Correlation of wing-leg identity in ectopic FGF-induced chimeric limbs with the differential expression of chick *Tbx5* and *Tbx4*

Hideyo Ohuchi[1],*, Jun Takeuchi[2],*, Hidefumi Yoshioka[1], Yoshiyasu Ishimaru[1], Keiko Ogura[2], Naoki Takahashi[2], Toshihiko Ogura[2] and Sumihare Noji[1],†

[1]Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minami-jyosanjima-cho, Tokushima 770, Japan
[2]Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-01, Japan
*The first two authors contributed equally to this work
†Author for correspondence (e-mail: noji@bio.tokushima-u.ac.jp)

SUMMARY

It has been reported that members of the fibroblast growth factor (FGF) family can induce additional limb formation in the flank of chick embryos. The phenotype of the ectopic limb depends on the somite level at which it forms: limbs in the anterior flank resemble wings, whereas those in the posterior flank resemble legs. Ectopic limbs located in the mid-flank appear chimeric, possessing characteristics of both wings and legs; feather buds are present in the anterior halves with scales and claws in the posterior halves. To study the mechanisms underlying the chimerism of these additional limbs, we cloned chick *Tbx5* and *Tbx4* to use as forelimb and hindlimb markers and examined their expression patterns in FGF-induced limb buds. We found that *Tbx5* and *Tbx4* were differentially expressed in the anterior and posterior halves of additional limb buds in the mid-flank, respectively, consistent with the chimeric patterns of the integument. A boundary of *Tbx5/Tbx4* exists in all ectopic limbs, indicating that the additional limbs are essentially chimeric, although the degree of chimerism is dependent on the position. The boundary of *Tbx5/Tbx4* expression is not fixed at a specific position within the interlimb region, but dependent upon where FGF was applied. Since the ectopic expression patterns of *Tbx5/Tbx4* in the additional limbs are closely correlated with the patterns of their chimeric phenotypes, it is likely that *Tbx5* and *Tbx4* expression in the limb bud is involved in determination of the forelimb and hindlimb identities, respectively, in vertebrates.

Key words: Fibroblast growth factor, FGF, Limb identity, T-box genes, *Tbx4*, *Tbx5*, Chick, Additional limb

INTRODUCTION

In vertebrates, two pairs of limb buds are formed from the lateral plate mesoderm at specific anteroposterior positions and grow out to form the limbs. The morphology of the anterior limb, the forelimb, is quite diverse among vertebrates; from wings in birds to hands in primates. Although the difference between the forelimb and the posterior limb, the hindlimb, appears small in rodents, the hindlimb is usually larger than the forelimb as it serves to support the body, especially in avians and humans.

One of the important questions in phylogeny and developmental biology has been how morphological and functional differences among different limbs are determined. Interesting implications can be drawn from recent experiments using chick embryos in which ectopic limb formation is induced in the flank by application of members of the fibroblast growth factor (FGF) family (for a review; Cohn and Tickle, 1996). The extra limbs thus formed exhibited wing-like phenotypes when induced in the anterior flank, whereas those in the posterior flank resemble legs (Cohn et al., 1995; Ohuchi et al., 1995).

This suggests that determination of limb identity may depend on the axial level of the limb. Furthermore, Cohn et al. (1997) recently showed that activation of the expression of a certain set of *Hox9* paralogous genes in the lateral plate mesoderm may be correlated with the specification of each limb.

In addition, several factors have been found to exist specifically in the forelimb or hindlimb region. For example, one of the *Hox* gene products, Hoxc-6 protein is specifically distributed in the forelimb region (Oliver et al., 1988; Nelson et al., 1996), whereas *Hoxc-9, Hoxc-10* and *Hoxc-11* are expressed in the hindlimb bud (Peterson et al., 1994, Nelson et al., 1996). However, the expression of these Hox proteins or RNA are further restricted within the limb bud, for example, to either the anterior or posterior region. Recently it has been shown that some T-box genes are expressed throughout the entire domain of either limb bud. The T-box genes belong to a family of putative transcription factors, characterized by a region of homology to the *T* (*Brachyury*) locus gene product (Herrmann et al., 1990; Bollag et al., 1994). It was found that the expression of two vertebrate T-box genes, *Tbx5* and *Tbx4* are predominantly restricted to the forelimb and hindlimb buds,

respectively (Chapman et al., 1996; Gibson-Brown et al., 1996; Simon et al., 1997). Furthermore, *TBX5* mutations have been reported to cause Holt-Oram syndrome, resulting in some defects of the upper limb (Basson et al., 1997; Li et al., 1997), implying that Tbx5 may be involved in forelimb development. Tbx5 and Tbx4 thus represent good marker genes for forelimb and hindlimb identity, respectively.

To understand the mechanisms of determination of limb identity, we analyzed the phenotypes of the additional limb induced by FGF in the flank. We found that the anterior half of an ectopic limb has characteristics of the forelimb, whereas hindlimb features are present in the posterior half, demonstrating the chimeric character of the ectopic limb. We used *Tbx5* and *Tbx4* as marker genes to examine the identity of the additional limbs. We found that the expression of both *Tbx5 and Tbx4* are induced by FGF in the flank and that there exists a clear boundary between *Tbx5/Tbx4*-expressing and non-expressing domains in the extra limb bud. The *Tbx* expression boundary in the additional limb appears to correspond to its future line of demarcation between the wing and leg phenotypes. Thus, the *Tbx5/Tbx4* expression appears correlated with the limb identity in the additional limb, suggesting that *Tbx5/Tbx4* may be involved in determination of limb identity in vertebrates.

MATERIALS AND METHODS

Retrovirus production

Two types of retroviruses were used for the present study, one is a replication-defective variant of the spleen necrosis virus (RDSNV; Dougherty and Temin, 1986), and the other a replication-competent variant of the avian leukemia virus with splice acceptor site, Bryan high titer polymerase, and A-envelope subgroup (RCASBP(A); Hughes et al., 1987). Construction of the RDSNV containing the human *Fgf4* cDNA and propagation of the recombinant viruses and that of the RCASBP(A) containing the chick *Fgf8* cDNA were described previously (Ohuchi et al., 1995, 1997a; Itoh et al., 1996).

Cell grafting

Fertilized chick eggs were purchased from Yamagishi Co. (Tokushima, Japan) or from Nisseiken Co. (for specific pathogen free chicks, line M; Yamanashi, Japan) and incubated at 37.5-38.5°C until the desired stages (Hamburger and Hamilton, 1951). Chick embryonic fibroblasts (CEFs) were grown as described previously (Ohuchi et al., 1997b). For grafting experiments, CEFs were first infected with the viruses, cultured for 3 days, and harvested by a light trypsinization and centrifugation for 5 minutes at 800 revs/minute. The resulting cell 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 and a piece of cell pellet was grafted into the prepared slit. The eggs were resealed and returned to the incubator. Embryos were incubated for further 48 hours to 11 days depending on the experiment.

