J. Anat. (2001) **199**, pp. 195–204, with 7 figures Printed in the United Kingdom

Evolutionary aspects of positioning and identification of vertebrate limbs

KOJI TAMURA[1], RITSU KURAISHI[2], DAISUKE SAITO[1], HIDEKI MASAKI[1], HIROYUKI IDE[1] AND SAYURI YONEI-TAMURA[1]

[1] *Biological Institute, Graduate School of Science, and* [2] *Asamushi Marine Biological Station, Tohoku University, Japan*

(*Accepted 9 April 2001*)

ABSTRACT

Emerging developmental studies contribute to our understanding of vertebrate evolution because changes in the developmental process and the genes responsible for such changes provide a unique way for evaluating the evolution of morphology. Endoskeletal limbs, the locomotor organs that are unique to vertebrates, are a popular model system in the fields of palaeontology and phylogeny because their structure is highly visible and their bony pattern is easily preserved in the fossil records. Similarly, limb development has long served as an excellent model system for studying vertebrate pattern formation. In this review, the evolution of vertebrate limb development is examined in the light of the latest knowledge, viewpoints and hypotheses.

Key words: Limb; limb bud; development; vertebrate; evolution; AER; FGF; Tbx.

INTRODUCTION

It is thought that the ancestors of terrestrial vertebrates arrived on land hundreds of millions of years ago, and that their fins—originally used for swimming gradually—evolved into limbs used for locomotion on land. This was the birth of the tetrapod. Among the tetrapod vertebrates, limb morphology was further modified to adapt to various environments. Birds developed wings to fly, and marine mammals such as dolphins and whales reformed their limbs into fins to swim in the sea again. Humans evolved a complicated configuration of hands and fingers that enabled them to create and use many kinds of tools. On the other hand, some species such as snakes and caecilians evolved a limbless morphology.

While each taxonomic group has different limb morphologies, limbs generally have a common skeletal pattern: 1 bone element in the upper arm, 2 in the forearm, and typically 5 in the hand (the number of fingers is 5 or fewer in modern tetrapods). Moreover, the process and molecular mechanisms of limb development are believed to be analogous. The primordial structure of the limb, the limb bud, is composed of lateral plate mesoderm surrounded by an ectodermal layer, and emerges laterally from specific positions of the embryonic body. During its outgrowth, mesenchymal cells in the limb bud differentiate into chondrocytes that make proximal cartilage elements and, by the addition of progressively more distal elements, the final limb pattern forms. This pattern of chondrogenesis depends on the positional identity of cells that corresponds to the nested expression of *Hox* genes common to many vertebrates. In addition, vertebrate embryos share the same molecular mechanisms for pattern formation during limb/fin development. For instance, a zone of polarising activity (ZPA), the region responsible for anterior-posterior axis formation, has been identified at the posterior margin of the limb bud in many tetrapods, and *Sonic hedgehog* (*Shh*), which is essential and sufficient for the ZPA function, is expressed in the ZPA in the limb/fin bud of a number of vertebrate groups, including mammals (Echelard et al. 1993), birds (Riddle et al. 1993), anurans (Endo et al. 1997), urodeless (Imokawa & Yoshizato, 1997; Torok et al. 1999, and teleosts (Akimenko & Ekker, 1995).

Fish possess paired pectoral and pelvic fins that are considered homologous to the tetrapod forelimb and hindlimb, respectively. Pectoral and pelvic fins in teleost fishes possess internal skeletal elements in their proximal region whose pattern does not completely

Correspondence to Professor Koji Tamura, Associate Professor, Biological Institute, Graduate School of Science, Tohoku University, Aobayama Aoba-ku, Sendai 980-8578, Japan. Tel: +81 22 217 3489; fax: +81 22 217 3489; e-mail: tam@biology.tohoku.ac.jp

correspond to that in tetrapods, as well as a peculiar distal structure, ectoskeleton, which tetrapods lack. Despite the different structural motifs of limbs and fins, anatomical and molecular observations reveal that they share many common mechanisms in their development, for example *Shh* expression. The fact that in embryogenesis limbs/fins appear to be produced by common developmental mechanisms suggests that modern vertebrates inherited the process of limb development from their common ancestor. What then have the various modern vertebrate groups acquired, altered or lost during the process of limb evolution from that ancestral structure? In other words, what is homologous and what is distinct between limbs and fins? Although we do not understand the precise nature of evolution that occurred in prehistoric times, we are still hopeful that emerging information based on the study of developmental processes will ultimately lead to such an understanding since the basis of final morphology is necessarily constructed during embryogenesis.

Hypotheses on the origin of the paired fin

Although there is still no clear evidence about when and how the original paired fins (pectoral and pelvic) developed in an ancestral fish, a number of hypotheses have been advocated. Probably the oldest hypothesis for the origin of paired fins is the 'gill-arch' theory that posits the origins of pectoral fins from the branchial arches. This hypothesis, which is based on the skeletal similarities between dipnoan branchial arches and fins, is not supported by other morphological and embryological considerations (see Coates, 1994, for a review). Except for some species whose fins are secondarily degenerate, all modern fish have 2 sets of paired fins. Nevertheless, some fossil fish have only 1 set of paired fins, supporting a hypothesis that the origin of 2 sets of paired fins proceeded from an ancestor that possessed a single set of fins at the pectoral level. This hypothesis is widely accepted and supported by fossil records showing that the earliest fins seen in some fossil agnathans such as *Hemicylaspis* are pectoral fins. This view is also supported by speculations from genetic and developmental bases (Coates & Cohn, 1998; Ruvinsky & Gibson-Brown, 2000). Nonetheless, this hypothesis can be challenged by the possibility that the fins themselves in these agnathans originated from a lineage that had already developed 2 sets of paired fins, and that the pelvic ones had been lost secondarily (see Tabin, 1992). Alternatively, 'the fin fold theory' proposes that 2 sets of paired fins were evolved from a pair of lateral fin folds extending along the lateral body of hypothetical ancestral fish (reviewed by Jarvik, 1980). This theory is also widely accepted and is supported by recent evidence from the fossil record (Shu et al. 1999), the existence of fin fold-like structures in amphioxus, and descriptions of paired fin development in cartilaginous fishes (reviewed in Jarvik, 1980). Evidence obtained from some experimental embryological studies also support this theory as described below.

