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Original Article

Effects of FGFs on the morphogenic potency and AER-maintenance activity of cultured progress zone cells of chick limb bud

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ABSTRACT The apical mesodermal region of chick limb buds (progress zone, PZ) which is essential for limb pattern formation contains uncommitted cells that change their positional values instructed by the apical ectodermal ridge (AER). Reciprically, the PZ cells maintain the AER activity. FGF-2 and FGF-4 can substitute for the AER to maintain normal outgrowth and gene expression in the limb bud. We examined the effects of FGF on the maintenance of PZ cells characteristics in culture by making recombinant limbs with anterior PZ cells that were pre-cultured in the presence of FGF-2 or FGF-4 and analyzed their morphogenic potency and responsiveness to positional cues arising from the zone of polarizing activity (ZPA). The limb buds expressed distal *Hox* genes and could form a segmented digit. Recombinant limb buds consisting of anterior PZ cells cultured without FGF failed to express *Hox* genes and formed instead a small cartilage nodule. These results indicate that addition of FGF-2 or FGF-4 to cultured PZ cells maintains their competence for *Hox* gene expression and digit formation, but not their responsiveness to positional cues from the ZPA. We also found that when anterior PZ cells which had been pre-cultured with FGF-2 or FGF-4 were implanted underneath the AER, they could maintain *Fgf-8* expression in the AER, whereas this expression was not detected in the AER on the grafted anterior PZ cells that had been pre-cultured without FGF, indicating that FGF maintains AER-maintenance activity of PZ cells in culture.

KEY WORDS: *FGF, PZ cells, AER maintenance, recombinant limb, in vitro*

Introduction

During the development of chick limb buds, a reciprocal interaction takes place between the apical ectodermal ridge (AER) and the underlying mesoderm. The AER is responsible for the induction of wing outgrowth and the maintenance of distal limb bud cells in a labile and uncommitted state. These undifferentiated but proliferating cells underlying the AER compose a region referred to as the progress zone (PZ), which is the site of positional fate assignment in the limb bud (Summerbell *et al.*, 1973). The limb bud mesoderm, on the other hand, maintains both the specialized morphology and functional properties of the AER (Hinchliffe and Johnson, 1980; Fallon *et al.*, 1983). Therefore, though the importance of AER-mesodermal interaction in limb pattern formation is now well established, the underlying mechanisms are not fully understood.

Members of the fibroblast growth factor (FGF) family are expressed in the AER, including *Fgf-2* (Savage *et al.*, 1993; Dono and Zeller, 1994; Savage and Fallon, 1995), *Fgf-4* (Niswander and Martin, 1992; Suzuki *et al.*, 1992), and *Fgf-8* (Ohuchi *et al.*, 1994; Crossley and Martin, 1995; Mahmood *et al.*, 1995; Crossley *et al.*, 1996; Vogel *et al.*, 1996). Limb bud outgrowth is curtailed following AER removal, but treatment of limb buds lacking AER with FGF-2, FGF-4, or FGF-8 results in the maintenance of distal outgrowth and normal pattern formation (Niswander *et al.*, 1993; Fallon *et al.*, 1994; Mahmood *et al.*, 1995). Thus, these endogenous FGF activities produced by the AER have been implicated in the control of distal outgrowth and the maintenance of the PZ. *Fgf-10*, on the other hand, expresses in the distal mesoderm of limb bud (Ohuchi *et al.*, 1997). Ectopic application of FGF-10 can induce expression of *Fgf-8* in non-AER ectodermal cells and maintain it in the AER-removed limb bud, indicating that *FGF-10* is a possible candidate for the AER maintenance factor (Ohuchi *et al.*, 1997). Therefore, FGFs play important roles in AER-mesodermal interaction during limb pattern formation.

The homeobox-containing genes are believed to regulate the process of pattern formation in the developing limb buds. The *msh-*

Abbreviations used in this paper: AER, apical ectodermal ridge; ZPA, zone of polarizing activity; PZ, progress zone; FGF, fibroblast growth factor.

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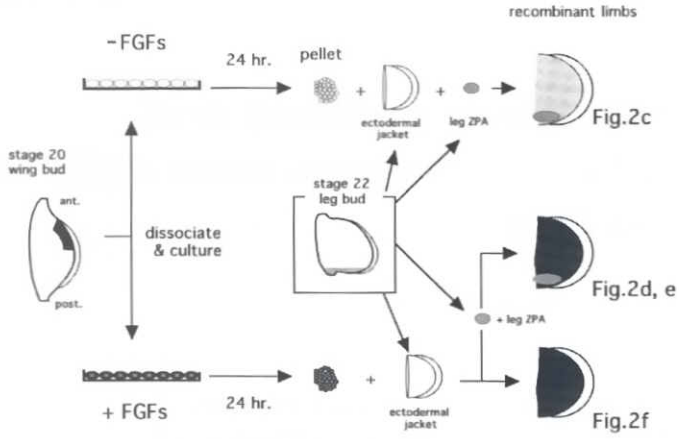

Fig. 1. Scheme showing the procedure to make recombinant limbs with cultured PZ cells. *Three types of recombinants were constructed. The labels for each recombinant with Fig. 2a (polarized recombinant made from PZ cells cultured without FGF), Fig. 2d and e (polarized recombinant made from PZ cells cultured with FGF), and Fig. 2f (non-polarized recombinant made from PZ cells cultured with FGF) correspond to the results shown in Figure 2.*

