

Maintenance of ZPA signaling in cultured mouse limb bud cells

Rosalie Anderson, Michael Landry and Ken Muneoka

Department of Cell and Molecular Biology and The Molecular and Cellular Biology Program, Tulane University, New Orleans, LA 70118, USA

*Author for correspondence

SUMMARY

The positional signal localized to the posterior (zone of polarizing activity or ZPA) region of the vertebrate limb is transiently expressed during development and a decline in ZPA signaling is accelerated when posterior cells are dissociated and cultured in vitro. The evidence that cultured posterior cells display a precocious decline in ZPA signaling when compared to in vivo studies suggests that a factor present in the limb bud maintains or stabilizes ZPA signaling during limb outgrowth and that this maintenance factor is lost and/or exhausted in in vitro studies. We have developed a new culture technique, 'microdissociation', which preserves extracellular components that we have found to be necessary for ZPA signal maintenance. Our data

suggest that the limb bud ectoderm produces a maintenance activity that becomes stored in the extracellular matrix where it acts on limb bud cells to stabilize the activity of the ZPA signal. Using our initial characterization of this maintenance activity, we have identified a growth factor, FGF-2 (bFGF), that can replace all of the ZPA signaling maintenance activity observed in microdissociate cultures. The existence of various members of the FGF family in the developing limb strongly argues a role for FGF in stabilizing ZPA signaling in vivo.

Key words: limb development, pattern formation, FGF-2

INTRODUCTION

Patterning of the vertebrate limb is thought to result from position-specific cell-cell interactions that take place early in development, and these interactions are best exemplified when positionally disparate cells are placed in contact through grafting. In the chick limb bud, posterior (ZPA) limb bud cells grafted into the anterior margin stimulates the formation of supernumerary digits, which are composed primarily of anterior cells (Honig, 1983a; Javois and Iten, 1986; Wanek et al., 1991). Thus, patterning of the developing chick limb involves a directional and non-reciprocal interaction; a posterior-derived positional signal modifies the developmental fate of anterior limb bud cells. This interaction makes the anterior region of the developing chick limb bud an excellent assay for determining the signaling ability of introduced cells. It is important to note that the 'zone' of polarizing activity is defined empirically by grafting studies and that there is confusion regarding the spatial extent of this zone (see Javois and Iten, 1981). There is also controversy regarding the nature of the ZPA signal itself, for example, whether it is a diffusible morphogen. There is, however, no controversy with regard to the existence of a positional signal that can be assayed in ZPA grafting studies. Thus, in using the acronym ZPA, we wish only to emphasize the existence of an activity without implying that a specific 'zone' exists or whether the signaling molecules are diffusible or not.

There is now considerable evidence that positional sig-

nals, like that from the posterior region of the limb bud, are conserved evolutionarily and also during embryogenesis. For example, interspecific grafts of posterior limb bud tissues from mouse, humans and a variety of reptilian limb buds into the anterior margin of the chick limb bud results in a supernumerary response (see Muneoka and Sassoon, 1992 for review). In these studies, the quality of the response is, however, reduced when compared to chick-chick posterior grafting results; supernumerary digit patterns of **4-3-2**-2-3-4 (supernumerary digit patterns are underlined and in bold) or **4-3**-2-3-4 or **4-3**-3-4 are observed in chick-chick studies whereas digit patterns of **3-2**-2-3-4 or **3-2**-3-4 or **2**-2-3-4 are typically observed in cross-species studies (MacCabe and Parker, 1976; Tickle et al., 1976; Fallon and Crosby, 1977; Honig, 1984; Wanek and Bryant, 1991). There are reports of the occurrence of a supernumerary digit 4 following mouse-chick posterior tissue grafts; however, these are the minority of the duplications induced (Fallon and Crosby, 1977; Izpisua-Belmonte, 1992). Thus, while the ZPA signal itself appears to be conserved, species-specific differences in the effectiveness of signal transmission are apparent. Similarly, a ZPA-like signal is also found in other developing tissues; ZPA-like signaling has been detected in embryonic flank, mesonephros and somites from the chick limb region (Saunders, 1977), tail mesoderm (Saunders, 1977; Saunders and Gasseling, 1983), Hensen's node (Hornbruch and Wolpert, 1986) and most recently the floor plate of the neural tube (Wagner et al., 1990) and the genital tubercle (Dolle et al.,

1991). Thus, a signal similar to that derived from the posterior limb bud appears to be utilized extensively throughout embryogenesis.

In the limb bud, ZPA signaling is controlled both spatially and temporally. Using chick tissue, maps of ZPA signaling have been established for different developmental stages of the chick wing bud (MacCabe et al., 1973; Honig and Summerbell, 1985); ZPA signaling is localized to the posterior region of the early limb bud and moves distally with the outgrowth of the limb bud. At earlier and later developmental stages, the quality of the positional response is reduced when compared to the period of maximal activity (stages 19-25); grafts result in an incomplete complement of supernumerary digits. A similar spatial-temporal pattern of ZPA signaling is observed when mouse limb bud tissues are assayed in the chick wing bud (Wanek and Bryant, 1991). These studies are consistent with the idea that the production of a positional signal from posterior tissue is transient during limb development and that its decline is not abrupt but gradual.

