

# The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor

Hideyo Ohuchi<sup>1</sup>, Takashi Nakagawa<sup>1</sup>, Atsuyo Yamamoto<sup>1</sup>, Akihiro Araga<sup>1</sup>, Takeshi Ohata<sup>1</sup>, Yoshiyasu Ishimaru<sup>1</sup>, Hidefumi Yoshioka<sup>1</sup>, Takashi Kuwana<sup>2</sup>, Tsutomu Nohno<sup>3</sup>, Masahiro Yamasaki<sup>4</sup>, Nobuyuki Itoh<sup>4</sup> and Sumihare Noji<sup>1,\*</sup>

<sup>1</sup>Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, Tokushima 770, Japan

<sup>2</sup>Pathology Section, National Institute for Minamata Disease, Minamata, Kumamoto 867, Japan

<sup>3</sup>Department of Molecular Biology, Kawasaki Medical School, Kurashiki, Okayama 701-01, Japan

<sup>4</sup>Department of Genetic Biochemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto 606-01, Japan

\*Author for correspondence (e-mail: noji@bio.tokushima-u.ac.jp)

## SUMMARY

Vertebrate limb formation has been known to be initiated by a factor(s) secreted from the lateral plate mesoderm. In this report, we provide evidence that a member of the fibroblast growth factor (FGF) family, FGF10, emanates from the prospective limb mesoderm to serve as an endogenous initiator for limb bud formation. *Fgf10* expression in the prospective limb mesenchyme precedes *Fgf8* expression in the nascent apical ectoderm. Ectopic application of FGF10 to the chick embryonic flank can induce *Fgf8* expression in the adjacent ectoderm, resulting

in the formation of an additional complete limb. Expression of *Fgf10* persists in the mesenchyme of the established limb bud and appears to interact with *Fgf8* in the apical ectoderm and *Sonic hedgehog* in the zone of polarizing activity. These results suggest that FGF10 is a key mesenchymal factor involved in the initial budding as well as the continuous outgrowth of vertebrate limbs.

Key words: FGF10, lateral plate mesoderm, limb initiation, FGF8, epithelial-mesenchymal interaction

## INTRODUCTION

The chick limb stands as an ideal model system to elucidate the mechanisms that coordinate growth and patterning during vertebrate development. In most vertebrates, the limb emerges as two pairs of bulges, limb buds, from the thickened lateral plate mesoderm at the axial levels of the cervical-thoracic and lumbosacral boundaries (Burke et al., 1995). The ectoderm surrounding the distal tip of the limb bud is then induced by the mesenchyme to thicken and form a specialized epithelial structure, the apical ectodermal ridge (AER; Saunders, 1948). Once the limb bud is formed, the cartilaginous elements are formed according to positional information established by signaling centers such as the AER and the zone of polarizing activity (ZPA) in the posterior mesoderm (Saunders and Gasseling, 1968). Recent studies have revealed much about the role of signaling molecules during limb pattern formation; Sonic hedgehog (SHH) most likely acts as a mediator of ZPA polarizing activity (Riddle et al., 1993), whereas members of the fibroblast growth factor (FGF) family can mimic the function of the AER (Niswander et al., 1993; Fallon et al., 1994; Crossley et al., 1996). Furthermore, with regard to patterning along the dorsoventral (DV) axis of the limb bud, several factors, such as WNT7a, LMX1 and EN1, have been shown to be involved in concert with other signaling molecules (Yang and Niswander, 1995; Riddle et al., 1995; Vogel et al., 1995; Loomis et al., 1996).

Many investigations have also focused on elucidating the cellular and molecular events in the initial phase of limb development. In the chick embryo, it has been demonstrated that, when prospective limb mesoderm is implanted into the host embryonic flank, an extra limb is formed in the flank through induction of a new AER (Saunders and Reuss, 1974). Furthermore, it was shown that the implanted prospective limb mesoderm can recruit host flank cells to become a part of the extra limb (Dhouailly and Kieny, 1972). However, prospective flank mesoderm does not induce an extra limb upon implantation in a host flank, indicating that this limb-forming ability is restricted to the mesoderm at the axial levels of the prospective limbs at stages 12-17 (Hamburger and Hamilton, 1951). From these experimental results, it seems likely that a certain factor present in the prospective limb mesoderm acts to induce limb bud formation. Since in other animals such as newts, the primordia of the ear, nose and pituitary gland can induce additional limbs when implanted in the embryonic flank, it has been suggested that the limb-inducing factor is not tissue specific (for a review, Balinsky, 1965).

Recently several enlightening studies have been done to clarify molecules involved in limb induction. Members of the FGF family, which have been shown to possess a ridge function, can induce additional limbs in the chick embryonic flank, upon implantation as FGF beads or *Fgf*-expressing cells. Such an ability has been shown for FGF1, FGF2, FGF4 and FGF8 (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al.,

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chick FGF10 1:MWKWLITNGASAFSHLP--CCCLLLLFLVSSVPVTC HDLGDQDMSPEATN-SSSSSSSSSF 57
rat FGF10 1:MWKWLITHCASAFPHLPGCCCFLLFLVSSVPVTC QALGDQDMSPEATNSSSSSSSSSS 60
mouse FGF7 1:MRKWILT--RILPTLLY--RSCFHLVCLVGTISLAC---NDM-SPEQT-----AT 42

chick FGF10 58:PSSFSSPSSAGRHRVRSYNHLQ-GDVRKRKLYSYNKYFLKIEKNGKVS GTKKENCPSILE 116
rat FGF10 61:SSSFSSPSSAGRHRVRSYNHLQ-GDVRWRKLFSTFKYFLKIEKNGKVS GTKKENCPSILE 119
mouse FGF7 43:SVNCSSP---ERHTRS YDYMEGGDIRVRLFCRTQWYLRIDKRGVKGTQEMKNSYNIME 99

chick FGF10 117:ITSVEIGVVAVKSIKSNYYLAMNKKGVYGSKEFNSDCKLKERIEENGYNTYASLNWKHN 176
rat FGF10 120:ITSVEIGVVAVKAINSNYYLAMNKKGKLYGSKEFNNDCKLKERIEENGYNTYASFNWQHN 179
mouse FGF7 100:IRTVAVGIVAIAKGVSEYYLAMNKEGKLYAKKECNECDNFKELILENHYNTYASAKWTHS 159

chick FGF10 177:GRQMFVALNGRGATKRGQKTRRKN TSAHFLPMVVMSS 212
rat FGF10 180:GRQMYVALNGKGAPRRGQKTRRKN TSAHFLPMVVHS 215
mouse FGF7 160:GGEMFVALNQKGI PVKGGKTKKEQKTAHFLPMAIT- 194