Implications of the dorsal AER of chick embryos

The timing of the start of limb bud development in the embryo varies depending on species. In the chick embryo, the limb bud starts to grow about 22 days after incubation, while in mice, the limb bud becomes visible in the embryo from about E9.5. Some studies using embryological manipulations in chick embryos (Saunders & Reuss, 1974; Carrington & Fallon, 1984) have demonstrated (see Fig. 1*A*) that an appropriate region of the lateral plate mesoderm is first restricted into 'the limb mesenchyme' and that the limb mesenchyme then induces a particularly thickened ectoderm, the apical ectodermal ridge (AER), in the overlying ectoderm. The limb mesenchyme and the AER interact with each other, resulting in distal limb bud growth and formation of the limb skeletal pattern; in the other words the AER keeps the underlying mesenchyme in an undifferentiated state and the mesenchymal cells maintain the AER structure. This is one of the most important epithelial–mesenchymal interactions for limb development.

Several fibroblast growth factors (FGFs) are known to be involved in this epithelial–mesenchymal interaction. *Fgf8* is specifically expressed in the AER (Fig. 1*B*), and the application of FGF protein can rescue the distal truncation that occurs after AER removal (Niswander et al. 1993; Fallon et al. 1994). *Fgf10* starts to be expressed in the limb field of the lateral plate mesoderm before the AER is constructed, and the protein has the ability to induce the ectopic AER in the flank region (Ohuchi et al. 1997; Yonei-Tamura et al. 1999) as shown with other FGFs (Cohn et al. 1995). Targeting disruption of the mouse *Fgf10* gene results in complete defect of the limb (Min et al. 1998; Seikine et al. 1999), strongly suggesting that FGF10 is an essential factor for AER induction and maintenance of its function. Moreover, FGF7 and FGF10, which share the same receptor and have similar functions (Ohuchi et al. 1997; Igarashi et al. 1998), can induce the AER directly in the ectoderm,

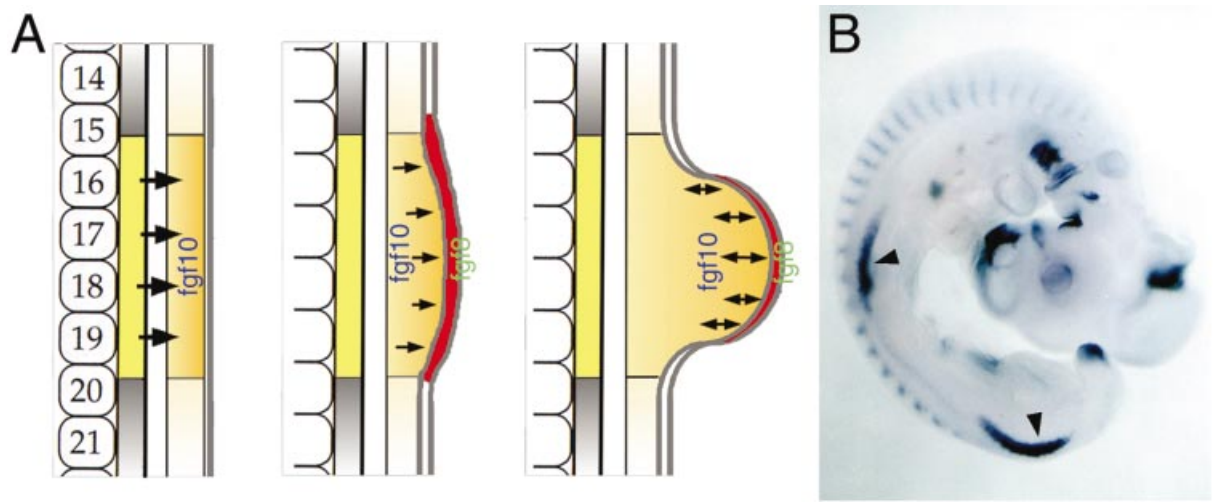

Fig. 1. (*A*) Schematic representation of limb initiation and epithelial-mesenchymal interaction for limb development. See text for the detail. (*B*) Expression pattern of *fgf8*. Arrowheads indicate *fgf8* expression in the AER of E3 chick embryo.

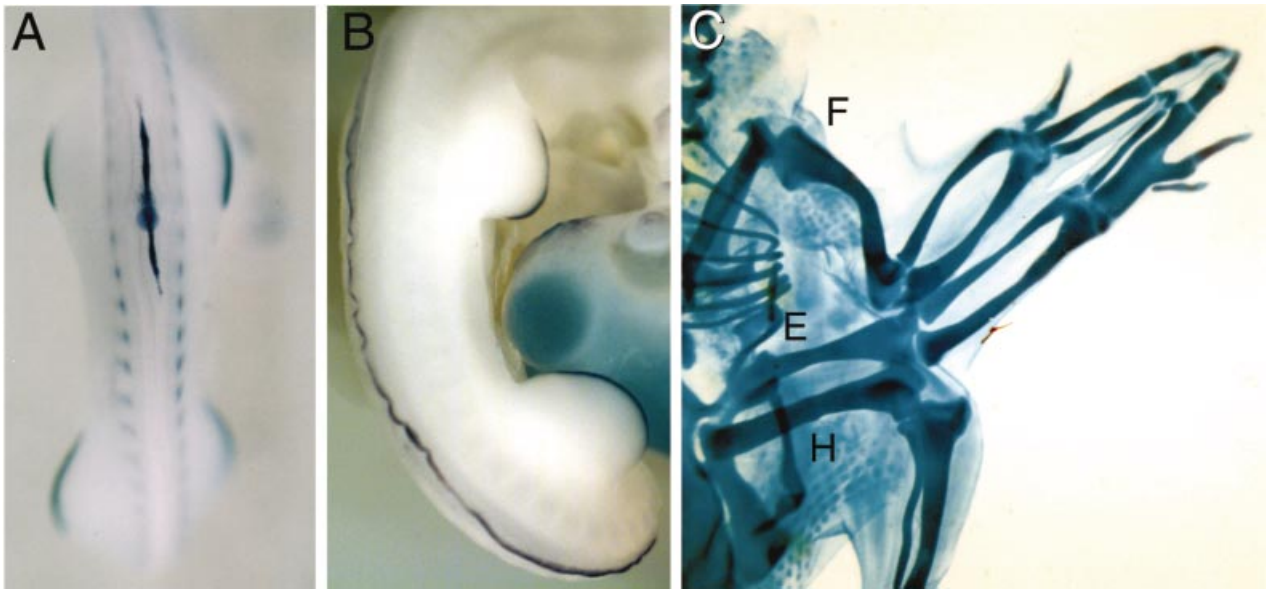

Fig. 2. (*A*) Ectopic expression of *fgf8* in the back induced by FGF7. in this sample, FGF7 was applied by a bead as a carrier. (*B*) Ectopic expression of *fgf8* in the back induced by FGF10. *Fgf10* containing R-CAS virus solution was injected into the central canal of the neural tube. (*C*) An extra limb (E) formed between forelimb (F) and hindlimb (H) after implantation of the AER-like structure induced in the back.