Studies in which posterior tissue/cells are tested for ZPA signaling following *in vitro* culture show that signaling declines rapidly and precociously when compared to the activity *in vivo*. A ZPA signal is not detectable from explant cultures (37°C) of chick or quail posterior tissue after 48 hours (Carlson, 1984) and dissociated monolayer cultures of chick posterior cells do not signal after 30 hours (Honig, 1983b). Similarly, culturing dissociated posterior cells of mouse and chick limb buds in high density micromass cultures does not improve the maintenance of the ZPA signal (Hayamizu and Bryant, 1992). Based on the *in vivo* mapping studies for ZPA signaling described above, Honig (1983b) has estimated the expected period of signaling to be 70-80 hours, and it is clear that in all studies to date, signaling declines precociously when posterior cells are cultured. This evidence suggests that an activity present in the limb bud maintains or stabilizes ZPA signaling during limb outgrowth and that this maintenance activity is lost and/or exhausted *in vitro*.

To characterize the *in vitro* conditions necessary for the maintenance of ZPA signaling, we have modified culture conditions for posterior mouse limb bud cells that preserve signaling for a period of time that is comparable to that predicted from *in vivo* studies. The major alteration from standard culture conditions is the development of a microdissociation protocol in which ectodermal fragments and extracellular components are not eliminated from the high density culture. This finding indicates that the decline of ZPA signaling does not normally occur in a cell autonomous manner and supports the idea that a maintenance activity stabilizes the signaling ability of limb bud cells during development. We also provide evidence that this maintenance activity is present in supernatant from microdissociation cultures and also in medium conditioned by isolated ectoderms. Finally, we have found that all of the maintenance activity that we observe in microdissociate cultures can be accounted for by culturing standard micromasses of posterior cells in medium containing FGF-2. These studies support the idea that the limb bud ectoderm provides a source of an FGF-like factor, which is bound to the matrix and acts on limb bud cells by either

maintaining the production of the ZPA signal, directly or indirectly, or by modulating the activity of this signal.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn chicken eggs (Trustlow Farms, Chestertown, MD) were incubated in a humidified incubator at 38°C. On the third day of incubation eggs were windowed and 2-3 drops of antibiotic solution (0.25 mg/ml penicillin, 0.25 mg/ml streptomycin sulfate) were added to each egg. Windows were covered with removable tape and eggs were returned to the incubator. The following day eggs were staged (Hamburger and Hamilton, 1951) and allowed to cool prior to grafting.

Swiss Webster mice (CFW, Charles River) were maintained at a constant temperature (21°C) and on a 10 a.m.-10 p.m. light/dark cycle. Mated females were checked for vaginal plugs each afternoon and plug day was designated embryonic day 1 (E 1). Timed pregnant female mice were killed on E 11 and embryos were removed under aseptic conditions and placed on ice in Hanks' balanced salt solution (HBSS). Fore and hind limb buds at stages 2/3 (Wanek et al., 1989) were collected and cut into 3 regions (anterior, central and posterior) as previously described (Shi and Muneoka, 1992). In this study, posterior fragments and anterior fragments were used.

Grafting

Operations were performed on stage 19-21 chick wing buds. Limb buds were stained on their dorsal surface with Nile blue sulfate (1% in 2% agarose) and an incision at the base of the apical ectodermal ridge (AER) in the anterior region of the bud was made with a sharpened tungsten needle. The AER was teased up and stretched to create a graft site. Grafts were then placed between the loosened AER and the anterior mesoderm; grafts were held in place by the AER. Control grafts into the posterior region of the wing bud were made in a similar manner with the exception that the graft site was created by teasing up the AER over the posterior region of the bud. For tissue grafts, regions of the posterior mouse limb bud (stages 2/3) were isolated as small cubes of tissue and grafted under the AER in the anterior region of the limb bud. For the most part, grafts for this study consisted of fragments of high density cultures of posterior limb bud cells (see below). After grafting, windows were sealed with parafilm (American Can Co.) and eggs were returned to the incubator. The first day after grafting embryos were examined for retention of the graft. After 7 days (11 days incubation), embryos were removed from their shells and both wings were excised and fixed in Bouin's fixative for 1-2 days. Wings were stained with Victoria blue (Bryant and Iten, 1974), dehydrated and cleared in methyl salicylate.

Standard dissociation cultures

Posterior limb bud fragments were collected and placed in 5 ml of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA, GIBCO) for 6 minutes at 37°C (Fig. 1) as described by Shi and Muneoka (1992). The trypsin solution was removed and the fragments were gently triturated in 5 ml of culture medium which consisted of Dulbecco's modification of Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO) and gentamicin (50 µg/ml). Following dissociation, the cell suspension was filtered through 2 layers of Nitex 20 (20 µm) to obtain a single cell suspension. The concentration of cells in suspension was determined using a hemacytometer, then adjusted to 4×10^7 cells/ml (Hayamizu and Bryant, 1992). 10 µl drops of cell suspension were plated in 35 mm tissue culture dishes (Falcon) and placed in a humidified incubator with 5% CO₂ at 37°C for 2 hours to allow cells to attach. After this period, 2 ml of culture medium