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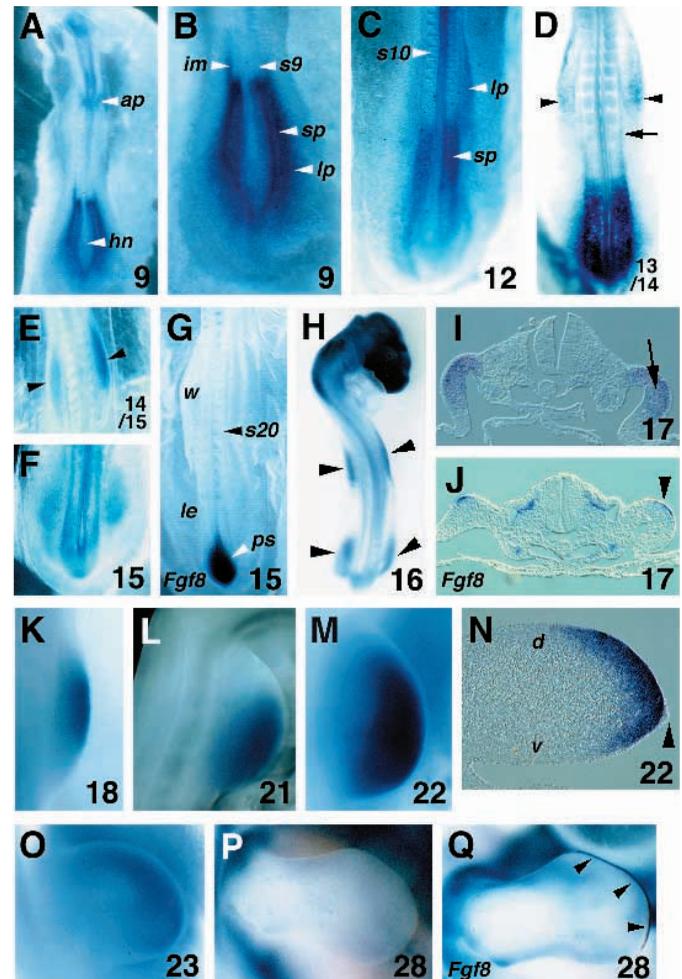
**Fig. 1.** Predicted amino-acid sequence of the chick FGF10 protein in comparison with rat FGF10 and mouse FGF7. Identical residues are enclosed by shaded boxes and dashes represent gaps inserted to allow alignment of homologous residues.

1996; Vogel et al., 1996; for a review, Cohn and Tickle, 1996). However, with the exception of FGF8, none of the other FGF members are likely to function as endogenous signaling factors for limb bud induction as their expression domains are not restricted to the prospective limb territories in chick and mouse embryos (Savage et al., 1993; Niswander and Martin, 1992; Ohuchi et al., 1994; Crossley and Martin, 1995; Mahmood et al., 1995; reviewed by Slack, 1995). Therefore, it is likely that ectopically applied FGFs may merely be mimicking the function of the endogenous limb-inducing factor. In the case of FGF8, Crossley et al. (1996) demonstrated that it is expressed in the intermediate mesoderm but not in the lateral plate mesoderm, and plays a key role in the induction and initiation of chick limb development. They also suggested that the FGF8 in the intermediate mesoderm may be responsible for the induction of its own expression in the prospective apical

ectoderm indirectly through the lateral plate mesoderm. Thus, the possibility exists that there is an unidentified endogenous factor in the lateral plate mesoderm that induces expression of *Fgf8*.

During the course of our search for the endogenous limb-inducing factor, a new FGF member was identified in rat embryos (Yamasaki et al., 1996). To test whether this new FGF member might be an endogenous initiator of limb bud formation, we cloned a chick *Fgf10* cDNA and examined its expression pattern. In this paper, we show that *Fgf10* is initially widely expressed in the lateral plate mesoderm of early chick embryos, becomes subsequently restricted to the prospective limb mesoderm and, finally, is restricted to the definitive limb mesenchyme. We also demonstrate that implantation of *Fgf10*-

**Fig. 2.** *Fgf10* expression in chick early embryos and developing limbs. For comparison, *Fgf8* expression is shown (G,J,Q). (A-H) Embryos are viewed dorsally; (A-H, K-M, O-Q) with anterior to the top. The numbers in the bottom corners of each panel indicate the embryonic stage. (A,B) *Fgf10* is expressed in the posterior region where neurulation is still taking place. Low levels of expression are observed in the auditory placodes (ap). hn, Hensen's node. (B) Higher magnification of A. *Fgf10* is expressed in the intermediate mesoderm (im), segmental plate (sp) and lateral plate mesoderm (lp); s9, somite 9. (C) *Fgf10* is expressed in the lateral plate mesoderm at, and posterior to, the level of somite 10 (s10). (D) Arrowheads indicate a weak *Fgf10* expression in the prospective forelimb mesoderm at the level of somite 19. *Fgf10* expression in the prospective interlimb region is downregulated. The arrow indicates *Fgf10* expression in the mesonephros. *Fgf10* is expressed intensely in the caudal segmental plate. (E,F) *Fgf10* is distinctly expressed in the prospective forelimb mesoderm (arrowheads, E) and in the prospective hindlimb mesoderm (F). (G) *Fgf8* has yet to be expressed in the prospective wing (w) and leg (le) regions, while it can be detected in the primitive streak region (ps). s20, somite 20. (H) *Fgf10* is expressed in the developing head region and prospective limb mesoderm (arrowheads). (I,J) Cross sections through the wing buds. *Fgf10* is expressed in the mesenchyme of the limb bud (arrow, I) while *Fgf8* is expressed in the limb ectoderm at the dorsoventral boundary, where the AER will develop (arrowhead, J). (K) *Fgf10* is expressed in the apical mesenchyme of the wing bud. (L) *Fgf10* is expressed preferentially in the wing posterior mesenchyme. (M) Intense *Fgf10* expression in the wing bud mesenchyme. (N) Cross section through the wing bud. *Fgf10* expression is predominantly expressed in the dorsal (d) mesenchyme. The arrowhead indicates the AER. v, ventral. (O) The level of *Fgf10* expression decreases by stage 23. *Fgf10* RNA becomes undetectable in the wing bud (P) but *Fgf8* RNA can be detected in the regressing AER (arrowheads, Q).



expressing cells gives rise to an extra limb in the competent embryonic flank through induction of *Fgf8* expression in the ectoderm. Moreover, FGF10 can induce expression of *Fgf8* in the ectoderm and *Shh* in the posterior mesoderm of the AER-removed limb bud. These results suggest that FGF10 is an endogenous mesenchymal factor involved in the initial budding and the continuous outgrowth of vertebrate limb buds.

## MATERIALS AND METHODS

### Isolation of the chick *Fgf10* cDNA

A 555 bp fragment of the 5' coding region of *Fgf10*, cf10-111 was isolated from stage 23 chick limb bud cDNA by polymerase chain reaction (PCR). Degenerate PCR primers were designed to target the amino acids, MWKWILT (5' primer) and MYVALNG (3' primer), which are highly conserved between rat FGF10 (Yamasaki et al., 1996) and mouse FGF7 (Mason et al., 1994). The entire coding region of the chick *Fgf10* cDNA was obtained by means of 5'- and 3'-rapid amplification of cDNA ends (RACE; Frohman et al., 1988) (MarathonTM cDNA Amplification Kit, Clontech). The obtained clone encoded a protein with 87% amino acid identity to the rat FGF10 (Fig. 1) and its expression pattern closely matched that of rat *Fgf10*, which we examined briefly (data not shown). As is the case for rat FGF10, chick FGF10 has the highest amino acid sequence identity with mouse FGF7 and chick FGF3 in the conserved core region (50-55%; amino acids 79-170 and 181-209) and the similarity with mouse FGF7 persists even outside this conserved region (Fig. 1). The nucleotide sequence of the chick *Fgf10* cDNA is deposited in the DDBJ/EMBL/GenBank database under the accession number: D86333.

