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Ectodermal Ridge in Chick Embryos

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During vertebrate limb development, the apical ectodermal ridge (AER) plays a vital role in both limb initiation and distal outgrowth of the limb bud. In the early chick embryo the prelimb bud mesoderm induces the AER in the overlying ectoderm. However, the direct inducer of the AER remains unknown. Here we report that FGF7 and FGF10, members of the fibroblast growth factor family, are the best candidates for the direct inducer of the AER. FGF7 induces an ectopic AER in the flank ectoderm of the chick embryo in a different manner from FGF1, -2, and -4 and activates the expression of *Fgf8,* an AER marker gene, in a cultured flank ectoderm without the mesoderm. Remarkably, FGF7 and FGF10 applied in the back induced an ectopic AER in the dorsal median ectoderm. Our results suggest that FGF7 and FGF10 directly induce the AER in the ectoderm both of the flank and of the dorsal midline and that these two regions have the competence for AER induction. Formation of the AER of the dorsal median ectoderm in the chick embryo is likely to appear as a vestige of the dorsal fin of the ancestors.

Key Words: **apical ectodermal ridge (AER); FGF7; FGF10; limb morphogenesis.**

INTRODUCTION

Tetrapod limbs evolved from paired fins (pectoral and pelvic fins) of fish and appear to inherit embryonic development from fins (Akimenko *et al.,* 1995; Akimenko and Ekker, 1995). For example, a common specialized ectoderm, which is required for skeletogenesis of the appendage, appears in the distal margin of the limb and fin primordia (Hall, 1991). One such structure is in the tetrapod limb bud which appears as a ridge-shaped thickening in the apical epidermis known as the apical ectodermal ridge (AER). In the early limb bud stages, the AER is induced by the lateral plate mesoderm of the limb field (Saunders and Reuss, 1974; Carrington and Fallon, 1984). Experiments using avian embryos have revealed that the AER promotes proliferation and directed outgrowth of subridge mesodermal cells of the developing limb bud (Saunders, 1948; Saunders *et al.*, 1976), while mesenchymal factor(s) are responsible for maintaining the AER as limb outgrowth proceeds (Zwilling and Hansborough, 1956).

These interactions between the AER and limb mesenchyme are fundamental to the pattern formation of the limb bud. Some molecules involved in these epithelial–mesenchymal tissue interactions have been reported. Fibroblast growth factor (FGF) family members FGF2, -4, and -8 have been reported to be the best candidates for the AER-derived signal since their transcripts are expressed in the AER, thus replacing the role of the AER (Fallon *et al.,* 1994; Niswander *et al.,* 1994; Laufer *et al.,* 1994; Mahmood *et al.,* 1995; Vogel *et al.,* 1996). Exogenous insulin and insulin-like growth factor I (IGF-I) maintain the thickness and activity of the AER (Dealy and Kosher, 1995) and, therefore, are candidates for the AER maintenance factor. Moreover, recent studies showed that FGF10 is expressed in the limb mesenchyme and suggested a role of FGF8 and FGF10 in the reciprocal interaction between the AER and limb mesenchyme

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(Ohuchi *et al.*, 1997; Xu *et al.*, 1998). On the other hand, although it is known that applications of several FGFs (FGF1, -2, -4, -8, and -10) to the flank can induce an additional limb bud with an ectopic AER (Cohn *et al.*, 1995; Crossley *et al.*, 1996; Vogel *et al.*, 1996; Ohuchi *et al.*, 1997), there is little evidence about the direct inducer of the AER derived from the presumptive limb bud mesenchyme.

In the present study, in order to identify the direct inducer of the AER, we investigated the role of several FGFs in AER induction. Particularly, we focused on FGF7 and FGF10. FGF7 is known as a diffusible mesenchymal mediator of epithelial–mesenchymal tissue interactions in several organs (Rubin *et al.*, 1995; Post *et al.*, 1996). FGF10, which is expressed in the presumptive limb mesenchyme, is thought to be most similar to FGF7 in both amino acid sequence and functions (Yamasaki *et al.*, 1996; Ohuchi *et al.*, 1997; Igarashi *et al.*, 1998). Our results demonstrate that FGF7 induces the AER in the flank in a different manner from FGF1, -2, and -4 and that FGF7 and FGF10 can induce the formation of an additional AER in the ectoderm of the dorsal midline where there is no underlying cell of lateral mesoderm. FGF7 can activate the expression of *Fgf8* in the ectoderm isolated from the underlying mesoderm *in vitro* but FGF1, -2, and -4 cannot. Thus, it is likely that FGF7 and FGF10 induce the AER directly while AER induction by FGF1, -2, and -4 may be mediated by mesodermal cells. Our data also suggest that there may be a correlation between the dorsal median AER induced by FGF7 and FGF10 and the dorsal median fin in amphibian and fish larva.

MATERIALS AND METHODS

Experimental Manipulation

Chick embryos were staged according to Hamburger and Hamilton (1951). Stage 13/14 chick embryos were used as hosts. The ectoderm at somite 20 level was cut and partially peeled to be separated from a mesodermal layer toward somite 23/24 level in the flank. Affi-gel heparin beads (Bio-Rad) soaked in 0.5 mg/ml of several FGFs as described below were inserted in the cavity and placed between the respective regions of the ectoderm and mesoderm. FGF1 (recombinant human, Boehringer-Mannheim), FGF2 (bovine, R & D System), FGF4 (recombinant human, R & D System), and FGF7 (recombinant human, Promega) were used for this experiment. This operation was performed with meticulous care to prevent making a scrape on the ectoderm around the bead. When grafting the presumptive limb bud mesoderm of stage 16 chick embryos, the transplants were isolated using 0.5% trypsin in Tyrode at 4°C and inserted in the back using the same procedure as bead application (Fig. 4A). For retroviral infection, RCASBP(A) retrovirus containing full-length chick *Fgf10* was produced as previously described (Vogel *et al.*, 1996). The virus was injected in the neural tube at stages 12–14 (Fig. 5A).

After manipulations, the eggs were sealed and returned to the incubator for subsequent analyses.