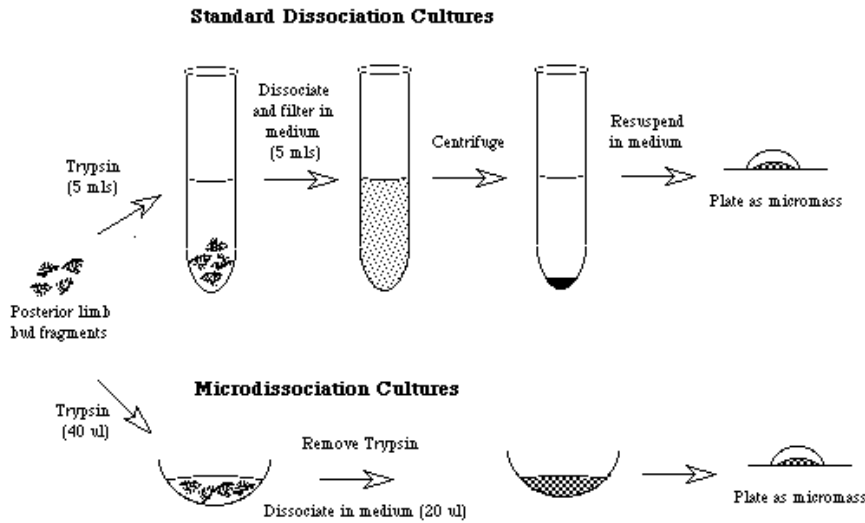


Fig. 1. Standard dissociation and microdissociation protocols. Utilizing the standard dissociation culture procedure posterior tissue fragments (one-third of the limb bud) are trypsinized and dissociated in large volumes prior to filtering. Cells are then centrifuged and resuspended in fresh culture media and plated as micromasses. All trypsin-sensitive extracellular components are lost by dilution in this procedure. By comparison, the microdissociation protocol utilizes only small volumes of trypsin and culture medium and the cell suspension is not filtered but immediately plated.

was added to each dish. Cultures were returned for incubation until grafted. For standard dissociation cultures treated with FGF-2, dissociated cells were plated in culture medium containing bovine FGF-2 (R&D Systems) at 0.1 ng/ml, 1.0 ng/ml, 10 ng/ml, 25 ng/ml, 50 ng/ml and 100 ng/ml. After a 2 hour period to allow for cells to attach, these cultures were flooded with culture medium containing a similar concentration of FGF-2. For cultures that were assayed at 72 and 96 hours, culture medium was replaced at 48 hours with fresh FGF-2-containing culture medium.

For grafting, micromasses were removed with a cell scraper and cut into small pieces with a tungsten needle. Approximately 10–15 grafts were obtained from each culture. Grafts were maintained in HBSS on ice, then transferred to the eggs with a pipet for grafting. In some cases the degree of chondrogenesis was assessed in high density micromass cultures at 96 hours. These cultures were fixed in formalin and stained with Alcian blue following a protocol described by Paulsen and Solursh (1988).

Microdissociation cultures

Fragments of posterior mouse limb buds were isolated as described above. Fragments were collected and placed in small watchglass wells in HBSS on ice (Fig. 1). 40 limb bud fragments were collected per group. HBSS was removed and 40 µl of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA, GIBCO) was placed in each well. Fragments were then placed in an incubator at 37°C for 5 minutes. Trypsin was carefully removed and any remaining trypsin activity was neutralized with 20 µl of culture medium. Tissue was dissociated by gentle pipetting and two 10 µl drops of cell suspension were plated in individual 35 mm tissue culture dishes. Cultures were incubated at 37°C in a humidified incubator with 5% CO₂ for 2 hours to allow cells to attach and then 2 ml of culture medium was added to each dish. Tissue culture dishes were returned to the incubator where they remained until grafted. Prior to grafting, the cultures were gently removed from the dishes using a cell scraper and cut into small fragments for grafting as described above.

Limb bud ectoderms were isolated and ectoderm-free microdissociate cultures were made as follows. Tissue fragments were collected and incubated in dishes containing 1% crude trypsin in HBSS for 1 hour at 4°C. Fragments were transferred to a Petri dish (60 mm, Falcon) containing 7 ml of culture medium and ectoderms were removed by gently agitating the culture medium with a pipet. Ectoderms were transferred to another dish containing culture medium and held on ice until needed. Ectoderm-free limb bud fragments (approximately 40) were placed in a glass well with 20 µl of fresh culture medium and gently triturated. This cell sus-

pension was plated in individual wells of a 24-well plate in 10 µl drops and cells were allowed to attach for 2 hours. After this attachment period a culture plate insert (Millicell-CM 4 µm insert, Millipore) was carefully placed on top of each micromass. Ectoderms (approximately 40) with culture medium were pipetted into each insert. The volume of culture medium in each well was approximately 1.5 ml. Plates were then returned to the incubator until needed.

Supernatant from microdissociated cells

Posterior supernatant was collected from microdissociate preparations as follows. 40 posterior fragments were microdissociated as described above and resuspended in 20 µl of culture medium. This single cell suspension was transferred into a sterile microcentrifuge tube and centrifuged at 4°C for 5 minutes at 14,000 revs/minute in an Eppendorf microfuge (model no. 5415). Supernatant was removed and used to resuspend posterior cells that had been microdissociated and diluted or posterior cells that had been dissociated using the standard dissociation protocol.

Analysis

For each experiment the frequency of the supernumerary response was determined by dividing the number of limbs displaying a supernumerary digit(s) by the total number of limbs grafted. In addition, the quality of the supernumerary response, as indicated by the digit pattern that formed, was also documented and is presented as the number of limbs displaying that digit pattern divided by the total number of limbs grafted. The frequency of the supernumerary response, as well as the quality of that response, are provided in tables. This method of analysis allows for the data to be presented in its entirety. We chose not to use the Strength of Activity Index (SAI, Honig et al., 1981; Honig and Summerbell, 1985), an alternative method of analysis, because it assumes that a single signal is responsible for all of the digit types that form and that varying concentrations of this signal determines the digits that form. As a result, the SAI does not take into the number of supernumerary digits that form per limb, only the most posterior supernumerary digit that forms. We note that the SAI for this study can be determined based on the data presented in our tables.

