in chimeric limbs that articulated near the normal leg and consisted of proximal leg elements and wing digits (Figures 1B and 1E). Unexpectedly, one of the beads placed at the wing level induced an extra leg.

Only one of the additional limbs with digits had a normal AP polarity, and one limb developed two unidentifiable digits. In all other cases, the AP axis was clearly reversed (16 of 18 cases; Figures 1A–1C, 1E, and 1F). Figure 1A shows a good example; the additional wing has a sequence of digits 4-3-2, reading from anterior to posterior, which is reversed compared with the sequence of normal wing digits 2-3-4. In Figure 1C, the additional leg has a reversed sequence of toes IV-III-II-I, compared with the normal leg pattern I-II-III-IV.

Most of the limbs that developed wing digits following implantation of FGF-2 beads in anterior or midflank (somites 20–23) also had an extra digit 3 inserted into the pattern (6 of 9 cases). This gave, for example, patterns such as 4-3-3-2 reading anteriorly to posteriorly (Figures 1B and 1F). Limbs with reversed polarity that resulted from beads in the posterior flank never had extra digits (Figures 1A and 1C). Additional limbs could be fused with the normal wing. Figure 1F shows an example of such a fusion that produced a mirror-image digit pattern of 2-3-4-4-3-3-2, with posterior digits in the middle. In some cases, forearm bones at the interface of the two limbs were fused into one wide element and posterior digits did not form (Figure 1E).

Histological analysis reveals the presence of muscles, tendons, and nerves in additional limbs, and the tendon pattern in feet of such limbs indicates that the dorsoventral axis is not reversed (M. C. and D. D'Souza, unpublished data).

In addition to causing ectopic limbs to develop, application of FGFs could cause the wing to shift posteriorly along the body axis (Figure 1D). Occasionally, wing and leg appeared to be drawn together, even when additional limbs did not form, and this could be accompanied by fusion of

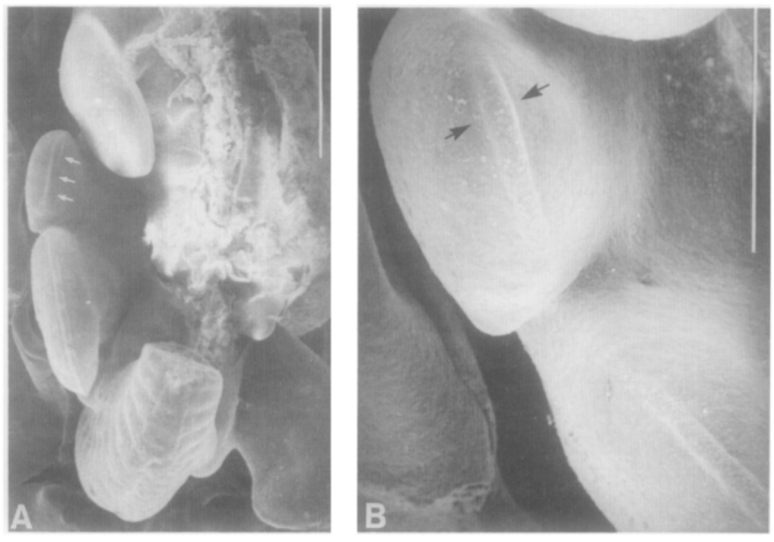

Figure 3. Scanning Electron Micrograph of Embryo with Ectopic Limb Bud 48 hr after Bead Implantation (Ventral View)

(A) Low power view showing position of ectopic bud between wing bud and leg bud. Thickened apical ridge on ectopic bud marked by arrows. Note tail posteriorly. Scale bar, 1 mm.

(B) High power view of ectopic bud (upper bud) showing AER (arrows). Compare with ridge of leg bud at bottom right. Scale bar, 500 μm.

pelvic and shoulder girdles. FGF beads could also cause a single lateral fin-like outgrowth to develop along the trunk, rather than discrete limb buds. This led to development of a single outgrowth containing leg and wing skeletal elements. The skeletal pattern of this specimen was unlike that seen in bud fusions, in that proximal elements began as a single large bone that branched to give a humerus and a very wide femur. Similarly, the ulna and tibia began as one large bone that bifurcated at midshaft, but the radius and fibula appeared normal. There were no wing digits, but the foot was complete.