FGF2-bead implantation

Heparin acrylic beads (H5263, Sigma), 125-250 μm in diameter, were soaked in 10 μl of human recombinant FGF2 (1 mg/ml; kindly provided by Takeda Pharmaceutical Company) for 1 hour at room temperature prior to implantation. Just before application to the embryos, the FGF2 beads were transferred to tissue culture medium to avoid application of highly concentrated FGF2-drops. A bead was inserted into the slit in the lateral plate mesoderm at the appropriate

anteroposterior position. Embryos were incubated for a further 7-11 days for cartilage staining or observation of cutaneous structures. To observe the epithelial patterns of extra limbs, the manipulated embryos were fixed in 4% formaldehyde at embryonic day 12-13. We confirmed that FGF2-soaked beads and *Fgf*-expressing cells have essentially the same effect on the flank cells in inducing an extra limb as reported previously (Crossley et al., 1996; Vogel et al., 1996; Ohuchi et al., 1997b).

Cartilage staining

After manipulation, embryos were allowed to develop for 7-9 days, then fixed in 4% paraformaldehyde and stained for cartilage using 0.1% alcian blue. After dehydrating and clearing with methyl salicylate, the cartilage structures of the embryos were observed with a Leica MZ-series microscope.

Isolation of chick *Tbx* cDNAs

To isolate chick *Tbx5* cDNA, mRNA was extracted from chick wing buds at embryonic day 3 using the QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech). 2 μg mRNA was reverse transcribed using the Ready-To-Go T-Primed First-Strand Kit (Pharmacia Biotech). Polymerase chain reaction (PCR) was performed using degenerate oligonucleotide primers, which were derived from highly conserved regions within the T-box and correspond to the following peptide sequences: YVHPDSP for sense and AVTSYQN for antisense (Agulnik et al., 1996). PCR products were gel purified, cloned into pCR2.1 according to the manufacturer's instructions (TA cloning Kit, Invitrogen Corporation). Sequencing analysis revealed that one of the plasmids contained a 252 bp fragment of chick *Tbx5* cDNA. To obtain a longer *Tbx5* clone, rapid amplification of cDNA ends (Frohman et al., 1988) was performed using the Marathon Kit (Clontech Laboratories, Inc.). The resultant 1.3 kb clone, *cTbx5-3* was used for in situ hybridization analysis.

Chick Tbx4 cDNA was obtained by screening a chick limb bud (stage 20-23) cDNA library prepared in lambda ZAPII phage vector (Stratagene) with a human *Tbx2* cDNA clone (a kind gift from Dr Christine E. Campbell). Among the 13 positive clones isolated was a clone for chick *Tbx4* (*cTbx4-N;* 2.3 kb). *Tbx4* RNA riboprobe was synthesized from *cTbx4-N* for in situ hybridization analysis.

Whole-mount in situ hybridization

Embryos were fixed in 4% paraformaldehyde 48 hours after surgical manipulation and prepared for whole-mount in situ hybridization, which was performed according to Wilkinson (1992) with minor modifications (Ohuchi et al, 1997b). Digoxigenin-UTP riboprobes were generated from the T-box sequence, coding sequences 3′ to the T-box, and 3′ untranslated sequences of *cTbx4-N* and *cTbx5-3*. To visualize the somites, a chick cDNA encoding a muscle transcription factor, MRF4 (1.1 kb; Fujisawa-Sehara et al., 1992) was used. According to Burke et al. (1995), the chick forelimb bud extends from somites 18-19 through somites 21-22 by stage 24. When *Mrf4* was used for a somite marker, the posterior-most level of the forelimb bud was counted as somites 20-21 in the stage 22-24 embryos that we examined. Therefore the somite levels reported here can be calibrated, plus one somite, compared with the report by Burke et al (1995).

To identify feather buds, a probe for chick *Sonic hedgehog* (*Shh*) (1.3 kb; Ohuchi et al., 1997a) was used on 10-day additional limbs and contralateral wings and legs as reported previously (Nohno et al., 1995).

RESULTS

Three types of skeletal patterns of the additional limb in the flank

We designated the FGF-induced extra limb in the flank as

Dasoku (snake leg); a Japanese word for a superfluous limb of the snake (Ohuchi et al., 1995). To analyze the patterns of cartilage and integument of these *Dasokus*, we implanted *Fgf*-expressing cells (FGF4-cells or FGF8-cells) or an FGF2-soaked bead in the prospective interlimb region of stage 13-15 chick embryos. At 7 days after implantation, we analyzed the cartilage pattern of the *Dasokus*. As reported previously (Cohn et al., 1995; Ohuchi et al., 1995), *Dasokus* exhibit reversed anteroposterior polarity, e.g., the sequence of digits in *Dasokus* read **4**-**3**-**2** from anterior to posterior, which is reversed compared with the sequence of normal wing digits **2**-**3**-**4**. Furthermore, the skeletal pattern of *Dasokus* changes according to their positions along the anteroposterior body axis. We classified 68 cases of *Dasoku* into three types according to their skeletal patterns; wing-type is defined as a *Dasoku* articulating with an authentic forelimb skeletal element such as scapula or humerus (Fig. 1A,B), intermediate type as a *Dasoku* which does not articulate with a forelimb or hindlimb element, but with fused ribs (Fig. 1C,D), and leg-type as a *Dasoku* articulating with an authentic hindlimb element such as pelvis or femur (Fig. 1F,G,H). All the FGF members that we examined, FGF2, FGF4 and FGF8, were similarly effective in the induction of all three types of additional limbs. It is noticeable that when FGF-cells were applied in the flank at the level of somites 22-23 of stage 14-15 embryos (34 cases), all three types of *Dasokus* were formed (Table 1). A possible explanation may be that, because of the very rapid growth rate of the embryonic cells, it was too difficult for us to apply FGF to the embryos under the exact conditions every time. Since the competence of the lateral plate mesoderm to respond to the FGF signal may change along the body axis due to the cephalocaudal gradient, i.e., the competence is lost earlier in more anterior flank, FGF could therefore induce a more leg-like extra limb even if FGF was applied to the relatively anterior flank of a more developed embryo.

Cartilage staining revealed that a full range of skeletal

Table 1. Cartilage pattern of FGF-induced additional limbs

Phenotypes	FGF4 n	FGF8 n	Total n
Wing type	7	4	11
truncation	1	2	3
duplication	6	2	8
Intermediate type	3	2	5
Leg type	13	5	18
Total	23	11	34

n, number of experimental samples.

FGF was applied to the lateral plate mesoderm at the level of somite 22/23 of stage 14-15 embryos.