while the other FGFs such as FGF1, 2, and 4 require the mesenchyme to induce the AER (Yonei-Tamura et al. 1999). Since it is likely that *fgf7* is not expressed in the appropriate position in the chick embryo (Yonei-Tamura et al. 1999), FGF10 must be the endogenous AER inducer. The results of *Fgf10* knock-out studies in mouse limb (Min et al. 1998; Sekine et al. 1999), embryological studies in the chick (Ohuchi et al. 1997; Yonei-Tamura et al. 1999), and the demonstration that epithelial–mesenchymal interactions important for limb formation in amphibians is mediated by FGF10 and FGF8 (Yokoyama et al. 2000, 2001) suggest that FGF10 is a common mediator of limb initiation and development in all vertebrates, although the function of FGF10 in fin development in fish remains unclear.

Intriguingly, additional application of FGF7 or FGF10 in the medial back of thick embryos induces the formation of an AER-like structure in the dorsal midline (Yonei-Tamura et al. 1999; see Fig. 2*A*, 2*B*). When taken from the midline and implanted into the flank region, this AER-like structure can induce an additional limb in the flank region (Fig. 2*C*), suggesting that this structure not only expresses several gene markers (*fgf8*, *msx1* and *msx2*, Yonei-Tamura et al. 1999) but also has intact AER function.

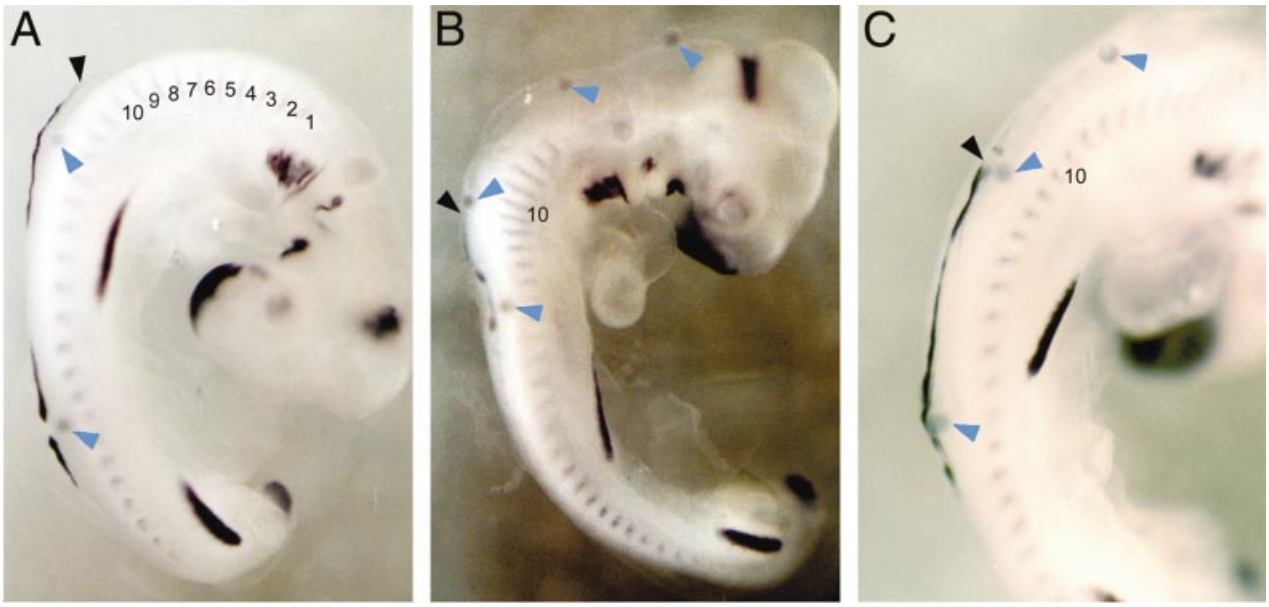

Fig. 3. When beads soaked in FGF7 were implanted at several positions (blue arrowheads) along the rostral–caudal axis of midline, the extra AER in the back is induced more posteriorly than the 10th somite (10). Note that ectopic expression of *fgf8* can be detected more posteriorly than back arrowheads in 3 examples (*A–C*).

Since the extra AER is only seen more posterior than the 10th somite (Fig. 3), the competence for AER induction in the median ectoderm appears to lie more caudally than the 10th somite in the chick embryo. These findings, together with the induction of AER formation in the flank region, indicate that 2 areas of the embryonic ectoderm have the competence for AER formation (Fig. 5*B*). One of them is the border between the dorsal and ventral portions of the trunk region from the anterior end of the forelimb bud to the posterior margin of the hindlimb bud. The other is the dorsal midline of the back extending from the neck to the tail (the caudal end of the competence is unclear). The induced AER in the dorsal midline disappears within 72 h and never forms an additional limb structure, probably because there are no limb competent mesenchymal cells in the back. If the limb-region mesoderm without an overlying ectoderm is inserted between the dorsal ectoderm and the neural tube (Fig. 4*A*), the implanted mesoderm induces an AER in the middorsal ectoderm, resulting in an additional limb on the back (Fig. 4*B* and 4*C*). Interesting, this extra limb (we call this wing 'an angel wing') appears to be double-dorsal since both sides of the limb express the dorsal-specific gene *Lmx-1* (Yonei-Tamura et al. 1999).

In contrast to the paired wings of an angel, the induced 'angel wing' on the back of the chick embryo is not paired; however, this single ectopic wing implies a great deal to us. The regional specificity of AER-forming ability shows that there are some commonalities in the 'epithelium of the dorso-ventral boundary' and the 'epithelium of the back' in the torso region of the embryonic body. We propose that the existence of these commonalities in modern tetrapod vertebrates originate from the ability to grow appendages in ancestral vertebrates. In other words, these commonalities suggest that a distant ancestor of the chicken, which is the common ancestor of amphibians and reptiles, had appendages bilaterally and along with middorsal back. The common ancestor must be a kind of fish forerunner, and existing fishes have median fins such as a dorsal fin, a caudal fin, and an anal fin. Amphibians, which first arrived on land as tetrapods, have a medium fin (fin fold) during their lives in water as tadpoles. There is no direct evidence that the AER-forming ability in the back is a trace or vestige of the ability to form median appendages, but some comparative descriptions on their development would support this idea. The embryonic precursor of median fins, the fin fold, in teleost fishes exhibits a specific structure, the apical fold, which has been found to express an AER marker, *fgf8* (Yonei-Tamura et al. 1999). The data in Figure 3, showing that the extra AER on the back is only induced more posteriorly than the neck region, is connected to the fact that the median fins of fishes and amphibians are formed only in the torso. Another similarity can be found between the median fin fold and the AER on the back. The apical fold in the median fins is known to recruit neural crest cells for forming the median fin mesenchyme (Smith et al. 1994), and, comparably,