### Whole-mount RNA in situ hybridization

Whole-mount in situ hybridization was performed essentially as described by Wilkinson (1992) and Riddle et al. (1993), except that embryos were dehydrated and rehydrated through an ascending or a descending ethanol series in PBT. The following probes were used for in situ hybridizations: *Fgf10*, cf10-111; *Fgf8*, a 495 bp fragment including coding sequences (Ohuchi et al., 1997); *Shh*, a 1.3 kb fragment including coding sequences (Nohno et al., 1995). Whole embryos were observed using a Leica zoom stereomicroscope. Selected embryos were processed for paraffin sections as described by Sasaki and Hogan (1993). Sections were observed with Nomarski optics using a Leica DMR microscope.

### Recombinant retroviral construction and production

The coding regions of the rat *Fgf10* (Yamasaki et al., 1996), chick *Fgf8b* (Ohuchi et al., 1997) and chick *Shh* (Nohno et al., 1995) cDNAs were subcloned into a ClaI2Nco shuttle vector (Hughes et al., 1987) and the resultant plasmids designated as Cla-*Fgf10*, Cla-*Fgf8b* and Cla-*Shh*, respectively. Subsequently, these plasmids were digested with ClaI and the inserts were subcloned into an avian retrovirus vector RCASBP(A) (Hughes et al., 1987), generating RCAS-*Fgf10*, RCAS-*Fgf8b* and RCAS-*Shh*. RCASBP(A) contains an A-type envelope protein that is able to infect embryonic fibroblasts derived from a specific pathogen-free (SPF) White Leghorn chick embryo (Nisseiken Co., Tokyo) but unable to infect the strain (Yamagishi Co., Tokushima) used as host embryos in this study as confirmed in control experiments (data not shown). Chick embryo fibroblast (CEF) cultures were grown and transfected with retroviral vector DNA as described (Kuwana et al., 1996; Fekete and Cepko, 1993). The supernatants of CEF cultures transfected with the viral DNAs were aliquoted and stored at -80°C until further use.

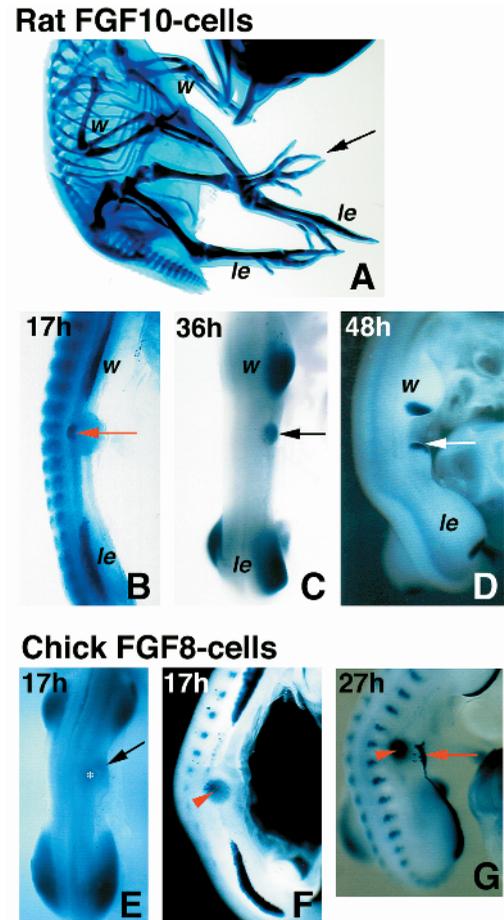
### Cell implants

12.5 cm<sup>2</sup> flasks containing SPF-CEFs infected with either RCASBP/AP(A) (Fekete and Cepko; 1993), RCAS-*Fgf10*, RCAS-

*Fgf8b* or RCAS-*Shh* were grown to 100% confluence, lightly trypsinized and processed for preparation of cell implants as described by Riddle et al. (1993). We confirmed that implantation of CEFs alone or those infected with RCASBP/AP(A) has no effect on any of the embryos examined.

### Experimental manipulations of chick embryos

Fertilized chicken eggs were incubated at 38°C and the embryos were



**Fig. 3.** Induction of additional limb formation by FGF10 and analysis of *Fgf10* and *Fgf8* expression in FGF-induced limb buds.

(A) Skeletal preparation of a whole embryo following implantation of FGF10 cells in the prospective interlimb region at stage 13. An extra leg-like limb is indicated by arrow. w, wing; le, leg. (B) Lateral view of an embryo 17 hours after the implantation at stage 13. Ectopic *Fgf8* expression in the flank ectoderm is indicated by arrow. (C) Dorsolateral view of an embryo 36 hours after the implantation at stage 12/13. The arrow indicates ectopic *Fgf10* expression in the flank mesenchyme. (D) Dorsolateral view of an embryo 48 hours after the implantation at stage 13. The arrow indicates ectopic *Shh* expression in the anterior mesenchyme of the additional limb bud. (E-G) Dorsolateral (E,G) and lateral (F) views of embryos following implantation of FGF8 cells at stages 14-15. The arrowheads in F,G indicate *Fgf8* expression in the implanted cells. (E) Detection of chick *Fgf10* RNA 17 hours later. Ectopic *Fgf10* expression in the flank mesenchyme on the implanted side is indicated by arrow. To reveal the site of the implanted cells, we used CEFs expressing the bacterial *lacZ* gene. The asterisk indicates the cells stained with X-gal. (F,G) Detection of chick *Fgf8* RNA 17 hours, 27 hours later, respectively. (F) No ectopic *Fgf8* expression in the flank ectoderm. (G) Note that ectopic *Fgf8* expression in the ectoderm of the nascent additional limb bud (arrow).

staged according to Hamburger and Hamilton (1951). *Fgf*-expressing cells were implanted into the lateral plate mesoderm of chick embryos at stages 12-15 as described (Ohuchi et al., 1995). Alternatively, *Fgf*- or *Shh*-expressing cells were applied to the mesoderm of the wing bud of stage 19-20 embryos with or without the AER as described (Niswander et al., 1993; Riddle et al., 1993). The embryos were examined the next day and the position of the cells within the flank or the limb bud was recorded. Embryos in which the cells were no longer present in the flank or the limb bud were excluded. Embryos at appropriate stages were fixed in 4% paraformaldehyde in PBS and either processed for RNA in situ hybridization or stained with alcian blue to visualize the cartilage structures as described previously (Cohn et al., 1995).

## RESULTS

### *Fgf10* expression becomes restricted to the prospective limb mesoderm

The pattern of *Fgf10* expression in chick embryos was revealed by whole-mount in situ hybridization. In addition to the developing limbs, *Fgf10* is expressed in the developing brain and sense organs, but this study focuses mainly on the analyses of the limb bud expression. At stage 8/9, *Fgf10* can first be seen in the segmental plate from which somites arise (data not shown) and subsequently can be found in the adjacent intermediate and lateral plate mesoderm (Fig. 2A,B). By stage 12, *Fgf10* is expressed in the segmental plate at high levels and increases in the lateral plate mesoderm (Fig. 2C). Down regulation of *Fgf10* expression in the prospective flank mesoderm at and below the level of somite 20 can be seen at stage 13/14 (Fig. 2D). Expression becomes more localized to the prospective forelimb mesoderm at stage 14/15 and to the prospective hindlimb mesoderm at stage 15 (Fig. 2E,F). By stage 16, its expression can be clearly observed in the prospective mesoderm of both limbs (Fig. 2H). In this manner, *Fgf10* expression progresses from its broad expression in early mesoderm to become restricted to the prospective limb mesoderm.

