

Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb

Astrid Vogel*, Concepción Rodríguez* and Juan-Carlos Izpisua-Belmonte

Gene Expression Laboratory, The Salk Institute For Biological Studies, 10010 N. Torrey Pines Rd, La Jolla, CA 92037, USA

*The first two authors contributed equally to this work

SUMMARY

Fibroblast Growth Factors (FGFs) are signaling molecules that are important in patterning and growth control during vertebrate limb development. Beads soaked in FGF-1, FGF-2 and FGF-4 are able to induce additional limbs when applied to the flank of young chick embryos (Cohn, M.J., Izpisua-Belmonte, J-C., Abud, H., Heath, J. K., Tickle, C. (1995) *Cell* 80, 739-746). However, biochemical and expression studies suggest that none of these FGFs is the endogenous signal that initiates limb development. During chick limb development, *Fgf-8* transcripts are detected in the intermediate mesoderm and subsequently in the prelimb field ectoderm prior to the formation of the apical ectodermal ridge, structures required for limb initiation and outgrowth, respectively. Later on, *Fgf-8* expression is

restricted to the ridge cells and expression disappears when the ridge regresses. Application of FGF-8 protein to the flank induces the development of additional limbs. Moreover, we show that FGF-8 can replace the apical ectodermal ridge to maintain *Shh* expression and outgrowth and patterning of the developing chick limb. Furthermore, continuous and widespread misexpression of FGF-8 causes limb truncations and skeletal alterations with phocomelic or achondroplasia phenotype. Thus, FGF-8 appears to be a key signal involved in initiation, outgrowth and patterning of the developing vertebrate limb.

Key words: chick, limb development, limb initiation, apical ectodermal ridge, phocomelia, FGF-8, Shh

INTRODUCTION

Initiation of vertebrate limb development is one of the best examples of cell interactions as a primary mechanism of morphogenesis and pattern formation. Embryological studies have shown that the embryonic origin of the limb involves complex cell interactions between various tissues (e.g. somites, mesonephros and lateral plate mesoderm). Although much recent progress has been made regarding the molecular mechanisms responsible for these cellular interactions, the initial stimulus for limb development has not been identified.

Limb buds emerge as a result of a thickening of the somatic layer of the lateral plate mesoderm. It is believed that this is caused by a selective decrease in proliferation in the presumptive flank region (Searls and Janners, 1971). The limb mesenchyme signals the ectodermal cells covering it to elongate and to thicken, giving rise to a specialized structure called the apical ectodermal ridge (AER). Outgrowth and differentiation of the limb depend upon inductive interactions between the AER and the underlying limb mesenchyme. The AER maintains the mesenchymal cells underneath it in an undifferentiated and proliferating state (Globus and Vethamany-Globus, 1976; Reiter and Solursh, 1982), and, as a consequence, limb outgrowth proceeds (Saunders, 1972). Differentiation and outgrowth occur in an orderly progression with proximal structures forming first (e.g., shoulder) and distal last (e.g., digits). In addition to initiating limb outgrowth,

prospective limb mesoderm controls the anteroposterior polarity of the limb, because when grafted to the flank of a host embryo it induces a limb with a polarity determined by the orientation of the graft (Hamburger, 1938; Saunders and Reuss, 1974). This ability is later transferred to the posterior mesoderm within the developing bud, the so-called zone of polarizing activity (ZPA). Transplantation of a ZPA to the anterior margin of a host limb induces mirror-image duplication of the limb along the anteroposterior axis (Saunders and Gasseling, 1968). Transcripts of *Sonic hedgehog* (*Shh*) have been found to map to the polarizing region, and *Shh* has been proposed as the mediator of polarizing region signalling because it causes a ZPA-like duplication when misexpressed in the limb bud (Riddle et al., 1993; Chang et al., 1994; Lopez Martinez et al., 1995).

The AER expresses different genes that seem to play a pivotal role for limb outgrowth including some members of the Fibroblast Growth Factors (FGF) family (see Tickle and Eichele, 1994). The FGF family comprises at least nine members (Miyamoto et al., 1993). Three of them, FGF-2 (Dono and Zeller, 1994; Savage et al., 1993), *Fgf-4* (mouse: Niswander and Martin, 1992; Suzuki et al., 1992; chick: Laufer et al., 1994; Niswander et al., 1994a) and *Fgf-8* (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995) are found in the AER. In the chick, the role of the AER in limb outgrowth can be substituted by exogenous FGF-4 application (Niswander et al., 1993) or FGF-

2 (Fallon et al., 1994) in vivo, and in the mouse by FGF-2, FGF-4 (Niswander and Martin, 1993) and FGF-8 (Mahmood et al., 1995) in vitro. Furthermore, FGF-4 can support a functional ZPA (Vogel and Tickle, 1993) by maintaining *Shh* expression; in turn, *Shh* is able to induce *Fgf-4* in the AER (Laufer et al., 1994; Niswander et al., 1994a). These interactions, together with the progressive activation of *Hox* genes (Izpisua-Belmonte et al., 1991; Dollé et al., 1993), are crucial for continued limb outgrowth and patterning. Production of an FGF could be required for the initiation of limb budding; exogenous FGF-1, FGF-2 and FGF-4 placed in the presumptive flank of chick embryos induce formation of ectopic limb buds that form an AER and express *Shh* and *Hox* genes (Cohn et al., 1995, Ohuchi et al., 1995). These buds then develop into complete additional limbs, suggesting that normal limb bud formation is initiated by a local source of FGF. However, both FGF-1 (Jackson et al., 1992) and FGF-2 (Abraham et al., 1986) lack a secretory signal sequence. Furthermore, FGF-2 protein is widely found both in the limb and in flank cells (Savage et al., 1993), yet no additional limbs develop and, finally, *Fgf-4* transcripts can only be detected once an AER is formed (Niswander and Martin, 1992). These observations indicate that none of these FGFs is likely to be the endogenous signal involved in the initiation of limb development and lead to a search for other FGFs expressed in the appropriate spatiotemporal patterns. One good candidate is the *Fgf-8* gene.

We have cloned the chicken *Fgf-8* gene. Transcripts for the chicken *Fgf-8* gene, similarly to the mouse *Fgf-8* (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995), are found in different areas involved in outgrowth and patterning during embryogenesis. Classical embryological studies (see Stephens et al., 1993 for a review) have shown the existence of a medial-lateral progression in the limb-forming influence starting with Hensen's node and/or neural structures and notochord (stages 7-9 Hamburger and Hamilton, 1951), moving to the intermediate mesoderm (stages 12-14) and finally reaching a maximum in the lateral plate (stages 15-18). Strikingly, some aspects of the *Fgf-8* expression pattern coincide with the areas described above. Furthermore, transcripts for *Fgf-8* are detected in the pre-limb ectoderm before a morphologically distinct AER is formed and later on are restricted to the ridge. Formation and maintenance of the AER is critical for limb outgrowth because its removal causes limb truncations (Saunders, 1948; Summerbell, 1974). *Fgf-8* is also found in the otic vesicle and nose placode, areas that are able to induce the formation of additional limbs in newts (Balinsky, 1925; 1933). FGF-8 application into the presumptive flank at the time limbs are being formed induces the formation of ectopic limbs. Like FGF-4, FGF-8 can maintain and induce *Shh* expression and drive limb outgrowth after AER removal. Continuous and widespread *Fgf-8* misexpression during midembryogenesis causes forelimb and hindlimb skeletal alterations, as well as vertebrae, rib and tail anomalies accompanied in some cases by a generalized developmental delay. Together, these results suggest that the same gene is involved not only in the initiation, outgrowth and patterning of the limb, but also in axis formation. In the present study, we discuss the role of FGF-8 as a key signal involved in vertebrate limb initiation and propose that the concerted and balanced action of different FGFs controls limb outgrowth and patterning.

MATERIALS AND METHODS

cDNA library screening

Approximately 1 million phages of a λ ZAP II cDNA library prepared from stage 20-22 chick limb bud RNA were screened under standard conditions (42% formamide, 5 \times SSC, 1% SDS, 0.1 mg/ml denatured salmon sperm DNA, 42 C) by hybridization with a 200 bp chick genomic *Fgf-8* fragment (a kind gift from Drs B. Hogan and N. Wall, Vanderbilt). From the original λ ZAP-positive clones, pBluescript recombinant plasmids were excised according to the manufacturer instructions (Stratagene) and sequenced by the dideoxy chain termination method using a USB sequencing kit.