Observation of Skeletal Pattern

For skeletal pattern observation, embryos were incubated for 7 days after operation. Embryos were fixed in 10% formalin, stained in 0.1% Alcian blue in 70% acid alcohol, dehydrated in ethanol, and then cleared in methyl salicylate.

Whole-Mount in Situ Hybridization and Immunofluorescent Staining

Whole-mount *in situ* hybridization was performed as described (Yonei *et al.*, 1995, for chick embryos; Endo *et al.*, 1997, for *Xenopus* embryos; Schulte-Merker *et al.*, 1992, for zebrafish embryos). Antisense RNA probes for chick *Fgf8* (a kind gift from Dr. Sumihare Noji) and *Msx1* and *Msx2* (gifts from Dr. Tsutomu Nohno) were described previously (Yonei *et al.*, 1995; Hara *et al.*, 1997; Ohuchi *et al.*, 1997). Probes for zebrafish *Fgf8* and *Xenopus Msx2* were synthesized from plasmids containing an 800-bp fragment of zebrafish *Fgf8* (Furthauer *et al.*, 1997) and a 447-bp fragment of *Xenopus Msx2* (Su *et al.*, 1991). After *in situ* hybridization, some chick embryos were processed for embedding in OCT compound (Miles) and sectioned (10 μm). For observation of the neural crest cells, frozen sections were immunostained using HNK-1 monoclonal antibody (Becton–Dickinson).

Culture of Flank Ectoderm

The procedure for ectoderm culture is described in the legend to Fig. 2A. Flank ectoderm was cultured using the floating collagen gel culture method (Emerman *et al.*, 1977). The flank ectoderm including the lateral plate/paraxial mesoderm was dissected from stage 16 chick embryos. The ectoderm was isolated from the mesoderm using 0.5% trypsin in Tyrode at 4°C. Culture dishes were coated with collagen gel according to the accompanying procedure of Cellmatrix I-A (Nitta Gelatin). Explants were placed on collagen gel in Dulbecco's modified Eagle's MEM containing 10% FCS, 10 μg/ml insulin, and 5 μg/ml transferrin. The media level was lowered until a thin layer of the ectoderm covered the gel. Two hours after incubation at 37°C in the presence of 5% CO_2, collagen gel was allowed to float in fresh medium and then cultured with 10 ng/ml of FGF1, -2, -4, or -7 for 22 h (24 h in total).

RT-PCR Analysis

Total RNA was isolated from explants using an RNeasy total RNA kit (Qiagen). RT-PCR was performed with *Fgf8* specific primers (forward primer, 5′-GAT GTG CAC GCC AAG CTC ATC GTC GAG ACC-3′; reverse primer, 5′-GCT TCA TGA AGT GCA CCT CGC GTT GGT GCT-3′) and *βactin* specific primers (forward primer, 5′-TCT GAC TGA CCG CGT TAC TC-3′; reverse primer, 5′-CCA TCA CAC CCT GAT GTC TG-3′). These primer sets were based on the chick *Fgf8* mRNA sequence (GenBank No. U41467) and the chick *βactin* mRNA sequence (GenBank No. L08165). Southern blotting and detection were performed with respective DIG-labeled probes according to the procedure described in the manufacturer's instructions: "DIG System User's Guide for Filter Hybridization" (Boehringer-Mannheim).