### *Fgf10* expression in initiation of limb bud outgrowth

We compared the temporal expression of *Fgf10* in the prospective limb mesoderm in relation to emergence of *Fgf8* expression in the prospective limb ectoderm. *Fgf8* expression is not present in the prospective limb territories at stage 15 (Fig. 2G). It first emerges in the prospective forelimb ectoderm at early stage 16 and in the prospective leg ectoderm at late stage 16, as reported previously (Fig. 2G and data not shown; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Examination of cross sections of hybridized embryos confirmed a complementary expression of *Fgf10* and *Fgf8* in the limb mesoderm and ectoderm (Fig. 2I,J). Therefore *Fgf10* expression in the prospective limb mesoderm precedes *Fgf8* expression in the future limb ectoderm.

It has been suggested that one of the earliest indications of limb bud formation is emergence of *Fgf8* expression in the prospective limb ectoderm at the prospective DV boundary (Crossley et al., 1996; Vogel et al., 1996). Also, it has been postulated that this ectodermal expression of *Fgf8* is initiated by a limb inducer from the intermediate mesoderm through a signal from the lateral plate mesoderm (Crossley et al., 1996).

Therefore, FGF10 is a good candidate for the lateral plate mesoderm factor that induces *Fgf8* expression in the ectoderm.

### *Fgf10* expression in the established limb bud

Since our preliminary data revealed that *Fgf10* is distinctly expressed in the rat limb bud at later stages, we sought to determine whether, in chick, it is also expressed in established limb buds. The level of *Fgf10* expression in the limb mesoderm seemed to increase from stage 17, peak at stage 22 and gradually decrease (Fig. 2K-O). By stage 28, when digits begin to be separated by grooves, *Fgf10* expression in the limb mesenchyme is no longer detectable, while *Fgf8* expression can still be weakly observed in the regressing AER (Fig. 2P,Q).

*Fgf10* expression in the established limb bud was not uniform, but was detected at higher levels in the posterior region. This predominantly posterior expression can be observed at stages 20-21 (Fig. 2L), after which the domain of the expression expands anteriorly (Fig. 2M). In addition, at stage 22, a dorsal predominant expression can be found in the wing bud (Fig. 2N) and thereafter in the leg bud (data not shown). These graded expression patterns of *Fgf10* suggest that, in developing limbs, FGF10 may interact with posterior factors such as SHH and FGF4, and dorsal ones such as WNT7a and LMX1.

### *Fgf10*-expressing cells induce additional limb formation in the flank

From the aforementioned early expression pattern of *Fgf10*, we assumed that FGF10 is likely to be an endogenous initiator of limb bud formation in the lateral plate mesoderm. Therefore, we tested whether exogenous FGF10 can induce formation of an additional limb in the chick embryonic flank. For ectopic application of FGF10, we prepared rat FGF10-producing cells by infection of a recombinant replication-competent retrovirus. As a control, we also prepared chick *Fgf8*-expressing cells and implanted the cells in the chick embryonic flank. We observed that an ectopic limb was formed in the flank when the FGF8 cells were implanted at stages 14-15 (10 of 12 cases, 83%; Table 1) as previously reported (Vogel et al., 1996). These results are similar to those obtained by implantation of an FGF8 protein-soaked bead (Vogel et al., 1996; Crossley et al., 1996).

In a similar fashion, we implanted the rat FGF10 cells in the prospective flank region of chick embryos between stages 12 and 15 (Table 1) and found that when the implantation was done at stages 12-13, ectopic wing- and leg-like structures were induced after 7 days of incubation in 9 out of 21 cases (42%; Table 1 and Fig. 3A). Two of these ectopic limbs were clearly wing-like and 7 were leg-like (Table 1). In another 2 cases, digit-like structures were generated directly from the flank, articulated with the ribs. Another 3 cases resulted in induction of digit duplications in the authentic leg. Notably, when the implantation was done at stages 14-15, FGF10 had little effect on additional limb formation in the flank. The same experiment was performed with chick *Fgf10*-expressing cells and confirmed that chick FGF10 induces an ectopic limb when applied at stages 12-13 (5 of 8 cases; data not shown), but not at stages 14-15 ( $n=2$ ; data not shown). The expression pattern of *Fgf10* together with these results are consistent with the idea that FGF10 plays a key role in initial outgrowth of the prospective limb mesoderm in the chick embryo.

### Additional limb formation by FGF8 through induction of *Fgf10* expression and subsequent induction of *Fgf8* in the ectoderm

To determine whether formation of the additional limb by FGF10 involves the same mechanisms as authentic limb formation, we examined the expression patterns of *Fgf8*, *Fgf10* and *Shh* genes in FGF10-induced ectopic limb buds. Since essentially the same order of gene expression was observed during FGF4- and FGF8-induced limb bud formation by Crossley et al. (1996), we chose the FGF8-induced limb bud as a model for the FGF-induced limb buds reported so far. In the FGF8-induced limb bud, *Fgf10* RNA was detected around the implanted cells within 17 hours ( $n=7$ ; Fig. 3E). In contrast, *Fgf8* was not yet induced in the flank ectoderm at 17 hours ( $n=7$ ; Fig. 3F). In the 2 cases examined at 17 hours, *Fgf8* expression in the authentic leg ectoderm slightly elongated towards the ectopic limb bud (data not shown). At 27 hours, *Fgf8* expression was apparent in the ectoderm in 7 of 8 cases examined (Fig. 3G). In the case of the FGF10-induced ectopic limb buds, *Fgf8* was expressed in the flank ectoderm at 17 hours ( $n=3$ ; Fig. 3B). To determine whether ectopically applied FGF10 cells induce *Fgf10* expression in the surrounding mesoderm, we examined chick *Fgf10* expression in rat FGF10-induced limb buds. At 17 hours, chick *Fgf10* RNA was not detectable in the flank mesoderm ( $n=3$ ; data not shown). By 36 hours, ectopic chick *Fgf10* RNA was detected in the flank mesoderm ( $n=4$ ; Fig. 3C). In all cases examined, the expression patterns of these genes were normal on the contralateral side (data not shown). One interpretation of these data is that ectopically applied FGF10 initially induces ectopic *Fgf8* expression in the flank ectoderm and subsequently the induced *Fgf8* in the flank ectoderm reciprocally induces *Fgf10* expression in the underlying flank mesoderm. In contrast, in the case of FGF8-induced ectopic limb buds, exogenous FGF may induce *Fgf10* expression in the lateral mesoderm, which then induces *Fgf8* expression in the overlying flank ectoderm.

We observed abundant *Shh* expression in the anterior mesoderm of FGF10- and FGF8-induced prominent limb buds at 48 hours (3 of 4; Fig. 3D) and at 36 hours ( $n=2$ ; data not shown) after implantation, respectively. This result shows that the FGF10-induced limbs have a reversed polarity along the anteroposterior axis as is the case for those induced by other FGF members (Cohn et al., 1995; Ohuchi et al., 1995; Crossley et al., 1996; Vogel et al., 1996). This gene expression analysis during additional limb formation appears to indicate that mesodermal FGF10 induces *Fgf8* expression in the ectoderm and,

subsequently, *Shh* expression in the polarizing region of the additional limb bud. The order of gene expression of these signaling molecules during ectopic limb formation by FGF10 closely matches that during authentic limb formation, thus indicating that FGF10 may be the endogenous initiator for limb formation in the lateral plate mesoderm.

