Fgf-4 maintains polarizing activity of posterior limb bud cells in vivo and in vitro

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SUMMARY

The polarizing region is a major signalling tissue involved in patterning the tissues of the vertebrate limb. The polarizing region is located at the posterior margin of the limb bud and can be recognized by its ability to induce additional digits when grafted to the anterior margin of a chick limb bud. The signal from the polarizing region operates at the tip of the bud in the progress zone, a zone of undifferentiated mesenchymal cells, maintained by interactions with the apical ectodermal ridge. A number of observations have pointed to a link between the apical ectodermal ridge and signalling by the polarizing region. To test this possibility, we removed the posterior apical ectodermal ridge of chick wing buds and assayed posterior mesenchyme for polarizing activity. When the apical ectodermal ridge is removed, there is a marked decrease in polarizing activity of posterior cells. The posterior apical ectodermal ridge is known to express FGF-4 and we show that the decrease in polarizing activity of posterior cells of wing buds that normally follows ridge removal can be prevented by implanting a FGF-4-soaked bead. Furthermore, we show that both ectoderm and FGF-4 maintain polarizing activity of limb bud cells in culture.

Key words: apical ectodermal ridge, chick embryo, FGF, limb development, polarizing activity

INTRODUCTION

A major question in development is how structures arise in the appropriate places in the embryo. In vertebrates, this process appears to be controlled largely by signals that specify cell position. These signals are produced by specialized groups of cells and understanding the way in which this signalling is controlled is therefore of central importance. For example, the polarizing region is a pivotal signalling region in vertebrate limb development and appears to control the pattern of limb structures. Because the signalling properties of the cells of the polarizing region can be assayed by a simple graft to chick limb buds, this tissue provides a useful model system for analyzing the control and origin of signalling regions.

The polarizing signal appears to specify position across the anteroposterior axis of the limb and leads for example to a characteristic pattern of digits (Tickle et al., 1975). The polarizing region comprises a small group of mesenchyme cells at the posterior margin of the limb bud (Saunders and Gasseling, 1968). The structures in the limb are laid down in sequence as the bud grows out starting with proximal structures, those nearest to the body wall, and ending with distal structures, such as digits (Saunders, 1948). The outgrowth of the bud is controlled by a second set of signalling cells, the apical ectodermal ridge (Saunders, 1948; Summerbell, 1974a). The signal from the apical ridge also maintains at the tip of the limb bud a region of undifferentiated cells, known as the progress zone (Summerbell et al., 1973). The polarizing region signal operates on cells in the progress zone (Summerbell, 1974b).

Polarizing cells cannot be distinguished morphologically and their identity must be determined by in vivo assays. When cells from a polarizing region are grafted to the anterior margin of a chick limb bud, this results in duplication along the anteroposterior axis (Saunders and Gasseling, 1968). Indeed, additional digits can be formed following posterior grafts from mammalian limb buds to the anterior margin of chicken wing buds (Tickle et al., 1976; Fallon and Crosby, 1977; Wanek and Bryant, 1991), thus, indicating that the signal from the polarizing region is conserved between vertebrates. The additional digits that develop provide a measure for polarizing activity and the formation of specific digits is proportional to the number of implanted polarizing region cells, such that with increasing number of polarizing region cells more posterior digits develop (Tickle, 1981).

The way in which cells acquire polarizing activity during limb bud outgrowth and how this activity is controlled during the period of pattern formation are unknown. It is known that highest polarizing activity is found just proximal to the progress zone during bud outgrowth (MacCabe et al., 1973; Honig and Summerbell, 1985) and then disappears at the time that the ridge regresses (MacCabe et al., 1973).
Other studies indicated that grafts of polarizing region cells are more efficient when placed in contact with or under an intact apical ectodermal ridge (Tickle, 1981). When the mesenchymal cells of the polarizing region are isolated from limb buds and placed in culture, polarizing activity is rapidly lost (Honig, 1983; Hayamizu and Bryant, 1992). Together, these observations suggest that the apical ectodermal ridge may play a role in maintaining polarizing activity. We therefore set out to test this idea by determining the effect of apical ectodermal ridge removal on polarizing activity.