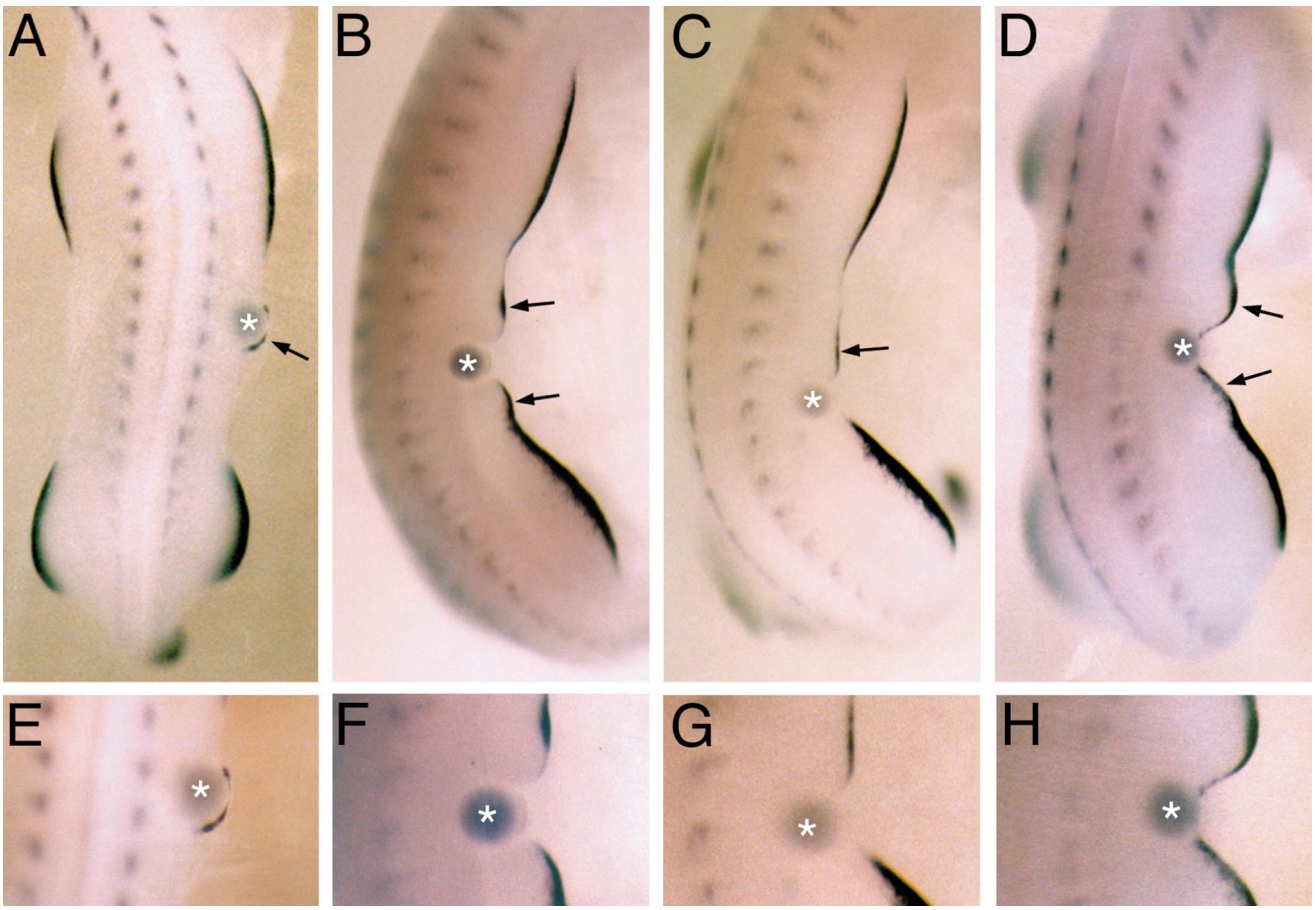


FIG. 1. Induction of *Fgf8* expression in the flank by FGFs 24 h after bead implantation onto the lateral plate mesoderm. Asterisks indicate beads. Arrows indicate ectopic expressions of *Fgf8.* A and E for FGF7, B and F for FGF1, C and G for FGF2, and D and H for FGF4. E–H are high magnifications of A–D, respectively.

RESULTS

AER Induction in the Flank by FGF7 Application

Figure 1A shows that FGF7 induced additional expression of an AER marker gene, *Fgf8,* in the flank ectoderm when a FGF7 bead was inserted between the lateral plate mesoderm and the flank ectoderm (8/9 cases). Although small ectopic outgrowth was observed in the flank region by the application of FGF7, an additional limb was not formed as previously described (Cohn *et al.*, 1995; and data not shown). The ectopic expression of *Fgf8* was induced within 16 h (not shown, 1/4 cases), significantly detectable in the samples after 24 h (Fig. 1A, 8/9 cases), and faded out within 72 h (not shown, 6/6 cases). Higher magnification revealed that *Fgf8* can be induced in the ectoderm over the FGF7 bead (Fig. 1E, 8/9 cases). In contrast, in the cases of FGF1, -2, and -4 applications, which were known to induce ectopic limbs in the flank (Cohn *et al.*, 1995), a new domain of *Fgf8* expression was not detected in the ectoderm over the beads but restricted to the ectoderm in contact with the lateral plate mesoderm (Figs. 1B and 1F for FGF1, 6/6 cases; Figs. 1C and 1G for FGF2, 5/5 cases; and Figs. 1D and 1H for FGF4, 6/6 cases).

Fgf8 Expression in Explants Cultured with FGF7

Expression of *Fgf8* was induced in the ectoderm over the FGF7 beads, while expression of *Fgf8* induced by FGF1, -2, and -4 was restricted to the ectoderm associated with the lateral plate mesoderm. These results suggest that FGF7 may induce the AER directly in the flank ectoderm, whereas FGF1, -2, and -4 require the mesoderm to induce it. To confirm this, we examined the expression of *Fgf8* in explants of the flank ectoderm cultured with each FGF *in vitro* (Fig. 2). Although the expression of *Fgf8* was slightly detected in the flank ectoderm at the beginning of the culture, this weak expression disappeared after 24 h in control culture (Fig. 2B). *Fgf8* was readily detectable in the

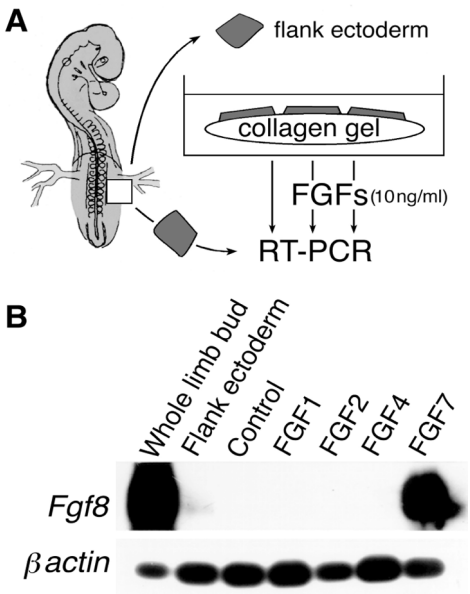


FIG. 2. FGF7 induces the expression of *Fgf8* directly in explants of the flank ectoderm *in vitro*. (A) Explants of the flank ectoderm were cultured for 24 h using the floating collagen gel culture method (Emerman *et al.*, 1977). (B) Expression of *Fgf8* was assayed by RT-PCR and Southern hybridization analysis. Expression of *Fgf8* was detected in a positive control of the whole limb bud containing the AER (stage 24), slightly in the flank ectoderm (stage 16), and strongly in explants cultured with FGF7. *βactin* amplification is shown as a control.

explants cultured with FGF7, but was not detected in the explants cultured with FGF1, -2, or -4 (Fig. 2B).