Transfection of chicken embryonic fibroblasts and virus production

Chicken embryonic fibroblasts were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum and 2 mM L-glutamine (DMEM-10%FBS, Gemini) and kept at 37C, 5% CO₂. Cells were seeded at 20-30% confluency in DMEM-10%FBS 24 hours before transfection with the *Fgf-8* gene inserted into a modified pRCMV vector (Invitrogen) containing the 5' and 3' untranslated sequences of the *Xenopus* β -globin gene (Amaya et al., 1991). Transfection was performed using DOTAP reagent (Boehringer Mannheim) according to manufacturer instructions. 24 hours after transfection, cells were replenished with DMEM-10%FBS, scraped off the substratum, and centrifuged and implanted into chick embryos.

For virus production and after transfection with the RCASBP(A) (Replication-Competent, Avian leukemia virus LTR, Splice acceptor, Bryan High Titer Polymerase, A-envelope subgroup) retrovirus containing the *Fgf-8* gene, chicken embryonic fibroblasts were grown as described previously (Morgan et al., 1992) for 8-12 days. Virus harvest and concentration was performed as described previously (Morgan et al., 1992).

Cell implants and virus injections

Chicken embryos were obtained either from MacIntyre Poultry (San Diego, California) or from SPAFAS (Norwich, Connecticut).

For grafting experiments, cells were scraped off the substratum and centrifuged for 3-5 minutes. The resulting pellet was then cut into pieces using tungsten needles. Before grafting, sterile black ink (Pelikan, 1:20 dilution) was injected below the blastoderm to visualize the embryo. Using fine tungsten needles, a small slit was made into the appropriate place of the lateral plate mesoderm. A piece of cells was then transferred into the egg and grafted into the prepared slit.

Concentrated viruses (approximately 10⁶ to 10⁷ infectious units/ml) were microinjected into the lateral plate of stage 14 chick embryos or in the limb field regions at stage 17 (Hamburger and Hamilton, 1951). For easier observation of the viral injection into the early chick limb bud, fast green (0.25%) was added to the virus stock prior to injection (1/10 dilution).

After cell implant or virus injection, the embryos were left to develop for a further 7-9 days, fixed in 5% trichloroacetic acid overnight and stained for cartilage using 0.1% alcian green. After dehydrating and clearing the embryos with methylsalicylate, the cartilage structures of the embryos were evaluated.

FGF-8 protein purification

The *Fgf-8* cDNA lacking the signal sequence (residues 1 to 24) was cloned into the pET23c expression vector (Novagen) such that the protein was expressed with a six-histidine C-terminal tag. Recombinant protein was purified from a 1 liter culture that was grown to OD 0.7 at 37°C prior to induction with 0.1 mM IPTG. After induction, the cells were grown for a further 2 hours at 30°C. The FGF-8 protein was found to be present in the insoluble pellet after cell lysis by sonication in buffer containing: 50 mM Hepes pH 8.0, 150 mM NaCl, 2.5 mM CaCl₂, 1 mM pepabloc protease inhibitor (Boehringer

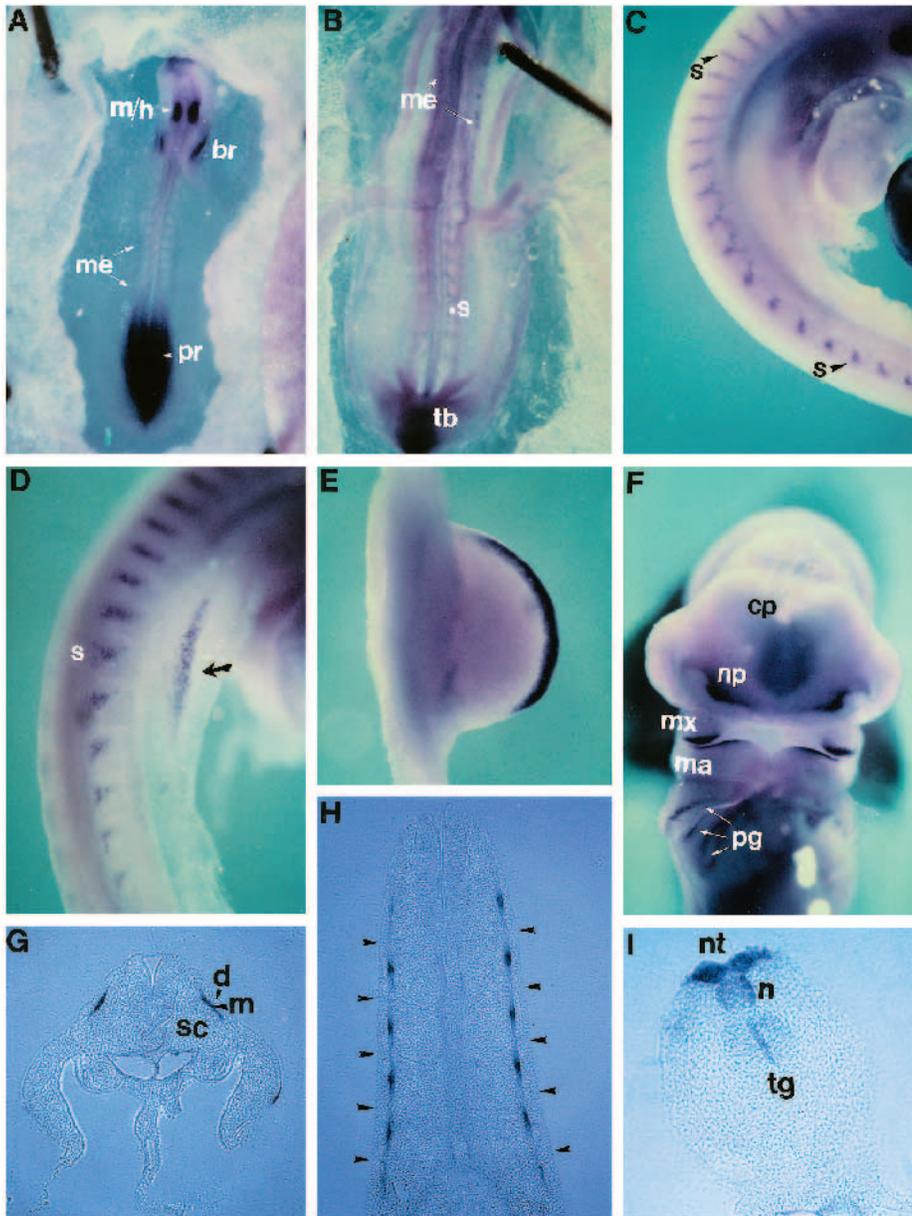


Fig. 2. Expression of *Fgf-8* during chick development. Gene expression of *Fgf-8* in whole mounts (A-F) and in sections (G-I) of developing chick embryos. (A) *Fgf-8* expression in the developing chick embryo at stage 10 (HH) is detected in the primitive streak, the unsegmented mesoderm, mesonephros/intermediate mesenchyme opposite somite 6-10 and opposite the unsegmented mesoderm (white arrows), the midbrain-hindbrain junction, the branchial arch region and the optic stalk. Ventral view. (B) *Fgf-8* expression in the mesonephros of the forelimb region and anterior to the forelimb region at stage 16 of chick development. Expression of *Fgf-8* is not yet detectable in the mesonephros of the flank region, but is detected in newly condensed somites (white star). *Fgf-8* transcripts are also strongly detected in the nascent tailbud. Ventral view. (C) *Fgf-8* expression during somite development. Note the anteroposterior and mediolateral wave of expression in parallel to the somite differentiation process. Dorsal view. (D) Expression of *Fgf-8* in the preridge ectoderm at stage 16 (black arrow) and in the somites. (E) *Fgf-8* expression in the apical ectodermal ridge of a stage 21 chick wing bud. (F) Frontal view of a stage 20 chick embryo. *Fgf-8* transcripts are detected in the commissural plate of the forebrain, nasal pits, maxillary and mandibular prominences of the first branchial arch and pharyngeal grooves. (G) Cross section of a stage 18 chick embryo. *Fgf-8* transcripts are detected in the lateral region of the myotomes and in the nascent AER. (H) Longitudinal section of a stage 18 chick embryo. *Fgf-8* expression is confined to the myotomal central region of each somite. (I) Cross section of a stage 17 chick embryo. *Fgf-8* transcripts are detected in the neural plate (except the floorplate), in the notochord

and in the endoderm of the tailgut. br, branchial arches; cp, commissural plate; d, dermatome; m, myotome; ma, mandibular prominence of the first pharyngeal arch; me, mesonephros; m/h, midbrain-hindbrain junction. mx, maxillary prominence of the first pharyngeal arch; n, notochord; np, nasal pits; nt, neural tube; pg, pharyngeal grooves; pr, primitive streak; s, somites; sc, sclerotome; tb, tailbud; tg, tailgut.