Definition of phenotypes: Wing type, a *Dasoku* articulating with an authentic forelimb skeletal element; Intermediate type, a *Dasoku* articulating with fused ribs; Leg type, a *Dasoku* articulating with an authentic hindlimb skeletal element.

elements from humerus to digits were formed in the wing-type *Dasokus.* Many exhibited duplication of the humerus and radius, and thickening of the ulna (Fig. 1B). In 3 out of 34 cases, the wing on the implanted side was truncated (Table 1; data not shown), possibly due to the action of FGF within the wing territory, as mentioned previously (Vogel et al., 1996). In the leg-type *Dasokus*, a full range of skeletal elements from pelvic girdle to digits were formed in which the ectopic femur articulated with an enlarged pelvic girdle (Fig. 1G,H). In the intermediate type *Dasokus*, skeletal elements from stylopods to autopods were formed, with the stylopods usually articulating directly with the fused ribs (Fig. 1D). In normal chick embryos, the ribs (which do not fuse) articulate directly with the sternum (data not shown). Occasionally, a small cartilage fragment was formed independently between two costal bones, which seemed to be an extra girdle (data not shown). In contrast to the 3 digits of the normal wing and the
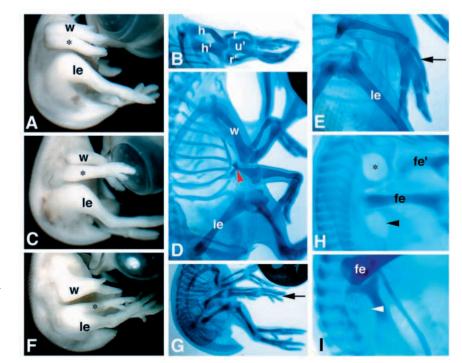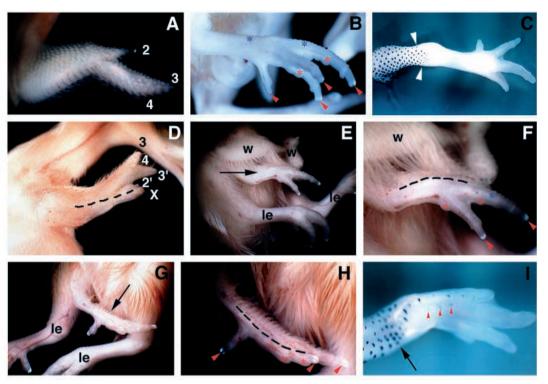

Fig. 1. Phenotypes of FGF4- (A,B,C,F) and FGF8- (D,E,G,H) induced additional limbs in the flank. (A,C,F) Whole-mount preparation of manipulated embryos at embryonic day 9 (E9), showing a wing-type (A), intermediate-type (C) and leg-type (F) *Dasoku*s (asterisks). w, wing; le, leg. (B,D,E,G) Skeletal patterns of three types of additional limbs. (B) Wing-type *Dasoku* fused with the wing, having two humeri (h, h′), two radiuses (r, r′) and a thickened ulna (u′). (D) Intermediate-type *Dasoku*, showing that an additional stylopod articulates with the fused ribs (arrowhead). (E) Additional limb (arrow) in D, with four digits. (G) Leg-type *Dasoku* (arrow). (H) Higher magnification of the pelvic girdle in G. An extra femur-like limb (fe′) articulates with an enlarged girdle with an additional ilio-ischial foramen (asterisk). The arrowhead indicates the authentic foramen. fe, femur. (I) Normal hip joint. The arrowhead indicates the ilio-ischial foramen.

Fig. 2. Epithelial patterns of normal limbs (A-C) and three types of FGF2-induced additional limbs (D-I). Embryonic stage is day 13 unless otherwise indicated. Anterior is to the top.
(A) Dorsal view of a normal wing with feather buds and three digits (**2** to **4**).
(B) Dorsolateral view of a normal leg showing the ventral curvature of the toe region. The dorsal side of the leg digits is covered with large scales called scuta (blue asterisks) and the ventral side with phalangeal pads (red asterisks). Developing claws are indicated by arrowheads.
(C) *Shh* expression in feather buds in the proximal region of a 10-day normal leg. To facilitate comparison, the photo of the unoperated left leg was reversed for developing. The arrowheads indicate the distalmost expression of *Shh*. (D) Wing-type *Dasoku* with three additional digits (**3′**, **2′**, X). The authentic digit **2** is not seen from this angle. No feather buds are seen in the caudal region of the limb, but instead, flat phalangeal pad-like structures. The broken line indicates the likely boundary between wing and leg phenotypes. (E) Intermediate-type *Dasoku* (arrow). w, wing; le, leg. (F) Higher magnification of the *Dasoku* in E. The cephalic region of the *Dasoku* has feather buds, whereas its caudal region has ventral phalangeal pads (red asterisks), dorsal scuta (blue asterisk) and developing claws (arrowheads). (G,H) Leg-type *Dasoku* (arrow) with three claws (arrowheads) and phalangeal pads (asterisks). The cephalic region of this *Dasoku* has both feather buds and a claw. (I) *Shh* expression in the feather buds of a 10-day *Dasoku*. The arrow indicates the distalmost position of the posterior leg-type region where the *Shh* expression fades out. The arrowheads indicate the caudalmost feather buds in the wing-type autopod region.

4 digits of the normal leg, the intermediate-type *Dasokus* had 2 to 5 digits (Fig. 1E), whose patterns do not seem to resemble either those of the wing or the leg. Eleven other cases displayed a combination of these phenotypes, such as formation of an additional humerus, thickening of the ulna, bifurcation of the tibia and presence of 5 digits in the common autopod of the duplicated wing and leg (data not shown).

Epithelial characteristics of the additional limb

Since the forelimb (wing) and the hindlimb (leg) in chicks can be discriminated more effectively by cutaneous structures than with the skeletal patterns, we examined the epithelial patterns of additional limbs at embryonic days 10-13. The normal chick embryonic wing has feather buds on the dorsal side of the distal autopod region in addition to the more proximal region (Fig. 2A). By contrast, no feather buds are present in the autopod region of the normal leg (Fig. 2B,C). The distal autopod region of the leg has several characteristics not found in the wing; scales, claws, ventral phalangeal pads and a ventral curvature of the toe region (Fig. 2B). In addition to the morphological analyses of epithelial structures in ectopic 13-day limbs, we examined ectopic 10-day limbs for feather bud formation by use of a molecular marker, *Shh* (Fig. 2I), since *Shh* has been reported to be expressed in the feather bud epithelium (Nohno et al., 1995; Ting-Berreth and Chuong, 1996).

In the intermediate-type *Dasokus* formed in the mid-flank,

the anterior half nearest the authentic wing had feather buds, whereas the posterior half near to the authentic leg had scales and claws (Fig. 2E,F). Interestingly, we found that even the wing-type *Dasokus* had phalangeal pad-like structures in their caudal margins, which are absent from feather buds (Fig. 2D) and the leg-type *Dasokus* had feather buds in their cephalic distal margins (Fig. 2G-I). In most cases, these feather bud and leg structure domains appeared to be separated by a boundary (Fig. 2D,F,H,I). It is noteworthy that the region of this boundary occasionally had characteristics of both wings and legs: there were feather buds in the autopod region and a claw in the distal tip of the digit (Fig. 2H). Although chimerism was not so obvious in the wing- or leg-type *Dasokus* by macroscopic observations of cartilage patterns, these epithelial characteristics of additional limbs indicated that the ectopic limb may be essentially a chimera of the forelimb and hindlimb.