like homeobox-containing gene, *Msx1*, is normally expressed in the AER and PZ of limb buds (Hill *et al.*, 1989; Davidson *et al.*, 1991; Monaghan *et al.*, 1991; Robert *et al.*, 1991; Yokouchi *et al.*, 1991a). The expression of the *Msx1* in the PZ is controlled by signals emanating from the AER and has been considered to be involved in the interactions that take place at the distal tip of the limb that are responsible for proximodistal growth (Robert *et al.*, 1991; Coelho *et al.*, 1992; Ros *et al.*, 1992). *Hox* genes are also involved in pattern formation in developing limbs. The expression pattern of the *Hox* genes suggests involvement of 5' *Hoxa* genes in the specification of the proximodistal axis (Yokouchi *et al.*, 1991b) and of 5' *Hoxd* genes in the patterning of the anteroposterior axis (Izpisua-Belmonte *et al.*, 1991; Nohno *et al.*, 1991; Yokouchi *et al.*, 1991b). Overexpression of *Hoxd-11* in chick leg buds results in the development of digit 1 with digit 2 morphology, presumably because of a change in the *Hox* code (Morgan *et al.*, 1992). Although loss of function experiments have yielded less dramatic phenotypes, possibly due to redundancy in function between paralogs, the location of the defects has been consistent with their domains of expression. In the limb buds lacking *Hoxa-11* (Small and Potter, 1993) or *Hoxd-11* (Davis and Capecchi, 1994) function, defects are observed in the forearm and wrist, which correspond to the region between the proximal border of normal expression and the proximal boundary of the next most 5' gene. In the *Hoxd-13* knockout mouse, defects are observed in the hand/foot regions where this gene is normally expressed (Dolle *et al.*, 1993). These *Hox* genes may be controlled by positional signals since *Hoxd* gene expression was altered by the grafting of ZPA or AER (Izpisua-Belmonte *et al.*, 1992a,b; Morgan *et al.*, 1992; Dolle *et al.*, 1993). These results suggest that the combination of *Hox* genes expressions in the PZ could be involved in the limb patterning process directly.

In vitro studies provide an important means of investigating the responses of mesodermal cells to signaling molecules. In this work, we used two unique experimental *in vitro* approaches to examine the question of the role of FGFs in the PZ. First, we used the recombinant limb system introduced by Zwilling (1964), which until this time had not been used for the study of limb bud cells in culture, to analyze the expression pattern of *Hoxa* and *Hoxd* genes and the cartilage pattern in recombinant limbs formed by ectodermal jackets and mesodermal cells that had been previously cultured with or without FGFs. Secondly, we used the micromass culture system of anterior PZ cells of stage 20 wing bud (Ide and Aono, 1988: Watanabe and Ide, 1993). The PZ cells cultured with or without FGF-2 or FGF-4 were reaggregated and grafted underneath the AER of the host limb bud. We analyzed the *Msx1* expression in the grafted cells and also maintenance of *Fgf-8* expression in the host AER overlying the grafted cells. These experiments provided a unique opportunity to analyze the action of FGF-2 or FGF-4 upon the PZ cells *in vitro*.

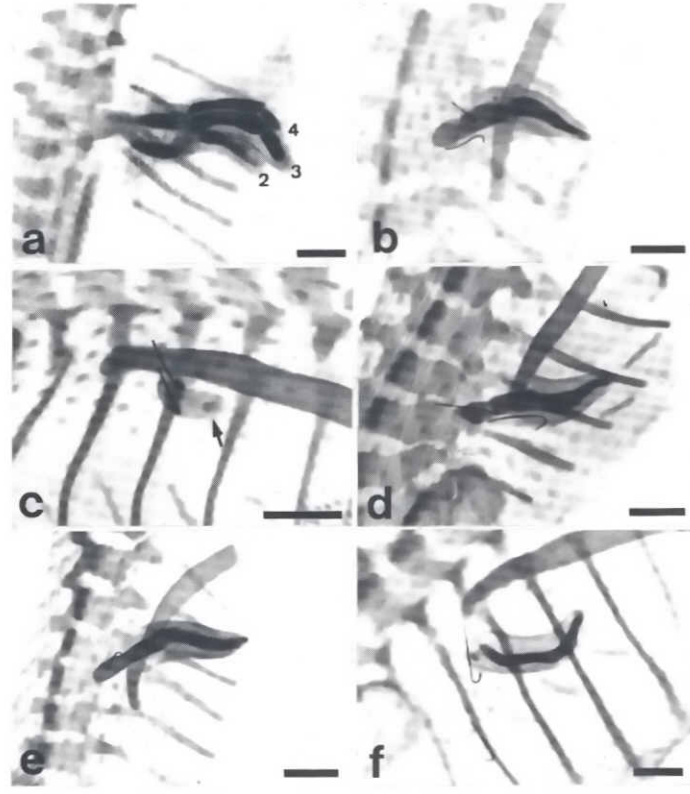

Fig. 2. Chondrogenic activity of the anterior PZ cells in recombinant limb buds. *The cartilage pattern was examined 7 days after grafting. Alcian blue staining. (a,b) Cartilage patterns in the polarized recombinants made from the anterior PZ cells of (a) stage 20 and (b) stage 25 wing buds.* **(a)** *An asymmetrical cartilage pattern. Humerus, radius, and ulna and hand cartilage elements exhibited an anteroposterior order. Numbers show digit number.* **(b)** *A digit with phalanges. (c-f) Cartilage patterns in three types of recombinants with cultured cells shown in Figure 3.* **(c)** *A polarized recombinant limb made from anterior PZ cells that had been cultured for 24 h without FGF-2 developed a small cartilage nodule (arrow). (d-f) Well-developed digit-like structures were formed in the polarized* **(d,e)** *and non-polarized* **(f)** *recombinants made from PZ cells cultured with FGF-2. Note that both type of recombinants formed single well-grown cartilage structures. Bars, 1mm.*

Results

Cartilage pattern formation in polarized recombinants with cultured anterior PZ cells

Some previous studies have reported on recombinant limbs made from the anterior half-limb mesoderm (Ros *et al.*, 1994; Hardy *et al.*, 1995). In the present experiment we analyzed the morphogenic capabilities of recombinant limb buds made with cultured anterior PZ cells (Fig. 1)

We confirmed the results of Wada *et al.* (1993) and Ros *et al.* (1994), in which polarized recombinants made with freshly-prepared anterior PZ cells at stage 20 gave rise to limb-like structures with polarized cartilage elements. The patterns of digits obtained in eight polarized recombinants were 234 (2 cases), 34 (4 cases) and 2 non-polarized limbs. Some of them formed possible proximal structures like humerus, radius, and ulna. Figure 2a shows an example of such polarized recombinant limbs with two skeletal elements at the zeugopodium level and three digits at the autopodium level, which could be clearly identified as digit-2, digit-3, and digit-4. In contrast, the polarized recombinants with stage 25 anterior PZ cells gave rise to poor limb-like structures with a single digit each with one or two phalanges (4/5). An example of these limbs is shown in Figure 2b. One recombinant gave rise to a single unidentified cartilage rod.