We have found that polarizing activity decreases dramatically in the absence of the ridge. Since Fgf-4 has been found to be expressed in the posterior apical ectodermal ridge (Niswander and Martin, 1992) and also has been shown to stimulate proliferation of limb bud mesenchyme in organ culture (Niswander and Martin, 1993), we then tested whether FGF-4 could substitute for the apical ectodermal ridge in providing a signal for maintenance of polarizing activity. Finally, we demonstrate that both limb bud ectoderm and FGF-4 can also function to maintain polarizing activity in limb mesenchyme cultured in vitro.

**MATERIALS AND METHODS**

**Removal of the posterior apical ectodermal ridge and the effects on polarizing activity**

The posterior apical ectodermal ridge of stage 19-20 chicken wing buds (Hamburger and Hamilton, 1951) was removed by lifting the posterior ridge from the underlying mesenchyme, using tungsten needles and cutting off the ridge, using fine forceps. After 24 and 30 hours, posterior mesenchyme was assayed for polarizing activity, as described below.

In a second set of experiments, FGF-4 was applied to the posterior part of the bud after apical ectodermal ridge removal. Heparin acrylic-beads (H5263, Sigma) of a size of 200 to 250 µm were soaked in 2 µl of 1 mg/ml FGF-4 for at least 1 hour, before transferring into the limb. To implant the FGF-4-soaked beads, a small cube of mesenchyme was removed. To keep the bead in place, we used sterile staples formed out of platinum wire (0.025 mm, Goodfellow). In a small series of experiments, FGF-4 was not applied immediately after the apical ectodermal ridge had been removed, but 16 or 24 hours later. In these cases, the beads were placed posteriorly between mesenchyme and ectoderm, by making a cut along the base of the ectoderm and pulling this away from the mesenchyme to make a loop. After these FGF-4 beads had been implanted, posterior mesenchyme was assayed as above, at 24 hours and 30-32 hours after ridge removal.

**Assaying polarizing activity of posterior chick limb bud cells**

The operated embryos were transferred into medium and the limbs isolated and treated for 45-60 minutes in 2% trypsin at 4°C. After removing the ectoderm, posterior pieces of mesenchyme were dissected from the wing, using tungsten needles and tested for polarizing activity by grafting them to the anterior margin of a stage 20/21 chick wing bud. After 6 days, the chicken embryos were fixed in 5% trichloracetic acid and stained for cartilage with Alcian Green (26 Gurr) in acid alcohol. After transferring the embryos into acid alcohol and dehydrating in 100% alcohol, the embryos were cleared with methyl salicylate to evaluate the cartilage structure of the wing. To measure polarizing activity, the development of a wing with no additional digits was scored as 0 (no polarizing activity, Fig. 1A). The appearance of a small knob of extra cartilage next to digit 2, which was not sufficiently well developed to be counted as a duplicated digit 2 was scored 0.5 (12.5% polarizing activity, Fig. 1B). The development of digit 2 scored 1 (25% polarizing activity, Fig. 1C), the development of digit 3 scored 2 (50% polarizing activity, Fig. 1D) and the development of an additional digit 4 scored 4 (100% polarizing activity, Fig. 1E,F). The number of points scored by each wing in an experimental series was added up and divided by the maximum possible score (number of limbs multiplied by 4). The scoring is based on quantitative data that relate the strength of the polarizing region signal and the number of polarizing region cells (Tickle, 1981).

The percentage of limbs with digit duplications was also calculated to give an indication of how many limbs had a changed digit pattern.