Analysis of the Dorsal Median AER

When FGF7 beads were inserted between the paraxial mesoderm of the flank and the overlying ectoderm, the ectopic domain of *Fgf8* expression was mostly restricted to the dorsoventral boundary of the flank ectoderm covering the lateral plate mesoderm (4/5 cases). Surprisingly, the application of FGF7 onto the paraxial mesoderm caused an additional expression of *Fgf8* in the dorsal median ectoderm overlying the neural tube (5/5 cases). Figure 3A shows that a FGF7 bead placed on the paraxial mesoderm induced two stripes of expression of *Fgf8* along the longitudinal axis (arrows in Fig. 3A). One of these was in the dorsoventral boundary of the flank ectoderm and the other was in the dorsal median ectoderm of the trunk. Both domains had ridge-like configurations which have stratified columnar epithelium (Figs. 3B and 3C). When the FGF7 bead was inserted between the dorsal median ectoderm and the neural tube, a new domain of expression of *Fgf8* was also formed (Fig. 3D, 15/16 cases). The expression of *Fgf8* in the dorsal median ectoderm was as intense as that in the normal AER. Although FGF1 induced a faint expression of *Fgf8* in the dorsal median ectoderm (arrow in Fig. 3E, 3/6 cases), FGF2 and FGF4 could not induce the expression of *Fgf8* in the dorsal median ectoderm (Fig. 3F for FGF2, 6/6 cases; and Fig. 3G for FGF4, 6/6 cases). FGF7 did not induce ectopic expression of *Fgf8* anteriorly to the somite 10 level of the dorsal median ectoderm (not shown). We examined the expression of some other AER markers in the AER-like structure on the back. *Msx1* (Fig. 3I) and *Msx2* (Fig. 3J), which are normally expressed in the AER, are significantly detected in the AER-like structure (compare Figs. 3I and 3J with 3H for *Fgf8*), suggesting that the AER-like structure may be a functional one. The AER-like structure induced an additional limb when implanted onto the flank mesoderm (data not shown, 18/26 cases).

Henceforth, the fact that FGF7 is able to induce the formation of an AER-like structure in the dorsal median ectoderm indicates that the dorsal median ectoderm has a competence for AER induction. When the mesoderm of a presumptive limb bud (the tissue which produces the AER inducer during normal limb development) was inserted between the dorsal median ectoderm and the neural tube, an additional limb bud with *Fgf8* expressing distal ectoderm was generated from the dorsal midline (Fig. 4B). These limb buds seem to have a double-dorsal aspect because *Lmx-1*, a dorsal marker, was expressed on both sides of the limb buds (Fig. 4C). Some additional limbs had a full range of skeletal elements, from a girdle to digits, although their patterns were not whole (the zeugopod was shortened and some digits were missing; Figs. 4D and 4E, 4/16 cases). In other cases, the limbs lacked distal structure (all digits were missing; not shown, 12/16 cases).

AER Induction in the Back by FGF10 Application

FGF10 has the highest amino acid sequence identity to FGF7 (Yamasaki *et al.*, 1996; Ohuchi *et al.*, 1997) and is known to be expressed in the limb bud from prelimb bud stages of chick embryos (Ohuchi *et al.*, 1997). To investigate whether FGF10 has the same capability as FGF7 to induce ectopic *Fgf8* expression in the back, we injected *Fgf10*-RCAS virus into the central canal in the neural tube of stage 12–14 embryos (Fig. 5A). Twenty-four hours after injection, a long line of AER-like structures was detected in the dorsal median ectoderm (7/12 cases), all of which were *Fgf8* positive (Figs. 5B and 5C). The *Fgf8*-positive AER-like structures were seen only in the trunk region. These results suggest that FGF10 as well as FGF7 could induce the AER directly in the ectoderm apart from the mesoderm.

Similarities in the Developmental Processes between Vertebrates

Some cells were noted at times to be clustered beneath the AER-like structure of the dorsal median ectoderm (Fig. 6C; see also Fig. 3C). These cells are believed to be derived from the neural crest cells since they were HNK-1 positive (Figs. 6A and 6B). It is known that the dorsal fins in larval