We next analyzed the polarized recombinants made from stage 20 anterior PZ cells that had been cultured for 24 h (stage 25 in normal embryo) with or without FGF-2 or FGF-4. Ten polarized recombinant limbs with ZPA and cultured PZ cells in the absence of FGF barely outgrew and gave rise to little mesodermal masses with small cartilage nodules, but failed to form limb-like structure with digits. An example of these limbs is shown in Fig. 2c. In contrast, 11 polarized recombinants made from PZ cells which had been cultured with FGF-2, gave rise to a single well-developed digit. Four of them consisted of a digit with phalanges (Fig. 2d) and 7 recombinants gave rise to single unidentified cartilage structures without segmentation (Fig. 2e). Similar unidentified cartilage structures without segment were observed in the recombinants made from the anterior PZ cells cultured with FGF-4 (6/8), whereas two recombinants gave rise to a single digit with segmented cartilage elements (not shown). No asymmetrical digit patterns were formed in the polarized recombinant limbs made from the anterior PZ cells that had been cultured for 24 h with FGF-2 or FGF-4. However, the non-polarized recombinants made from the PZ cells cultured with FGF also gave rise to a single digit with segments (5/8) (for example, Fig. 2f). It has also been demonstrated that the implantation of a small fragment of ZPA in a recombinant limb will impose polarity in the recombinant limbs with freshly-prepared PZ cells (MacCabe *et al.*, 1973). However, in the present recombinant experiments with cultured PZ cells in the presence of FGF, no obvious differences in growth and digit pattern between the polarized and non-polarized recombinants were demonstrated.

TABLE 1

GENE EXPRESSION OF *HOXA-11, HOXA-13, HOXD-12* AND *HOXD-13* IN RECOMBINANT LIMBS MADE FROM THE PZ CELLS THAT HAD BEEN CULTURED IN THE PRESENCE OF FGF-2

Time at which expression was analyzed (h)	*Recombinant limbs	n	Hoxa-11	Hoxa-13	Hoxd-12	Hoxd-13
0	non-polarized	2	—	—	—	—
	polarized	3	+	±	±	—
20	non-polarized	2	+	±	±	—
	polarized	3	+	+	+	+
50	non-polarized	6	+	+	+	+

*The recombinant lims were polarized by the implantation of a small piece of ZPA.
—, negative; ±, barely detectable; +, positive

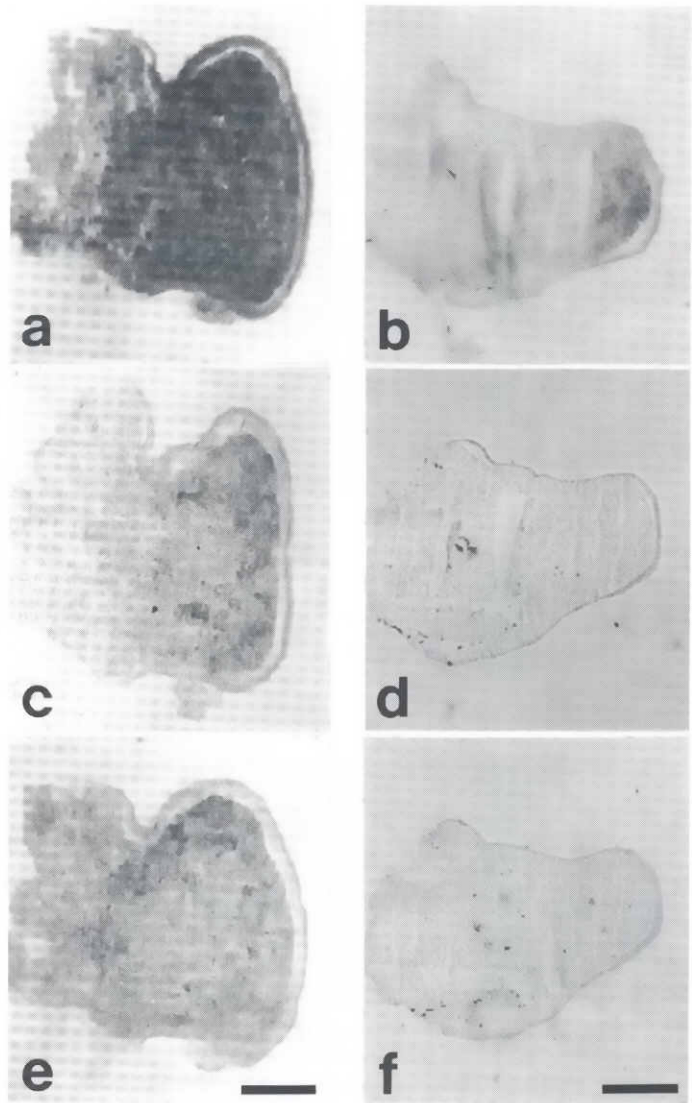

Fig. 3. Expression patterns of *Hoxa-11* (a,b), *Hoxa-13* (c,d) and *Hoxd-12* (e, f) in the non-polarized recombinant limbs made from anterior PZ cells that had been cultured for 24 h with (a,c,e) or without (b,d,f) FGF-2; hybridized at 20 h after grafting. *Note that Hoxa-11 was expressed in all cells, and Hoxa-13 and Hoxd-12 were barely expressed in the recombinant made from cells cultured with FGF-2, while no expression of these genes was detectable in the cells that had been cultured without FGF-2. Bars, 200 μm.*