**Cell cultures and composition of media**

Micromass cultures were prepared from the posterior one third of limb buds of 9.5-11 day mouse embryos (strain C57 black and tan and H-2K<sup>b</sup>-tsA58 transgenic mice) following the technique described for chick limbs by Cottrill et al. (1987). The mouse embryos were staged according to Martin (1990). The embryos were placed in MEM medium (GIBCO) supplemented with 10% foetal calf serum (FCS, GIBCO), 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone (antibiotic/antimycotic, GIBCO) and 2 mM L-glutamine (GIBCO). The hindlimb or forelimb buds were dissected from the embryos and the ectoderm removed after soaking the limbs in 2% trypsin (GIBCO 1:250) in calcium- and magnesium-free saline (Hanks Buffered Salt Solution, GIBCO), pH 7.4 for 45-60 minutes at 4°C. After transferring the limb buds in MEM medium supplemented as described, the posterior thirds of the limb buds, which contain the polarizing region, were dissected and the mesenchyme was disaggregated and the cells centrifuged for 5 minutes to form a pellet. The cells were resuspended in medium, the concentration was determined with a hemacytometer and the final concentration adjusted to 10<sup>7</sup> cells/ml with serum-containing CMRL medium. One 10 µl drop of the cell suspension containing 10<sup>5</sup> cells was plated per well, using 4-well multidishes (Nunclon Delta), and the cells allowed to attach for 1-1.5 hours at 37°C. The high density (micromass) cultures were then flooded with 300 µl CMRL medium (GIBCO) containing 10% FCS (GIBCO), 2 mM L-glutamine (GIBCO) and 1% antibiotic/antimycotic (GIBCO). Cultures were incubated at 37°C with 5% CO<sub>2</sub> for 1, 2, 3 or 4 days. The medium was replaced daily.

In one series of experiments, posterior mesenchyme cells in micromass were cocultured with ectoderm. The ectoderm jackets were kept after the treatment of the limb buds with trypsin then transferred into CMRL medium and placed in 5-10 µl drops (containing one to three ectoderms) around one micromass culture in one well of a 4-well dish. Up to 10 separated ectoderm jackets were placed around individual micromass cultures. The cells were incubated at 37°C for 1.5 hours and then flooded with 300 µl serum-containing CMRL medium. Usually not all the ectoderm jackets plated attached to the substratum. Ectoderm jackets that did attach, started to flatten after about 24 hours and, in some experiments, there were between one and three ectodermal cell sheets in contact with the edge of the micromass culture.

To culture tissue fragments, the posterior tissue of mouse limb buds was isolated and one fragment of mesenchyme with its covering epithelium placed into 4-well multidiish. The tissue of posterior mesenchyme with or without (after treatment with trypsin) overlying ectoderm was incubated at 37°C, with the mesenchyme facing the substratum. After several hours in a drop of CMRL medium, the dish was flooded with serum-containing culture medium.

**Growth factors**

Fibroblast growth factors (FGF-4), heparan sulphate (Sigma) and...
acetylated BSA (New England Biolabs) were kindly provided by Dr Lee Niswander (University of California, San Francisco). FGF-2 was added at a concentration of 1, 10 and 100 ng/ml to the serum-containing culture medium. When FGF-4 or FGF-2 was added to the culture medium, heparan sulphate was also added for stabilization of the FGF-protein. 30 µl of heparan sulphate stock (1 µg/ml) and 30 µl of FGF-4 protein (1 µg/ml) was added to 300 µl serum-containing CMRL medium, which gives a final concentration of approximately 80 ng/ml heparan sulphate and FGF-4 protein in the culture medium. FGF-2 was a gift from M. Noble (purchased from British Biotechnology Ltd, Oxford) and prepared as described by the manufacturers.