RESULTS

Maintenance of ZPA signaling in vitro

Our in vivo assay for ZPA signaling of mouse tissue is

Table 1. ZPA signalling in vitro

Method of culture	Time (hrs)	n	Normal limbs 2-3-4	Limbs with supernumerary digits			Frequency
				<u>2</u> -2-3-4	<u>3</u> -2-3-4	<u>3-2</u> -2-3-4	
ZPA tissue not cultured	0	10	0 (0%)	2 (20%)	2 (20%)	6 (60%)	100%
Anterior tissue not cultured	0	15	15 (100%)	0 (0%)	0 (0%)	0 (0%)	0%
Standard	24	9	2 (22%)	6 (67%)	1 (11%)	0 (0%)	78%
	45	10	7 (70%)	2 (20%)	1 (10%)	0 (0%)	30%
	52	8	8 (100%)	0 (0%)	0 (0%)	0 (0%)	0%
Microdissociate	24	9	0 (0%)	0 (0%)	2 (22%)	7 (78%)	100%
	48	14	2 (14%)	10 (71%)	2 (14%)	0 (0%)	86%
	72	16	7 (44%)	9 (56%)	0 (0%)	0 (0%)	56%
	96	12	5 (42%)	7 (58%)	0 (0%)	0 (0%)	58%

based on the formation of supernumerary digits resulting from grafts placed under the AER in the anterior margin of the chick wing bud. This assay is similar to that used by Izpisua-Belmonte et al. (1992) but differs somewhat from that used in previous heterospecific studies involving grafts of mouse ZPA into the chick wing bud. Other studies have used either grafts that replace anterior tissue with posterior tissue (Tickle et al., 1976) or wedge grafts in which posterior tissue is added into the anterior wing bud (Wanek and Bryant, 1991). However, Tickle (1981) has shown that grafts of posterior cells placed beneath the AER are more effective in specifying supernumerary digits. To determine a baseline response for mouse ZPA signaling, we assayed small pieces of posterior one-third mouse limb bud tissue by grafting them under the AER. Posterior tissue grafts result in the formation of supernumerary digits in 100% of the cases ($n=10$). The quality of the supernumerary digits (see Table 1) is comparable to that described by Wanek and Bryant (1991), that is, 80% of the limbs forming either two supernumerary digits (digit pattern 3-2-2-3-4, Fig. 2B) or a single supernumerary digit 3 (digit pattern 3-2-3-4, Fig. 2C) and the remaining limbs forming a supernumerary digit 2 (digit pattern 2-2-3-4, Fig. 2D). In all cases, the supernumerary digits were of chick origin. These results differ considerably from those reported by Fallon and Crosby (1977) and Izpisua-Belmonte et al. (1992) in that we never observe the formation of a supernumerary digit 4 nor do we observe multiple consecutive supernumerary digit types from a single experimental limb. The reason for these differences is currently under investigation. Frequently, ectopic cartilage nodules were observed in grafted limbs which presumably resulted from differentiation of the donor tissue (Fig. 2B). Anterior mouse limb bud tissues grafted in a similar manner resulted in normal limbs ($n=15$, Table 1).

We have repeated experiments by Hayamizu and Bryant (1992) in which dissociated mouse posterior cells have been cultured for varying lengths of time at micromass density then assayed for ZPA signaling by grafting into the chick wing bud. Using our standard dissociation protocol (Fig. 1), we find that signaling is relatively high (78%) at 24 hours, but declines rapidly within the next day of culture (Fig. 3). At 45 hours we observe signaling in 30% of the grafts and at 52 hours we find that ZPA signaling is completely absent. These data differ somewhat from those found by Hayamizu and Bryant (1992) in that we observe signaling in our cultures for a slightly longer period; however, both studies demonstrate a precocious decline in sig-

naling associated with in vitro culture at micromass density. Monolayer cultures of posterior cells display a similar decline in signaling in that no activity is present in 48 hour cultures (Shi, 1991). These data are consistent with analogous studies using posterior limb bud cells derived from the chick (Honig, 1983b; Carlson, 1984, 1988).

To investigate the precocious decline in ZPA signaling observed in vitro, we altered the way in which we collected cells from posterior limb bud fragments so as to minimize the loss of extracellular components removed in the standard dissociation protocol. Posterior limb bud fragments were dissociated in very small volumes (microdissociation, Fig. 1) and immediately plated as high density micromass cultures. The signaling profile (Fig. 3) of microdissociate cultures indicate that ZPA signaling is maintained for a longer period than that observed in previous studies. Microdissociation cultures differ from our standard dissociation cultures in a number of ways. First, in microdissociate cultures, we can only estimate the number of cells plated in each culture based on previous cell counts of similar stage posterior limb bud fragments (see Shi and Muneoka, 1992). Microdissociating 40 posterior limb bud fragments is estimated as yielding 10^6 posterior cells which are distributed into two high density cultures; we estimate that each microdissociate culture initially contains between 3.3 and 5.0×10^5 total cells. We have carried out similar experiments with a two-fold difference in the number of limb bud fragments without any variation in the frequency or quality of the resulting supernumerary response (not shown); thus we are confident that subtle variations in the number of posterior cells initially plated in microdissociate cultures do not dramatically influence the response. Second, microdissociate cultures are not diluted and filtered during the dissociation procedure, thus the extent of dissociation can only be ascertained by visual examination. Our protocol for microdissociation was developed empirically based on the complete dissociation of the limb bud mesenchyme but not the limb bud ectoderm. For this reason, undissociated fragments of ectoderm are present in these cultures. Standard dissociate cultures do contain some single ectodermal cells as identified by immunoperoxidase staining of cultures with a cytokeratin antibody, Troma-1 (Brulet et al., 1980; Kemler et al., 1981), specific for embryonic limb ectoderm (R. Anderson, unpublished). Thus, both standard dissociate and microdissociate cultures contain some dissociated ectodermal cells; however, microdissociate cultures contain in addition visible fragments of undissociated