### Interaction between FGF10 and FGF8 in the established limb bud

The distinct expression of *Fgf10* in the established limb bud prompted us to study its function during later limb development. Since *Fgf10* is expressed in the limb mesenchyme beneath the AER, we tested whether *Fgf10* expression is dependent on the presence of the AER. Within 7 hours after AER removal at stage 20, the level of *Fgf10* expression decreased (data not shown) and is no longer detectable at 10 hours (Fig. 4A), in contrast to the unmanipulated contralateral side (Fig. 4B). Thus, it seems that *Fgf10* expression is AER dependent. Since *Shh* expression is also reported to be AER dependent (Laufer et al., 1994), the loss of *Fgf10* expression after extirpation of the AER could either reflect the direct requirement by *Fgf10* for the AER, or be an indirect consequence of the dependence of *Shh* expression on the AER. To distinguish between those possibilities, we tested whether the loss of *Fgf10* expression can be rescued by ectopically applied FGF8 in the anterior half of the limb bud, where *Shh* is not usually expressed. When the anterior half of the AER is removed, the limb bud becomes deformed due to underdevelopment of the anterior region (Fig. 4E), resulting in the loss of anterior bones such as the radius and digit 2 (compare Fig. 4C, D and H; Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1981). Under this condition, *Fgf10* was not expressed in the anterior mesoderm, while its expression remained in the posterior mesoderm (compare Fig. 4F and G). However, when *Fgf8*-expressing cells were implanted in the anterior mesoderm after removal of anterior AER (Fig. 4I), *Fgf10* expression was induced within 24 hours (Fig. 4J). Although the direction of limb outgrowth seemed to be altered laterally, anterior bones were restored and an almost normal bone pattern was observed at 10 days (Fig. 4L; Table 2). Therefore, it seems that the AER is required for *Fgf10* expression and that FGF8 is able to substitute for the AER to maintain *Fgf10* expression in limb mesenchyme.

Conversely, to see the effect of FGF10 on the AER, we implanted *Fgf10*-expressing cells in the anterior mesoderm after removal of anterior AER (Fig. 4M). We checked *Fgf8*

**Table 1. Effects of FGF-cells implanted in the lateral plate mesoderm of chick embryos between stages 12 and 15**

Host stages	FGF application	Additional limb development						
		Total <i>n</i>	S+Z+digits <i>n</i>	Z+digits <i>n</i>	Digit-like structure in the flank <i>n</i>	Only digit duplications in authentic limbs <i>n</i>	Others <i>n</i>	No additional limbs or digit duplications <i>n</i>
12-13	FGF10	21	1 <sup>a</sup>	8 <sup>a</sup>	2	3	1 <sup>b</sup>	6
12-13	None <sup>c</sup>	6	0	0	0	0	0	6
14-15	FGF10	17	0	0	0	1	1 <sup>d</sup>	15
14-15	FGF8	12	6	3 <sup>a</sup>	0	0	1 <sup>e</sup>	2 <sup>f</sup>

S, stylopod; Z, zeugopod; *n*, number of experimental samples.

<sup>a</sup>One zeugopod was formed in every specimen.

<sup>b</sup>Digit 1 was absent and digit 3 was thickened in the authentic leg of one specimen.

<sup>c</sup>CEFs without infection of the FGF-viruses were implanted as a control.

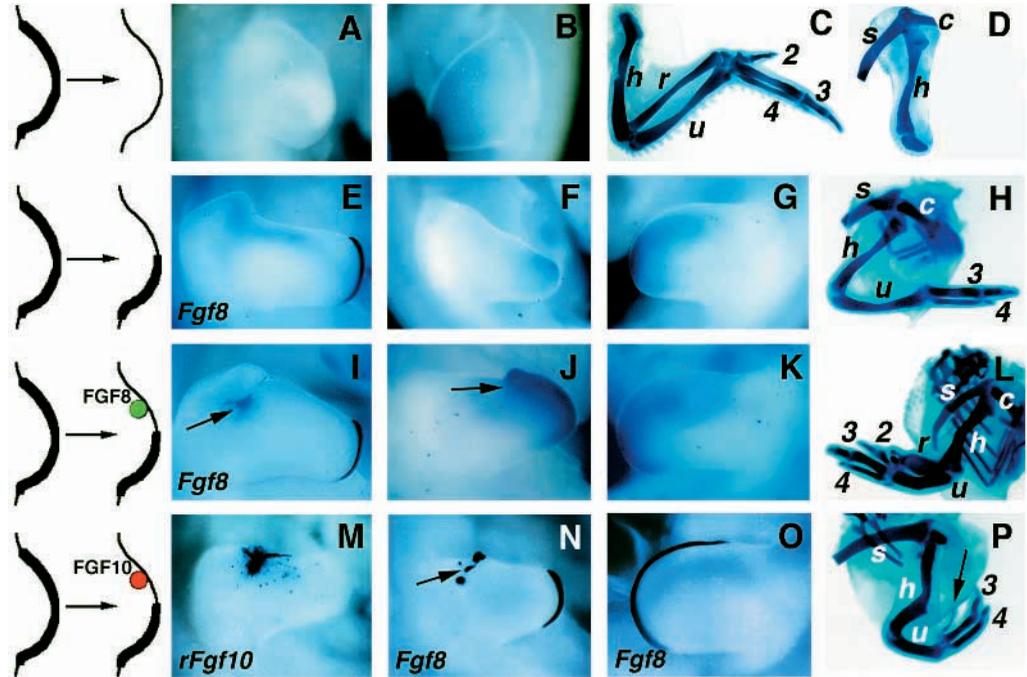
<sup>d</sup>One specimen developed an extra femur with no additional zeugopod nor digits.

<sup>e</sup>One specimen developed an extra femur and zeugopod with no additional digits.

<sup>f</sup>Wing truncation was seen in two specimens.

**Fig. 4.** *Fgf10* expression is dependent on the AER and can be rescued by FGF8 cells in the AER-removed wing bud.

Individual surgical protocols are indicated schematically on the left; the thickened line is the AER and the circles represent *Fgf*-expressing cells. Embryos were harvested after 10 hours (A,B), 24 hours (E-G, I-K, M-O) or 7 days (C,D,H,L,P) and processed for in situ hybridization or cartilage staining. Limb buds other than indicated were hybridized with the chick *Fgf10* probe. (A) *Fgf10* expression is lost in the wing bud mesenchyme. (B) Contralateral control wing bud for comparison. (C) Normal wing skeletal pattern at 10 days of incubation, showing a stylopod (h, humerus), two zeugopods (r, radius; u, ulna) and three digits (the digit number is 2 to 4, anterior to posterior). (D) Total AER removal at stage 19/20 results in a truncated wing at the proximal level of the zeugopod. c, coracoid; s, scapula. (E) *Fgf8* expression in a wing bud in which the anterior half of the AER was removed. (F) The anterior domain where *Fgf10* is usually expressed is lost after anterior AER removal. The contralateral wing bud is shown in G. (H) Anterior AER removal results in the absence of the radius and digit 2. (I) *Fgf10* expression in the implanted cells (arrow) and posterior AER. Note the mesenchymal outgrowth in the vicinity of the cells, compared with the wing bud in E. (J) The arrow indicates that *Fgf10* expression rescued by FGF8-cells, compared with the wing bud in F. The contralateral wing bud is shown in K. (L) FGF8 cells restore the cartilage pattern after 10 days of incubation. (M) Rat *Fgf10* is expressed in the implanted cells. (N) FGF10 cells induce a novel *Fgf8* expression in the adjacent ectoderm. The novel *Fgf8* expression domain is discontinuous to the posterior AER. (O) *Fgf8* expression in the contralateral wing bud. (P) FGF10 cells cannot restore the radius. The arrow indicates a thin digit 2.