**Assaying polarizing activity of cultured posterior mouse limb bud cells**

Polarizing activity of the cultured cells was assayed by grafting pieces of the cell culture to a stage 19 to 21 chicken wing bud (Hamburger and Hamilton, 1951). The cultured cells were scraped off the substratum using a silicone rubber policeman and the resulting cell sheet cut into 5-20 pieces. The apical ectodermal

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**Fig. 1.** Whole mounts of embryonic chick wings stained with alcian green to show digit patterns. (A) Normal wing (score 0); (B) wing with small anterior blip resulting from a graft of a piece of micromass of posterior mouse limb bud cells cultured for 24 hours (score 0.5); (C) duplicated wing, digit pattern 2234, resulting from a graft of micromass cocultured with ectoderm for 96 hours (score 1). Note knobs of cartilage (arrowed) that developed from the cultured cells. (D) Duplicated wing, digit pattern 32234, resulting from a graft of a piece of micromass of posterior cells cultured for 24 hours (score 2); (E) duplicated wing, digit pattern 43334, resulting from a graft of a piece of micromass of posterior cells cultured for 24 hours in the presence of FGF-4 (score 4); (F) duplicated wing, digit pattern 43234, resulting from a graft of polarizing region cut directly from mouse limb bud (score 4).
ridge of stage 20 to 21 chicken wing buds was lifted from the underlying mesenchyme anteriorly and a piece of the cell culture placed under the loop. 6 days after performing the grafts, the thorax with limbs of the embryo were fixed and stained for cartilage as described above.

RESULTS

Effects of the apical ectodermal ridge and FGF-4 on polarizing activity in limb buds of chicken embryos

Removal of posterior apical ectodermal ridge is followed by a decline in polarizing activity of the limb bud. 24 hours after removal of the posterior part of the ridge of early wing buds, posterior outgrowth was clearly inhibited in most cases (13/17; Fig. 2A). Buds with a skewed shape resulted because some outgrowth continued anteriorly. Polarizing activity in all regions of these skewed buds was reduced compared with that of the mesenchyme from the posterior margin of the contralateral limb (stage 24/25) which was 100% (8 cases; Fig. 2B). The region from the posterior tip (B) showed highest polarizing activity (52%) and 69% of the grafted wings had duplicated digits, whereas piece D taken most anteriorly had the lowest activity, 2% (Fig. 2A and Table 1A). In a few cases (4/17), some outgrowth occurred posteriorly, presumably due to incomplete removal of the ridge (see also Todt and Fallon, 1987) and grafts of pieces of the mesenchyme from the posterior margin of these buds had 100% polarizing activity. At 30 hours after posterior ridge removal, most buds were even more stunted and polarizing activity was reduced still further. Grafts of piece B had only 25% polarizing activity (50% of the limbs had duplicated digits; 6 cases).

The addition of FGF-4 to posterior mesenchyme in the absence of the ridge maintained polarizing activity. When the posterior part of the ridge was removed and a bead soaked in 1 mg/ml FGF-4 inserted posteriorly, bud outgrowth at the posterior margin continued and polarizing activity did not decrease. At 24 hours, polarizing activity of posterior cells (piece A) was 95% (100% limbs with digit duplications) and grafts of piece B, more distal posterior mesenchyme, had a polarizing activity of 82% (100% limbs with digit duplications; Fig. 2C and Table 1B).

FGF-4 had to be applied by 16 hours after ridge removal to rescue polarizing activity of the posterior mesenchyme of

(A) Limb buds after posterior ridge removal:

(B) Controls (contralateral limb):

(C) Limb buds after posterior ridge removal and addition of FGF-4-soaked bead:

<table>
<thead>
<tr>
<th>Pieces grafted</th>
<th>Polarizing activity</th>
<th>Duplications</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>17%</td>
<td>33%</td>
</tr>
<tr>
<td>B</td>
<td>52%</td>
<td>69%</td>
</tr>
<tr>
<td>C</td>
<td>21%</td>
<td>30%</td>
</tr>
<tr>
<td>D</td>
<td>2%</td>
<td>14%</td>
</tr>
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</table>