Early Development of the Additional Limbs

By 24 hr after insertion of an FGF-2 bead in the flank, a small swelling was seen adjacent to the bead. By 48 hr, an additional limb bud was present between wing and leg buds (Figure 3A). Initial formation of additional limb buds was slightly delayed in comparison to normal limb buds, and this is why additional buds can appear smaller than the normal buds (Figure 3A). The bud was capped with a well-developed AER (Figures 3A and 3B). At this time, the bead could be found proximal to the bud. The shape of additional buds was variable. Normal-shaped buds developed into complete limbs, while very narrow buds gave rise to truncated limbs. In some embryos, as the extra bud continued to grow, the wing bud remained small and often took on an abnormal, pointed shape. These buds lagged behind the extra bud and the leg bud and resulted in truncated wings often consisting of only a humerus or a humerus and radius. Occasionally, the additional limb bud was fused with the wing bud, and the types of patterns seen in Figures 1E and 1F were obtained.

A series of experiments was performed in which beads were implanted and later removed at several timepoints (Table 2). We found that removal of the the FGF-2 bead as early as 2 hr after implantation, at stage 14/15, did not interfere with bud outgrowth, and the bud went on to develop into an additional limb.

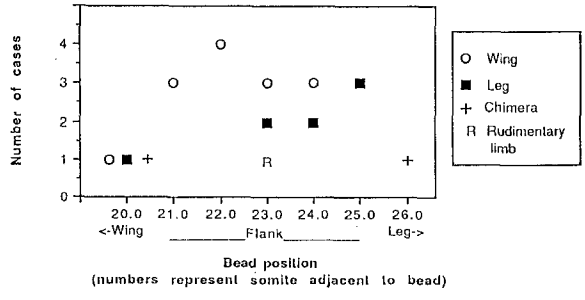

Figure 4. Type of Additional Limb at Each Somite Level

FGF-2 beads were placed in the lateral plate mesoderm opposite a particular somite along the body axis. Distribution of additional limbs is shown according to limb type. This figure was prepared from the data in Table 1. Note the flank extends from somite 21 through 25.

The Additional Limb Buds Express *Sonic hedgehog* and *Hoxd-13*

We investigated polarity of early limb buds by looking at expression of two genes, *Shh* and *Hoxd-13*, which are normally expressed posteriorly (Figure 2). Whole-mount in situ hybridization was carried out between 4 and 48 hr after FGF-2 bead implantation (Table 2). The earliest point at which *Shh* transcripts could be detected near the FGF bead is 24 hr after bead implantation (1 of 2 cases). All embryos examined from 25.5–48 hr after FGF bead implantation were positive for *Shh* in the additional bud (n = 7; Figure 2). *Shh* transcripts could not be detected in the flank of any of the embryos examined between 4 and 23.5 hr after bead implantation (n = 6), nor in the second embryo examined at 24 hr. *Shh* expression in the notochord was used as a control, and each specimen was positive for *Shh* in the notochord.

Similarly, low levels of *Hoxd-13* expression could be detected locally near the bead by 24 hr after application of FGF. At 48 hr after FGF-2 application, additional limb buds had been established, and *Shh* and *Hoxd-13* expression

Table 2. Effects of Removing FGF-2 Beads at Different Timepoints on Limb Induction and Timing of Onset of *Shh* and *Hoxd-13* Expression

Hours after Bead Implanted	2	4	20	20.5	21	21.5	23	23.5	24	25.5	30.5	48
Extra limb induced when bead removed at this timepoint	+ (2)	+ (1) − (1)				*			+			
Shh expressed		− (2)	−	−	−			−	+ (1) − (1)	+	+	+ (5)
Hoxd-13 expressed		−					−		+ (1) − (1)			+ (3)

Plus indicates limb induction; detectable expression.
Minus indicates no limb induction; no detectable expression.
Asterisk indicates wing and leg drawn together; additional pelvic structures.
Numbers in brackets indicate cases when n > 1.

domains were clearly seen (Figure 2). The expression pattern of both genes was restriced to the anterior margin of the additional limb buds, which is reversed compared with that of the normal buds.

Discussion

Local application of FGF protein to the flank of the chick embryo establishes a new axis of proximodistal outgrowth and initiates development of a limb bud. The limb bud acquires a polarizing region and an apical ridge and can then develop independently into a complete limb. These results suggest that local production of an FGF determines limb position along the body axis. The additional limbs have reversed polarity, and the type of limb that forms depends on the position of the FGF bead along the flank.