Regarding the developing limb, *Fgf-8* transcripts are first detected in the ectoderm of the limb field at stage 16 just before a morphologically distinct apical ectodermal ridge has formed (Fig. 2D). Expression appears to be diffuse in the ectodermal cells, but soon after formation of the apical ectodermal ridge transcripts are restricted to the ridge cells (Fig. 2E). *Fgf-8* expression is maintained throughout the AER until it starts to regress. Expression is not detectable by the time the ridge has regressed, as has been reported for the mouse (Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995).

Other areas of expression, as in the *Fgf-8* mouse gene, include rostral neural fold forebrain and commissural plate, midbrain-hindbrain junction, developing optic stalk, nasal pits,

and maxillary and mandibular components of the branchial arches (Fig. 2E, see also Wall and Hogan, 1995). In *Fgf-8* expression is also detected in the otic vesicle of stage 15 and 16 chick embryos (data not shown).

Limb pattern alterations induced by FGF-8 beads

Beads soaked in FGF-8 (see Materials and Methods for FGF-8 purification and Fig. 1B) and applied to the presumptive flank of chick embryos, as reported for other FGF-family members (Cohn et al., 1995; Ohuchi et al., 1995), lead to the development of additional limbs. FGF-8 beads were placed at different somite levels along the lateral plate mesoderm and at different stages of chick development ranging from stage 11 to stage 17. Position of the beads and stage of implantation was found to

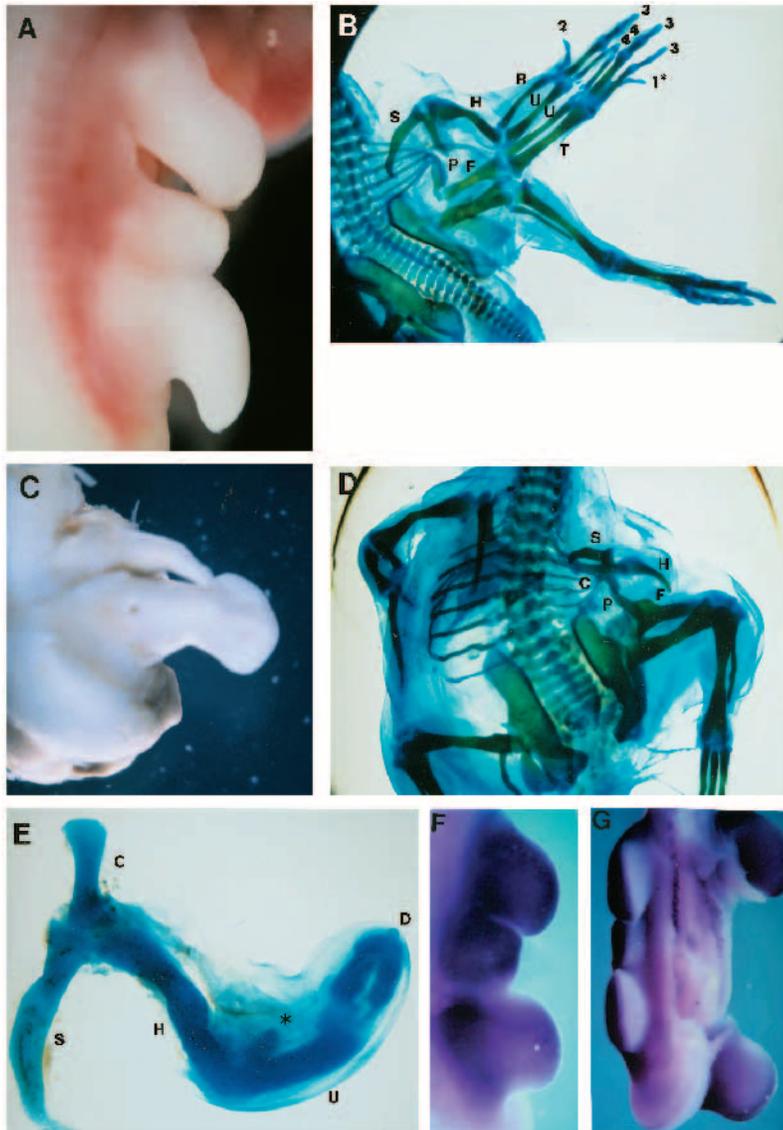


Fig. 3. Limb pattern alterations induced by FGF-8 protein beads. (A) Outgrowth of flank mesenchyme of a stage 26 chick embryo, 2 days after application of an FGF-8-soaked bead to the flank (opposite somite 24) of a stage 15 chick embryo. (B) Additional limb with reversed chimeric appearance (wing and leg skeletal structures are present), which developed following FGF-8 bead application opposite somite 23 of a stage 15 chick embryo. This limb developed an additional girdle structure, femur, ulna and tibia and a digit pattern 4-3-3-1 (compare to Fig. 5A). The most posterior digit (digit 1) appears to be a leg digit. (C) Following application of FGF-8 opposite somite 21 (stage 18), wing and leg are drawn together. (D) Skeletal pattern of a chick embryo (10 days) where a truncated wing developed following application of an FGF-8 bead to the flank of a stage 14/15 chick embryo (compare to Fig. 5A). The scapula is shifted ventrally and only a partial humerus developed. An additional pelvis structure and femur are present and the proximal part of the femur is connected with the coracoid. Note the presence of an additional rib on the left side (arrow), and the absence of posterior vertebrae (+). (E) Following entire ridge removal and application of FGF-8 to the posterior margin of a chick wing bud, humerus, ulna and several posterior-like digits developed. Anterior structures, except of the proximal part of the radius (star) did not develop. (F) *C-Lmx-1* expression in the additional limb bud that developed 48 hours following FGF-8 application to the lateral plate mesenchyme opposite somite 23/24 of a stage 15 chick embryo. Expression of *C-Lmx-1* is detected in the entire dorsal mesenchyme of the wing and leg and in the additional limb bud induced. The additional limb bud developed an AER. Dorsal view. (G) Same embryo as in F showing the absence of *C-Lmx-1* transcripts in ventral mesenchyme. Note that the limbs on the operated side appear to be drawn together compared to the limbs on the contralateral side. Ventral view. C, coracoid; D, digits; F, femur; H, humerus; P, pelvis; S, scapula; T, tibia; U, ulna.

Table 1. Effects of FGF-8 soaked beads implanted in the lateral plate mesenchyme of stage 11 to 17 chick embryos

Stage of operation	Somite level	Wing truncations	Additional proximal structures (e.g. girdle, femur or humerus)	Additional limbs	Cases
11	12	0	0	0	1
12	16	1*	1	0	1
13	18/19	2	1	0	2
14/15	20-22	9	5	0	11
14/15	23-25	1	3	11†	14
16	23-25	0	4	3	7
17	22-26	0	3	0	5

*Digit 2 was missing in the wing.

†5 additional legs and 6 chimeric limbs developed.

be critical. Table 1 and Fig. 3 summarizes some of the results obtained.

Following application of FGF-8-soaked beads to the lateral

plate of stage 14/15 chick embryos, opposite somite 23 to 25, limb bud outgrowth was observed (Fig. 3A), which resulted in the development of additional limbs (11/14 cases; Table 1). The additional limbs that developed were either legs (5 cases) or chimeric limbs composed of leg and wing skeletal elements with one to four digits (6 cases, Fig. 3B). In all 11 cases, additional shoulder and pelvic girdles were formed; 4 of the ectopic limbs shared the femur, which was broadened in size. In the other three cases, where no complete additional limbs formed, additional girdle structures and femurs were observed and one chick developed also a truncated wing (Table 1). In two cases, the bead was applied into the lateral plate mesenchyme opposite somite 21 at stage 15 and this resulted in the development of a truncated wing (Table 1). The additional limbs induced were always reversed in polarity along the anteroposterior limb axis, as determined by the localization of *Shh* expression in anterior mesenchyme and by the position of the tibia/fibula, tibia/ulna and the order of wing and leg digits (Fig. 3B; data not shown). In a few cases, we observed that *Shh* expression was lost or down-

regulated in posterior mesenchyme of the original wing (which was reduced in size) compared to the contralateral or ectopic limb bud induced (data not shown). The dorsal ventral polarity of the additional limbs induced, based on *C-Lmx-1* expression, a gene localized to the dorsal mesenchyme of the developing limb (Riddle et al., 1995, Vogel et al., 1995), and on morphological criteria, conformed to the polarity of the host limbs (3/3 cases; Fig. 3F,G).