The chick *Tbx5* and *Tbx4* genes are differentially expressed in the prospective and definitive limb regions

To further verify their chimeric character, we wished to examine the expression patterns of wing- or leg-specific genes in the *Dasoku* limb buds. It has been reported that two members of the T-box gene family, *Tbx5* and *Tbx4*, are expressed in the mouse forelimb and hindlimb buds, respectively (Chapman et
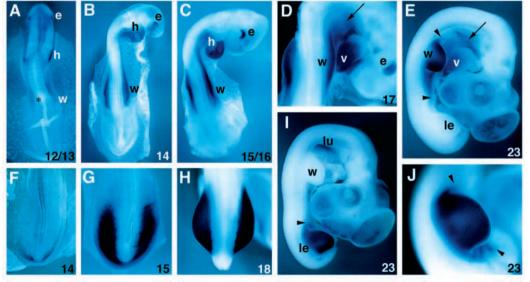
```
mTbx4    ----------MIITKAGRRMFPSYKVKVTGMNPKTKYILLIDIVPADDHRYKFCDNKWMVAGKAEP.MP
cTbx4    LWKKFHEAGTEMIITKAGRRMFPSYKVKVTGMNPKTKYILLIDIVPADDHRYKFCDNKWMVAGKAEPAMP
mTbx5    LWLKFRSGVTEMIITKAGGRMFPSYKVKVTGLNPKTKYILLMDIVPADDHRYKFADNKWSVTGKAEPAMP
cTbx5    LWLKFHEVGTEMIITKAGRRMFPSYKVKVTGLNPKTKYILLMDIVPADDHRYKFADNKWSVTGKAKPAMP
```

```
mTbx4    GRLYVHPDSPATGAHWMRQLVSFQKLKLTNNHLDPFGHIILNSMHKYQPRLHIVKADENNAFGSKNTAFC
cTbx4    GRLYVHPDSPATGAHWMRQLVSFQKLKLTNNHLDPFGHIILNSMHKYQPRLHIVKADENNAFGSKNTAFC
mTbx5    GRLYVHPDSPATGAHWMRQLVSFQKLKLTNNHLDPFGHIILNSMHKYQPRLHIVKADENNGFGSKNTAFC
cTbx5    GRLYVHPDSPATGAHWMRQLVSFQKLKLTNNHLDPFGHIILNSMHKYQPRLHIVKADENNGFGSKNTAFC
```

```
mTbx4    THVFPETSFISVTSYQNHKITQLKIENNPFAKGFRGSDDSDLRVA
cTbx4    THVFPKTSFISVTSYQNHKITQLKIENNPFAKGFRGSDDSDLRVA
mTbx5    THVFPETAFIAVTSYQNHKITQLKIENNPFAKGFRGSDDLELHR-
cTbx5    THVFPKTAFIAVTSYQNHKITQLKIENNPFAKGFRGSDDMELHRM
```

al., 1996; Gibson-Brown et al., 1996), so we decided to use these *Tbx* genes as wing- and leg-specific markers. To obtain the chick *Tbx* genes, we employed RT-PCR using degenerate oligonucleotide primers in addition to cDNA library screening. Fig. 3 shows the deduced amino acid sequences of the chick Tbx4 and 5 T-box region, aligned with those of the mouse homologues. Within this conserved region, the amino acid identity for each gene compared with the corresponding mouse homologue is 99% for *Tbx5* and *Tbx4*.

Prior to analysis of their expression in ectopically induced limb buds, we checked the mRNA localization of the *Tbx5* and *Tbx4* genes during normal chick limb development. At stage 11, the earliest stage examined, *Tbx5* was expressed in the developing heart but not in the lateral plate mesoderm, while *Tbx4* expression was not yet detectable (data not shown). By stage 12/13, *Tbx5* expression became detectable in the lateral plate mesoderm at and below the level of the heart, and in the prospective wing mesoderm (Fig. 4A). The onset of *Tbx4* expression began slightly later, at stage 13, in the caudal-most region of the lateral plate mesoderm (data not shown; Fig. 3F at stage 14). By stages 14-15, before demarcation of the wing and leg bud from the lateral plate mesoderm, high levels of *Tbx5* and *Tbx4* expression can be seen in the prospective wing and leg mesoderm, respectively (Fig. 4B,C,G). At stages 17-18, when the limb buds have begun bulging outwards, these two *Tbx* genes are differentially expressed in the wing and leg buds, respectively (Fig. 4D,H). Transverse sections of embryos stained by whole-mount situ hybridization revealed that neither *Tbx5* nor *Tbx4* are expressed in the limb bud epithelium (data not shown). In contrast with the reported sustained expression

Fig. 4. Expression of *Tbx5* (A-E) and *Tbx4* (F-J) in normal chick embryos. Embryonic stages are indicated in the bottom corners of each panel. Anterior is to the top. (A-C) *Tbx5* expression is detected in the prospective wing region (w), the developing heart (h) and the developing eye (e). At stages 12-16, *Tbx5* is also expressed in the lateral plate mesoderm at and below the somite levels of the heart. Somite 15 is indicated by an asterisk in A. (C) Expression in the eye becomes restricted to the dorsal retina. (D) *Tbx5* expression in the early wing bud, the ventricle (v), foregut mesenchyme (arrow) and the eye. (E) *Tbx5* expression in the wing bud, flank mesenchyme, the ventricle, and lung and tracheal mesenchyme (arrow). The arrowheads indicate the anterior and posterior limits of *Tbx5* expression in the wing and flank mesenchyme, respectively. *Tbx5* expression in the flank mesenchyme disappears by stage 25. (F) *Tbx4* expression begins in the posteriormost region of the lateral plate mesoderm and appears in the prospective leg mesenchyme at the level of somite 26-29 (G). This expression expands to the whole leg bud (H-J). (I) *Tbx4* expression in the leg (le) and lung (lu) mesenchyme. The arrowhead indicates the anterior limit of *Tbx4* expression in the flank. (J) Higher magnification of the leg bud in I. The arrowheads indicate the anterior and posterior limits of *Tbx4* expression in the flank and in the mesoderm caudal to the leg bud, respectively. In contrast to the strong signals in the mesenchyme, neither gene was expressed in the AER or in the ectoderm of wing and leg buds (J) throughout all stages of limb bud development.

of *Tbx4* in mouse tail mesenchyme (Chapman et al., 1996), expression of chick *Tbx4* in the tail bud significantly decreased from stage 18 onwards (Fig. 4H-J). This is interesting from the view of comparable biology, because chickens lack tails whereas mice have quite prominent tails. At stage 23, *Tbx5* and *Tbx4* are still specifically expressed in the prominent wing and leg buds, respectively (Fig. 4E,I,J), but not in the apical ectodermal ridge (AER) (Fig. 4E,J), as reported in the mouse (Gibson-Brown et al., 1996). In 4-day embryos, *Tbx5* expression was also present in the flank mesoderm at and anterior to the level of somite 25-26 (Fig. 4E). In contrast, *Tbx4* expression was restricted to the mesenchyme in the vicinity of the leg bud at this stage (Fig. 4J).

As has been observed in mouse embryos (Chapman et al., 1996), chick *Tbx5* is also expressed in the developing eye and the mesenchyme surrounding the foregut, lung buds and trachea (Fig. 4A-E), and *Tbx4* in the lung and tracheal mesenchyme (Fig. 4I).