Fig. 2. Maps of polarizing activity of limb buds 24 hours after posterior ridge removal. 24 hours after removal of the posterior ridge and showing the effects of adding FGF-4. (A) removal of the posterior ridge; (B) controls showing polarizing activity of unoperated limb buds (stage 24/25); (C) removal of the posterior ridge and adding FGF-4-soaked beads. After 24 hours the limb buds were trypsinized to remove the ectoderm and the posterior pieces of bud mesenchyme were cut as shown in diagrams and assayed for polarizing activity by grafting to the anterior margin of a host wing bud. The results of these grafts are shown on the right.
the bud. To test whether shorter exposures to FGF-4, given at various times after posterior ridge removal, were also effective in maintaining polarizing activity, the posterior ridge of the chick wing bud was removed and beads soaked in FGF-4 were implanted at later times. When beads were implanted 15-16 hours after ridge removal, outgrowth of the posterior part of the chicken limb bud appeared to resume 6 hours later. Grafts of piece A, the more distal part of the newly formed posterior outgrowth resulted in 100% polarizing activity (2 cases; one gave an additional digit 2 and the other gave no additional digit). When beads soaked in FGF-4 were implanted a little later, 24 hours after ridge removal, no posterior outgrowth was observed 5-6 hours after bead insertion. Even though the contralateral left wing bud (stage 25/26) still had full polarizing activity (100% of 10 cases), little polarizing activity was detected in operated buds. Grafts of tissue from the posterior edge (piece A) and from the edge of the truncated posterior margin (B) both had 20% polarizing activity (50% and 40% of the wings had duplicated digits; 6 cases each). Thus, application of FGF-4, 24 hours after ridge removal, had no effect on maintaining polarizing activity.

Polarizing activity of mesenchyme cells cultured from the posterior region of mouse limb buds and the effects of ectoderm and FGF

Polarizing activity was rapidly lost when cells are placed in culture. When mesenchyme from the posterior third of mouse limb buds was either explanted as intact fragments or disaggregated and placed in micromass culture, polarizing activity was completely lost after 96 hours. The relationship between polarizing activity and time in micromass culture is shown in Fig. 3A. Even after 24 hours, polarizing activity of cells in micromass cultures was much reduced. The polarizing activity of cells cultured for 24 hours was 20% although 71% of the wings were duplicated (Fig. 3B). This compares with a polarizing activity of 50% (87% wings with digit duplications), which was obtained with pieces of posterior mesenchyme from mouse limb buds grafted directly into chick limb buds. With longer times in culture, the polarizing activity was reduced still further. The difference in extent of the polarizing activity of the cells at 24 hours and 72 hours is shown by comparing the character of the additional digits produced; at 72 hours only 1 out of 6 wings showed marked changes in pattern and an additional digit 2 formed, whereas at 24 hours, 4 out of 7 wings had either an additional digit 2 or an additional digit 3 (Table 2A).

Coculture with ectoderm maintained polarizing activity of cultured cells

The experiments in vivo showed that posterior apical ridge maintained polarizing activity in the limb bud. To investigate this in culture, fragments of posterior third mouse limb buds with ectoderm attached were cultured and ectoderm jackets were cocultured with micromasses of posterior mesenchyme cells. With fragments of posterior limb with attached ectoderm, polarizing activity of the mesenchyme could still be detected at 96 hours; 2 out of 12 grafts gave duplications. With micromass cultures, there was also maintenance of polarizing activity after 96 hours: 38% of the limbs were duplicated after receiving a graft of micromass cocultured with ectoderm (Figs 1C, 3A, B and Table 2B), whereas when mesenchyme was cultured in the absence of ectoderm, no duplications were obtained after grafting at this time point. However, at 72 hours of coculture, the mesenchyme had 5% polarizing activity (18% of wings with digit duplications).