The competence to form additional limbs appears to peak at stage 15. Application of FGF-8-soaked beads to the lateral plate mesenchyme, opposite somite 20 to 22 at stage 14, lead to the development of truncated wings (7/9 cases; Table 1). The limbs in the affected embryos were often drawn together (Fig. 3C,D). The truncations were mainly at the level of the humerus (Fig. 3D and compare to Fig. 5A) and, in 5 cases, duplication of the wing and leg girdle was observed. The scapula and pelvis were connected in 2 cases and an additional 8th rib developed on the left side of the embryo in 4 cases (Table 1, Fig. 3D). Thus, application of FGF-8-soaked beads in or close to the presumptive wing field leads to truncations of the wing. When the bead was applied to stage 16 chick embryos, opposite somite 23-25, additional legs with reversed anteroposterior polarity developed in 3 out of 7 cases. In the other 4 cases, girdle structures and proximal limb parts developed (Table 1). After stage 16, the competence to induce additional limbs was reduced. FGF-8 beads implanted opposite somite 22-26 at stage 17 did not induce an additional limb although proximal limb structures developed in 3 out of 5 cases (Table 1). In 2 cases, the development of an additional rib on either the left or on both sides of the embryo was observed and the last vertebrae did not form resulting in tailless chicken (Fig. 3D). In another case, where the bead was applied into the presumptive leg field (opposite somite 26/27) duplicated leg digits developed. In this limb, the tibia was broadened and shortened in size. Control beads (PBS-soaked) did not induce any abnormal phenotype.

In summary, complete limbs developed when FGF-8 beads where implanted opposite somites 23 to 25 at stage 14/15. This coincides approximately with the time at which *Fgf-8* transcripts are detected in the intermediate mesoderm at the level of the limb field. However, when the FGF-8 bead is placed in or close to the wing field, limb truncations occur and, after stage 16, no additional limbs are obtained.

The fact that the position of the bead and the stage of implantation dictates the outcome of the limbs obtained seems to indicate that presumptive flank and limb cells respond differently to FGF-8 protein and that this response changes in time. To further investigate this and to study whether a continuous source of FGF-8 had a different effect than a transient and localized application, we generated *Fgf-8*-transfected cells and an *Fgf-8* replication competent avian retroviral vector. They were then either grafted or injected into the developing chick embryo.

Limb pattern alterations induced by grafts of *Fgf-8*-transfected cells

In order to evaluate the effects of providing a localized and continuous source of FGF-8, we transiently transfected chicken embryonic fibroblasts with *Fgf-8* and grafted them along the primary axis of stage 13 to 17 chicken embryos at different somite levels. Results are summarized in Table 2 and Figs 4 and 5.

Fgf-8-transfected cells grafted to the flank region at stage 15 to 17 of chick development, at the level of somites 21-25, affected the development of the forelimbs and/or hindlimbs in 100% of the cases (9) (Table 2). Severe shortening of the proximal skeletal elements (e.g. humerus, radius and ulna in the forelimb and femur, tibia and fibula in the hindlimb) were mainly observed and often accompanied by thickening of the epiphysis of the humerus/femur and tibia (Fig. 5B,C,E). Fusions were often observed at the level of the joints, especially between humerus and radius/ulna. Digit abnormalities, mainly reduction in size and failure of the phalanges to separate, in the forelimb and/or hindlimb were also observed (88%; Table 2; Fig. 5B,C,E). The general appearance of the limbs was similar to the shortened limbs observed in humans after thalidomide administration to pregnant mothers (Fig. 4F; Sucheston and Cannon, 1973) and/or to the genetically associated disease achondroplasia (Fig. 4D, Smith and Jones, 1982). Frequently, featherbuds appeared later in the affected limbs when compared to the contralateral limb, suggesting a general developmental delay (Fig. 4D,F). In 33% of the cases, an additional digit was found posterior to digit 4 of the forelimb (Table 2). The skeletal pattern of both limbs of the operated side and in 66% of the cases also the contralateral limb was affected (Table 2, Figs 4D,F, 5B,C). Abnormalities

Table 2. Skeletal elements of both fore- (operated side/contralateral side) and hindlimbs (operated side/contralateral side) affected after implanting *Fgf-8* transfected cells or viral *Fgf-8* injection

Stage of operation	Position of graft/inject.	Skeletal abnormalities								
		Forelimb					Hindlimb			
		Cases	Humerus	Radius/ulna	Digits	Extra digit	Femur	Tibia/fibula	Digits	Extra digits
15-17	Flank	9	9/3	9/3	8/2	3/0	6/4	5/3	6/4	0/0
14-17	Limb field	12*	4/0	4/0	4/0	3†/0	5/2	6/2	5/2	5‡/0
18	Forelimb¶	7	7/0	7§/0	4/0	1/0	0/0	0/0	0/0	0/0
17	Hindlimb¶	1	0/0	0/0	0/0	0/0	1/0	1/0	1/0	0/0

Generally affected proximal skeletal elements of fore- and hindlimb were mainly reduced in size and/or abnormally shaped, and affected digits were smaller in size and/or the phalanges did not separate.

*6 cases each to the forelimb and hindlimb regions.

†The graft was placed in the forelimb region.

‡Graft was placed in the hindlimb region.

§Ectopic cartilage formed on the ventral side of the proximal part of radius/ ulna in two cases.

¶Viral injection of *Fgf-8*.

in pelvis and shoulder girdle, vertebrae and ribs were also observed (100%; Fig. 5B-E). Ribs, scapula and pubis were often thickened and deformed on either the operated side or on both sides of the embryo (Fig. 5B-E). The lumbar and sacral vertebrae did not separate properly in 55% of the cases, resulting in the development of dwarf-like chickens (Figs 4D, 5B,C). In 2 cases, the most posterior vertebrae did not form resulting in tailless embryos (Fig. 5B). Condensation of additional rib-like cartilage structures were found either on the operated side or on both sides of the embryo (2 cases).

With the aim of targeting the limb field rather than the flank region, in another set of experiments, the grafts were placed into stage 14 to 16 chick embryos opposite the unsegmented mesoderm. In 66% of the cases additional cartilage elements developed (Table 2). When the graft was placed just below somite 20/21 of a stage 14 chicken embryo, a posterior digit developed (50%, 3 cases). In the other 3 cases, the embryos developed normal limbs, although fusion, splitting and thickening of the ribs were observed. When the graft was placed in the anterior part of the presumptive leg field at stage 16, just below somite 26, well-formed legs developed with the appearance of 5 to 6 digits (75%, 3 cases). In one case, 2 femuri developed, the tibia was thickened and shortened and 6 digits developed (Fig. 5D; see also Riley et al., 1993). In the other case where no additional digits developed, the leg was shortened and deformed. When the graft was placed directly into the presumptive leg field at stage 14 and 16, both severe shortening of the proximal skeletal structures and the development of additional limbs was observed (2 cases, Fig. 4E,H). In both cases, the phalanges of all digits did not separate and one of the embryos had a dwarf-like phenotype. Untransfected chicken embryonic fibroblast grafted to the presumptive flank region at stages 14-16 did not show any abnormal phenotype, except that in two cases parts of the pubis were slightly deformed (2/5 cases).

To study whether the development of shortened or smaller limbs following grafts of *Fgf-8*-transfected cells could be correlated with the size and formation of the AER, we assayed *Fgf-8* expression (as a marker for the AER) in the affected limbs. When *Fgf-8*-transfected cells were placed just below the wingfield, opposite somite 21/22 at stage 14, a limb developed that was shortened in size compared to the contralateral wing and *Fgf-8* expression was absent in the posterior part of the limb (2/2 cases; Fig. 6E). When the transfected cells were placed directly into the hindlimb field of stage 16 embryos, *Fgf-8* expression was more diffuse (Fig. 6F) and transcripts were also detected on both the dorsal and ventral side of the shortened limb buds (2/2 cases).

In summary, the grafting of *Fgf-8*-transfected cells leads to severe skeletal anomalies that range from deletions, reductions and fusions of the more proximal bones to the appearance of extra digits. Furthermore, the primary body axis was also affected, showing, in some cases, extreme shortening of all skeletal elements, appearance of extra ribs, fused vertebrae and tail bud defects.

Limb pattern alterations induced by viral injection of *Fgf-8*

Since it is possible that a widespread source of FGF-8 could give different results (see for instance Ohuchi et al., 1995 and Riley et al., 1993) and, in order to deliver high levels of FGF-

8 protein to the developing embryo, we employed the RCASBP(A) retroviral vector (Hughes et al., 1987).