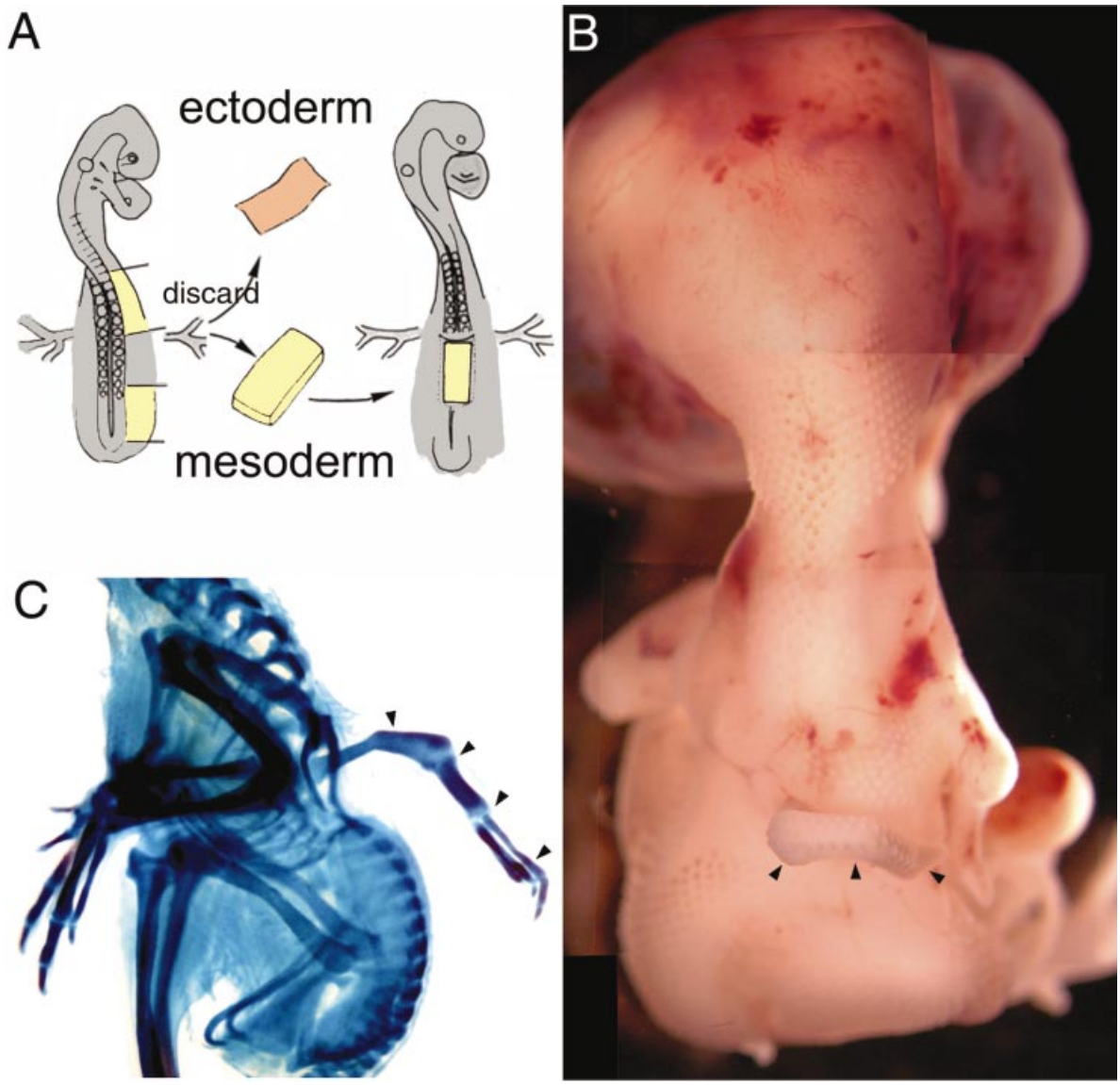

Fig. 4. An additional limb (an ''angel wing'') is formed in the back of the chick embryo. (*A*) Schematic representation of implantation. After discarding an overlying ectodermal tissue, only presumptive limb mesenchymal layer was implemented into a space between the neural tube and the overlying back ectoderm. (*B*) Five days after the operation, an additional limb outgrowth (arrowheads) can be observed on the back. (*C*) After eight days, well patterned limb cartilage (stained with Alcian blue) is produced on the back.

neural crest cells can be observed under the AER induced in the back of the chick embryo (Yonei-Tamura et al. 1999). A significant difference between them is whether the crest cells can differentiate into mesenchyme or not. The neural crest cells under the dorsal AER in the chick never produce cartilage, probably only the crest cells in the head region have the ability to form skeletogenic mesenchymal tissue. These anatomical and molecular similarities provide an opportunity to consider evolutionary aspects of similarity and diversity between various shapes and positions of the fin and limb.

On the other hand, the chick flank region can initiate a limb bud by application of FGFs such as FGF1, 2, 4, 8 and 10 (Cohen et al. 1995; Ohuchi et al. 1995; Crossley et al. 1996; Vogel et al. 1996; Yonei-Tamura et al. 1999), suggesting that the dorsoventral boundary in the flank, as well as the dorsal midline, has the ability for AER induction. Similar results of FGF application have been reported in mouse embryos (Abud et al. 1996; Tanaka et al. 2000). Even if FGF is not applied, a chick lateral flank tissue can form a limb when it is isolated from the embryo (Stephens et al. 1989). Classical embryological manipulations in amphibian embryos have shown extra limb formation in their flank region (Balinsky, 1925). It is possible that the limb-growing ability in all tetrapods is latently provided in the flank region. The