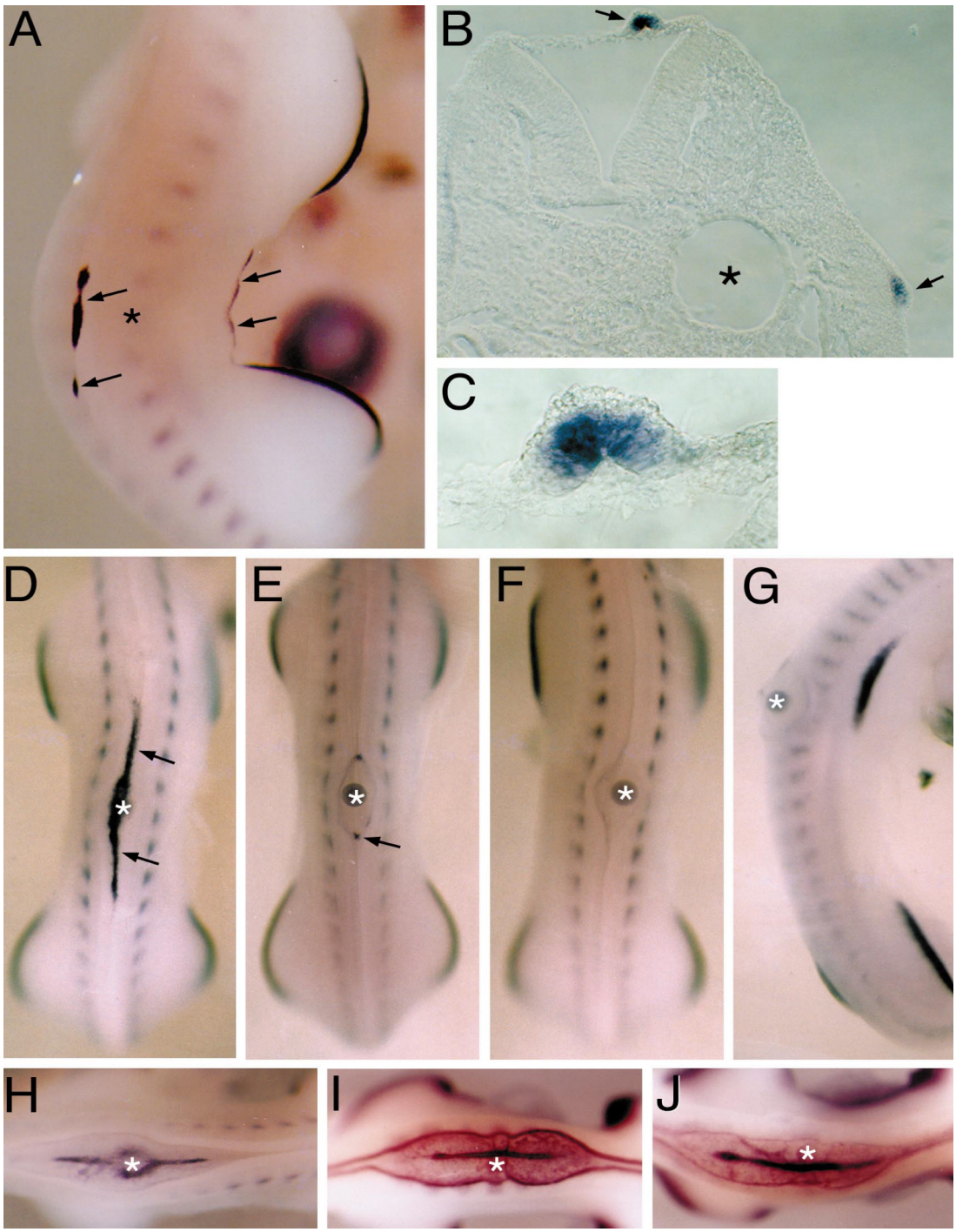


FIG. 3. Additional expression of *Fgf8* in the dorsal median ectoderm by FGF7. Asterisks indicate implanted beads. Arrows indicate ectopic expressions of *Fgf8*. (A) Two stripes of Fgf8 expression are induced 36 h after FGF7 bead application onto the paraxial plate mesoderm. (B, C) Vertical sections of A. (D–G) Samples 24 h after applications of FGF7 (D), FGF1 (E), FGF2 (F), and FGF4 (G) under the dorsal median ectoderm are shown. (H–J) Comparison of expression of three AER marker genes in the AER-like structures induced by FGF7. H, *Fgf8*. I, *Msx1*. J, *Msx2*.

amphibians and fish consist of a mesenchyme of neural crest origin (DuShane, 1935; Bodenstein, 1952; reviewed by Eisen and Weston, 1993). Our data (Figs. 6A and 6B) seem to suggest that the AER-like structure and the dorsal fins might share a kind of phylogenetical resemblance. To obtain some molecular description supporting this idea, we checked the expression of some AER marker genes in the dorsal fins. *Fgf8* was expressed not only in the pectoral fins (Fig. 6D) but also throughout the ridge of the dorsal fin in zebrafish (Fig. 6E) as briefly described by Furthauer *et al.* (1997). *Msx2* was considerably detectable in the dorsal fin of a *Xenopus* larva (Fig. 6F).

DISCUSSION

Roles of FGF7 and FGF10 in Chick Limb Development

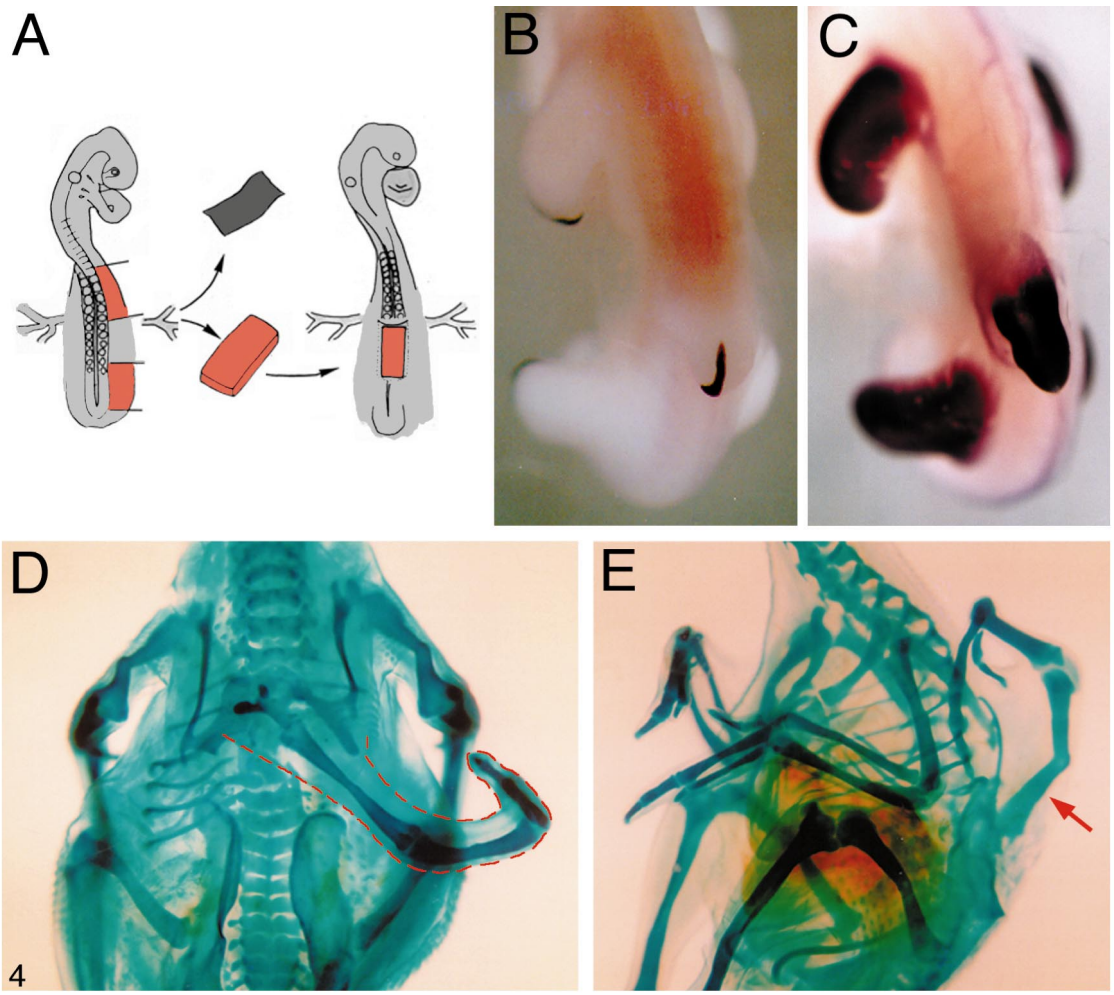
In the present study we have demonstrated that FGF7 and FGF10 induce the formation of an additional AER in the ectoderm of the chick embryo in a distinct manner from FGF1, -2, and -4. Since induction of *Fgf8* takes 16 h after application of FGF7, several steps of gene activation may be involved to induce *Fgf8* expression. However, FGF7 seems to work on the ectodermal cells directly, inasmuch as it activated the expression of *Fgf8* in the flank ectoderm *in vitro*. This is supported by the results that FGF7 induced the AER in the flank ectoderm over the implanted bead and that the ectodermal ridge was formed in the dorsal midline where the lateral plate mesoderm was absent. Since the injection of *Fgf10*-RCAS virus induced the AER in the dorsal median ectoderm, it is likely that FGF10 may have the same property in terms of AER induction. Although both FGF7/10 and prelimb bud mesoderm can induce a dorsal median AER, a limb bud develops only with the mesoderm. This means that, in addition to the AER, the limb mesoderm is necessary to develop the extra limb bud in the back. This also indicates that FGF7/10 cannot induce limb bud mesoderm in surrounding tissues, although they strongly induce the AER in the dorsal ectoderm. On the other hand, in the cases of FGF1, -2, and -4 applications that induce ectopic limbs in the flank (Cohn *et al.*, 1995), expression of *Fgf8* was not detected in the flank ectoderm over the implanted bead but was restricted to the ectoderm in contact with the lateral plate mesoderm. FGF2 and -4 did not induce the expression of *Fgf8* in the dorsal median ectoderm, though FGF1 showed slight induction. Additionally, expression of *Fgf8* disappeared in explants of the flank ectoderm cultured with FGF1, -2, and -4. These results suggest that FGF1, -2, and -4 required the lateral plate mesoderm to induce AER in the ectoderm. Most likely, these FGFs act on the lateral plate mesoderm of the flank and give limb-forming ability to the mesoderm, which is responsible for the subsequent induction of an additional AER.