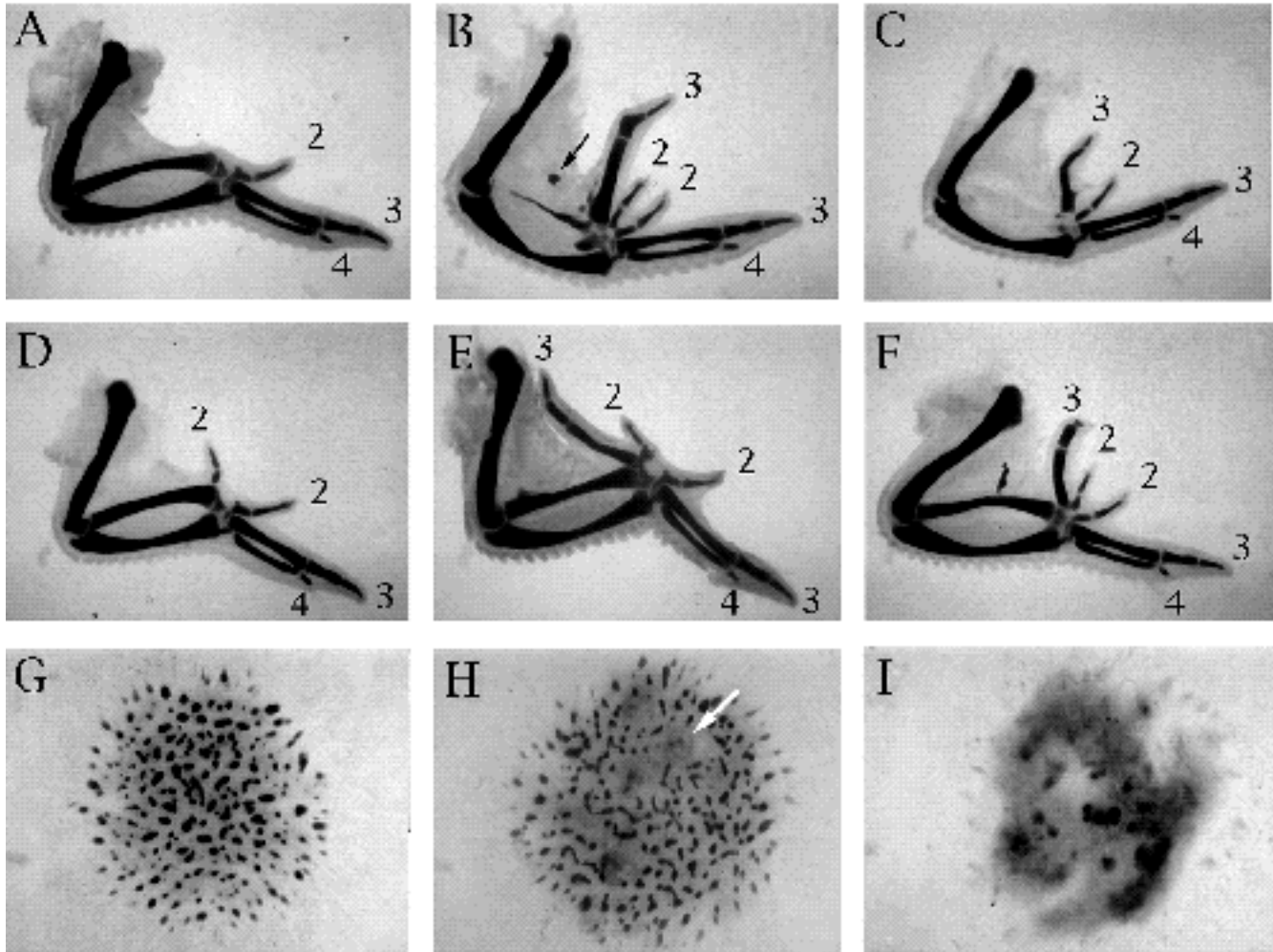


Fig. 2. (A-F) Dorsal view of chick limbs stained with Victoria blue to reveal the skeletal pattern. (A) The normal pattern of the chick wing, digits 2, 3 and 4 are indicated. Digit patterns resulting from mouse posterior grafts: (B) the maximum response, digit pattern **3-2-2-3-4**, arrow indicates ectopic cartilage presumably resulting from differentiation of the grafted tissue, the radius is reduced, (C) digit pattern **3-2-3-4**, the radius is absent, (D) digit pattern **2-2-3-4**. The radius was often affected after grafting mouse posterior tissue or 24 hour posterior microdissociate cultures (50% and 70% of grafted limbs, respectively). With prolonged culturing, this occurrence drops off dramatically. In addition this abnormality is always associated with supernumerary digit formation. Grafted limbs that develop normal patterns never display radial defects. (E) Digit pattern (**3-2-2-3-4**) resulting from a graft of ectoderm-free posterior microdissociated cells cultured transfilter with posterior ectoderms. (F) Supernumerary digit pattern (**3-2-2-3-4**) induced by grafting FGF-2-treated (100 ng/ml) standard dissociate cultures. The tip of supernumerary digit 3 is out of the plane of the camera. (G-I) Patterns of cartilage differentiation in 96 hour cultures stained with Alcian blue: (G) posterior standard dissociate culture, (H) microdissociate culture in which ectodermal fragments (arrow) are present and (I) FGF-2-treated standard dissociate cultures (dark regions are areas of high cell density). (A-F, $\times 5$; G-I, $\times 8$).

ectoderm. Third, because microdissociate cultures are not diluted or filtered, they retain extracellular components released by mild trypsinization and lost by dilution, filtration and centrifugation when posterior cells are collected using the standard dissociation protocol.