FGF-4 maintained polarizing activity of cultured posterior limb bud cells

To see whether FGF-4 can maintain polarizing activity in

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**Table 1. Digit pattern of wings following grafts of posterior mesenchyme**

(A) 24 hours after removal of the posterior apical ectodermal ridge

<table>
<thead>
<tr>
<th>Stage of operation</th>
<th>Piece grafted</th>
<th>n</th>
<th>Digit pattern of limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal 2 3 4</td>
</tr>
<tr>
<td>19</td>
<td>A 1</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B 2</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C 2</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>20</td>
<td>A 5</td>
<td>5</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B 11</td>
<td>11</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C 11</td>
<td>7</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>D 7</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

(B) 24 hours after removal of the posterior apical ectodermal ridge and placing an FGF-4-soaked bead at the posterior margin of the wing bud

<table>
<thead>
<tr>
<th>Stage of operation</th>
<th>Piece grafted</th>
<th>n</th>
<th>Digit pattern of limbs</th>
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<tbody>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
<td>19</td>
<td>A 1</td>
<td>1</td>
<td>–</td>
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<td></td>
<td>B 2</td>
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<tr>
<td>20</td>
<td>A 10</td>
<td>10</td>
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<td></td>
<td>B 9</td>
<td>9</td>
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vitro just as it did in intact buds, FGF-4 was added to micromass cultures of posterior mesenchyme cells. After 24 hours, polarizing activity was 53% and 100% of wings had duplicated digits, compared with 20% polarizing activity (71% wings with digit duplications) for mesenchyme cells cultured for the same time period without FGF-4. The maintenance of polarizing activity was shown by development of a duplicated digit (Fig. 1E; Table 2C), a result that was never obtained in the absence of the growth factor. Even after 72 hours, the activity with FGF-4 was 19% and three wings with an additional digit developed (Table 2C), whereas without FGF, cultured cells had only 4% polarizing activity. However, by 96 hours, no polarizing activity could be detected even with FGF-4 (Fig. 3A,B).

FGF-4 had an effect on cultured posterior cells only when added at the start of culture. When FGF-4 was added to the cultures after 24 hours, polarizing activity after a further 24 hours was 20% (40% of the wings had duplicated digits) and after 72 hours was zero. FGF-4 was added to the cultures together with heparan sulphate, but heparan sulphate alone had no effect on polarizing activity (13% polarizing activity at 24 hours, 4 cases).

FGF-2 can also maintain polarizing activity of cultured posterior cells. In the presence of 1 ng/ml of FGF-2, cells cultured for 24 hours had a polarizing activity of 50% (100% of the wings had duplicated digits, 5 cases). In the presence of 10 ng/ml and 100 ng/ml, grafts had 43% and 46% polarizing activity (100% of wings with digit duplications at both concentrations: 9 and 14 cases, respectively).

**DISCUSSION**

The maintenance of polarizing activity in mesenchyme cells at the posterior margin of the limb bud is dependent on a signal from the apical ectodermal ridge. We found that, when the posterior apical ridge is removed or the mesenchyme cells are taken from the bud and placed in culture, polarizing activity declines. An interaction between the apical ectodermal ridge and the polarizing region would link patterning across the anteroposterior axis with bud outgrowth. Thus as successive structures along the proximodistal axis are specified in the progress zone, their anteroposterior character would be controlled by a signal from the polarizing region cells. However, there is some controversy about whether the polarizing region signal is required throughout the patterning process because structures can

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**Table 2. Digit pattern of chick wings following grafts of micromass cultures of posterior mesenchyme cells of mouse limb buds**

(A) Posterior mesenchyme cells cultured in micromass

<table>
<thead>
<tr>
<th>Time in culture</th>
<th>Number of grafts</th>
<th>Digit pattern of limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>Normal 234 &lt;sup&gt;1&lt;/sup&gt;</td>
<td>2 3 4 1 3 2 3 4 3 1 4 3 3 4</td>
</tr>
<tr>
<td>48 h</td>
<td>7 2 4 1 3 2 3 4 3 1 4 3 3 4</td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>6 5 3 1 4 3 3 4</td>
<td></td>
</tr>
<tr>
<td>96 h</td>
<td>18 18 3 3 1 4 3 3 4</td>
<td></td>
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</tbody>
</table>