Hoxa and Hoxd expressions in the cultured anterior PZ cells of the recombinant limbs

To examine whether FGF in the medium can maintain responsiveness of the cultured PZ cells to positional cues, we analyzed the *Hox* gene expression in the recombinant limbs made from the

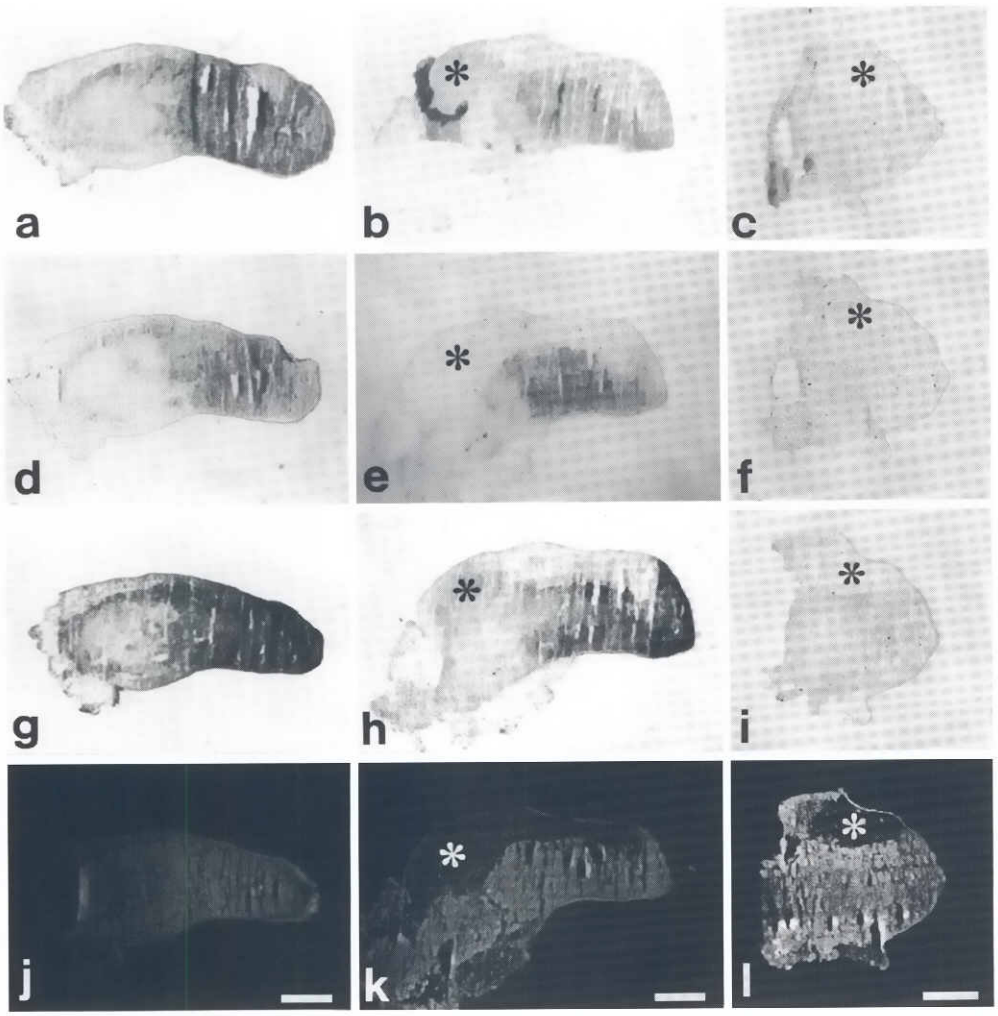

Fig. 4. Distribution of *Hoxd-12* (a,b,c), *Hoxd-13* (d,e,f) and *Hoxa-13* (g,h,i) transcripts, and grafted cells (j,k,l) in frontal adjacent sections of the recombinant limbs made from anterior PZ cells that had been cultured for 24 h with (a,b,d,e,g,h,j,k) and without (c,f,i,l) FGF-2; hybridized at 50 h after grafting. *Note that the expression patterns of these genes are similar in both non-polarized (a,d,g) and polarized (b,e,h) recombinants made from cells cultured with FGF, while no expression was detected in the polarized recombinant made from cells cultured without FGF (c,f,i). (j,k,l) A223 staining. Asterisks show grafted ZPA fragments which were A223-negative. Bars, 200 μm.*

anterior PZ cells that had been cultured for 24 h with or without FGF-2. Results are summarized in Table 1.

At 0 h (i.e., before grafting) no expressions of *Hoxa* and *Hoxd* were present in the recombinant mesoderm that had been cultured both with or without FGFs (not shown).

Twenty hours after grafting, *Hoxa-11* was expressed in the whole mesoderm of the non-polarized recombinants which had been cultured with FGF (Fig. 3a). The expression of *Hoxa-13* was barely detectable in the distal region (Fig. 3c). The expressions of *Hoxd-12* and *Hoxd-13* were very weak or absent (Fig. 3e). Similar expressing patterns were also observed in the polarized recombinants (not shown). On the other hand, in the recombinant limbs made from cultured cells without FGF, the expression of *Hoxa*-11 was rudimentary and no expressions of *Hoxa-13* and *Hoxd* genes were detected (Fig. 3b, d and f).

Fifty hours after grafting, the area of *Hoxd-12* expression expanded into the sub-distal region in the non-polarized recombinants made from cultured cells in the presence of FGF (Fig. 4a). The expression domain of *Hoxd-13* almost overlapped with that of *Hoxd-12* (Fig. 4d). The expressions of these two most 5' members of the *Hoxd* cluster in the recombinants were considered to be *de novo* expression since the anterior mesoderm would never have expressed them in the normal limb bud. Similar expression patterns of *Hoxd-12* and *Hoxd-13* were observed in all non-polarized recombinant limbs examined. At this time the expression of *Hoxa-11* was maintained in the whole mesoderm (not shown), and the *Hoxa*-13 expression was expanded over the sub-distal region of the recombinant (Fig. 4g). The proximal core region in which cartilage differentiation was undergoing lacked the expression of *Hoxa* and *Hoxd* genes.