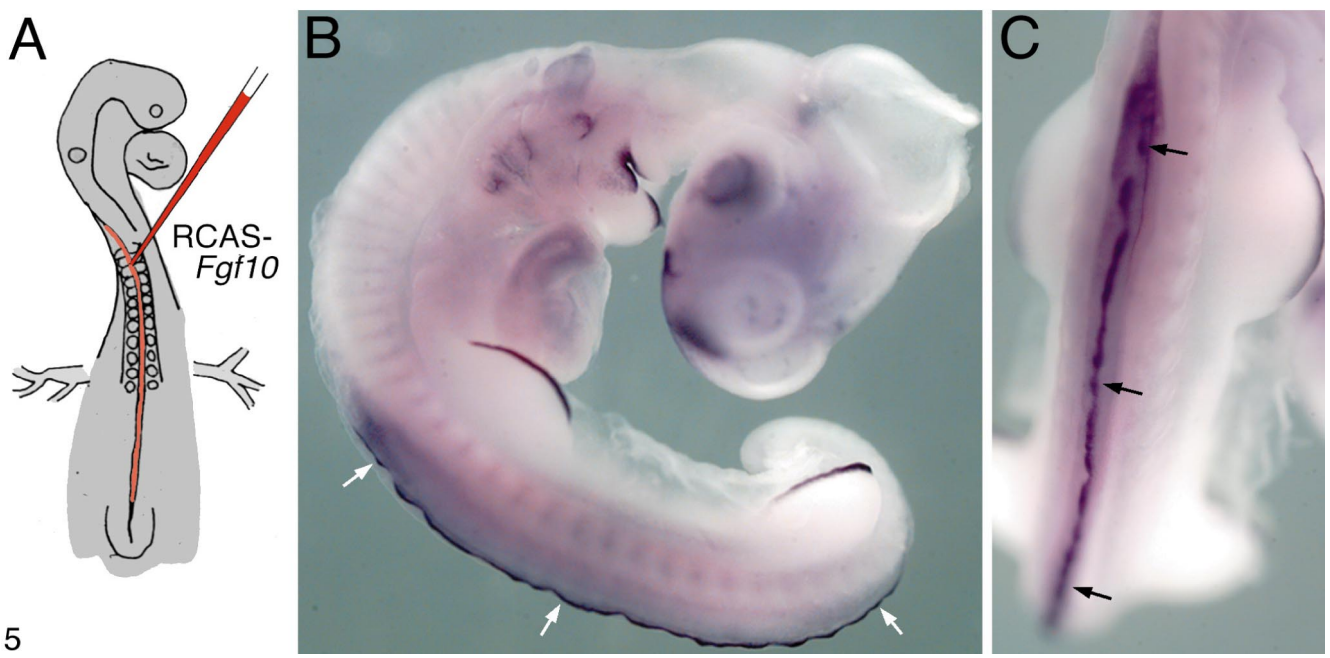
The different inducibility between FGF7/10 and the other FGFs may be due to the distinct affinities in their receptors (FGFRs). Four distinct FGFR genes are known (FGFR1–FGFR4). FGFR1–FGFR3 genes encode multiple isoforms generated by alternative splicing (FGFR1b, -1c, -2b, -2c, -3b, and -3c; Hou *et al.*, 1991), and each isoform has unique ligand binding properties. FGFR1c can bind FGF1, -2, and -4, and FGFR2c, FGFR3c, and FGFR4 can bind FGF1, -2, -4, and -8 (FGF8 also induces the additional limbs in the flank; see Crossley *et al.*, 1996; Vogel *et al.*, 1996), but not bind FGF7 and FGF10 (Dionne *et al.*, 1990; Mansukhani *et al.*, 1990; Werner *et al.*, 1992; Ornitz *et al.*, 1996; Igarashi *et al.*, 1998). These three FGFRs are expressed in the embryonic mesenchyme (Noji *et al.*, 1992; Orr-Urtreger *et al.*, 1993; MacArthur *et al.*, 1995). Taken together, FGF1, -2, -4, and -8 are likely to signal through some of these FGFRs to induce additional limbs, whereas the FGF7/10 receptor for AER induction is probably ectoderm specific.