Both *Tbx5* and *Tbx4* are differentially expressed in the additional limb bud

Since the normal expression patterns of *Tbx5* and *Tbx4* in chick embryos correlate well with those observed in mice, it appears that these *Tbx* genes can function as good wing- or leg-specific markers. We next examined their expression patterns in three types of FGF-induced extra limb buds. *Tbx5* was expressed in the cephalic half of the intermediate-type *Dasoku* limb bud, while *Tbx4* was expressed in its caudal half (Fig. 5B,E). Although we had speculated that *Tbx4* would not be expressed in the wing-type *Dasoku* and *Tbx5* absent from the leg-type, we observed *Tbx4* expression in the caudal margin of the wing-type *Dasoku*, in which *Tbx5* was predominantly expressed (Fig. 5A,D). Correspondingly, *Tbx5* was also expressed in the cephalic margin of the leg-type *Dasoku*, which otherwise almost entirely expressed *Tbx4* (Fig. 5C,F). These *Tbx5/Tbx4* expression patterns imply that the cephalic region of the additional limb identifies itself as a forelimb, but its caudal region as a hindlimb, and that all of these FGF-induced extra limbs in the flank may be more or less chimeric even those formed near the authentic limb. In addition, this result indicates that FGF can induce or maintain expression of *Tbx5* and *Tbx4* genes in the flank mesenchymal cells, which are usually not expressed in the flank at such high levels (Fig. 4E,I). Since the differential expression patterns of *Tbx5* and *Tbx4* in the additional limb buds correlates well with the chimeric characteristics of their future morphology, it is likely that *Tbx5* and *4* genes are involved in determination of chick limb identity.

The axial level of the boundary between the forelimb phenotype (*Tbx5*-expressing domain) and hindlimb phenotype (*Tbx4*-expressing domain) is not fixed but can be altered by FGF

To examine whether the somite level of the phenotypic boundary built by FGF application is fixed or variable, we performed in situ hybridization on *Dasoku* embryos using *Tbx5* and *Tbx4* RNA probes, together with a myotome marker gene, *Mrf4,* to mark the somites (Fujisawa-Sehara et al., 1992). We could thereby determine the level of the somite opposite the boundary in *Dasoku* limb buds. Typical results are shown in Fig. 6, where the boundary is seen at somite 23 (Fig. 6B), 23/24 (Fig. 6D) or 25/26 (Fig. 6F), indicating that the forelimb-
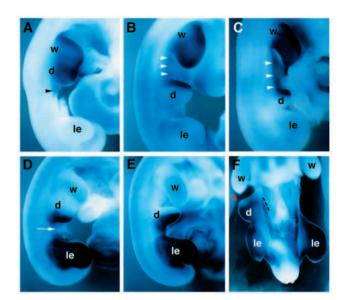

Fig. 5. Expression of *Tbx5* (A-C) and *Tbx4* (D-F) in three types of *Dasoku*s induced by FGF8. As judged from their positions, (A,D) are wing-type, (B,E) are intermediate-type and (C,F) are leg-type. w, wing bud; d, *Dasoku* limb bud; le, leg bud. Anterior is to the top. (A) *Tbx5* is expressed in the wing and *Dasoku* limb buds. The arrowhead indicates the posterior limit of *Tbx5* expression in the flank. (B,C) *Tbx5* is expressed in the cephalic region of the *Dasoku* limb buds. The arrowheads indicate *Tbx5* expression in the flank. (D,E) *Tbx4* is expressed in the leg bud and caudal region of the *Dasoku* limb buds. *Tbx4* is not expressed in the flank between the *Dasoku* and the leg bud (arrow in D). (F) Ventral view of an embryo with a *Dasoku* on the right side. *Tbx4* is expressed in the leg and *Dasoku* limb buds. *Tbx4* expression appears weak in the cephalic margin of the *Dasoku* limb bud (arrowhead). The broken line indicates the expanded domain of *Tbx4* expression on the right side, compared with the left.

hindlimb boundary is not fixed at a particular somite level. We also defined the position of the additional limb bud as the somite level corresponding to the middle of the additional limb bud and examined its correlation with the level of the boundary. As shown in Fig. 7, the extent of the expression domains of *Tbx5* and *Tbx4* genes are limited; *Tbx5* can be expressed in the authentic forelimb region through the flank region to somite 26, whereas *Tbx4* can be expressed in the flank region from somite 22 through the leg region. We found that the somite level of the boundary varies depending upon the position of the additional limb bud; when the extra limb is formed in more anterior flank, the boundary exists at a more anterior somite level, whereas when the additional limb is established in the more posterior flank, the boundary exists at more posterior somite levels (Fig. 7). The boundary of the *Tbx5* expression (open circles in Fig. 7) differs slightly from that of *Tbx4* expression (closed circles in Fig. 7). This result implies that the expression domain of *Tbx5* can slightly overlap with that of *Tbx4*. This is consistent with the fact that the wing-leg boundary regions of *Dasoku*s occasionally exhibit a mixture of both limb phenotypes (Fig. 2H).

Since the posterior end of the chick wing bud is at the level of somite 21-22 and the anterior end of the leg bud is at somite 29-30 by stage 24 (Burke et al., 1995), our results indicate that the somite level of the FGF-induced forelimb-hindlimb boundary can be altered within the interlimb region between
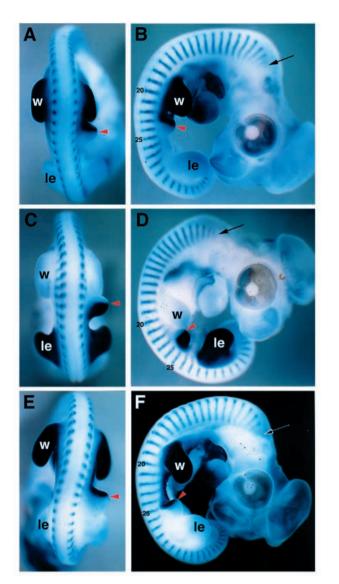
Fig. 6. *Tbxs* and *Mrf4* expression in embryos with FGF8-induced additional limb buds in the right flank. (A,C,E) Posterior views. (B,D,F) Lateral views of the embryos in A,C,E, respectively. Anterior is to the top. The arrowheads indicate the boundaries in the additional limb buds between *Tbx5*- (A,B,E,F) or *Tbx4*- (C,D) expressing and non-expressing domains. The arrows indicate the anteriormost somites that express *Mrf4*. w, wing bud; le, leg bud. The numbers in B,D,F indicate the somite level along the anteroposterior axis. Judged from their position, (A,B) are wing-type, (C,D) intermediate-type and (E,F) leg-type. The boundaries are at the level of somite 23 (A,B), somite 23/24 (C,D) and somite 25/26 (E,F).
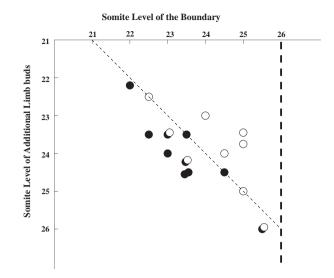

Fig. 7. Dependence of the somite level of the boundary between *Tbx*-expressing and non-expressing cells in *Dasoku* limb buds on their anteroposterior positions. FGF2 or FGF8 was applied to the flank cells. To determine the somite level of the boundary (abscissa), we examined *Tbx5* expression (open circles) in 9 cases of *Dasoku*-embryos and *Tbx4* expression (closed circles) in another 10 cases. The ordinate shows the position of *Dasoku* limb buds, defined as the level of opposite somites corresponding to the middle of the limb bud. For reference, the thoracic-lumbar boundary (Burke et al., 1995) is shown by the broken line. The dotted line indicates the ideal linear relation between the *Dasoku* position and the axial level of the boundary, which exists in the mid-level of the additional limb bud.

the level of somites 22-26 (Fig. 7). This suggests that FGF signaling is required for *Tbx5* and *Tbx4* to be expressed in the prospective limb regions, but the expression domains of each *Tbx* gene are further determined by additional factors.