Viral injection of *Fgf-8*, similarly to the *Fgf-8*-transfected cell graftings, was followed by reduction of proximal skeletal limb structures in addition to digit abnormalities resembling the phocomelic (Fig. 5E) phenotypes observed with cell implants. When *Fgf-8* virus was injected into stage 17 forelimb buds, shortened forelimbs developed (100% cases). In those limbs, the humerus, radius and ulna were mainly affected (Table 2, Fig. 5E). The digits appeared to be reduced in size and the phalanges did not separate in 57% of the cases. In one case a complete additional rib formed on both sides of the embryo (Fig. 5E). The *Fgf-8* effect was also extended to the tail bud where a fin-like outgrowth was observed (Fig. 4G). In one case, viral injection into the hindlimb bud (stage 18) was followed by the development of a phocomelic leg with severe shortening of the long bones (Table 2). Similar alterations were observed when the virus was injected at stage 10 to 14 (5/11 cases) in the presumptive limb area. In general, the embryos were reduced in size and presented anomalies in limbs, vertebrae, ribs and scapula. In the other 6 cases, the embryos died shortly following the operation. 2 days following viral injection into stage 14 chick embryos in the presumptive flank region, we detected outgrowths of flank mesoderm (Fig. 4A), but in no case was the outgrowth capped with an AER and, on the next day, the initial swelling started to regress (Fig. 4B).

Viral spread was tested following viral injection of FGF-8 into the flank of stage 10-14 chick embryos using a specific riboprobe for the viral vector. We found that the entire forelimb and hindlimb as well as the flank and somite region were infected 2 to 3 days after the operation. Two days following viral FGF-8 injection into stage 17 forelimb buds, virus was localized almost throughout the forelimb and little patches of virus were found in some somites, mostly in the tail region (data not shown). In control experiments, RCASBP(A) vector containing the alkaline phosphatase gene was injected into the flank of stage 14 chick embryos and this did not lead to any morphological changes.

FGF-8 maintains limb outgrowth and *Shh* expression

Following AER removal, FGF-4 can maintain *Shh* expression, outgrowth and patterning of the developing chick limb (Niswander et al., 1993; Laufer et al., 1994; Niswander et al., 1994a). To determine whether FGF-8 can induce and/or maintain *Shh* expression, we removed the AER of stage 20 limb buds, which abolishes *Shh* expression after 20 hours (Laufer et al., 1994; Niswander et al., 1994) and applied an FGF-8-soaked bead to posterior mesenchyme. This operation resulted in maintenance of *Shh* expression 24 hours following the operation (3/3 cases; Fig. 6C). Moreover, FGF-8 application can substitute for the AER to allow formation of distal posterior skeletal elements. When the entire ridge of a stage 20 limb bud was removed and an FGF-8-soaked bead was implanted in the posterior mesenchyme, a humerus, an ulna and several posterior-like digits developed (Fig. 3E), as has been reported for FGF-4 (Niswander et al., 1993). In another set of experiments, after removal of the ridge, a FGF-8 bead was placed in the posterior proximal mesenchyme at stage 22 to 23 and *Shh* expression was analyzed 24 hours after the operation (Fig. 6B). After stage 23 during normal develop-

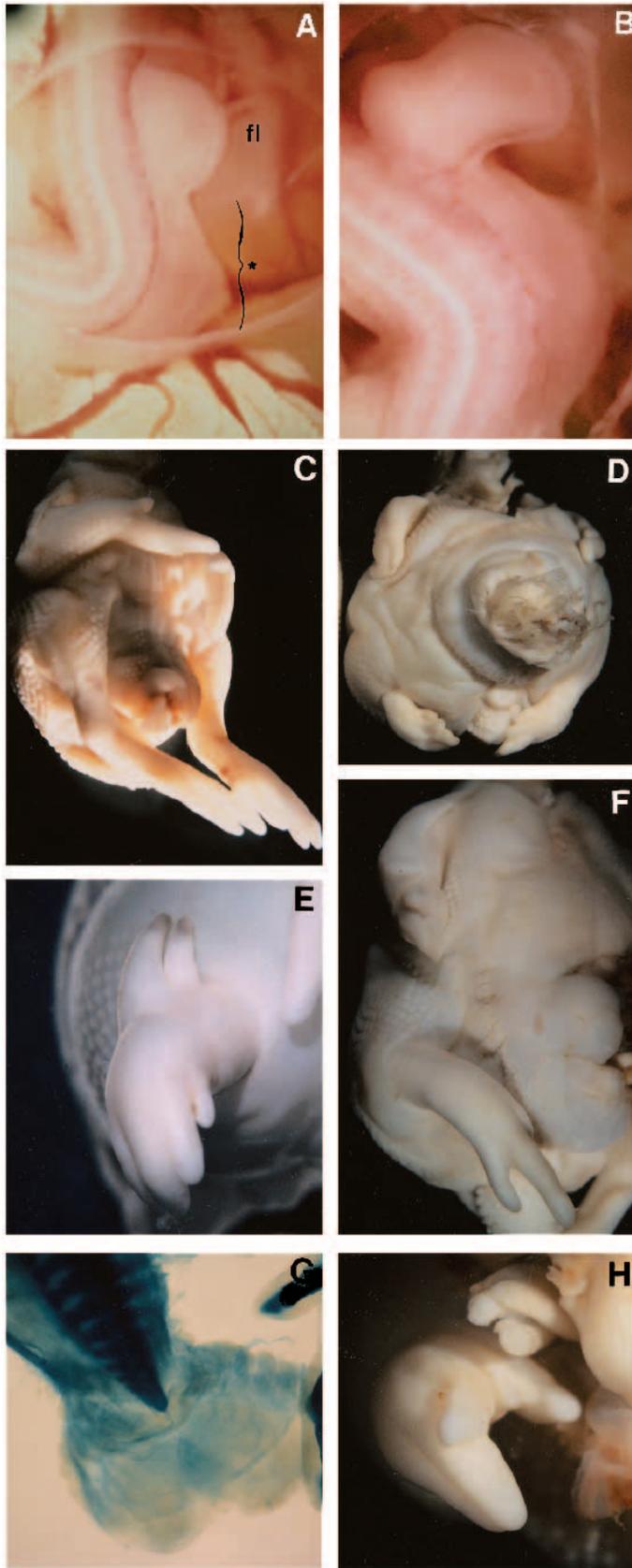


Fig. 4. Limb pattern alterations induced by FGF-8 misexpression. (A) Dorsal view of an outgrowth of the flank observed after viral injection of *Fgf-8* into the flank of stage 14 chick embryos. The size of the outgrowth is similar to the normal forelimb bud. (B) The following day the outgrowth started to regress. Whole mounts of fixed chick embryos after grafting cells transfected with *Fgf-8* to the flank (D, F) or into the presumptive limb-forming region (E, H) at stages 14-16. (C) Normal chick embryo where no manipulation had been carried out, fixed after 10 days of incubation (ventral view). Note that the specimen is one day younger than the embryo shown in D. (D) Dwarf-like embryo that developed following a graft of *Fgf-8*-transfected cells into the flank region of a stage 14 embryo, fixed after 11 days. Notice the extreme reduction of body size and the shortened limbs compared to the non-operated embryo, which was fixed a day earlier (ventral view). (F) Chick embryo were both the forelimb (achondroplasia) and hindlimb (phocomelic) was affected on the operated side. Notice that the forelimb seems to be dorsoventrally thickened and no featherbuds developed (ventral view). (E, H) Following grafts of *Fgf-8*-transfected cells into the presumptive limb field, severe shortened polydactylous limbs developed. (E) Two additional digits developed on the dorsal side of the limb. The femur, tibia and fibula were extremely shortened in this limb (ventral view). One day following the operation the grafted cells were localised at the base of the outgrowing limb bud in a more posterior localisation. (H) Seven digits developed in the severely shortened leg. Partially shown is a big outgrowth that developed out of the abdominal cavity stomach, which stained heavily for cartilage. In all cases, the digits of the affected limbs appear to be delayed in development. (G) Fin-like outgrowth of the tail of a chick embryo after viral injection of *Fgf-8* into the forelimb region of a stage 18 chick embryo (dorsal view)

4 applied to proximal limb mesenchyme is able to ectopically induce *Shh* expression (Yang and Niswander, 1995; Kostakopoulou et al., 1996). In a similar way, proximal FGF-8 application maintained and induced *Shh* expression in proximal mesenchyme (Fig. 6B). Thus, FGF-8, like FGF-2 and FGF-4, is able to substitute for the ridge to maintain outgrowth and patterning of the limb.