Initiation of a Limb by Application of FGF

Application of FGF elicits limb formation from the flank, which has a limb-forming potential that is normally not realized. This suggests that normal limb bud formation is initiated by a local source of FGF (Figure 5). Evidence that constitutive FGF-4 expression in chimeric mice stimulates limb bud outgrowth from the flank further supports this view (H. A. et al., unpublished data). We propose that, under the influence of FGF, lateral plate mesoderm cells continue to proliferate and also that FGF leads to local activation of *Shh* in cells with potential polarizing activity, thereby converting them into a polarizing region. A signal from the polarizing region then cooperates with FGF to establish a progress zone, and *Hoxd* genes are activated. Cells of the progress zone produce a signal that induces an AER in the overlying epithelium. These initial effects of FGF establish a limb bud with a polarizing region, a progress zone, and an AER. The newly induced AER then produces FGF-4, which maintains the polarizing region and proliferation of distal mesenchyme cells in the progress zone. Cells of the progress zone then maintain the AER, and outgrowth and patterning of the bud ensues.

FGF could have a primary effect in maintaining cell proliferation in the region in which limb buds will form (see Searls and Janners, 1971). FGFs can promote proliferation of limb mesenchyme cells (Niswander and Martin, 1993; Niswander et al., 1993), and an FGF bead could

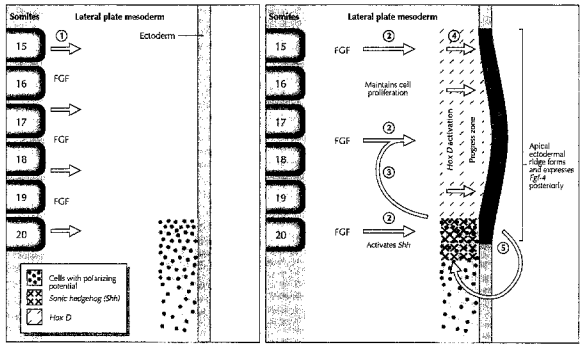

Figure 5. Diagram to Show How a Local Source of FGF Could Initiate Formation of a Limb Bud

Signals 1–5 are proposed to operate sequentially. (1) A signal controls local production of FGF at the appropriate axial level, e.g., for wing, between somites 15 and 20 (can be side stepped in flank by applying an FGF bead). (2) FGF signal maintains cell proliferation and leads to activation of *Shh* in cells with polarizing potential. (3) Polarizing signal acts in conjunction with FGF to establish progress zone and activate *Hoxd* genes. (4) Signal from progress zone acts on epithelium to induce AER. (5) Posterior part of apical ridge produces FGF-4, which acts back on the mesenchyme to maintain cell proliferation and polarizing region. These steps may be direct or indirect, and several other gene products are involved.

maintain cell proliferation in the flank as an early step in producing an ectopic bud.

Another effect of FGF in the formation of both normal and ectopic buds is the activation of *Shh*, which is central to establishing the polarizing region. Additional limbs developed even when the FGF bead was removed as early as 2 hr after bead implantation, and 22–23 hr prior to activation of *Shh*. The late response of *Shh* suggests that intermediate steps are involved and that *Shh* is not directly activated by the FGF bead. However, the possibility that FGF released from the bead could be sequestered in the extracellular matrix (reviewed by Klagsburn and Baird, 1991) and act after the bead has been removed can not be excluded.

It seems likely that only cells with potential polarizing activity can be induced by FGF to express *Shh* and form a polarizing region. Since cells with this potential lie at the posterior edge of the region that normally forms the limb bud, activation of *Shh* in these cells would lead to development of a discrete polarizing region. In later limb buds,

both retinoic acid and FGF are required for ectopic activation of *Shh*. Therefore, retinoids may underlie potential polarizing activity, and FGF allows this potential to be realized.

Potential for polarizing activity also extends to cells in anterior and midflank in chick embryos, but becomes progressively weaker posteriorly (Hornbruch and Wolpert, 1991). Although this activity normally remains dormant, the cells can produce a polarizing region signal when transplanted to a limb bud. *Shh* is not expressed in the flank, but transcripts first appear as the extra limb bud begins to emerge around 24 hr after bead implantation. Ectopic buds may possess a discrete polarizing region because FGF is applied locally. This contrasts with the effects of overexpressing FGF globally, when it is possible to activate *Shh* throughout the flank (H. A. et al., unpublished data).

It has been proposed that a polarizing region signal and an FGF cooperate to establish a progress zone (Niswander et al., 1993). Once a polarizing region has been established in the presumptive limb, mesenchyme cells under the influence of FGF will form a progress zone. Grafts of *Shh*-expressing cells to the normal limb bud induce *Hoxd-13* expression in the progress zone (Riddle et al., 1993). Thus, activation of *Shh* could lead to activation of *Hoxd* genes in the presence of FGF. The progress zone cells also acquire the ability to induce an AER. Although flank mesoderm cannot normally support an AER (Searls and Zwilling, 1964), mesoderm of the presumptive limb region can induce formation of an apical ridge when grafted under flank ectoderm (Saunders and Reuss, 1974). The posterior part of an apical ridge expresses FGF-4 (Niswander and Martin, 1992; Suzuki et al., 1992; Niswander et al., 1994). The induced apical ridge could, therefore, take over control of mesenchyme cell proliferation and also maintain the polarizing region so that, for example, *Shh* and *Hoxd-13* continue to be expressed (Fallon et al., 1994; Niswander et al., 1994).