In contrast to FGF1, -2, -4, and -8, FGF7 and FGF10 have a high affinity only for FGFR2b (Miki *et al.*, 1991; Ornitz *et al.*, 1996; Igarashi *et al.*, 1998), which is known to be expressed exclusively by epithelial cells (Orr-Urtreger *et al.*, 1993). The data suggest that FGF7 and FGF10 could bind FGFR2b that is expressed in the ectodermal epithelial cells and function to induce the AER directly in the ectoderm. FGFR2b is expressed in the limb bud ectoderm including the AER (Noji *et al.*, 1992; Min *et al.*, 1998). FGFR2 mutant mice (both FGFR2b and FGFR2c have mutations in these mice) fail to express *Fgf8* and do not form limb buds (Xu *et al.*, 1998). These studies support the fact that AER induction is also mediated by FGFR2b in normal limb development. Biochemical experiments showed that FGFR2b binds FGF1 and FGF3 with a high affinity as well as FGF7 (Ornitz *et al.*, 1997). However, according to our data, FGF1 has little activity for the direct induction of AER formation. It is

FIG. 4. Additional limb buds induced by presumptive limb bud mesoderm in the dorsal midline. (A) A schematic procedure for implantation of the presumptive limb bud mesoderm into the dorsal region. (B, C) Expression of *Fgf8* (B) and *Lmx-1* (C) 48 h after implantation of the presumptive limb bud mesoderm under the dorsal median ectoderm. (D, E) Independent two samples of skeletal patterns of the additional limb induced by the presumptive limb bud mesoderm implanted under the dorsal median ectoderm. The skeletal elements indicated by broken lines in D and by an arrow in E are the ectopic limbs on the back. In most cases, the identities of skeletal elements were not identified.

FIG. 5. *Fgf10*-RCAS virus injected into the central canal of the neural tube (drawn in A) gives rise to an ectopic AER-like structure with *Fgf8* expression (arrows in B and C) in the dorsal median ectoderm after 24 h.





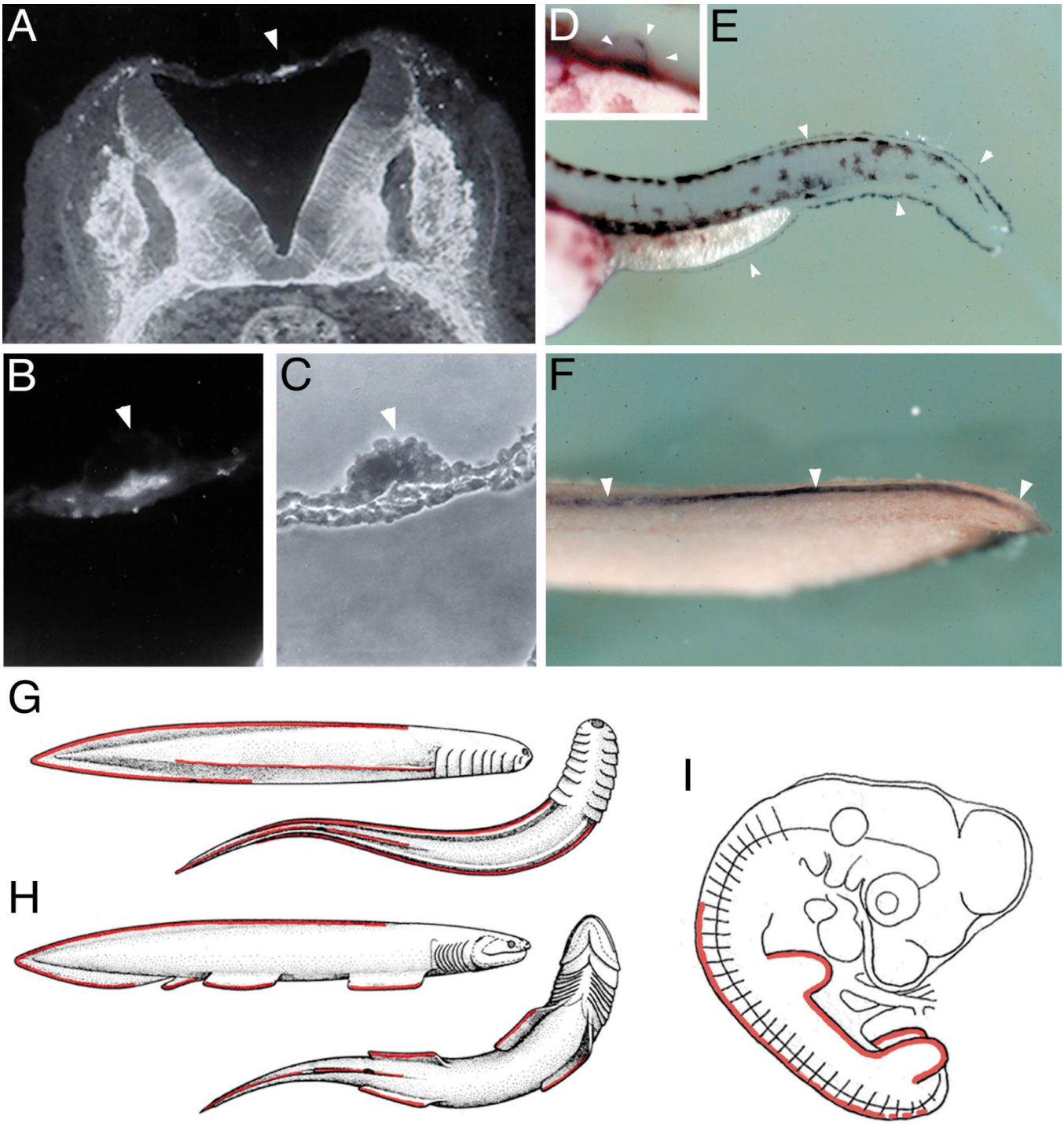


FIG. 6. (A–C) Vertical sections of an embryo which has the ridge-like structure formed by FGF7 application (indicated by arrowheads). (A, B) Staining with HNK-1 monoclonal antibody. (C) Phase contrast of B. (D, E) Expression of *Fgf8* mRNA in the pectoral fin (D) and dorsal fin (E) of larval zebrafish. (F) Expression of *Msx2* in the dorsal fin of *Xenopus* tadpole. (G–I) Hypothetical ancestral fish (G, H) and extant chick embryo (I). According to the hypothesis (see text in detail), the earliest fish (G) had continuous fin folds in the dorsal median and ventrolateral regions. In the later evolutionary stage (H), intermediate regions of each continuous fold were lost and the remainder became median fins (dorsal, caudal, and anal fins) and paired fins (pectoral and pelvic fins). Domains that have AER-forming ability in the chick embryo (red lines in I) correspond with the fin-forming regions in the earliest fish (red lines in G and H). The fish drawings were adapted from Jarvik (1981).