The expression of *Hoxd-12* and *Hoxd-13* was also detected in the polarized recombinants made from cultured cells in the presence of FGF (Fig. 4b,e). These *Hoxd* transcripts were not detected in the implanted piece of ZPA since the piece was obtained from a stage 20 limb bud at the boundary region between the posterior margin and body wall where polarizing activity was maximum (MacCabe *et al.*, 1973). We noted that this expression of the *Hoxd* genes was not restricted to the posterior-distal region as in normal limb bud, but expanded over the entire distal region in a uniform manner along the anteroposterior axis as in the case of non-polarized recombinants. In contrast, neither expression of *Hoxa* nor *Hoxd* was detectable in the recombinants made from the anterior PZ cells which had been cultured without FGF (Fig. 4c,f and i).

Effect of FGF-2 or FGF-4 on the maintenance of Fgf-8 expression in the AER

In the limb bud at stage 22, *Fgf-8* is expressed strongly in the whole AER (Fig. 5a). To investigate if local contact between the AER and underlying mesoderm was necessary for the maintenance of *Fgf-8* expression in the AER, the AER-mesodermal contact was interrupted by the insertion of a bead soaked in PBS underneath the AER of stage 22 wing bud. We found that the expression was reduced in the AER which was interrupted from direct contact with mesoderm by the implanted bead (Fig. 5b). The inhibition was always observed regardless of the kind of inserted beads, and thus the *Fgf-8* expression in AER seems to require a local signal from the underlying mesoderm (data, not shown).

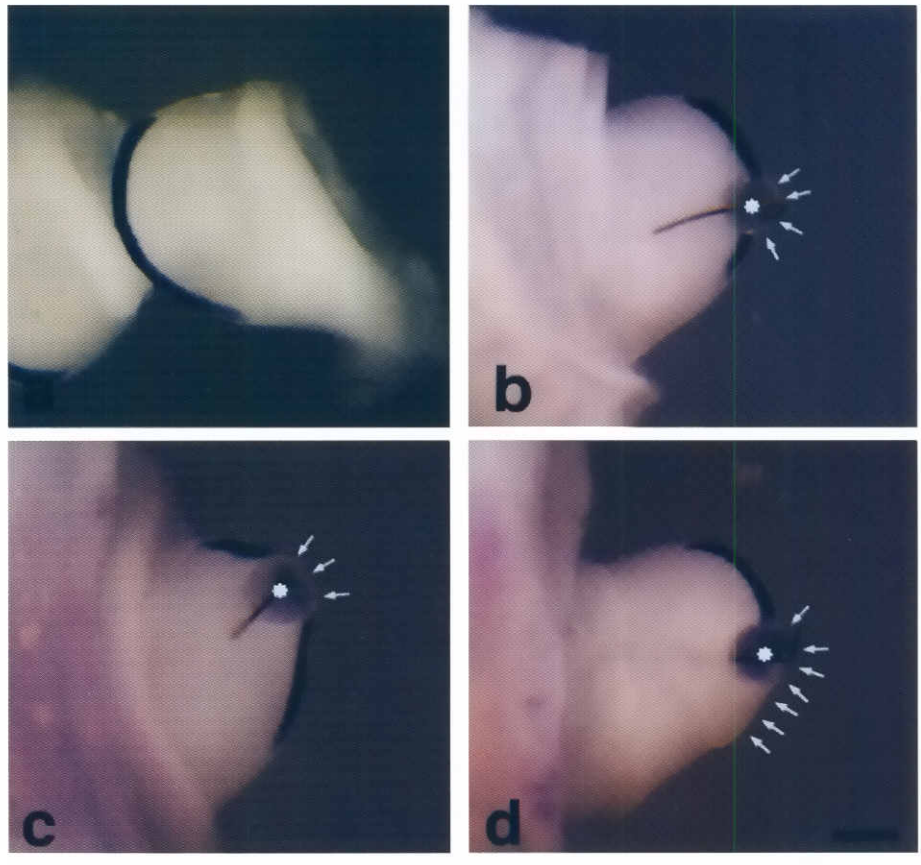

Fig. 5. Maintenance of *Fgf-8* expression in the AER by local contact with the underneath mesoderm. (a) *Fgf-8 was strongly expressed in the whole AER of stage 22 wing bud. (b-d) No Fgf-8 expression was detected in the AER overlying implanted beads presoaked in PBS* **(b)**, *0.1 mg/ml FGF-2* **(c)** *and 1mg/ml FGF-2* **(d)**, *6 h after implantation of the beads underneath the AER. Note that the disappearance of Fgf-8 was restricted in the AER overlying low FGF beads (arrows in b and c), but expanded over more of the anterior region overlying high FGF bead (arrows in d). Asterisks show implanted beads. The posterior side of the limb buds are oriented toward the top of the figures. Bar, 200 μm.*

FGF-2 distributes in the mesoderm underneath the AER (Savage *et al.*, 1993). We then examined the expression of *Fgf-8* in AER overlying implanted FGF-soaked beads. Expression was not found in the AER with the beads soaked in 0.01 mg/ml FGF-2 (not shown), nor 0.1 mg/ml (arrowheads in Fig. 5c). Similar results were observed in the case of 0.1 or 0.01 mg/ml of FGF-4 (not shown). The inhibition of *Fgf-8* expression expanded further over the more anterior region of the AER with the bead soaked in 1 mg/ml FGF-2 (arrows in Fig. 5d). Thus, neither FGF-2 nor FGF-4 could maintain *Fgf-8* expression in AER directly and in fact may even be inhibitory at high concentrations.

Effects of cultured anterior PZ cells on maintenance of Fgf-8 expression in the AER

We tested whether cultured PZ cells could maintain *Fgf-8* expression in the AER. The anterior PZ cells were cultured with or without FGF-2 or FGF-4, grafted underneath the AER of stage 22 wing buds and after 20 h the expression of *Msx1* in the grafted cells and of *Fgf-8* in the AER were examined (Table 2).