Reversed Polarity of the Additional Limbs

A striking feature of additional limbs is that they almost always have a reversed AP polarity. In ectopic buds, *Shh* and *Hoxd-13* expression is found at the anterior margin (the reverse of the normal limb), and this correlates with the reversed pattern of digits. Occasionally, the ectopic bud is continuous with the normal wing bud, and these develop into limbs that have a mirror-image pattern of digits, with posterior digits in the middle. This pattern is consistent with signaling from a shared polarizing region.

To explain the reversed polarity, the idea that *Shh* can be activated only in flank cells with potential polarizing activity is important. Because potential polarizing activity is higher in anterior flank, it is these anterior flank cells that are readily converted into polarizing cells that express *Shh*. Cells from more posterior positions in the flank that are incorporated into the ectopic bud are exposed to the signal from the *Shh*-expressing cells. The pattern of digits that develops is orientated with respect to the polarizing region and, thus, is reversed. Even in buds that develop entirely from anterior flank, only a discrete region expressing *Shh* is induced; however, the limbs often have additional digit 3's. The gradient of potential polarizing activity is shallow in anterior flank (Hornbruch and Wolpert, 1991); therefore, cells next to *Shh*-expressing cells could have weak polarizing activity that specifies the additional digit(s).

Which FGF Member Initiates Limb Development in the Normal Embryo?

We have shown that three members of the FGF family can induce limb development from the flank. Although FGF-1, FGF-2, and FGF-4 are able to elicit bud formation, none of these are likely to be the signal that normally initiates limb development. *Fgf-4* transcripts can only be detected once a bud is formed (Niswander and Martin, 1992). FGF-2 lacks a recognizable secretory signal sequence (Abraham et al., 1986), and several systems have shown that it is not released from the cell unless the cell death or damage occurs (reviewed by Thomas, 1993). FGF-1 also lacks a known signal sequence; however, cells can be experimentally induced to release the protein in response to heat shock (Jackson et al., 1992). Unless there are special mechanisms that locally release FGF-1 or FGF-2 in the appropriate places, it is unlikely that either is the endogenous limb initiation factor. FGF-2 protein has been detected in flank cells (Savage et al., 1993) yet no additional limbs develop, further suggesting that endogenous FGF-2 is not a probable candidate. FGF-8 has recently been shown to be expressed in the limb ectoderm as buds begin to emerge in the mouse, suggesting a possible role in very early stages of limb budding (Ohuchi et al., 1994; Crossley and Martin, 1995).

Several FGF receptors (FGFRs) are expressed in the emerging limb buds. FGFR-1 is expressed in mesenchymal cells, and FGFR-2 is expressed in the overlying ectoderm, including the AER (Orr-Urtreger et al., 1991; Peters et al., 1992). Both receptors can bind FGF-1, FGF-2, and FGF-4, but not FGF-7 (Johnson et al., 1990; Mansukhani et al., 1990; Miki et al., 1992; Werner et al., 1992; Orr-Urtreger et al., 1993), which failed to induce additional limbs. FGF-7 is the ligand for KGF receptor (KGFR), a splicing variant of FGFR-2 encoded by the *bek* gene (Miki et al., 1992), which is expressed only in the ectoderm (Orr-Urtreger et al., 1993). Failure of additional limbs to develop when the ectoderm-specific FGFR is targeted suggests that mesenchymal stimulation is required for additional limb development.

Position and Identity of Limbs

The results suggest that local production of an FGF controls limb position (Figure 5). The nature of the signal that switches on FGF production at a particular axial level and how its production is controlled are also unknown. The position at which this signal is produced could be encoded by the pattern of *Hox* gene expression along the body axis. For example, in mice, frogs, and zebrafish, upper limbs (fins) develop at a level that corresponds approximately to the anterior limit of expression of *Hoxc-8* (Oliver et al., 1988; Molven et al., 1990). It is interesting that an anterior shift of the expression domain of *Hoxb-8* in the primary

body axis of the mouse led to development of an additional polarizing region in the anterior forelimb and could also cause ectopic bud formation anterior to the forelimb (Charité et al., 1994).