DISCUSSION

Tbx5/Tbx4 may be involved in determination of limb identity

We demonstrated that three types of skeletal patterns of additional limbs, wing-type, leg-type and intermediate-type can be formed by ectopic application of FGF in the chick embryonic flank. Although the wing-leg phenotypic boundary is not clear in skeletal elements, most of the FGF-induced limbs were found to be chimeric in integumental characteristics and to exhibit a clear boundary in the integumental pattern. We also showed that the anterior half of the additional limb expresses *Tbx5*, whereas the posterior half expresses *Tbx4* in the intermediate type. Since the expression domains of *Tbx5* and *Tbx4* in the extra limb bud seem to correspond to the future cephalic wing-like limb and the caudal leg-like limb, respectively, concomitant expression of *Tbx5* and *Tbx4* may be involved in forelimb and hindlimb chimerism.

The expression of *Tbx5/Tbx4* genes could be closely correlated to establishment of the ectodermal structures rather than that of limb-type-specific skeletal features, because chimerism is not so obvious in cartilaginous elements (Table 1; Fig. 1), as observed in the pattern of epithelial structures (Fig. 2) and the pattern of *Tbx5/Tbx4* expression (Fig. 5) in additional limbs. However, the skeletal patterns of the additional limbs are clearly different from those of the wing and leg, especially in intermediate-type *Dasokus* (Fig. 1), although it is difficult to verify their chimerism. If there were any appropriate molecular markers for limb-type-specific cartilaginous elements, one might be able to clarify whether Tbx5/Tbx4 specifies limb-type skeletal pattern. This idea is supported by the fact that skeletal abnormalities affect the upper limbs in Holt-Oram syndrome, caused by mutations in the human *TBX5* gene (Basson et al., 1997; Li et al., 1997). Thus, *Tbx5/Tbx4* may also be involved in specification of the

skeletal pattern. Taken together with the expression pattern in normal chick (this study) and mouse embryos (Gibson-Brown et al., 1996), Tbx5 in the forelimb and Tbx4 in the hindlimb are most likely to be involved in determination of normal limb identity.

FGF may change the axial level of the limb through activation of *Tbx5/Tbx4*

We found that a boundary exists between the *Tbx5/Tbx4*-expressing and non-expressing domains in the additional limb bud in the flank. In normal chick embryos by stage 24, the most posterior end of *Tbx5* expression domain in the flank is at somite level 25/26 (Fig. 4E). This axial level corresponds to the thoracolumbar transition site of chick vertebrae by stage 25 (Burke et al., 1995). Therefore, we expected that the axial level of the *Tbx* expression boundary would be fixed at the level of the thoracolumbar transition even in the induced flank limb bud. However, we found that the level of this boundary varies with the site of FGF application between the levels of somites 22 and 26, not necessarily coinciding with the thoracolumbar boundary in the paraxial mesoderm. Thus, it appears that FGF activation of *Tbx5* and *Tbx4* gene transcription in the lateral plate mesoderm occurs independently of influence from the paraxial mesoderm. Furthermore, this implies that the position of the forelimb and hindlimb in tetrapods may be determined according to the site of FGF action in the lateral plate mesoderm. Recently, Cohn et al. (1997) showed that FGF can induce particular combinations of *Hox9* paralogous gene expression in the lateral plate mesoderm reflecting the types of additional limbs formed in the flank. Therefore, it is likely that a Hox code may also constitute this FGF-Tbx signaling cascade.

Correlation of FGF with activation of the *Tbx5* and *Tbx4* genes is reminiscent of the upregulation of expression of other T-box-containing genes, such as *TbxT* and *Tbx6L*, by FGF4 (Knezevic et al., 1997). Furthermore, another T-box-containing gene *Brachyury* and embryonic FGF (eFGF) were shown to be components of the same regulatory loop in *Xenopus* (Schulte-Merker and Smith, 1995). Although T-box-containing genes are quite divergent outside of the T-box, it is possible that similar reciprocal interactions might be present between FGF and *Tbx5/Tbx4* cognates in vertebrate limb specification.

Pinot (1970) reported that the prospective limb mesoderm can autonomously form a limb and determine its own limb identity depending upon its origin, but the prospective flank mesoderm cannot form a limb by itself nor specify limb identity. This phenomenon may be explained as follows: the prospective limb mesodermal cells can induce a limb bud in the presence of a mesenchymal FGF, such as FGF10 (Ohuchi et al., 1997), and determine limb identity by FGF-activated *Tbx5/Tbx4* expression. By contrast, the flank mesodermal cells could form a *Dasoku* if they were supplied with FGF, thus inducing the concomitant expression of *Tbx5* and *Tbx4*.

Tbx5/Tbx4 may regulate production of secreting factors to specify epithelial phenotype

Classical experiments demonstrated that transplantation of leg mesoderm underneath the wing AER produces a leg distal structure in the host wing (Saunders et al., 1959). This implied that a secreted molecule(s) derived from limb mesoderm may be directly responsible for limb epithelial specification (for

review see Gilbert, 1994). We showed that the mesenchymal expression patterns of *Tbx5/Tbx4* closely correlate to its epithelial pattern: cephalic wing epithelium (feather buds) and caudal leg epithelial structures (scales, claws, etc). This observation suggests that the forelimb and hindlimb epithelial phenotypes may be specified by mesenchymal factors induced by Tbx5/Tbx4. Therefore, it is likely that Tbx5 and Tbx4 regulate particular downstream secreting factors to specify limb epithelium. Recently, it was shown that bone morphogenetic protein (BMP) signaling is required for scale formation in chicks (Zou and Niswander, 1996). In addition, a *Tbx* homologous gene, *optomotor-blind* was found to be regulated by *decapentaplegic* (a *Bmp* homologue) in *Drosophila* (Grimm and Pflugfelder; 1996). Since such homologous gene sets seem to be used in many developmental processes in insects and vertebrates, it is reasonable to predict *Bmp* as a candidate target gene for Tbx4. It has also been shown that FGF signaling is involved in feather bud development in chicks (Noji et al., 1993; Song et al., 1996; Widelitz et al., 1996). Therefore, FGF stands as another candidate target factor of Tbx5, at least in the case of wing epithelial specification. Since there exists a clear boundary between wing- and leg-epithelial phenotypes and between *Tbx*-
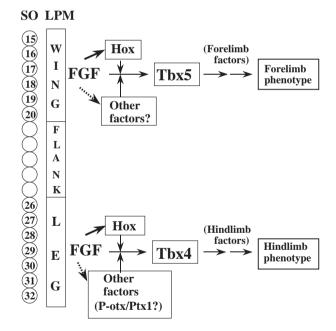