In the microdissociate cultures, posterior cells maintained ZPA signaling in 96 hour cultures (Fig. 3). For comparison, posterior cells cultured under standard conditions ceased to signal after 52 hours in culture and estimates of ZPA signaling in vivo from similar stage limb buds range from 70-80 hours (Honig, 1983b). The profile of ZPA signaling of microdissociate cultures shown in Fig. 3 indicates that the frequency of signaling responses was 100% at 24 hours, 86% at 48 hours, 56% at 72 hours and 58% at 96 hours. Our data show that signaling does decline in

microdissociate cultures; however, the rate and magnitude of this decline, based on the frequency of supernumerary responses, is substantially lower than responses observed in standard dissociate cultures. The fact that signaling in 96 hour cultures is still relatively high suggests that microdissociate cultures maintain ZPA signaling for a longer period than that expected in vivo. To ensure that the patterning response that we observe from microdissociate cultures is indeed a position-specific effect, we grafted cells from 24 hour posterior microdissociate cultures which display a high level of signaling into the posterior region of the chick wing bud, and in no case ($n=7$) was a supernumerary response observed.

In addition to the increase in frequency of the supernumerary response, we also find that the quality of the

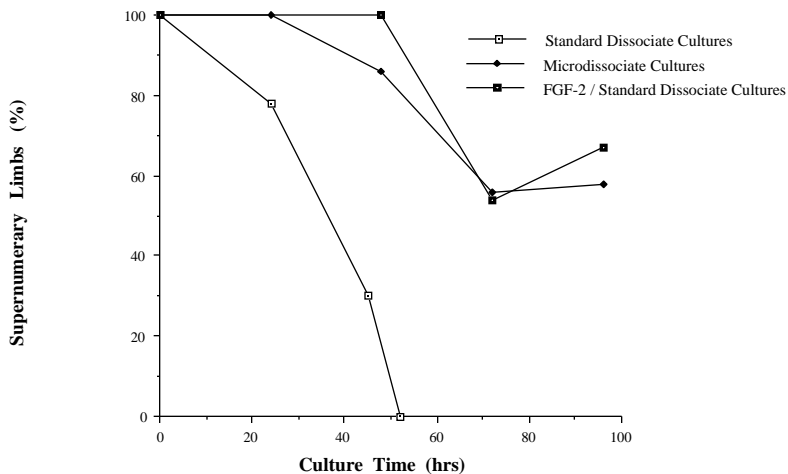


Fig. 3. Profile of ZPA signaling in posterior microdissociate, standard dissociate and FGF-2-treated (100 ng/ml) standard dissociate cultures. ZPA signaling declines rapidly in posterior standard dissociate cultures until no activity is assayable at 52 hours. Microdissociate cultures maintain a relatively high level of signaling even after 96 hours as do FGF-2-treated posterior standard dissociate cultures.

response in terms of the number of supernumerary digits that form is enhanced in microdissociate cultures (Table 1). Microdissociate cultures at 24 hours stimulated the formation of two supernumerary digits (digit pattern **3-2-2-3-4**) in 78% (7/9) of the cases and the remaining limbs formed a single supernumerary digit 3 (2/9, 22%, digit pattern **3-2-3-4**). Grafting cells from standard dissociate cultures at 24 hours never resulted in the formation of two supernumerary digits, but rather formed a single supernumerary digit in all positive cases; 11% (1/9) formed a supernumerary digit 3 (digit pattern **3-2-3-4**), and 67% (6/9) formed a supernumerary digit 2 (**2-2-3-4**). Since the maximum response from posterior mouse tissue grafted into the chick wing bud is the formation of two supernumerary digits, our results show that at 24 hours microdissociate cultures, but not standard dissociate cultures, display a supernumerary response that closely mimics that expected from undissociated posterior tissue grafts. Microdissociate cultures at 48 hours induced a single supernumerary digit in all cases. By comparison, 48 hour microdissociate cultures most closely mimicked the signaling response (both in terms of frequency and quality) of 24 hour standard dissociation cultures. The frequency as well as the quality of supernumerary digit formation remained approximately the same between 72 hours and 96 hours.

Characterization of a ZPA signaling maintenance activity

Our finding that ZPA signaling can be maintained in microdissociate cultures is consistent with the idea that an extracellular factor is involved in maintaining or stabilizing ZPA signaling by posterior cells. To investigate this possibility further, we performed experiments to establish critical differences between the standard dissociation and the microdissociation protocols. Two obvious differences that are not mutually exclusive were investigated. First, compared to standard dissociate cultures, microdissociate

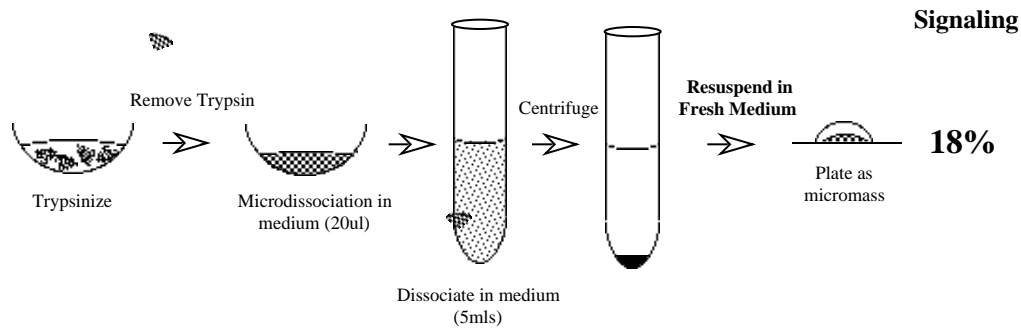
cultures are trypsinized and dissociated in small volumes and never centrifuged; thus, maintenance activity could be lost in standard dissociate cultures by dilution. Second, microdissociate cultures, but not standard dissociate cultures, contain fragments of undissociated ectoderm that may be providing maintenance activity. To investigate the first of these two possibilities posterior fragments were trypsinized (40 μ l) and microdissociated in a small volume (20 μ l) then diluted into a much larger volume (5 ml) with fresh culture medium (Fig. 4A). These microdissociated and diluted cells were either filtered to remove undissociated ectodermal tissue or left unfiltered and both groups were then concentrated by centrifugation. Cells were resuspended in fresh culture medium and plated as micromasses. We assayed these cultures at 48 hours since this was a time point at which standard dissociate cultures displayed a low level of activity (less than 30%) and the activity of microdissociate cultures was high (86%). Our results show that ZPA signaling of these cultures dropped to levels predicted for standard dissociate cultures regardless of whether ectodermal fragments were filtered out (27% activity) or not (18% activity). Thus, the maintenance of ZPA activity was lost by a 250-fold dilution (20 μ l to 5 ml) of microdissociated cells in culture medium, suggesting that this maintenance activity was soluble and present in the dissociation medium of microdissociated cells.