Msx1 was expressed in the PZ cells that had been cultured without FGF for 6 h and grafted back into the host limb (Fig. 6a). *Msx1* expression was restricted to the cells just underneath the host AER. Strong expression of *Fgf-8* was maintained in the AER overlying the grafted cells (Fig. 6e). On the other hand, no expression of *Msx1* was found in the grafted cells that had been cultured for 20 h in the absence of FGF (Fig. 6b), whereas *Fgf-8* expression was maintained in the AER normally (Fig. 6f). *Fgf-8* was not found in the AER overlying the grafted cells that had been cultured for 40 h without FGF (Fig. 6g), whereas the AER could express *Msx1* (Fig. 6c). However, *Fgf-8* expression was detected in the AER with the grafted cells that had been cultured for 40 h with FGF-2 (Fig. 6h) and FGF-4 (not shown), whereas *Msx1* expression could not be found in these grafted cells (Fig. 6d).

TABLE 2

MSX1 EXPRESSION IN THE ANTERIOR PZ CELLS THAT HAD BEEN CULTURED IN THE PRESENCE OR ABSENCE OF FGFS WHEN GRAFT BACK UNDERNEATH AER, AND FGF-8 EXPRESSION IN THE HOST AER ON THESE GRAFTED CELLS

Culture time (h)	FGF application*	n	Msx1 positive graft	Fgf-8 positive AER
6	none	13	8 (61%)#	13 (100%)
6	FGF-2	2	0	2 (100%)
6	FGF-4	4	0	4 (100%)
20	none	18	0	15 (83%)
20	FGF-2	2	0	2 (100%)
20	FGF-4	3	0	3 (100%)
40	none	12	0	4 (33%)
40	FGF-2	10	0	10 (100%)
40	FGF-4	8	0	7 (88%)

*None, application of heparan sulfate (100 ng/ml). FGF-2, FGF-4, 100 ng/ml together with hearan sulfate (100 ng/ml).