Fig. 8. A model for roles of FGF, Tbx5 and Tbx4 in determination of limb identity. FGF upregulates expression of *Tbx5* and *Tbx4* in the prospective wing (forelimb) and leg (hindlimb) mesoderm, respectively. Which *Tbx* expression is upregulated may be determined by Hox codes and other factors. According to Cohn et al. (1997), vertebrate limb position may be determined by a particular combinational expression of *Hox9* paralogous genes, which can be induced by FGF. By contrast, Tbx5/Tbx4 specifies each limb phenotype such as skeletal and integumental elements via subsequent signalling cascade. In the hindlimb, *P-otx/Ptx* may be included in 'other factors' because of its expression pattern (Szeto et al., 1996; Lanctôt et al., 1997) and FGF activation (our unpublished results). Constant reciprocal interactions between limb mesenchyme and epithelia may be crucial for establishment of each limb phenotype. SO, somites; LPM, lateral plate mesoderm.

expressing domain and non-expressing domain in additional limbs, we speculate that putative hindlimb and forelimb factors may suppress expression of *Tbx5* and *Tbx4*, respectively, as observed in the case of Radical fringe and En-1 in establishment of the dorsal-ventral boundary during the AER formation (Rodriguez-Esteban et al., 1997; Laufer et al., 1997).

A molecular model for vertebrate limb specification

We propose a model for vertebrate limb specification as shown in Fig. 8. The present study demonstrated that FGF is involved in activation of *Tbx5* and *Tbx4*, which then appear to function in determination of the forelimb and hindlimb identities, respectively. Mutual interaction between FGF and the Tbxs may be indispensable for initial limb specification, as has been shown in the case of Brachyury and eFGF in *Xenopus* (Schulte-Merker and Smith, 1995). Recent work has indicated that an initial endogenous FGF is most likely to be FGF10 (Ohuchi et al., 1997); however, as the main targets of FGF10 appear to be epithelial cells (Ohuchi et al., 1997), the effects of FGF10 on the mesenchymal cells might be indirect. FGF8 and FGF4, whose mRNAs are detected in the AER (Crossley et al., 1996; Niswander et al., 1993), seem to be dispensable for *Tbx5/Tbx4* expression, because AER removal did not affect *Tbx* expression (H. Ohuchi, H. Yoshioka and S. Noji, unpublished results). Since FGF can upregulate expression of both *Tbx5* and *Tbx4*, it would appear that FGF does not determine limb identity by itself. However, it has been shown that FGF can regulate the expression of specific *Hox9* paralogous genes in the lateral plate mesoderm depending upon the somite level of FGF action (Cohn et al., 1997). This suggests that, in authentic limb formation, endogenous FGFs may determine the position of the limb by regulating the expression of *Hox* genes. Thus, expression of *Tbx5/Tbx4* should be regulated at least by both FGF and Hox9 paralogous gene products. It has been found that a homeobox gene, *P-otx/Ptx1* is specifically expressed in the hindlimb (Szeto et al., 1996; Lanctôt et al., 1997) and preliminary data reveals that it can be induced by FGF in the leg-type additional limb bud (H. Ohuchi, H. Yoshioka and S. Noji, unpublished results). Therefore, in determination of hindlimb identity, P-otx/Ptx1 may be involved in addition to the *Hox* genes and FGF, suggesting that these factors are likely to work cooperatively with Tbx5/Tbx4 in vertebrate limb specification. Subsequently, Tbx5 and Tbx4 activate their own sets of target genes to establish individual phenotypes with each limb identity. In determination of bone patterning, some factors may regulate Hox9-Hox13 codes for establishing cartilage pattern formation. For determination of epithelial phenotypes, mesenchymal cells expressing *Tbx5/Tbx4* may produce some secreting factors such as BMP and FGF which act on epithelial cells. The consequent epithelial-mesenchymal interaction would result in epithelial pattern formation.

REFERENCES

Agulnik, S. I, Garvey, N., Hancock, S., Ruvinsky, I., Chapman, D. L., Agulnik, I., Bollag, R., Papaioannou, V. and Silver, L. M. (1996). Evolution of mouse *T-box* genes by tandem duplication and cluster dispersion. *Genetics* **144**, 249-254.

Basson, C. T., Bachinsky, D. R., Lin, R. C., Levi, T., Elkins, J. A., Soults, J., Grayzel, D., Kroumpouzou, Traill, T. A., Leblanc-Straceski, J., Renault, B., Kucherlapati, R., Seidman, J. G. and Seidman, C. E. (1997). Mutations in human cause limb and cardiac malformations in Holt-Oram syndrome. *Nature Genet.* **15**, 30-35.

Bollag, R. J., Siegfried, Z., Cebra-Thomas, J. A., Garvey, N., Davidson, E. M. and Silver, L. M. (1994). An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the *T* Locus. *Nature Genet.* **7**, 383-389.

Burke, A. C., Nelson, C. E., Morgan, B. A. and Tabin, C. (1995). *Hox* genes and the evolution of vertebrate axial morphology. *Development* **121**, 333-346.

Chapman, D. L., Garvey, N., Hancock, S., Alexiou, M., Agulnik, S. I., Gibson-Brown, J. J., Cebra-Thomas, J., Bollag, R. J., Silver, L. M. and Papaioannou, V. E. (1996). Expression of the T-box family genes, *Tbx1-Tbx5*, during early mouse development. *Dev. Dyn.* **206**, 379-390.

Cohn, M. J., Izpisúa-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-746.

Cohn, M. J. and Tickle, C. (1996). Limbs: a model for pattern formation within the vertebrate body plan. *Trends in Genetics* **12**, 253-257.

Cohn, M. J., Patel, K., Krumlauf, R., Wilkinson, D. G., Clarke, J. D. W. and Tickle, C. (1997). *Hox9* genes and vertebrate limb specification. *Nature* **387**, 97-101.

Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84**, 127-136.

Dougherty, J. P. and Temin, H. M. (1986). High mutation rate of a spleen necrosis virus-based retrovirus vector. *Mol. Cell Biol.* **6**, 4387-4395.

Frohman, M. A., Dush, M. K. and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**, 8998-9002.

Fujisawa-Sehara, A., Nabeshima, Y., Komiya, T., Uetsuki, T., Asakura, A. and Nabeshima, Y. (1992). Differential trans-activation of muscle-specific regulatory elements including the myosin light chain box by chicken MyoD, myogenin, and MRF4. *J. Biol. Chem.* **267**, 10031-10038.

Gibson-Brown, J. J., Agulnik, S. I., Chapman, D. L., Alexiou, M., Garvey, N., Silver, L. M. and Papaioannou, V. E. (1996). Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mech. Dev.* **56**, 93-101.

Gilbert, S. F. (1994). Regional specificity of induction. In *Developmental Biology* (4th ed.), pp. 650-652. Massachusetts: Sinauer Associates, Inc.