***Fgf-8* is induced by FGF-2 application to the flank of young chick embryos**

Application of FGF-2-soaked beads to the flank of young embryos leads to the formation of ectopic limb buds and transcripts of *Shh* and *Hox d-13* start to appear 24 hours after FGF-2 implantation (Cohn et al., 1995). Since, during normal limb development, *Fgf-8* expression precedes the appearance of these two genes, we wanted to investigate whether FGF-2 mimics the same molecular cascade leading to the initiation of limb budding. We implanted FGF-2-soaked beads into the flank opposite somites 21-25 at stages 15 to 16. 19 hours after application of FGF-2 to the flank, and before an apical ectodermal ridge was formed, *Fgf-8* expression was observed (3/3 cases) while *Shh* and *hoxd-13* expression were not detected until 23 hours after FGF-2 application to the flank (1/3 cases; data not shown). As with FGF-8, we observed that, in two cases, *Shh* expression in the original wing, which developed into truncated wings, was reduced or absent, compared to *Shh* expression in the contralateral and ectopic limb buds (data not shown). In one case where the bead was applied to the right flank of the embryo, an additional limb bud also developed on the left side (Fig. 6D).

ment, *Shh* expression is absent from the distal posterior mesenchyme of the limb bud (Riddle et al., 1993; Fig. 6A). FGF-

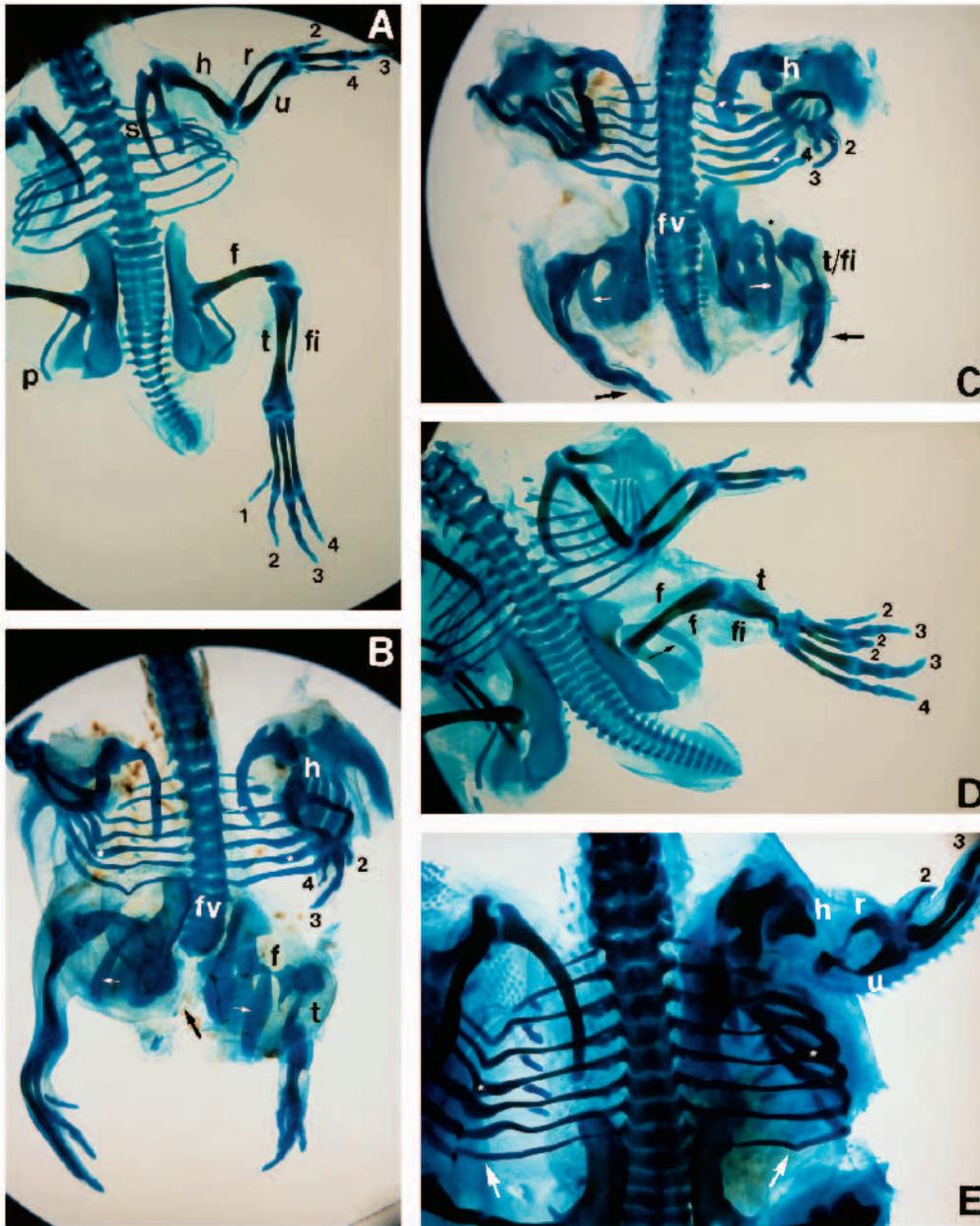


Fig. 5. Skeletal alterations after FGF-8 misexpression. Skeletal structures of alcian-green-stained embryos after grafting *Fgf-8*-transfected cells to stages 15-16 chick embryos (B-D) and following viral injection of *Fgf-8* into the limb at stage 18 (E). (A) Normal skeletal pattern of forelimbs and hindlimbs, and skeletal elements of the primary body axis (9 days of development). (B) A dwarf-like chick embryo (10 days) developed after grafting *Fgf-8*-transfected cells opposite somite 24/25 on the right side at stage 16. The forelimbs and hindlimbs on the operated side are reduced in size and the phalanges are fused. Approximately the last 20 vertebrae of the embryo did not form (black arrow) and the last-forming vertebrae are fused. The pelvis of both sides is abnormal and the ribs (white star), scapula and pubis of either the operated or both sides are thickened (white arrow). (C) Skeletal structure of the embryo shown in Fig. 4D that developed after grafting *Fgf-8*-transfected cells into the flank region. The forelimb and hindlimb of the operated side are severely shortened. Note that digit 4 is thickened and reduced in size and the phalanges of digit 2 and 3 are not separated. The digit's phalanges did not separate (black arrows). Defects are also observed on the unoperated side, but not as severe. The ribs (white star), scapula and pubis (white arrows) are, either on both sides or only on the operated side, thickened. The vertebrae are not properly separated. (D) Skeletal

pattern of an embryo where the graft had been placed into the anterior part of the prospective hindlimb region (below somite 26) at stage 16. The femur is duplicated, the tibia is greatly reduced and the fibula is connected to the ankle. In this specimen, 6 digits developed with a digit pattern 2,3,2,2,3,4. The pelvis including the pubis (black arrow) is also affected. (E) Skeletal pattern of an embryo following viral injection of *Fgf-8* into the forelimb at stage 18. The forelimb is greatly reduced and only a stump of the humerus (compare to the humerus of the contralateral limb) and the distal parts of the radius and ulna developed. Digit 3 and 4 appear of normal size, whereas digit 2 is reduced. The scapula was shortened on the operated side. The ribs were thickened on both sides and an additional rib formed on both sides of the embryo (white arrows). All the skeletal structures are dorsal views. f, femur; fi, fibula; fv, fused vertebrae; h, humerus; p, pubis; r, radius; s, scapula; u, ulna.

DISCUSSION

Fgf-8 expression and limb bud initiation

Classical embryology experiments have shown that the formation of the vertebrate limb involves a complex set of interactions between the different tissues located at the presumptive limb-forming region, among which are the mesonephros, somites and lateral plate mesoderm. Various

FGF family members (FGF-1, FGF-2 and FGF-4) can, in part, mimic these interactions. FGF application to the flank, the area between the two future limb buds, triggers the mechanism that initiates limb outgrowth and subsequently the development of a complete limb (Cohn et al., 1995; Ohuchi et al., 1995). However, none of these FGF family members is expressed at the right time or the right place to be the endogenous limb inducer. FGF-2 is widely expressed throughout the lateral plate

mesenchyme and ectoderm and *Fgf-4* transcripts can only be detected once an AER is formed (Savage et al., 1993; Niswander and Martin, 1992).

Furthermore, both FGF-1 and FGF-2 lack a recognizable secretory signal sequence (Abraham et al., 1986; Jackson et al., 1992). Taken together this suggests that, either these FGFs are mimicking the role of another factor, or another FGF-family member is responsible for the initiation of limb development (Cohn et al., 1995; see also Slack, 1995; Tabin, 1995; Tickle, 1995).