To confirm that maintenance activity was soluble and present in the dissociation medium of microdissociated cells, we collected supernatant from microdissociated posterior cells and used this medium to resuspend and plate high density micromass cultures of microdissociated and diluted posterior cells (Fig. 4B). These 10 μ l spot cultures were flooded after 2 hours with 2 ml of culture medium (a 200-fold dilution) and assayed after 48 hours. We found that the ZPA signaling of these cultures returned to levels close to that of microdissociate control cultures; we observe a supernumerary response in 78% (7/9) of the grafted limbs. The quality of the supernumerary digits that formed was also indicative of a maintenance of ZPA signaling similar to that observed in microdissociate cultures, 33% (3/9) of the limbs formed a supernumerary digit 3 (digit pattern **3-2-3-4**) with the remaining limbs (4/9, 44%) forming a supernumerary digit 2 (digit pattern **2-2-3-4**). Since the rescued cells were exposed to the posterior microdissociate supernatant for only 2 hours (during plating of the micromass culture) prior to flooding with culture medium (a 200-fold dilution, 10 μ l to 2 ml), we conclude that maintenance activity is plating out along with the posterior cells. We performed a similar microdissociate supernatant rescue experiment using 48 hour cultures of standard dissociation posterior cells and found that these cultures induced the formation of supernumerary digits at a level only slightly higher than control cultures (5/11, 45%). These results suggest that ZPA signaling maintenance requires at least two components. One of these components can be diluted from microdissociated cells and the second component remains associated with microdissociated and diluted cells but is absent in standard dissociated cells.

ZPA signaling and the ectoderm

Because posterior cells plated as microdissociates are not

A) Microdissociation and Dilution



B) Microdissociation Supernatant

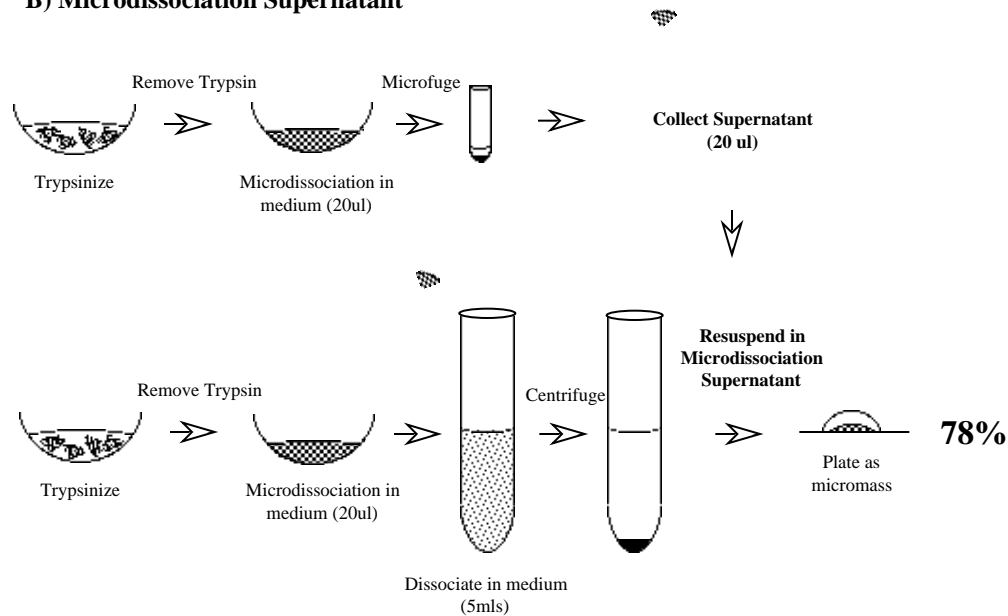


Fig. 4. Characterization of a ZPA signaling maintenance activity. (A) Posterior one-third limb bud fragments were microdissociated as described in Fig. 1 then diluted in 5 ml of culture medium. Cells were centrifuged, plated in fresh culture medium as micromasses and assayed for ZPA signaling after 48 hours in culture. The frequency of supernumerary digit induction remained relatively the same whether the cells were filtered prior to centrifugation (27%) or not (18%). This level of ZPA signaling indicates that activity is lost upon dilution of microdissociated cells suggesting that the maintenance activity is found extracellularly. (B) To confirm the presence of maintenance activity in the dissociation medium of microdissociated cells posterior one-third limb bud fragments were microdissociated and centrifuged in a microfuge tube and the supernatant collected. This supernatant was used to resuspend posterior cells that had been microdissociated and diluted. After 48 hours in culture these cells were assayed for ZPA signaling. The frequency of the supernumerary response indicates that maintenance activity is present in the microdissociation medium of microdissociated cells and this maintenance activity plates out with limb cells prior to flooding with culture medium.