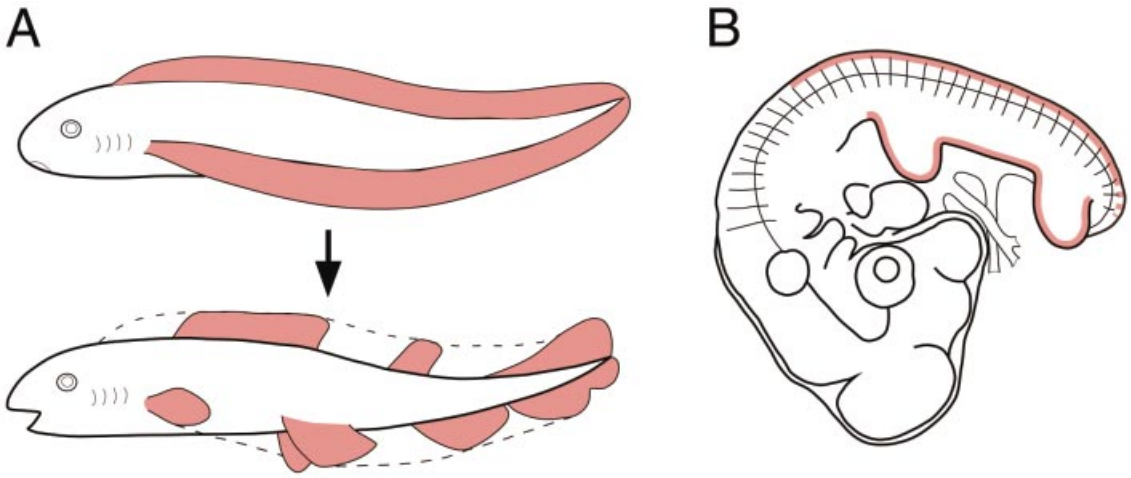

Fig. 5. (*A*) The fin-fold theory, which proposes that a pair of lateral fin folds extending along the lateral body of a hypothetical ancestral fish evolved into 2 sets of paired fins and that unpaired fins such as a dorsal fin, a caudal fin, and an anal fin in existing fishes were evolved from a dorsal fin fold. (*B*) Pink lines in the dorsal and lateral regions of a chick embryo indicate the regions that possess competence for AER induction.

fin-fold theory proposes that ancestral fishes had one set of continuous lateral fin fold that ran the length of the torso, from which paired appendages evolved, resembling the manner in which the dorsal fin fold became median fins. The theory that fins originated in the flank region is supported by fossil evidence (Shu et al. 1999). In addition, as mentioned above, the lines of fin fold in the ancestral fish (Fig. 5*A* represented in pink) and the lines that point out the competence for the AER formation in the chick embryo (Fig. 5*B*, represented by pink lines) agree.

Chondrichthyans (cartilaginous fishes) are thought to have arrived as a major separate groups early after the emergence of jawed vertebrates; they also have the most primitive paired fin structure of modern vertebrates. Interestingly, an adult skate has a set of enlarged pectoral fins (Fig. 6*A*) and a set of small but distinct pelvic fins (Fig. 6*B*), but it does not appear to have any space (flank region) between the pectoral and pelvic fins. Observation of embryos of the skate reveals that the pectoral and pelvic fin buds emerge side-by-side (Fig. 6*C*, 6*D* and 6*E*), suggesting that their paired fins might be derived by using whole lateral competences for limb/fin formation. Experiments in which the cell proliferation of the lateral plate mesoderm in chicken embryos was measured indicate that budding of limb buds arose because of a local decrease in the cell proliferation rate in the flank region, rather than a specific increase in cell growth in the presumptive limb region (Searls & Janners, 1971). The competent regions in the flank and back that can form the AER in various animals may indicate the ancestral fin regions that fell away during the process of the limb evolution in the fin-fold theory. In addition, the interspecies difference in limb/fin positioning might depend on where a species opens a window to grow the limb/fin by choosing specific positions from the regions of AER-forming competence located in the entire lateral and dorsal body. Further anatomical, molecular and experimental analyses of the skate embryo and comparative embryological studies on cartilaginous fishes and tetrapods should provide more evidence of this scenario.

Limb/fin identity and Tbx5/4 *genes*

Limbs in human, needless to say, are the arms (forelimbs) and legs (hindlimbs). Despite the clear difference in morphology (limb identity) between human limbs, the forelimb bud and the hindlimb bud at early stages of embryogenesis are very homologous or even identical, as seen in many other vertebrate embryos. Classical transplantations of chick fore and hindlimb bud revealed that these young limb buds have already been identified (Saunders & Gasseling, 1959; Isaac et al. 1998), signifying that there must be regulatory mechanisms and responsible genes for the establishment of limb identity. Some mutations in mice and humans display phenotypes with a different manner or severeness in forelimb and hindlimb, suggesting that they have different gene regulations, probably in the developmental process. Indeed, a human mutation, Holt-Oram syndrome, which displays forelimb (hand)-specific abnormalities or defects in heterozygotes, was discovered to have mutations in the first forelimb-specific gene, *Tbx5* (Basson et al. 1997; Li et al. 1997). *Tbx5* transcripts are found