(E) *Fgf8* expression in a wing bud in which the anterior half of the AER was removed. (F) The anterior domain where *Fgf10* is usually expressed is lost after anterior AER removal. The contralateral wing bud is shown in G. (H) Anterior AER removal results in the absence of the radius and digit 2. (I) *Fgf10* expression in the implanted cells (arrow) and posterior AER. Note the mesenchymal outgrowth in the vicinity of the cells, compared with the wing bud in E. (J) The arrow indicates that *Fgf10* expression rescued by FGF8-cells, compared with the wing bud in F. The contralateral wing bud is shown in K. (L) FGF8 cells restore the cartilage pattern after 10 days of incubation. (M) Rat *Fgf10* is expressed in the implanted cells. (N) FGF10 cells induce a novel *Fgf8* expression in the adjacent ectoderm. The novel *Fgf8* expression domain is discontinuous to the posterior AER. (O) *Fgf8* expression in the contralateral wing bud. (P) FGF10 cells cannot restore the radius. The arrow indicates a thin digit 2.

expression as an AER marker and found that *Fgf8* was induced in the ectoderm adjacent to the implanted cells ( $n=2$ ; Fig. 4N). Histological analysis showed that the ectoderm where *Fgf8* was ectopically expressed was thickened (data not shown), suggesting that an AER-like structure had been induced by ectopic FGF10. Since FGF10 is distributed widely in the mesenchyme of the normal limb bud but the AER is formed only in the DV boundary, there seem to be some mechanisms in the boundary region to prevent the dorsal and ventral ectoderm from forming extra AERs. However, once the distinct DV boundary is removed due to AER removal, those suppressing factors are likely eliminated, allowing exogenous FGF10 to give rise to an ectopic AER. Under those conditions, however, anterior bones were only partially rescued: often the radius was

missing (Fig. 4P; Table 2). This partial rescue by FGF10 may be attributed to insufficient induction of *Fgf8* expression in the ectoderm (compare Fig. 4N and O), that is, incomplete restoration of the AER.

#### Interaction between FGF10 and SHH

Since *Fgf10* is predominantly expressed in the posterior mesenchyme of the limb bud as shown in Fig. 2L, we suspected some interaction between FGF10 and SHH may occur. To study this possible interaction, we implanted *Shh*-expressing cells in the anterior margin of the wing bud to examine whether *Fgf10* expression could be induced by SHH. By 27 hours, the domain of *Fgf10* expression was found to expand to the anterior mesenchyme of the bifurcating wing bud ( $n=3$ ;

**Table 2. Analysis of skeletal elements formed after experimental manipulation of stage 20 limb buds**

Ridge removal	Cell position	FGF application	<i>n</i>	Humerus	Radius	Ulna	Digit 2	Posterior digit-like elements
Anterior	Anterior	FGF8	13	13	11 <sup>b</sup>	12 <sup>b</sup>	4 <sup>b</sup>	11 <sup>b</sup>
		FGF10	9	9	0	9 <sup>c</sup>	4 <sup>d</sup>	8
		None <sup>a</sup>	2	2	0	2	0	2
Posterior	Posterior	FGF8	8	8	8	8	8	5
		FGF10	8	8	8	6 <sup>e</sup>	8	4 <sup>f</sup>
		None <sup>a</sup>	3	3	3	1	0	0

*n*, number of experimental samples.

<sup>a</sup>No cells in any of the positions.

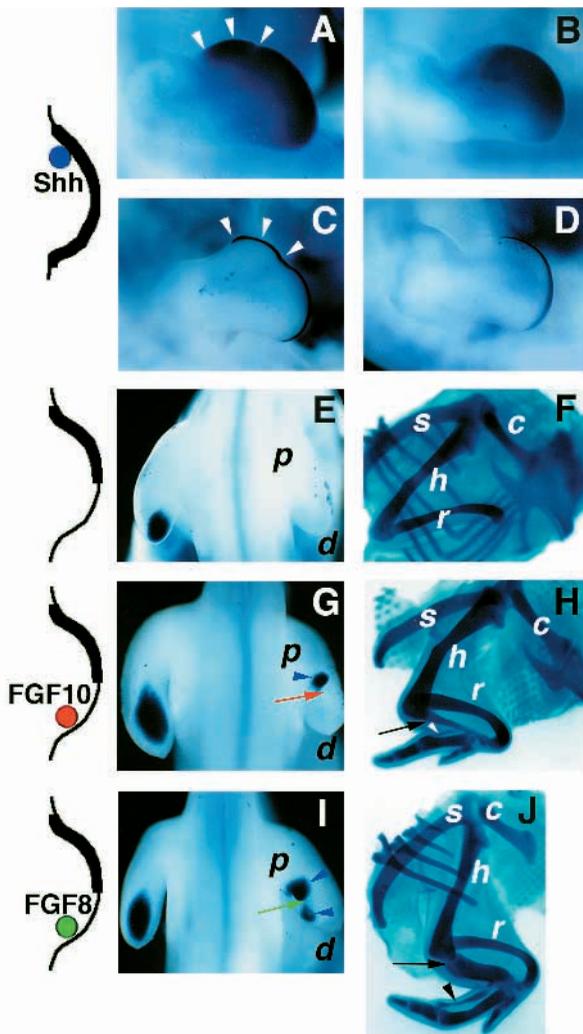
<sup>b</sup>Thickening of the radius, ulna, and all digits.

<sup>c</sup>One specimen developed a thickened ulna.

<sup>d</sup>Formation of thin digits 2.

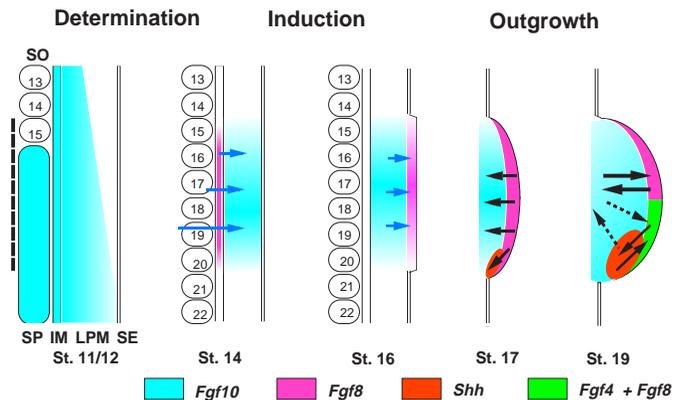
<sup>e</sup>Formation of partial ulna.

<sup>f</sup>Formation of partial digits 4.