Grimm, S. and Pflugfelder, G. O. (1996). Control of the gene *optomotor-blind* in *Drosophila* wing development by *decapentaplegic* and *wingless*. *Science* **271**, 1601-1604.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **94**, 257-265.

Herrmann, B. G. Labeit, S., Poustka, A., King, T. R. and Lehrach, H. (1990). Cloning of the *T* gene required in mesoderm formation in the mouse. *Nature* **343**, 617-622.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**, 3004-3012.

Itoh, N., Mima, T. and Mikawa, T. (1996). Loss of fibroblast growth factor receptors is necessary for terminal differentiation of embryonic limb muscle. *Development* **122**, 291-300.

Knezevic, V., De Santo, R. and Mackem S. (1997). Two novel chick T-box genes related to mouse *Brachyury* are expressed in different, non-overlapping mesodermal domains during gastrulation. *Development* **124**, 411-419.

Lanctôt, C., Lamolet, B. and Drouin, J. (1997). The *bicoid*-related homeoprotein *Ptx1* defines the most anterior domain of the embryo differentiates posterior from anterior lateral mesoderm. *Development* **124**, 2807-2817.

Laufer, E., Dahn, R., Orozco, O. E., Yeo, C.-Y., Pisenti, J., Henrique, D., Abbott, U. K., Fallon, J. F. and Tabin, C. (1997). Expression of Radical *fringe* in limb-bud ectoderm regulates apical ectodermal ridge formation. *Nature* **386**, 366-373.

Li, Q. Y., Newbury-Ecob, R. A., Terrett, J. A., Wilson, D. I., Curtis, A. R. J., Yi, C. H., Gebuhr, T., Bullen, P. J., Robson, S. C., Strachan, T., Bonnet, D., Lyonnet, S., Young, I. D., Raeburn, J. A., Buckler, A. J., Law, D. J. and Brook, J. D. (1997). Holt-Oram syndrome is caused by mutations in *TBX5*, a member of the *Brachyury (T)* gene family. *Nature Genetics* **15**, 21-29.

Nelson, C. E., Morgan, B. A., Burke, A. C., Laufer, E., DiMambro, E., Murtaugh, L. C., Gonzales, E., Tessarollo, L., Parada, L. F. and Tabin, C. (1996). Analysis of Hox gene expression in the chick limb bud. *Development* **122**, 1449-1466.

Niswander, L., Tickle, C., Vogel, A., Booth, I. and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579-587.

Nohno, T., Kawakami, Y., Ohuchi, H., Fujiwara, A., Yoshioka, H. and Noji, S. (1995). Involvement of the *Sonic hedgehog* gene in chick feather formation. *Biochem. Biophys. Res. Commun.* **206**, 33-39.

Noji, S., Koyama, E., Myokai, F., Nohno, T., Ohuchi, H., Nishikawa, K. and Taniguchi, S. (1993). Differential expression of three chick FGF receptor genes, *Fgfr1, Fgfr2* and *Fgfr3*, in limb and feather development. In *Limb Development and Regeneration* (ed. J. F. Fallon, P. F. Goetinck, R. O. Kelley and D. L. Stocum), pp. 645-654. New York: Wiley-Liss, Inc.

Ohuchi, H., Nakagawa, T., Yamauchi, M., Ohata, T., Yoshioka, H., Kuwana, T., Mima, T., Mikawa, T., Nohno, T. and Noji, S. (1995). An additional limb can be induced from the flank of the chick embryo by FGF4. *Biochem. Biophys. Res. Commun.* **209**, 809-816.

Ohuchi, H., Shibusawa, M., Nakagawa, T., Ohata, T., Yoshioka, H., Hirai, Y., Nohno, T., Noji, S. and Kondo, N. (1997a). A chick *wingless* mutation causes abnormality in maintenance of *Fgf8* expression in the wing apical ridge, resulting in loss of the dorsoventral boundary. *Mech. Dev.* **62**, 3-13.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N. and Noji, S. (1997b). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interactions with FGF8, an apical ectodermal factor. *Development* **124**, 2235-2244.

Oliver, G., Wright, C. V. E., Hardwicke, J. and De Robertis, E. D. (1988). A gradient of homeodomain protein in developing forelimbs of Xenopus and mouse embryos. *Cell* **55**, 1017-1024.

Peterson, R. L., Papenbrock, T., Davda, M. M. and Awgulewitsch, A. (1994). The murine Hoxc cluster contains five neighboring AbdB-related Hox genes that show unique spatially coordinated expression in posterior embryonic subregions. *Mech. Dev.* **47**, 253-260.

Pinot, M. (1970). Le rôle du mésoderme somitique dans la morphogenèse précoce des membres de l'embryon de Poulet. *J. Embryol. Exp. Morph.* **23**, 109-151.

Rodriguez-Esteban, C., Schwabe, J. W. R., De La Peña, J., Foys, B., Eshelman, B. and Izpisúa Belmonte, J. C. (1997). *Radical fringe* positions the apical ectodermal ridge at the dorsoventral boundary of the vertebrate limb. *Nature* **386**, 360-366.

Saunders, J. W. Jr., Gasseling, M. T. and Cairns, J. M. (1959). The differentiation of prospective thigh mesoderm grafted beneath the apical ectodermal ridge of the wing bud in the chick embryo. *Dev. Biol.* **1**, 281-301.

Schulte-Merker, S. and Smith, J. C. (1995). Mesoderm formation in response to *Brachyury* requires FGF signalling. *Curr. Biol.* **5**, 62-67.

Simon, H-G., Kittappa, R., Khan, P. A., Tsulfidis, C., Liversage, R. A. and Oppenheimer, S. (1997). A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate involved in vertebrate forelimb/hindlimb patterning. *Development* **124**, 1355-1366.

Song, H.-K., Wang, Y. and Goetinck, P., F. (1996). Fibroblast growth factor 2 can replace ectodermal signaling for feather development. *Proc. Natl. Acad. Sci. USA* **93**,10246-10249.

Szeto, D. P., Ryan, A. K., O'Connell, S. M. and Rosenfeld, M. G. (1996). P-OTX: a PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development. *Proc. Natl. Acad. Sci. USA* **93**, 7706-7710.

Ting-Berreth, S. A. and Chuong, C-M. (1996). Sonic hedgehog in feather morphogenesis: Induction of mesenchymal condensation and association with cell death. *Dev. Dyn.* **207**, 157-170.

Vogel, A., Rodriguez, C. and Izpisúa-Belmote, J. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737-1750.

Widelitz, R. B., Jiang, T.-X., Noveen, A., Chen, C-W., J. and Chuong, C-M. (1996). FGF induces new feather buds from developing avian skin. *J. Invest. Dermatol.* **107**, 797-803.

Wilkinson, D. G. (1992). Whole mount in situ hybridization of vertebrate embryos. In *In Situ Hybridization* (ed. D. G. Wilkinson), pp. 75-85, Oxford: Oxford University Press.

Zou, H. and Niswander, L. (1996). Requirement for BMP signalling in interdigital apoptosis and scale formation. *Science* **272**, 738-741.