#Expression in the grafted cells just underneath AER

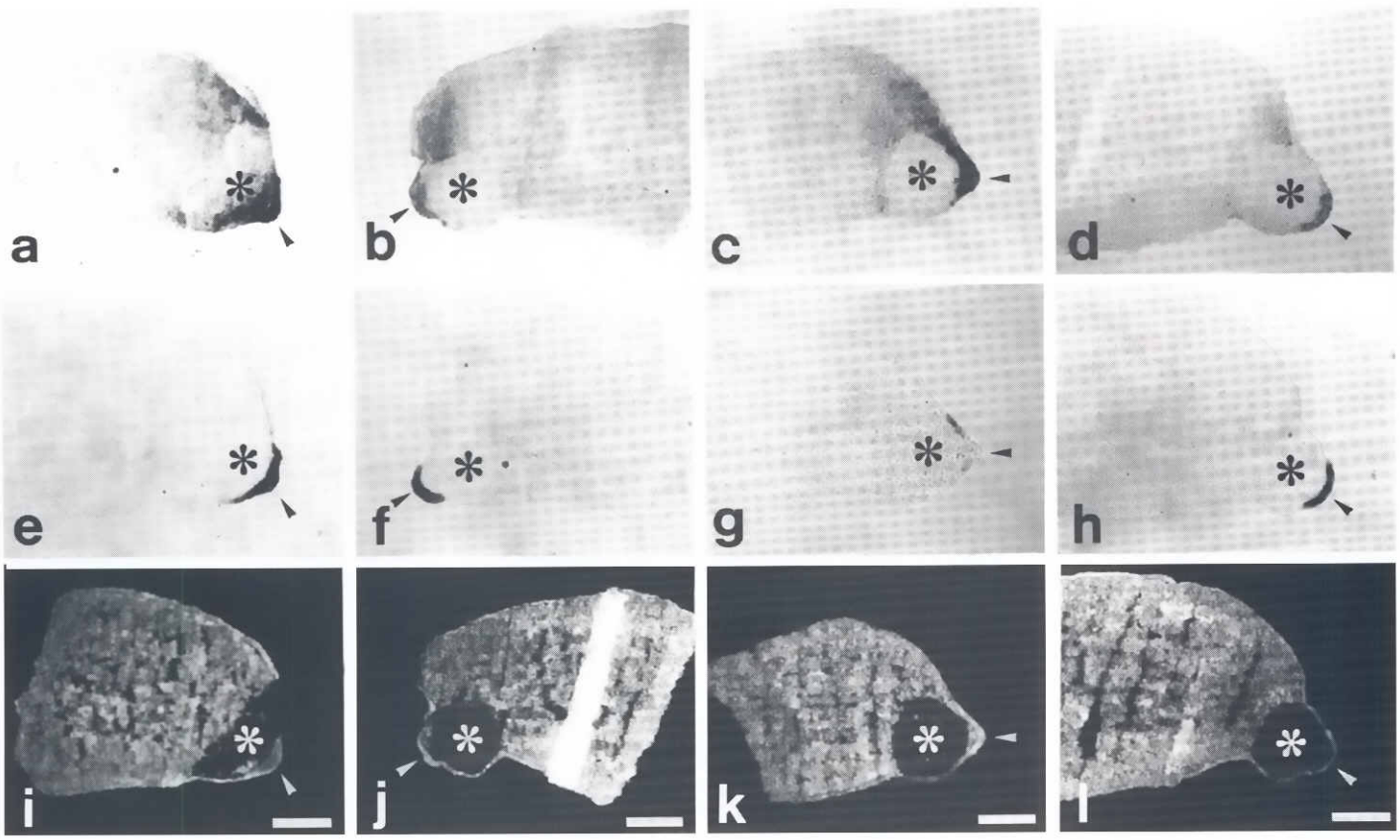

Fig. 6. Distribution of *Msx1* (a,b,c,d), *Fgf-8* (e,f,g,h) transcripts and grafted cells (i,j,k,l) in transverse adjacent sections of the operated limb buds 20 h after grafting of cultured anterior PZ cells that had been cultured without FGF for 6 h (a,e,i), 20 h (b,f,j), and 40 h (c,g,k), or with FGF-2 for 40 h (d,h,l). *(a) Msx1 expression was found in the distal part of grafted cells that had been cultured for 6 h without FGF-2, and (e) Fgf-8 expression was detectable in the AER on the graft. (b) No Msx1 expression was detectable in the grafted cells that had been cultured for 20 h, while in the AER on the graft (b) Msx1 and (f) Fgf-8 expressions were found. (c) The Msx1 expression was detectable in the AER on the grafted cells that had been cultured for 40 h without FGF-2, whereas (g) Fgf-8 expression was not detected in the AER. (d) Msx1 expression was not detected in the cells that had been cultured for 40 h in the presence of FGF-2, whereas (h) Fgf-8 expression was found in the AER on the grafts. (i,j,k,l) A223 staining. Asterisks show grafted mesoderm which was A223-negative. Bars, 200 μm.*

Discussion

FGF is not sufficient to maintain all the characters of PZ cells in vitro

The AER promotes the growth of underlying mesoderm and maintains it in an undifferentiated state (Summerbell *et al.*, 1973). If the AER is removed at any time, limb development ceases. However, treatment of limb buds lacking AER with FGF-2 or FGF-4 results in the maintenance of distal outgrowth and normal pattern, indicating that endogenous FGFs produced by the AER are involved in the control of distal outgrowth and the maintenance of PZ. The present study showed that cultured stage 20 PZ cells in the presence of FGF-2 or FGF-4 could proliferate and differentiate to cartilage in recombinant limbs, whereas the cells in the absence of FGFs could not, supporting the idea that FGFs can maintain the undifferentiated state of PZ cells *in vitro*. However, we also showed that stage 20 PZ cells formed a complete limb-like structure with normal digit pattern in the polarized recombinant limb, while stage 20 PZ cells cultured in the presence of FGFs only formed a poor limb-like structure with a single digit, indicating that these cultured cells in the presence of FGFs were clearly not the same as the stage 20 PZ cells.

We could not detect *Msx1* expression in the stage 20 PZ cells that had been cultured for 24 h with FGF-2 or FGF-4 when grafted back underneath the AER. *Msx1* is normally expressed in the PZ, and the expression is maintained by the AER. (Hill *et al.*, 1989; Davidson *et al.*, 1991; Monaghan *et al.*, 1991; Robert *et al.*, 1991; Yokouchi *et al.*, 1991a; Ros *et al.*, 1992). FGF-2 and FGF-4 have been shown to be one of the inducing factors of *Msx1* expression (Vogel *et al.*, 1995; Wang and Sassoon, 1995). Therefore, our failure to detect maintained expression of *Msx1* is surprising, especially in view of data suggesting that FGF-2 maintains the expression of *Msx1* in cultured PZ (Watanabe and Ide, 1993). One possible explanation for this result may be that lower levels of *Msx1* expression in the grafts are difficult to detect by *in situ* hybridization. In any case, our results indicate that the ability to respond to an AER signal and express *Msx1* could not be retained in the cultured PZ cells treated with the presence of FGFs compared to PZ cells of the unmanipulated limb buds.

Ros *et al.* (1994) showed that the anterior mesoderm of stage 20 wing bud could express the most 5' members of the *Hoxd* cluster *(Hoxd-12*, and *-13)* uniformly across the anteroposterior axis of a recombinant limb with no polarizing region present, whereas this anterior mesoderm would not express them in normal limb bud.

The present study showed that cultured anterior PZ cells in the presence of FGF could express these *Hoxd* genes and 5' *Hoxa* genes in non-polarized recombinant limbs, but cells cultured without FGF could not. FGF is thus able to maintain the ability of the anterior mesoderm of stage 20 wing bud *in vitro* to express *Hoxd* genes and respond to proximodistal positional cues in the developing recombinant limb. The PZ cells of early stage limb bud are thought to receive anteroposterior positional values, which are specified by the ZPA (Summerbell, 1974), and the recombinant limb made from the anterior third wing mesoderm re-established the nested pattern of *Hoxd* genes when the ZPA is present (Ros *et al.*, 1994). However, our present study showed that the anterior PZ cells when cultured for 24 h in the presence of FGFs lost the responsiveness to the positional cues from the ZPA, indicating that cells cultured with in the presence of FGFs were not the same as stage 20 PZ cells.

Our results, therefore, show that some but not all characteristics of PZ cells at the molecular and cellular level are maintained *in vitro* by FGF-2 and FGF-4. Since other growth factors, such as BMP-2, BMP-4, and WNT-7a, have been shown to be involved in pattern formation of developing limb bud in concert with FGFs (Niswander and Martin, 1993; Francis *et al.*, 1994; Yang and Niswander, 1995), the complete maintenance of PZ cell phenotype *in vitro* is likely to require additional factors.

FGF maintains the AER-maintenance activity of PZ cells in vitro

The PZ cells maintain both the specialized morphology and functional properties of the AER (Hinchliffe and Johnson, 1980; Fallon *et al.*, 1983), which to date has not been studied from the aspect of gene expression. We showed that the mesodermal cells underneath the AER are necessary for the maintenance of *Fgf-8* expression, whose product is thought to be one of the endogenous AER factors (Mahmood *et al.*, 1995). We also showed that PZ cells that had been cultured with FGF-2 or FGF-4 could maintain *Fgf-8* expression in the AER. This is the first report to show that FGFs maintains AER-maintenance activity of PZ cells in culture.