**Fig. 5.** *Shh*-expressing cells induce *Fgf10* expression and FGF10 cells maintain *Shh* expression in the posterior limb bud. (A–D) *Fgf10* (A,B) and *Fgf8* (C,D) expression after implantation of *Shh*-expressing cells in the anterior margin of stage 19/20 wing buds. The embryos were harvested 27 hours (A,B) and 30 hours (C,D) later, respectively. The arrowheads indicate ectopically induced *Fgf* genes. Photos of the contralateral wing buds (B,D) were developed inversely for a better comparison. (E,G,I) Posterior views of the embryos 24 hours after surgery, hybridized with a *Shh* RNA probe. p, proximal; d, distal. (E) *Shh* expression disappears in the right wing bud, where the posterior AER was removed. (F) Posterior AER removal results in the absence of the ulna and digits. (G) FGF10 cells maintain *Shh* expression (arrowhead) in the proximal region to the implantation site (arrow). (I) FGF8 cells maintain *Shh* expression (arrowheads) in the proximal and distal regions to the cells (arrow). (H,J) The FGFs restore posterior bones (arrows, ulna; arrowheads, digit 4) at 10 days.

compare Fig. 5A and B). Thus, it appears that, SHH induces expression of *Fgf10*. We observed that *Fgf8* expression was also induced in the anterior elongated AER by SHH ( $n=3$ , Fig. 5C,D), therefore the induction of *Fgf10* expression by SHH is likely AER dependent. To test this, the entire AER was removed and *Shh*-expressing cells were implanted in the wing anterior margin. Under this condition, *Fgf10* expression was still observed within 24 hours in the mesenchyme surrounding the cells, although the level of expression was much lower



**Fig. 6.** A molecular model of the early stages of limb formation. At stage 11/12, *Fgf10* RNA is widely distributed in the segmental plate (SP), intermediate mesoderm (IM) and lateral plate mesoderm (LPM). The dotted line indicates the axial level of the prospective forelimb territory. By stage 14, the definitive forelimb territory is determined by the restricted expression of *Fgf10* in the LPM. This process may be regulated by signals from the axial structures medial to the lateral plate mesoderm. At stage 16, *Fgf10* expression in the LPM leads to induction of *Fgf8* expression in the overlying surface ectoderm (SE) and initiates limb bud formation. By stage 17, FGF8 in the ectoderm acts on the underlying mesoderm and maintains *Fgf10* expression. It also induces *Shh* expression in the posterior margin of the nascent limb mesoderm. By stage 18, *Fgf4* is induced in the posterior apical ectoderm by SHH (not shown). By stage 19, interactions among FGF10, FGF8, SHH and FGF4 maintain outgrowth of the established limb bud. The dotted arrows indicate possible signaling pathway from SHH to FGF10 and FGF10 to FGF4. The molecules involved in pattern formation along the dorsoventral axis are not illustrated in the diagram. SO, somites.

( $n=2$ ; data not shown). Therefore, it appears that, although SHH alone can induce *Fgf10* expression, the induction is intensified by the presence of the AER.

On the contrary, members of the FGF family have been shown to be capable of maintaining *Shh* expression in the posterior limb mesenchyme (Laufer et al., 1994; Niswander et al., 1994; Crossley et al., 1996). Thus, we examined whether FGF10 can also maintain *Shh* expression. We observed that *Shh* expression was extinguished within 10 hours following posterior AER removal (Fig. 5E; Laufer et al., 1994), resulting in truncation of posterior bones, as reported previously (Fig. 5F; Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1981). When *Fgf10*-expressing cells were implanted in the posterior margin after posterior AER removal, *Shh* RNA was detected in the region proximal, but not distal, to the cells (Fig. 5G). This indicated that FGF10 is able to maintain *Shh* expression in the posterior mesenchyme of the limb bud. However, examination after 7 days of incubation revealed that the rescue of posterior bones was incomplete: the ulna was thin and the digit 4 was not formed (Fig. 5H; Table 2). For comparison, we performed the same experiment using *Fgf8*-expressing cells. We found that *Shh* expression was maintained in the regions both proximal and distal to the implanted cells (Fig. 5I), and that the rescue of posterior bones seemed to be more complete (Fig. 5J; Table 2). This more complete rescue of limb patterning by FGF8 may be due to its ability to maintain *Shh* expression in a broader domain than FGF10, at least as seen in our experimental system.

## DISCUSSION

We demonstrated here that a new member of the *Fgf* gene family, *Fgf10*, is expressed in the prospective limb territories of the somatic lateral plate mesoderm. Ectopic application of *Fgf10*-expressing cells into the prospective flank mesoderm of chick embryos induces expression of *Fgf8* in the nascent ectopic AER and, subsequently, the additional complete limb in the flank. *Fgf10* continues to be expressed in the limb mesenchyme and is able to interact with FGF8 from the AER and SHH from the ZPA. These results suggest that FGF10 is not only an endogenous initiator for limb formation in the lateral plate mesoderm, but also a mesenchymal factor that may be responsible for the epithelial-mesenchymal interaction necessary for limb bud outgrowth.

### Possible roles of FGF10 in pattern formation of the limb

On the basis of the data presented here, we propose some possible roles of FGF10 in limb pattern formation with emphasis on the FGF cascade. We divide our discussion into three parts, according to three phases of limb formation (Fig. 6): (1) determination of the limb territories (until stages 13-14), (2) induction of limb buds (stages 14-16) and (3) outgrowth of limb buds (from stage 17).

#### (1) Determination of the limb territories: regulation of *Fgf10* expression in the lateral plate mesoderm may be involved in the determination process of the limb territories

It has been thought that interactions within the mesoderm are necessary for the early lateral plate to form a limb. For example, prospective wing mesoderm taken before stage 11 could form a limb if accompanied by some somitic tissue (Pinot, 1970; Kieny, 1971). Also, Stephens et al. (1989, 1993) showed that limb-like structures could be generated from early lateral plate explants when combined with the surrounding tissues and placed in the body cavity of an older host embryo. From these results, it has been speculated that the axial structures medial to the prospective limb regions may produce some factor(s) capable of transforming the lateral plate into definitive limb territories. Crossley et al. (1996) and Vogel et al. (1996) postulated that FGF8 in the intermediate mesoderm may function as a forelimb inducer. On the contrary, our results show that exogenous FGF8 applied in the flank can induce *Fgf10* expression in the lateral plate mesoderm. Taken together, it is likely that, during authentic limb formation, FGF8 in the intermediate mesoderm is involved in upregulation of *Fgf10* expression in the prospective forelimb mesoderm (Fig. 6). Since we found that *Fgf10* is also expressed in the intermediate mesoderm, some interaction between FGF8 and FGF10 in the nephrogenic mesoderm may elaborate the forelimb induction.

Insulin-like growth factor-I (IGF-I) has been shown to play a role in the initial event of limb formation: explants of stage 10-12 lateral plate mesoderm treated by IGF-I protein can autonomously grow and differentiate into limb-bud-like structures (Dealy and Kosher, 1996). In addition, *Igf-I* RNA was found to be detected in rat presumptive limb mesoderm (Streck et al., 1992). Also, hepatocyte growth factor/scatter factor (HGF/SF) and T-box genes 5 and 4 (*Tbx5*, *Tbx4*) are expressed

at stages 13-15 in the prospective limb mesoderm and their expressions are induced during additional limb formation (Théry et al., 1995; Heymann et al., 1996; Gibson-Brown et al., 1996; H. O. et al., unpublished data). Together with this study, there may be some interplay among FGF10, IGF-I, HGF/SF and TBXs in the prospective limb mesoderm before induction of limb buds.