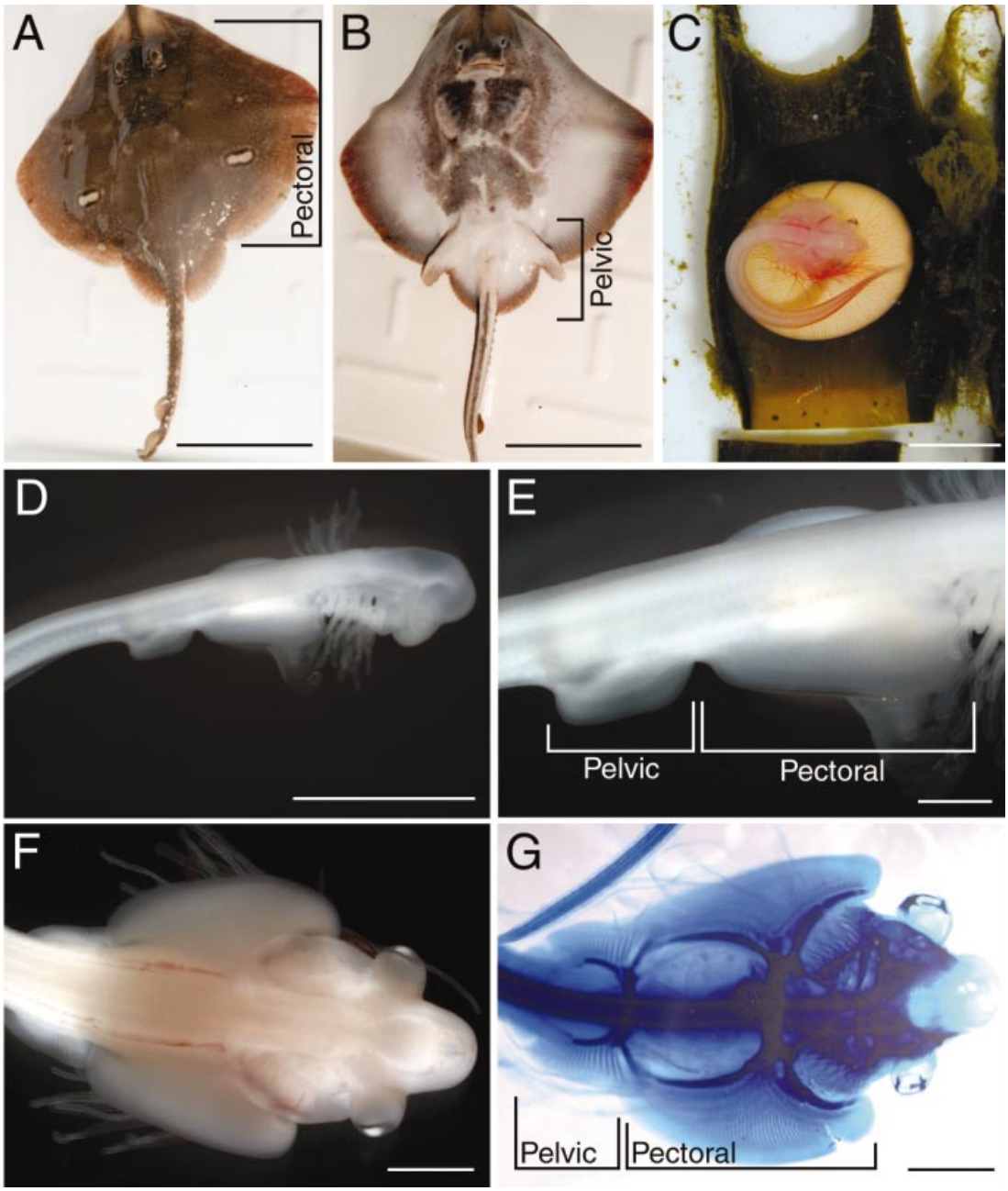

Fig. 6. (*A*, *B*) An adult skate, *Raja kenojer*; dorsal view (*A*) and ventral view (*B*). Scale bars, 10 cm. Distinct pelvic fins that are much small than pectoral fins can be observed from the ventral side (*B*). (*C–G*) Skate embryos (*C*) An egg with an embryo covered with a shell Bar, 1 cm. (*D*) An early stage embryo that starts to develop pectoral and pelvic fin buds side by side. Bar 500 μm. (*E*) Higher magnification of (*D*). Bar 100 μm. (*F*) An older embryo whose pectoral fin buds enlongate rostrally and caudally. Bar, 200 μm. (*G*) Cartilage pattern of the embryo in (*F*). Two individual sets of pectoral and pelvic fin rays are visible. Bar, 200 μm.

almost exclusively in the forelimb bud (Fig. 7*A*) in many tetrapod embryos, including human (Li et al. 1997), mouse (Chapman et al. 1996; Gibson-Brown et al. 1996), chick (Isaac et al. 1998; Logan et al. 1998; Ohuchi et al. 1998; Gibson-Brown et al. 1998), Xenopus (Takabatake et al. 2000), and newt (Simon et al. 1997) embryos. *Tbx4*, another member of the T-box gene family (see Papaioannou & Silver (1998) for a review of T-box gene family), has a complementary expression only in the hindlimb (Fig. 7*A*). The results of experiments on tissue transplantation and FGF-bead implantation (Isaac et al. 1998; Logan et al. 1998; Ohuchi et al. 1998; Gibson-Brown et al. 1998) and the results of functional analyses of these T-box genes (Takeuchi et al. 1999; Rodriguez-Esteban et al. 1999) in the chick embryo have reinforced the idea that these 2 T-box genes are responsible for the specification of limb identity.

By phylogenetic and mapping analyses, Agulnik et al. (1996) proposed an interesting model for the

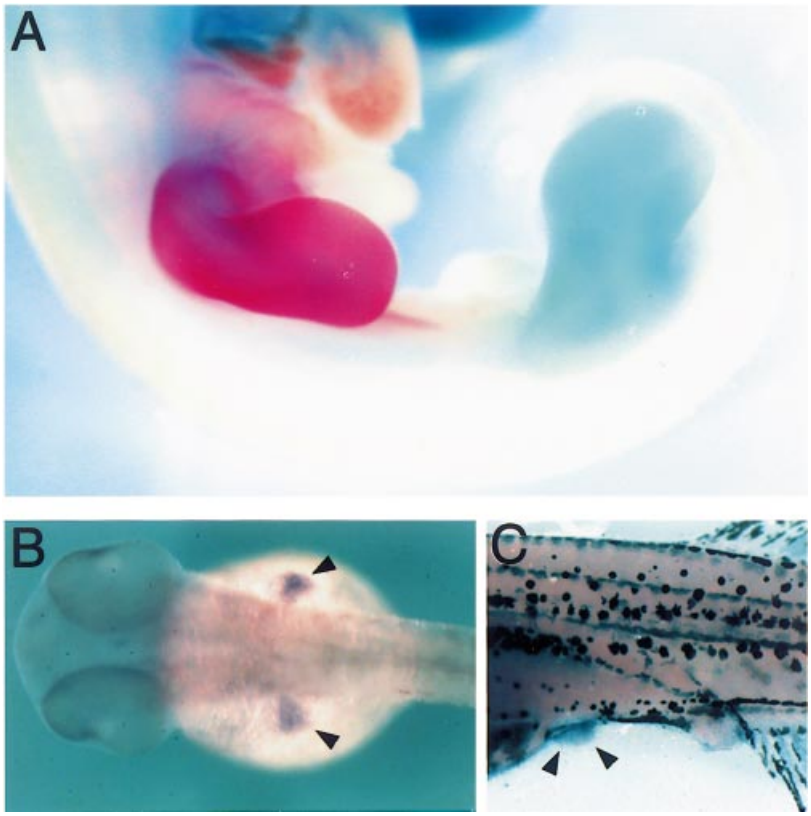

Fig. 7. Limb/fin identity-specific expression of two T-box genes. *Tbx5* is only found in forelimb buds of chick embryo (*A*, stained in red) and pectoral fin buds of zebrafish embryo (*B*, arrowheads), and *Tbx4* is exclusively expressed in hindlimb buds (*A*, shown in green) and pelvic fin buds (*C*, arrowheads).