Although the limb field is not capable of self differentiation until stage 12 during chick development, Stephens et al. (1991) have shown that between stages 7 and 9, the regressing Hensen's node/primitive streak can induce limb-like structures when grafted into the coelom of a host embryo. These authors have proposed that, at this stage, when Hensen's node is passing by the future wing region, some morphogenetic factor(s) move out of Hensen's node into the segmental plate. During stages 10-11 the 'limb influence' is located in the segmental plate and subsequently moves to the region of the intermediate mesoderm during stages 12-14. At the beginning of stage 15, the limb-forming ability is 'masked' in the neck and flank area and becomes circumscribed to the future wing and leg buds until stage 18. *Fgf-8* transcripts are detected in the primitive streak at stages 7-9 and, by stages 10-11, they are detected in the paraxial mesoderm exiting the primitive streak; in the following stages, transcripts are detected laterally in the somites. *Fgf-8* mRNA is also present in the mesonephros, and acquisition of limb-forming properties by the lateral plate partially depends on its interaction with the mesonephros because its ablation prevents limb outgrowth (Geduspan and Solursh, 1992). At stage 16, *Fgf-8* expression is detected in the ectodermal layer at the level of the wing and leg bud. Later, *Fgf-8* transcripts are restricted to the apical ectodermal ridge and are finally lost by the time the ridge regresses. The AER, which is capable of supporting limb outgrowth until stage 28, is unable to do so after stage 29, when grafted on top of a nude limb mesenchyme (Rubin and Saunders, 1972). Two other areas of *Fgf-8* expression in the developing embryo, the otic vesicle and the nose rudiment, induce additional limbs when grafted to the flank of newt embryos (Balinsky, 1925, 1933). The overlap of *Fgf-8* expression with the 'limb-forming regions' suggests that FGF-8 is involved in the initiation and outgrowth of limb development.

FGF-8 can initiate limb development

Application of FGF-8-soaked beads to the flank region of young chick embryos, as previously reported for FGF-1, FGF-2 and FGF-4 (Cohn et al., 1995), is also able to induce development of additional limbs. The additional limbs are inverted along the anteroposterior axis (see also Cohn et al., 1995; Ohuchi et al., 1995) but conform to the host limbs along the dorsoventral axis. This result, together with its spatial and temporal pattern of expression, implicates FGF-8 as a key molecule involved in the process of limb initiation.

When additional limbs develop following FGF-8 application to the flank, the initial swelling that occurs is followed by the formation of limb buds covered with an AER. Formation and maintenance of the AER is central for limb outgrowth because removal of the AER from a growing limb bud causes limb truncations (Saunders, 1948; Summerbell, 1974). The role of FGF-

8 in the formation of the AER remains an open question. It has been suggested that the fate of those cells expressing *Fgf-8* during budding is subsequently to form the AER (Ohuchi et al., 1994; Mahmood et al., 1995). However, although FGF-8 is able to induce an AER, flank mesenchyme can not maintain an AER (Zwilling, 1961, 1972; Searls and Zwilling, 1964), suggesting that the effects of FGF-8 beads have to be other than just providing an AER.

Based on evidence from a chick *limbless* mutant, Carrington and Fallon (1988) suggested that the initial budding of the mesenchyme and AER activity are two distinct and separate phases of limb initiation with limb budding preceding the formation of the AER. FGF-8 bead application seems to recreate interactions in the flank tissue that normally occur in the pre-limb territories by first inducing mesenchyme cells to proliferate, which will cause budding and, subsequently, the appearance of an AER. These two events are necessary to later induce *Shh* expression (about 24 hours later after bead application) which is central to establish a polarizing region and, hence, limb development (Cohn et al., 1995). Preliminary results suggest that these two initial effects (budding of the mesenchyme and AER formation) are indeed separate events and that their uncoupling prevents limb development from occurring: before budding, limb development is inhibited when mesoderm signaling is blocked by placing a foil barrier between the mesonephros and the lateral plate (Stephens et al., 1991) and limb outgrowth can not be rescued by application of FGF-8 or *Shh*/RA alone (data not shown). This is reminiscent of what happens later on, once the limb bud outgrowth has initiated, where the grafting of a ZPA, and either RA or *Shh* application at the anterior of the limb margin do not induce digit duplication or limb outgrowth, unless an AER or an FGF source is present (Izpisúa Belmonte et al., 1992; Koyama et al., 1993; Laufer et al., 1994; Niswander et al., 1994a).

FGF-8 could act on the mesoderm by ectopically inducing some gene(s), which in turn, directly or indirectly, would regulate polarizing potential in both limb and flank mesoderm. *Hox* genes are good candidates for such genes. In *Xenopus*, it is known that FGF-2 can activate some of the posterior *Hox* genes (Ruiz i Altaba and Melton, 1989; Cho and De Robertis, 1990) and, during mouse limb development, extending the expression limits of *Hoxb-8* to the anterior side of the forelimb bud causes digit duplications that are preceded by ectopic *Shh* expression at the anterior of the limb (Charité et al., 1994). It has been suggested that the activation of *Hox* genes is an intrinsic property of rapidly dividing cells (Duboule, 1994). Thus, FGF-8, acting as a mitogenic agent, could maintain mesenchyme cells at a high mitotic rate and, via *Hox* gene activation, convert flank cells with potential polarizing activity into actual polarizing cells through the activation of *Shh* (Cohn et al., 1995). This could explain how polarizing region signalling is established during normal limb initiation. *Fgf-8*, localized to the presumptive limb field ectoderm, could be a key component of this pathway.

Although FGF-8, by virtue of being expressed in the intermediate mesoderm at the limb field level, could be the limb-inducing signal, FGF-8 transcripts are also detected anterior to the wing field and in the flank mesonephros from stage 17 onwards, yet no limbs develop. This could be explained by postulating a mechanism that uncouples limb budding of the

mesenchyme from AER formation in the presumptive flank and neck regions. This model would involve the presence of a limb inhibitor or a reduced competence to form limbs in flank mesoderm. Ectopic application of FGF-8 beads between the intermediate mesoderm and ectoderm would overcome this repression or reduced competence, allowing limb development to occur.

Skeletal alterations induced by FGF-8

Apart from inducing ectopic limbs, misexpression of FGF-8 during limb outgrowth causes skeletal anomalies that range from truncations, deletions, reduction and fusions of mainly the more proximal bones to the appearance of extra digits. These effects were more pronounced when misexpression of FGF-8 was done by using a continuous and/or widespread source of FGF-8 (virus or transfected cells), although they were also observed with FGF-8 or FGF-2 bead implants. Viral infection into the presumptive flank was also able to induce flank outgrowth. However, after certain time, this outgrowth regressed and did not result in ectopic limbs. Mima et al. (1995) have shown a similar phenomenon after FGF-4 viral infection. Moreover, *in vitro*, mouse FGF-8 does not seem to maintain expression of *Shh* after AER removal (Mahmood et al., 1995) and extremely high concentrations of FGF-4 seem to inhibit limb growth (Niswander et al., 1994b). Our results show that application of FGF-8- and FGF-2-soaked beads in or close to the wing field results in the development of truncated wings, which correlates with loss or down regulation of *Shh* expression in posterior mesenchyme of the developing wing bud. Furthermore, *Fgf-8*-transfected cells grafted to the flank or into the limb field at early embryonic stages appear to influence the position and extent of AER formation in the future bud. Therefore, variations in the dose, mode, place and time of FGF application seem to be critical. Although the kinetics of uptake and release of FGF-8 by the beads is not known, they probably bind very little in comparison with the protein delivered by the viral construct or the transfected cells. Localized and transient misexpression of FGF-8 (bead implants) at a time at which *Fgf-8* transcripts appear in the limb ectoderm that will form the AER, induces complete limbs. A higher and continuous dose of FGF-8, or misexpression before *Fgf-8* expression is activated during normal development in the presumptive flank and limb regions have adverse effect for normal limb development.

Changes, either in the timing of mesenchymal cell condensation or in the quantity of mesoderm available to condense are a major source of morphological variations during vertebrate limb phylogeny (Hinchliffe and Johnson, 1980 and references therein). A constant stimulation of cell division caused by the continuous presence of FGF-8 (either by grafted cells or by viral infection) could alter the ratio of proliferating versus differentiating cells leading to shortening or full elimination of skeletal elements. Thus, the paradoxical phenotype of shortening or absence of the more proximal bones combined with extra digits could be interpreted as a shift in the boundaries of the prospective chondrogenic areas.