Msx1 is normally expressed in the PZ cells, although the role of the gene itself in the developing limb buds remains at present unclear. Some studies have shown that the mesodermal tissues which express or have the ability to express *Msx1* retain a high potential of developmental regulation and AER-signal transfer (Reginelli *et al.*, 1995; Hara and Ide, 1997). In this regard it has been reported that *Msx1* expression may keep cells in a proliferating and undifferentiated state in cells of the myogenic line (Song *et al.*, 1992; Woloshin *et al.*, 1995). However we could not detect *Msx1* expression in the PZ cells that had been cultured with FGF-2 or FGF-4 when grafted back underneath the AER, indicating the possibility that *Fgf-8* expression in AER may not be required for the *Msx1* expressing mesoderm.

It has been postulated that the PZ produces AER maintenance factors (Zwilling and Hansborough, 1956; Saunders and Gasseling, 1963). Previous studies showed that IGF-1 could support the AER maintenance activity and FGFs might allow diffusion of IGF-1 from the mesodermal cells underneath the AER (Dealy and Kosher, 1995,1996). FGF-10 is another possible candidate for the AER maintenance factor (Ohuchi *et al.*, 1997). Expression of *Fgf-10* persists in the PZ, and FGF-8 can rescue the expression of *Fgf-10* in the mesoderm of AER-removed limb buds. Conversely, ectopic application of FGF-10 can induce expression of *Fgf-8* and maintain it in the AER-removed limb bud, indicating the FGF-10 and FGF-8 may be involved in communication between the limb mesoderm and the AER. The extracellular matrix proteins (ECMs) are also strongly implicated in the signaling process of the AER-mesodermal interaction and in establishing morphological differences between the AER and non-ridge epithelia in the limb bud (Tomasek and Brier, 1986). The laminin receptor gene, *cLbp*, is normally expressed in the AER and PZ cells, and its expression is maintained by the AER and FGF (Hara *et al.*, 1997), indicating that the laminin-cLBP interaction is a candidate for maintenance of the special AER structure (Critchlow and Hinchliffe, 1994; Hara *et al.*, 1997). In this study, we showed that the PZ cells that had been cultured with FGF-2 or FGF-4 could maintain *Fgf-8* expression in the AER, although these FGFs themselves were not able to directly maintain the *Fgf-8* expression in the AER. Our results indicate that FGF-2 or FGF-4 can maintain cultured PZ cells in a state where they are able to produce these AER maintenance factors.

Materials and Methods

Application of FGF-2 and FGF-4 to wing bud

For FGF-2 or *FGF-4* application, Affi-Gel beads (200-250 μm diameter; Bio-Rad) were soaked in 2 μl of 1, 0.1, and 0.01 mg/ml FGF-2 or *FGF-4* (R&D systems) for at least 1 h at room temperature before application. The bead was inserted into a slit underneath the AER of stage 22 wing buds and fixed with a tungsten wire. At various time points after the operation, the embryos were fixed in 4% paraformaldehyde and used for whole-mount *in situ* hybridization.

Micromass cultures of chick limb bud cells and grafting underneath AER

The stage 20 anterior PZ (mesoderm within 100 μm underneath the anterior AER) of chick and quail wing buds were dissected out and mesodermal cells were isolated by the method described previously (Ide and Aono, 1988). The cells were suspended at 7.5×10^5 cells/ml in F12 medium containing 1% fetal calf serum, and 1 ml of the cell suspension was plated per well in a 24-well dish (Falcon). In some experiments, FGF-2 or *FGF-4* (R&D Systems) at 100 ng/ml, together with heparan sulfate (Sigma) at 100 ng/ml, were added to the serum-containing medium.

After culturing with or without FGF-2 or FGF-4, the cells were treated with CMF (Ca, Mg-free Tyrode's solution) at 37°C for 30 min, and dissociated in F12-10%FCS by pipetting. The suspended cells were pelleted by low-speed centrifugation in F12-10%FCS, and were incubated at 37°C for 1 h to promote cell adhesion. A piece of cell aggregate was then grafted to the apical margin of a chick wing in a slit underneath the AER. 12 to 24 h following the operation, the embryos were fixed in 4% paraformaldehyde, then embedded in O.C.T. compound (Miles) and sectioned for *in situ* hybridization.

To analyze the distribution of grafted cells in the host limb bud quantitatively, we used the chick-quail chimeric method (Wada *et al.*, 1993). The grafted quail cells in host chick limb bud were detected with A223, a chick specific mouse monoclonal antibody (Wada *et al.*, 1993; Ide *et al.*, 1994).

Preparation of recombinant limb buds

The recombinant limb buds were composed of an ectodermal jacket of chick leg buds and cultured PZ cells of chick wing buds. The aggregates of cultured PZ cells were prepared as described above and inserted into the ectodermal jacket. To obtain polarized recombinant limbs, a small fragment (100-150 μm) of ZPA from stage 20 leg bud was also inserted into the ectodermal jacket after the method of MacCabe *et al.* (1973). We used leg ectoderm and leg ZPA to rule out the contamination of mesodermal tissues from donor ectoderm or the ZPA fragment. Such contamination would be found by the formation of distal leg structures; these were never observed in our recombinant limbs and therefore, all structures were considered to be formed from the cultured wing mesoderm.

Ectodermal jackets were obtained from whole stage 22 leg buds by mild trypsin digestion. The cultured anterior-distal wing mesoderm was treated with CMF at 37°C for 30 min. Cells were then dissociated by pipetting and pelleted by low-speed centrifugation in F12-10%FCS. The recombinant limbs were assembled by introducing a fragment of the pelleted mesoderm into the ectodermal jacket.

The recombinant limb buds were grafted to the somite region at flank level of stage 20 host embryos and allowed to develop. Some recombinants were fixed in 4% paraformaldehyde 50 h after grafting for the assay of gene expression. The remaining recombinants were allowed to develop for 10 days to examine skeletal pattern after alcian blue staining.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described in Yonei *et al.* (1995). Digoxygenin-labeled RNA probes were prepared after Yokouchi *et al.* (1991a). The chick *Msx1* gene was kindly gifted by Dr. T. Nohno, Chick *Hoxd* and *Fgf-8* genes were kindly gifted by Dr. S. Noji.

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