evolution of some T-box genes, in which a gene ancestral to *Tbx5* and *Tbx4* (and two other T-box genes, *Tbx2* and *Tbx3*) went through a tandem duplication by uneven crossing-over, resulting in distinct genes that obtained different regulations for specific expressions and functions. One possibility for the time of origin of this separation into *Tbx5* and *Tbx4* in evolution could be that it diverged when tetrapods obtained their limbs from fish fins; that is, it occurred between amphibians and teleost fishes. However, the discovery of both *Tbx5* and *Tbx4* in zebrafish (Tamura et al. 1999; Ruvinsky et al. 2000) and their fin-specific expressions (*Tbx5* is found in the pectoral fin and *Tbx4* is found only in the pelvic fin; Fig. 7*B*, 7*C*) suggest that bony fishes have fin identity that is provided at least by these T-box gene expressions and that duplication occurred prior to teleost fishes. Recently, Ruvinsky and Gibson-Brown (2000) reported that amphioxus has a precursor gene *Tbx5/4* and suggested that the distinct genes, *Tbx5* and *Tbx4*, diverged after the separation of the cephalochordate and vertebrate lineages. Our preliminary data on the skate embryo suggest that the skate (Fig. 6), *Raja kenojei*, has at least the distinct *Tbx5* that is expressed only in the pectoral fin bud (K.T. and S.Y-T., unpublished data in preparation). It is possible that all modern vertebrates that have 2 sets of paired appendages may show differential genetic identity and that the separation into *Tbx5* and *Tbx4* occurred at a point between jawless vertebrates and cartilaginous fishes.

CONCLUSION

By focusing on 2 separate viewpoints (i.e. AER induction by FGF10 and positioning of the limb/fin field, and specification of limb identity regulated by selector genes, *Tbx5* and *Tbx4*), some recent findings and hypothetical insights regarding the evolution of limb development were reviewed in this article. The initiation of limb-type specific expression of 2 T-box genes is likely to be induced independently of, or parallel to, the AER induction by FGF10 because *Fgf10*-null mouse embryos initiate expression of both *Tbx5* and *-4* at early stages (Sekine et al. 1999). However, these 2 issues must be closely related in the evolutionary aspects of limb development, morphology and diversity. Efforts to study phylogeny using experimental embryological strategies must become a powerful means of understanding evolution, because studies on developmental processes (morphogenesis) provide a considerable amount of in-

formation on the evolution of morphology of organisms, the diversity of organs, and the origin of structures, as described in this review. Furthermore, it is obvious that not only comparative descriptions of gene expression patterns but also experimental embryological studies based on evolutionary working hypothesis are important to deduce vertebrate evolution and the origin of structural innovation.

ACKNOWLEDGEMENTS

We wish to thank Dr Ken Muneoka of Tulane University for his constructive criticism of the manuscript. The research work in this review was supported by research grants from Yamada Science Foundation, the Naito Science Foundation, Nissan Science Foundation, and the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

ABUD HE, SKINNER JA, McDONALD FJ, BEDFORD MT, LONAI P, HEATH JK (1996) Ectopic expression of Fgf-4 in chimeric mouse embryos induces the expression of early markers of limb development in the lateral ridge. *Developmental Genetics* **19**, 51–65.

AGULNIK SI, GARVEY N, HANCOCK S, RUVINSKY I, CHAPMAN DL, AGULNIK I, BOLLAG R, PAPIOANNOU V, SILVER LM (1996) Evolution of mouse T-box genes by tandem duplication and cluster dispersion. *Genetics* **144**, 249–254.

AKIMENKO MA, EKKER M (1995) Anterior duplication of the Sonic hedgehog expression pattern in the pectoral fin buds of zebrafish treated with retinoic acid. *Developmental Biology* **170**, 243–247.

BALINSKY BI (1925) Transplantation des ohrblaschens bei Triton. *Roux's Arch. Developmental Biology* **143**, 718–731.

BASSON CT, BACHINSKY DR, LIN RC, LEVI T, ELKINS JA, SOULTS J et al. (1997) Mutations in human TBX5 cause limb and cardiac malformation in Holt-Oram Syndrome. *Nature Genetics* **15**, 30–35.

CARRINGTON JL, FALLON JF (1984) The stages of flank ectoderm capable of responding to ridge induction in the chick embryo. *Journal of Embryological Experimental Molphoogy* **84**, 19–34.

CHAPMAN DL, GARVEY N, HANCOCK S, ALEXIOU M, AGULNIK SI, GIBSON-BROWN JJ et al. (1996) Expression of the T-box family genes, Tbx1-Tbx5, during early mouse development. *Developmental Dynamics* **206**, 379–390.

COATES MI (1994) The origin of vertebrate limbs. *Development* (Suppl.), 169–180.

COATES MI, COHN MJ (1998) Fins, limbs, and tails: outgrowths and axial patterning in vertebrate evolution. *BioEssays* **20**, 371–381.

COHN MJ, IZPISUA-BELMONTE JC, ABUD H, HEATH JK, TICKLE C (1995) Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **10**, 739–746.

CROSSLEY PH, MINOWADA G, MACARTHUR CA, MARTIN GR (1996) Roles for FGF8 in the induction, initiation, and Maintenance of chick limb development. *Cell* **84**, 127–136.

ECHELARD Y, EPSTEIN DJ, ST-JACQUES B, SHEN L, MOHLER J, McMAHON et al. (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417–1430.

ENDO T, YOKOYAMA H, TAMURA K, IDE H (1997) Shh expression in developing and regenerating limb buds of Xenopus laevis. *Developmental Dynamics* **209**, 227–232.

FALLON JF, LOPEZ A, ROS MA, SAVAGE MP, OLWIN BB, SIMANDL BK (1994) FGF-2: apical ectodermal ridge growth signal for chick limb development. *Science* **264**, 104–107.

GIBSON-BROWN JJ, AGULNIK SI, CHAPMAN DL, ALEXIOU M, GARVEY N, SILVER LM, PAPAIOANNOU VE (1996) Evidence of a role for T-box genes in the evolution of limb morphogenesis and the specification of forelimb/hindlimb identity. *Mechanisms of Development* **56**, 93–101.

GIBSON-BROWN JJ, AGULNIK SI, SILVER LM, NISWANDER L, PAPAIOANNOU VE (1998) Involvement of T-box genes Tbx2-Tbx5 in vertebrate limb specification and development. *Development* **125**, 2499–2509.

IGARASHI M, FINCH PW, AARONSON SA (1998) Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). *Journal of Biology and Chemistry* **273**, 13230–13235.

IMOKAWA Y, YOSHIZATO K (1997) Expression of Sonic hedgehog gene in regenerating newt limb blastemas recapitulates that in developing limb buds. *Proceedings of the National Academy of Sciences of the USA* **94**, 9159–9164.

ISAAC A, RODRIGUEZ-ESTEBAN C, RYAN A, ALTABEF M, TSUKUI T, PATEL K et al. (1998) Tbx genes and limb identity in chick embryo development. *Development* **125**, 1867–1875.