Application of FGF induces formation of a limb at a level where limbs do not usually form. The entire flank can form limbs, but no additional limbs form when FGF is applied more anteriorly in the neck, or posteriorly in the tail. This supports the view that the field with limb-forming potential extends from the anterior edge of the upper limb to the posterior edge of the lower limb.

It is interesting that when a single fin-like bud formed along the body axis, a single broad skeletal element formed proximally, rather than several serially spaced elements. Generally, anterior flank gives rise to wings and posterior flank to legs following FGF-2 application. There was only one exception, and only two chimeric limbs were observed. Taken together, this suggests that axial level determines limb identity.

A Molecular Explanation for Limb Induction

The present results cast new light on experiments carried out over 70 years ago. Locatelli (1924) and Kiortsis (1953) deflected brachial nerves to the flank of newts and showed that limbs could regenerate from this position. Interestingly, it has been suggested that early events in amphibian limb regeneration may be initiated by release of FGF-like molecules from traumatized nerves (Mescher and Gospodarowicz, 1979). Balinsky grafted otic vesicle (1925) and nose rudiment (1933) to the flank of newt embryos and induced formation of additional limbs that were often of reversed polarity. It is now known that the mouse otic vesicle expresses *Fgf-3* (Wilkinson et al., 1989) and that the nasal placode and epithelium express *Fgf-8* (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995). Thus, production of an FGF could be the molecular basis of limb-inducing ability.

Experimental Procedures

Application of FGF-2 Bead to Chick Embryos

Fertilized chicken eggs were incubated at 38°C, and the embryos were staged by counting somites according to Hamburger and Hamilton (1951). Experiments were performed on embryos between stages 10 and 17. To improve visibility, a small amount of India ink (Pelikan) diluted 1:4 in tissue culture medium was injected under the blastoderm, or a small chip of 1% neutral red in 1% agar was placed on the vitelline membrane. The vitelline membrane was torn away from the right side of the embryo, and a small transverse slit was made with electrolytically sharpened tungsten needles in the ectoderm covering the lateral plate mesoderm at a particular somite level. Heparin acrylic beads (H5263, Sigma), 125–250 μm in diameter, were soaked in a 2 μl drop of 1 mg/ml FGF-1 (132-FA-025, R&D Systems), 1 mg/ml FGF-2 (133-FB-025, R&D Systems), 1mg/ml FGF-7 (251-KG-010, R&D Systems), or 700 μg/ml FGF-4 for at least 1 hr at room temperature prior to implantation. A bead was inserted into the slit and manipulated into the appropriate AP position under the ectoderm. A few drops of medium were added to the egg before it was resealed and returned to the incubator. Embryos were incubated for a total of 9–10 days.

Whole-Mount Skeletal Preparations

Embryos were removed from the egg and washed in 1 × phosphate-buffered saline (PBS), and the membranes and internal organs were removed prior to overnight fixation in 5% trichloroacetic acid (TCA). They were then transferred to 0.1% Alcian green in acid alcohol for 6–16 hr. Alcian green was removed, and the embryos were differentiated in acid alcohol overnight, dehydrated in 100% alcohol, and cleared in methyl salicylate for analysis of skeletal patterns.

Whole-Mount In Situ Hybridization

Gene expression was assayed in embryos incubated for 4 to 48 hr after the operation, at which timepoints they were removed from the egg and washed in 1 × PBS. Embryos were fixed overnight in 4% paraformaldehyde at 4°C and dehydrated in a series of graded methanol washes. For *Hoxd-13*, processing and hybridization was according to Wilkinson (1992), using digoxygenin-labeled riboprobes for the chick *Hoxd-13* gene (Izpisúa-Belmonte et al., 1991). For *Shh*, processing and hybridization was performed essentially as described by Hemmati-Brivanlou et al. (1990) with some minor modifications (Izpisúa-Belmonte et al., 1993). The antisense *Shh* digoxygenin probe used corresponds to exon 3 of the chick *Shh*, spanning amino acids 251–501.

Scanning Electron Microscopy

Scanning electron microscopy was used to examine early morphology of treated embryos. Embryos were incubated up to 56 hr following FGF application, then removed from the egg into PBS for washing and removal of membranes. They were fixed and stored in modified Tyrode's solution (1% gluteraldehyde) at 4°C. After postfixation in 1% osmium in 0.1 M phosphate buffer for 1 hr, the specimens were dehydrated in graded ethanols and placed in amyl acetate. They were then dried by critical point-drying, mounted on metal studs, and sputter coated with gold particles. The embryos were observed using a Hitachi S-530 scanning electron microscope.

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