The restricted expression of *Fgf10* in the prospective limb territories led us to speculate that the *Fgf10* expression domain in the very early embryo might be correlated with the competence of that region for limb formation. For instance, the prospective neck and flank mesoderm of the lateral plate were found to possess limb-forming potential at stages 10-12 and 11-14, respectively (Stephens et al., 1989), where we have shown that *Fgf10* is expressed. Although it is unlikely that all *Fgf10*-expressing domains have the potential to form limbs, we propose that regulation of *Fgf10* expression in the lateral plate mesoderm might be involved in the determination process of the limb territories (Fig. 6). Nevertheless, we must await further elucidation of control mechanisms for *Fgf10* expression to understand the role of FGF10 at this phase of limb development.

#### (2) Induction of limb buds: FGF10 may be an endogenous initiator for limb formation

This study demonstrated that ectopic FGFs, such as FGF8 and FGF10, form an additional limb via *Fgf10* induction in the lateral plate mesoderm. Taking into consideration their expression patterns, FGF10 appears most likely to be the initiator of authentic limb formation. Since recent studies on a *limbless* mutant have revealed that the limb bud emerges without *Fgf8* expression in the limb ectoderm, it does not seem that FGF8 in the nascent limb ectoderm is involved in initial limb bud outgrowth (Ros et al., 1996; Grieshammer et al., 1996; Noramly et al., 1996). Thus, we propose that FGF10 rather than FGF8, is a key factor inducing the limb bud, or initiating limb bud outgrowth (Fig. 6). Since it was shown that the labeling index decreases in the flank region just after the induction of limb buds (Searls and Janners, 1971), FGF10 may control the mitotic activity in the lateral plate mesoderm during this period.

The analysis of FGF10-induced additional limb buds revealed that FGF10 acts specifically on the epithelium and induces *Fgf8* expression in the flank ectoderm. Furthermore, it seems likely that the effect of ectopic FGF10 on the flank mesenchyme is correlated with activation of epithelial factors such as FGF8. Thus, in authentic limb formation, endogenous FGF10 in the prospective limb mesoderm likely induces expression of *Fgf8* in the prospective limb ectoderm, the nascent AER (Fig. 6). Then, the *Fgf8* induced in the nascent AER reciprocally affects the underlying mesenchyme to maintain expression of *Fgf10* and induce expression of *Shh* in the posterior margin of the limb bud (Fig. 6). Such mutual interplay between FGF10 and FGF8 appears to be an essential process in epithelial-mesenchymal interactions during induction of limb buds.

Among the FGF members identified so far, FGF10 exhibits the highest amino acid sequence identity to FGF7 (See Materials and Methods; Yamasaki et al., 1996). FGF7 was originally discovered as keratinocyte growth factor (KGF) that binds specifically to the FGF receptor (FGFR) isoform 2b

(KGFR; IgIIIa/IgIIIb) that was shown to be expressed in the embryonic epithelia (Ornitz et al., 1996; Orr-Urtreger et al., 1993; Noji et al., 1993). Therefore FGF7 appears to affect epithelial cells. Deduced from this, it is likely that specific receptors for FGF10 may exist on epithelial cells and that FGF10 may affect epithelium rather than mesenchyme. Indeed, our preliminary data indicates that FGF10 acts on epithelial cells rather than mesenchymal fibroblasts *in vitro* (M. Y., N. I., unpublished data). Cohn et al. (1995) reported that FGF7 did not induce additional limb formation in the flank. Although FGF10 structurally resembles FGF7, FGF10 may differ from FGF7 in the ability to induce limb formation in the embryonic flank. It has been demonstrated that specific receptors for FGF8b, a functional isoform in limb development (Crossley et al., 1996), are FGFR2c (bek; IgIIIa/IgIIIc), FGFR3c and FGFR4, and are present in embryonic mesenchymal cells (Orr-Urtreger et al., 1993; Noji et al., 1993; MacArthur et al., 1995). These observations further support the idea that, in limb development, a mesenchymal signal is transmitted to the epithelium by the FGF10-FGFR system and an epithelial signal to the mesenchyme by the FGF8-FGFR system.

One might ask why ectopic FGF10 works only on earlier and not later stages to induce additional limb formation. Since FGF10 likely acts on epithelia rather exclusively, ectopic proliferation of the flank mesenchymal cells seems to be a secondary effect mediated through FGF8 in the flank ectoderm. We hypothesized that, in the case of FGF10 application at later stages, the competence of the flank mesenchymal cells to interact with the FGF8 signal may already be lost. In support of this, we observed that when FGF10 cells are implanted at stage 15, *Fgf8* expression is induced in the flank ectoderm but chick *Fgf10* and *Shh* RNA are not detected in the flank mesenchyme (H. O. et al., unpublished data). This observation implies that the flank ectoderm remains competent to express *Fgf8* whereas the flank mesoderm has already lost its competence to express some of mesodermal factors. On the contrary, other FGFs such as FGF2, FGF4 and FGF8 may act more directly on the mesenchyme, as deduced from the fact that their specific receptors are localized in the mesenchyme (Ornitz et al., 1996). This may be the reason that they are still able to induce additional limb formation even when applied at later stages. Alternatively, the relative amount of FGF protein produced by the cells that we used in this study may be less than that of the *Fgf8*- or *Fgf4*- (Ohuchi et al., 1995) expressing cells, because, at earlier stages, the requirement for the amount of FGF10 by the cells may be much less.

### (3) Outgrowth of limb buds: FGF10 and FGF8 may be involved in communication between the limb mesenchyme and the AER

Once the limb bud is established, *Fgf10* expression becomes AER dependent. Since FGF8 can rescue the expression of *Fgf10* in the mesoderm of AER-removed limb buds, FGF8 in the AER appears to be a key factor in maintaining expression of *Fgf10* in the mesoderm. Conversely, *Fgf8* expression in the AER is likely to depend on the presence of FGF10 in the mesoderm, because ectopic application of FGF10 can induce expression of *Fgf8* and maintain it in the AER-removed limb bud. It has been postulated that the mesenchymal cells underlying the AER produce some factor(s) to maintain the AER (AER maintenance factor; Zwillig and Hansborough, 1956;

Saunders and Gasseling, 1963). Our results imply that FGF10 is a possible candidate for this AER maintenance factor. Thus, we considered that the mutual interaction between FGF8 and FGF10 might be a molecular basis for epithelial-mesenchymal interactions between the AER and the underlying mesoderm of the established limb bud as well (Fig. 6).

In the posterior limb bud, a signaling loop between FGF10 and SHH is found: FGF10 maintains *Shh* expression and SHH induces *Fgf10* expression. Since the apical ridge factors, FGF8 and FGF4, also maintain *Shh* expression (Crossley et al., 1996 and this study; Laufer et al., 1994; Niswander et al., 1994), the coordinate FGFs-SHH signaling loop should be essential for the continuous patterned outgrowth of the normal limb bud (Fig. 6).

We have referred to the roles of the FGF10-FGF8 cascade and FGFs-SHH signaling loops in limb development, but it is tempting to speculate that similar regulatory systems involving the same gene families are used in other developmental processes, such as brain development.

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