JARVIK E (1980) *Basic Structure and Evolution of Vertebrates*. London: Academic Press.

LI QY, NEWBURY-ECOB RA, TERRETT JA, WILSON DI, CURTIS AR, YI CH et al. (1997) Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nature Genetics* **15**, 21–29.

LOGAN M, SIMON HG, TABIN C (1998) Differential regulation of T-box and homeobox transcription factors suggests roles in controlling chick limb-type identity. *Development* **125**, 2825–2835.

MIN H, DANILENKO DM, SCULLY SA, BOLON B, RING BD, TARPLEY JE et al. (1998) Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to drosophila branchless. *Genes and Development* **12**, 156–161.

NISWANDER L, TICKLE C, VOGEL A, BOOTH I, MARTIN GR (1993) FGF-4 replaces the apical ectodermal ridge and directs outgrowth and patterning of the limb. *Cell* **75**, 579–587.

OHUCHI H, NAKAGAWA T, YAMAUCHI M, OHATA T, YOSHIOKA H, KUWANA T et al. (1995) An additional limb can be induced from the flank of the chick embryo by FGF4. *Biochemical and Biophysical Research Communication* **209**, 809–816.

OHUCHI H, NAKAGAWA T, YAMAMOTO A, ARAGA A, OHATA T, ISHIMARU Y et al. (1997) The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124**, 2235–2244.

OHUCHI H, TAKEUCHI J, YOSHIOKA H, ISHIMARU Y, OGURA K, TAKAHASHI N et al. (1998) Correlation of winged identity in ectopic FGF-induced chimeric limbs with the differential expression of chick Tbx5 and Tbx4. *Development* **125**, 51–60.

PAPAIOANNOU VE, SILVER LM (1998) The T-box gene family. *Bioessays* **20**, 9–19.

RIDDLE RD, JOHNSON RL, LAUFER E, TABIN C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* **75**, 1401–1416.

RODRIGUEZ-ESTEBAN C, TSUKUI T, YONEI S, MAGALLON J, TAMURA K, IZPISUA BELMONTE JC

(1999) The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. *Nature* **398**, 814–818.

RUVINSKY I, GIBSON-BROWN JJ (2000) Genetic and developmental bases of serial homology in vertebrate limb evolution. *Development* **127**, 5233–5244.

RUVINSKY I, OATES AC, SILVER LM, HO RK (2000) The evolution of paired appendages in vertebrates: T-box genes in the zebrafish. *Development, Genes and Evolution* **210**, 82–91.

SAUNDERS JW, GASSELING MT (1959) The differentiation of prospective thigh mesoderm grafted beneath the apical ectodermal ridge of the wing bud in the chick embryo. *Developmental Biology* **1**, 281–301.

SAUNDERS JW, REUSS C (1974) Inductive and axial properties of prospective wing bud mesoderm in the chick embryo. *Developmental Biology* **38**, 41–50.

SEARLS RL, JANNERS MY (1971) The initiation of limb bud growth in the embryonic chick. *Developmental Biology* **24**, 198–213.

SEKINE K, OHUCHI H, FUJIWARA M, YAMASAKI M, YOSHIZAWA T, SATO T et al. (1999) Fgf10 is essential for limb and lung formation. *Nature Genetics* **21**, 138–141.

SHU DG, LUO HL, CONWAY MORRIS S, ZHANG XL, HU SX, CHEN L et al. (1999) Lower Cambrian vertebrates from south China. *Nature* **402**, 42–46.

SIMON HG, KITTAPPA R, KHAN PA, TSILFIDIS C, LIVERSAGE RA, OPPENHEIMER S (1997) A novel family of T-box genes in urodele amphibian limb development and regeneration: candidate genes involved in vertebrate forelimb/hindlimb patterning. *Development* **124**, 1355–1366.

SMITH M, HICKMAN A, AMANZE D, LUMSDEN A, THOROGOOD P (1994) Trunk neural crest origin of caudal fin mesenchyme in the zebrafish *Brachydanio rerio*. *Proceedings of The Royal Society London B*: *Biological Sciences* **256**, 137–145.

STEPHENS TD, BEIER RL, BRINGHURST DC, HIATT SR, PRESTRIDGE M, PUGMIRE DE et al. (1989) Limbness in the early chick embryo lateral plate. *Developmental Biology* **133**, 1–7.

TABIN CJ (1992) Why we have (only) five fingers per hand: hox genes and the evolution of paired limbs. *Development* **116**, 289–296.

TAKABATAKE Y, TAKABATAKE T, TAKESHIMA K (2000) Conserved and divergent expression of T-box genes Tbx2-Tbx5 in Xenopus. *Mechanisms of Development* **91**, 433–437.

TAKEUCHI JK, KOSHIBA-TAKEUCHI K, MATSUMOTO K, VOGEL-HOPKER A, NAITOH-MATSUO M, OGUR K et al. (1999) Tbx5 and Tbx4 genes determine the wing/leg identity of limb buds. *Nature* **398**, 810–814.

TAMURA K, YONEI-TAMURA S, BELMONTE JC (1999) Differential expression of Tbx4 and Tbx5 in Zebrafish fin buds. *Mechanisms of Development* **87**, 181–184.

TANAKA M, COHN MJ, ASHBY P, DAVEY M, MARTIN P, TICKLE C (2000) Distribution of polarizing activity and potential for limb formation in mouse and chick embryos and possible relationships to polydactyly. *Development* **127**, 4011–4021.

TOROK MA, GARDINER DM, IZPISUA-BELMONTE JC, BRYANT SV (1999) Sonic hedgehog (shh) expression in developing and regenerating axolotl limbs. *Journal of Experimental Zoology* **284**, 197–206.

VOGEL A, RODRIGUEZ C, IZPISUA-BELMONTE JC (1996) Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737–1750.

YOKOYAMA H, YONEI-TAMURA S, ENDO T, IZPISUA BELMONTE JC, TAMURA K, IDE H (2000) Mesenchyme with fgf-10 expression is responsible for regenerative capacity in Xenopus limb buds. *Developmental Biology* **219**, 18–29.

YOKOYAMA H, IDE H, TAMURA K (2001) FGF-10 stimulates limb regeneration ability in *Xenopus laevis*. *Developmental Biology* **233**, 72–79.

YONEI-TAMURA S, ENDO T, YAJIMA H, OHUCHI H, IDE H, TAMURA K (1999) FGF7 and FGF10 directly induce the apical ectodermal ridge in chick embryos. *Developmental Biology* **211**, 133–143.