Misexpression of *Fgf-8* not only causes limb abnormalities but also affects other skeletal processes. A high percentage of the operated embryos showed a reduction in overall body size and appeared to be developmentally younger compared to controls. Changes were observed in the number and mor-

phology of vertebral units and ribs. *Fgf-8* effects were also extended to the tail bud. As with the limb phenotype, the alterations observed suggest that FGF-8 can affect the formation of skeletal structures by interfering with local cell growth, differentiation or survival. The most extreme phenotype observed is shown in Fig. 4D, where the reduction of limb cartilage is accompanied by an extreme shortening of all skeletal elements of the rest of the embryo. This phenotype is clearly reminiscent of a human genetic disease, achondroplasia. This is the most common genetic form of dwarfism characterized by disproportionate shortening of the long bones of the limbs, exaggerated lumbar lordosis, and generalized skeletal abnormalities (Smith and Jones, 1982). Mutations in the FGF-receptor 3 are implicated in achondroplasia (Rousseau et al., 1994; Shiang et al., 1994; Bellus et al., 1995; Superti-Furga et al., 1995; Stoilov et al., 1995) and mutations in FGF-receptor genes appear to be responsible for several human disorders of bone growth and patterning (Jabs et al., 1994; Reardon et al., 1994; Lajeunie et al., 1995; Rutland et al., 1995; Tavormina et al., 1995; Wilkie et al., 1995a). It has been suggested that the biological consequences of mutations in the IgIII-domain of FGF receptors result from ligand-independent FGF signaling (see Wilkie et al., 1995b for a review) i.e. the receptors are hyperactive. Thus, continuous FGF signaling could be responsible for the observed skeletal anomalies. The different anomalies observed after FGF-8 misexpression might be a direct effect of FGF-8 or might result from an interference between FGF-8 and other members of the family, like FGF-2 or FGF-4.

FGFs and limb outgrowth and patterning

After stage 17 the outgrowth of the limb bud is controlled by the AER because its removal prevents further limb outgrowth and patterning. The ridge can be substituted by local application of FGF-2 and FGF-4 (Niswander et al., 1993, 1994a; Vogel and Tickle, 1993; Fallon et al., 1994; Laufer et al., 1994; Vogel et al., 1995). In this study, we show that FGF-8, like FGF-2 and FGF-4, can substitute for the ridge to maintain outgrowth and patterning of the developing chick limb. Application of FGF-8 stimulates local proliferation of chick limb mesenchyme *in vivo* (this study) and mouse mesenchyme cells *in vitro* (Mahmood et al., 1995). As it is the case for FGF-4 (Yang and Niswander, 1995; Kostakopoulou et al., 1996), FGF-8 is able to induce *Shh* expression in proximal mesenchyme of the chick limb. Following partial or entire ridge removal, FGF-8, like FGF-4 (Laufer et al., 1994; Niswander et al., 1994a), is also able to maintain expression of *Shh* and *Hoxd-13*. This is in contrast to the *in vitro* study by Mahmood et al. (1995) who have shown that, in the absence of the ridge, FGF-8 is able to maintain limb outgrowth, whereas *Shh* expression is not maintained. It is possible that this difference might be due to the different experimental set up or that in our experiment a higher FGF-8 concentration was delivered to posterior mesenchyme. Alternatively it could be due to the different isoform used. FGF-4, specifically localized to the posterior ridge, could be the endogenous signal that maintains *Shh* expression in posterior mesenchyme of the developing limb. However, *ld* mutant limb buds show low levels of *Shh* expression in posterior mesenchyme in the absence of *Fgf-4* but in the presence of low levels of *Fgf-8* transcripts in the AER. Thus, it is possible that other FGFs, possibly FGF-4,

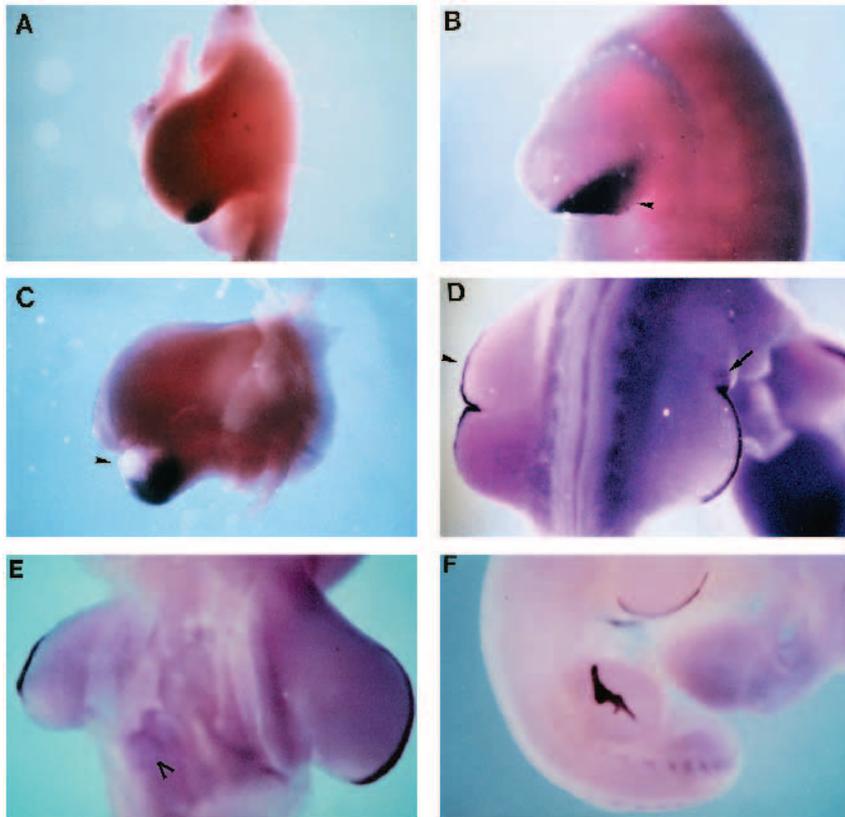


Fig. 6. *Fgf-8* and *Shh* expression pattern after FGFs application. (A) Normal expression pattern of *Shh* in a stage 24 wing bud. Expression is located to the posterior distal mesenchyme. (B) Following entire ridge removal of a stage 22 chick limb bud and proximal application of FGF-8, *Shh* expression is maintained/induced in the posterior mesenchyme 24 hours following the operation. Note the presence of *Shh* mRNA in the proximal part of the limb (arrow). (C) Maintenance of *Shh* expression in the posterior distal mesenchyme 24 hours following entire ridge removal and application of an FGF-8-soaked bead to the posterior part of the wing bud. (D) Following application of an FGF-2-soaked bead to the right flank of a stage 15/16 chick embryo an additional limb bud developed on the left side. By 20 hours after the bead implantation, the *Fgf-8* expression is covering the entire bud. Notice a small patch of ectopic *Fgf-8* expression on the opposite side, where the bead was applied (arrow). (E) *Fgf-8* expression in the developing forelimbs following a graft of *Fgf-8*-transfected cells. Cells were grafted opposite somite 21 of a stage 14 chick embryo. The forelimb on the operated side is shortened in size compared to the contralateral limb and *Fgf-8* expression (AER formation) is absent posteriorly. The arrow shows the localisation of the grafted cells, which do not appear to express *Fgf-8*. Ventral view. (F) *Fgf-8* expression in the developing leg

bud of a stage 24/25 chick embryo following a graft of *Fgf-8*-transfected cells that had been placed into the leg field at stage 16 of development. *Fgf-8* expression appears to be diffuse and the AER appears to be delocalised to a more dorsal position.

might be also involved in maintaining *Shh* expression (Chang et al., 1995; Hamaris et al., 1995).

The question now is which member/s of the FGF family is responsible for limb initiation, if any, and which one/s is required for the continued outgrowth and patterning of the vertebrate limb. Various studies, mainly in vitro, have shown that specific receptor types are used for specific FGFs. During limb development FGFR1 is expressed in limb mesenchyme and FGFR2 is expressed in limb ectoderm (Orr-Urtreger et al., 1991; Peters et al., 1992). Since both receptors can bind FGF-1, FGF-2 and FGF-4, and FGF-8 binds FGFR1 (Johnson et al., 1990; Mansukhani et al., 1990; Miki et al., 1992; Werner et al., 1992; Orr-Urtreger et al., 1993; Kouhara et al., 1994), most likely, ligand availability is determinant. The spatiotemporal pattern of expression of *Fgf-8* prior to limb outgrowth and the fact that FGF-2 beads induce *Fgf-8* expression in the additional limbs suggests that limb initiation involves, possibly together with a mesoderm signal/s, the activation of *Fgf-8*. Subsequently, once limb budding is on its way, FGF-8, in conjunction with FGF-2 and FGF-4, by means of their asymmetric expression, may control outgrowth and, concomitantly, patterning of the developing limb bud, by regulating the timing and extent of local growth and differentiation rates. The molecular basis of these interactions is not known, but changes in the temporal or spatial expression or in the relative ratios of the different FGFs will likely be a major cause of skeletal alterations by altering, either directly or indirectly, the expression of key genes like *Shh* and *Hox*. Elucidation of the molecular

nature of these interactions will not only provide a better understanding of some human skeletal malformations like achondroplasia or those induced by thalidomide, but will also be helpful to understand the evolutionary process leading to the appearance of the tetrapod limb diversity.

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