possible that this activity is regulated in a different way from the affinity for ligands, such as regulation by components of the extracellular matrix (ECM). Interestingly, under different ECM conditions, FGFs which have similar receptor binding properties cause deferent cell behaviors (Igarashi *et al.,* 1998). Alternatively, it could be that the FGF7 receptor, which is unknown and has a higher affinity for FGF7 and FGF10 than for FGF1, plays a role in AER induction (although this idea is unlikely because most data described above suggest that FGFR2b has a role for AER induction).

FGF7 and FGF10 applications induced the formation of an additional AER in the dorsal median ectoderm as with implants of the prelimb bud mesoderm, and they seem to play the same role as the factor secreted by the prelimb bud mesoderm at least in the induction of the ectopic AER, suggesting that FGF7, FGF10, or their homologous protein is an endogenous AER inducer. One possibility is that FGF7 may merely be mimicking the function of FGF10. At prelimb bud stages *Fgf10* expression is restricted in the presumptive limb mesoderm (Ohuchi *et al.,* 1997), and we have shown that *Fgf10* can induce an ectopic AER-like structure with *Fgf8* expression in the median ectoderm as well as FGF7. Additionally, a recent study on *Fgf10* knockout mice shows that *Fgf10* is required for *Fgf8* expression in the mouse limb buds and *Fgf10*$^{-/-}$ mice failed to develop the limbs (Min *et al.,* 1998). Taken together with the present data, these findings strongly suggested that FGF10 functions as an endogenous AER-inducing factor.

Ectodermal Competence of AER Induction

The ectodermal competence of AER induction may be variable under the influence of the presumptive limb mesoderm, since the presumptive limb mesoderm can induce the AER not only in the flank ectoderm but also in the paraxial ectoderm at least by stage 13 (Saunders and Reuss, 1974; Michaud *et al.,* 1997). On the other hand, our data have indicated that FGF7 strongly induces the AER in two restricted regions: the dorsoventral boundary and the dorsal midline of the trunk ectoderm. These results suggest that the two regions which originally have high competence for AER induction are defined under the influence of surrounding tissue such as the lateral plate mesoderm and the trunk neural tube. Interestingly, inserted prelimb bud mesoderm could induce ectopic *Fgf8* expression in the dorsal median ectoderm, resulting in the ectopic limb bud and limb skeletal structure. These limb buds seem to have a double-dorsal aspect because both sides of the limb buds are *Lmx-1* positive. Other experiments also indicated limb bud mesoderm having double-dorsal or double-ventral aspects that could induce normal AER (Riddle *et al.,* 1995; Vogel *et al.,* 1995; Michaud *et al.,* 1997). These results suggest that the boundary of dorsoventral properties of the limb bud mesoderm might be unessential for AER positioning.

Common expressions of two genes, *Msx2* (a homeobox gene) and *Bmp4* (a member of the transforming growth factor β superfamily), are seen in ectodermal regions of the lateral plate and the dorsal midline (Yokouchi *et al.,* 1991; Francis *et al.,* 1994). Interestingly, after the limb bud is established, both expressions of *Msx2* and *Bmp4* in the ectoderm of the limb region become confined to the AER (Yokouchi *et al.,* 1991; Francis, *et al.,* 1994). It is possible that the competence of the ectoderm for AER induction is partially characterized by original expressions of these AER marker genes. Moreover, these competent regions for AER induction seem to have a kind of phylogenetical resemblance. How paired appendages of vertebrates arose during the evolutionary history has long been the subject of speculation, and several ideas have been put forward (Hinchliffe and Johnson, 1980; Hall, 1991). According to a hypothesis as reviewed by Tabin (1992), a paired appendage is believed to be evolved from an ancestral, continuous lateral fin (Figs. 6G and 6H) resembling the embryonic fold precursing median fin (Kimmel *et al.,* 1995): That is, the loss of the intermediate fin fold has left two centers as paired fins. Although this fin-fold theory has some debatable points because it has little fossil evidence (Coates, 1994), the AER-forming potential of the flank and dorsal median ectoderm in the chick embryo seems to reflect the fin (appendage)-forming ability of the hypothetical ancestor (Figs. 6G–6I, red lines), most of which has been altered or lost in the process of limb evolution. This extrapolation is supported by our results that there is a cluster of neural crest cells beneath the ectopic dorsal median AER (Figs. 6A and 6B), because the dorsal fin ectoderm of larval amphibians attracts the neural crest cells for building a fin that consists of a mesenchyme of neural crest origin and an overlying ectodermal epithelium (DuShane, 1935; Bodenstein, 1952), and it is assumed that the same is the case for fish (reviewed by Eisen and Weston, 1993). Some previous studies have also implied similarities in the developmental processes of the median fin and the paired fin (Akimenko *et al.,* 1995; Eeden *et al.,* 1996). Moreover, dorsal fins in amphibians and fish share the expression of some marker genes, *Msx2* and *Fgf8,* with the AER in the limb bud. Of course, further embryological, molecular biological, and paleontological studies are necessary to support this concept. A comparative analysis of the dorsal median AER of the chick embryo and the specialized ectoderm of fin primordia of fishes and larval amphibians, however, should aid in understanding the evolution of the developmental processes of vertebrate appendages.

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