(B) Posterior mesenchyme cells in micromass cocultured with four to six ectodermal jackets

<table>
<thead>
<tr>
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<th>Digit pattern of limbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>72 h</td>
<td>Normal 234 &lt;sup&gt;1&lt;/sup&gt;</td>
<td>2 3 4 1 3 2 3 4 3 3 4</td>
</tr>
<tr>
<td>96 h</td>
<td>17 14 1 5 3 3 1 4 3 3 4</td>
<td></td>
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(C) Posterior mesenchyme cells in micromass cultured with FGF-4

<table>
<thead>
<tr>
<th>Time in culture</th>
<th>Number of grafts</th>
<th>Digit pattern of limbs</th>
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<tbody>
<tr>
<td>24 h</td>
<td>Normal 234 &lt;sup&gt;1&lt;/sup&gt;</td>
<td>2 3 4 1 3 2 3 4 3 1 4 3 3 4</td>
</tr>
<tr>
<td>48 h</td>
<td>7 2 4 1 3 2 3 4 3 1 4 3 3 4</td>
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</tr>
<tr>
<td>72 h</td>
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<td></td>
</tr>
<tr>
<td>96 h</td>
<td>18 18 3 3 1 4 3 3 4</td>
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![PostScript Picture](PostScript Picture)
FGF-4 appears to be a signal from the apical ridge that maintains polarizing activity of posterior limb bud mesenchyme. We showed that the addition of FGF-4 either in vivo or in vitro acts as a substitute for the ridge. Fgf-4 transcripts are present in the posterior apical ridge (Niswander and Martin, 1992) and, because FGF-4 is a readily secreted protein (Delli-Bovi et al., 1989), it could be released by the apical ectodermal ridge cells and diffuse into the underlying mesenchyme. FGF receptor-1 is expressed in the mesenchyme of the mouse limb bud (Orr-Urtreger et al., 1991; Peters et al., 1992) and an isoform of FGF-receptor 1 binds FGF-4 (Mansukhani et al., 1990). FGF-2 can also maintain polarizing activity in vitro (see also Anderson et al., 1993). FGF-2 has been isolated from limb buds (Seed et al., 1988; Munaim et al., 1988) and FGF-2 can also bind to FGF-receptor 1 (Dionne et al., 1990). Transcripts of Fgf-5 are only found in a small patch near the base of the developing limb (Haub and Goldfarb, 1991) and Fgf-3 does not appear to be expressed in the developing limb (Wilkinson et al., 1989).

FGF-4 could act in several ways to maintain polarizing activity. FGF-4 could act directly on polarizing cell signalling. For example, FGF could be required in order for the cells to produce a positional signal. However, if this is the mechanism of action, it seems unlikely that FGF is the only factor involved. The maintenance of polarizing activity of cultured cells by FGF may provide a model for identifying putative additional factors. It is perhaps interesting in this respect that apical ridge cells also contain transcripts of genes that code for other growth factors including bone morphogenetic proteins (Lyons et al., 1990; Jones et al., 1991).

A second possibility is that FGF-4 could simply promote survival and proliferation of limb mesenchyme cells (see also MacCabe et al., 1991). According to this idea, FGF-4 would have no specific role in regulating the polarizing region signal. When the posterior ridge is removed, outgrowth of the bud is clearly reduced (see also Todt and Fallon, 1987) and application of FGF-4 restores bud outgrowth. Fgf-4 is expressed in the posterior part of the apical ridge (Niswander and Martin, 1992). We propose that FGF-4 stimulates proliferation of posterior mesenchyme cells at the tip of the limb which are precursors of the polarizing region and which will, as they leave the progress zone, take on polarizing activity. This could explain how polarizing activity is maintained and also why, in normal limb development, highest polarizing activity is present proximal to the progress zone.

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