filtered and hence contain sheets of ectodermal cells, the possible role of the ectoderm in these cultures was examined. Ectoderm-free microdissociates were plated and assayed after 48 hours in culture. 47% (9/19) of the grafts from ectoderm-free microdissociate cultures induced the formation of a supernumerary digit. In all cases, the supernumerary response consisted of a single supernumerary digit 2 (digit pattern 2-2-3-4, Table 2). This represents an approximate two-fold decrease in the response frequency with a concomitant decrease in the quality of the supernumerary response. This suggests that the ectoderm is playing some role in ZPA signal maintenance. To characterize

further the function of the ectoderm in ZPA signaling in vitro, ectoderm-free posterior microdissociates were plated and isolated limb ectodermal tissue was cultured transfilter on small culture inserts. Ectoderms were isolated from similar numbers of posterior one-third fragments and the remaining anterior/central two-thirds fragments of the limb bud. Ectoderm-free microdissociates cultured for 48 hours transfilter with posterior ectoderms resulted in a supernumerary response in 93% (13/14) of the limbs grafted (Table 2) indicating that cocultures with posterior ectoderm caused the ZPA signaling in ectoderm-free microdissociate cultures to return to control levels. The quality of the supernumerary

Table 2. Maintenance of ZPA signalling in vitro

Method of culture	Time (hrs)	<i>n</i>	Normal limbs 2-3-4	Limbs with supernumerary digits			Frequency
				<u>2-2-3-4</u>	<u>3-2-3-4</u>	<u>3-2-2-3-4</u>	
Posterior ectoderm	0	6	6 (100%)	0 (0%)	0 (0%)	0 (0%)	0%
Not cultured*							
Anterior ectoderm	0	3	3 (100%)	0 (0%)	0 (0%)	0 (0%)	0%
Not cultured*							
Microdissociate	48	19	10 (53%)	9 (47%)	0 (0%)	0 (0%)	47%
No ectoderm							
+Posterior ectoderm	48	14	1 (7%)	8 (57%)	4 (29%)	1 (7%)	93%
+Anterior/central ectoderm	48	11	2 (18%)	4 (36%)	5 (45%)	0 (0%)	82%

*ectoderm was removed from anterior and posterior one-third limb bud fragments and immediately grafted into an anterior site beneath the AER of the chick wing bud.

Table 3. FGF-2 maintenance ZPA signalling in vitro

FGF-2 Concentration (ng/ml)	Time (hrs)	<i>n</i>	Normal limbs 2-3-4	Limbs with supernumerary digits			Frequency
				<u>2-2-3-4</u>	<u>3-2-3-4</u>	<u>3-2-2-3-4</u>	
100	48	11	0 (0%)	4 (36%)	2 (18%)	5 (45%)	100%
	72	13	6 (46%)	5 (38%)	1 (8%)	1 (8%)	54%
	96	12	4 (33%)	7 (58%)	1 (8%)	0 (0%)	67%
50	48	9	0 (0%)	2 (22%)	4 (44%)	3 (33%)	100%
	48	9	0 (0%)	4 (44%)	0 (0%)	5 (56%)	100%
10	48	9	0 (0%)	1 (11%)	3 (33%)	5 (56%)	100%
	48	14	5 (36%)	6 (43%)	3 (21%)	0 (0%)	64%
0.1	48	12	10 (83%)	2 (17%)	0 (0%)	0 (0%)	17%

ary response also returned to control levels (Table 2). Similarly, ectoderm-free microdissociates cultured for 48 hours transfilter with anterior/central ectoderms also resulted in the maintenance of signaling at approximately control levels (Table 2). Cocultures with anterior/central ectoderms induced the formation of supernumerary digits in 9 of 11 limbs (82%) and all supernumerary responses consisted of a single supernumerary digit (Table 2). Anterior/central ectoderms ($n=3$) and posterior ectoderms ($n=6$, Table 2) grafted directly into the anterior region of the limb did not stimulate the formation of supernumerary digits. Our results show that both posterior and anterior/central ectoderm cocultures with ectoderm-free microdissociates maintain ZPA signaling at similar levels, although the size of the ectoderms cultured in anterior/central versus posterior cocultures was not equal. Thus, while our results show that both posterior and anterior/central ectoderms augment the maintenance of ZPA signaling in vitro, we cannot exclude the possibility that there exists an asymmetry to the ectodermal effect with posterior ectoderm being more effective in signal maintenance.

Effect of FGF-2 on the maintenance of ZPA signaling

Our studies implicating the limb ectoderm with the maintenance of ZPA activity in vitro, in association with recent reports that FGF-4 mRNA transcripts are localized to the posterior half of the AER (Niswander and Martin, 1992; Suzuki et al., 1992), suggested that the putative maintenance factor might be a member of the FGF family. To investigate this possibility, standard dissociate posterior cells were cultured for 48, 72 and 96 hours in culture medium containing 100 ng/ml FGF-2. ZPA signaling of

standard dissociate posterior cells at 48 hours is very low and absent by 52 hours in culture. In the presence of FGF-2, however, ZPA signaling is maintained at a high frequency at all time points and parallels that of untreated microdissociate cultures (Fig. 3). At 48 hours, we obtain a supernumerary response in 100% of the grafted limbs ($n=11$) and the quality of the supernumerary response (Table 3) is comparable to ZPA grafts from the mouse into the chick and also to 24 hour microdissociate cultures. We observe the maximum response of two supernumerary digits in 45% (5/11) of these limbs (digit pattern 3-2-2-3-4, Fig. 2F). Limbs grafted with cells from 72 hour FGF-2-treated cultures induced the formation of supernumerary digits in 54% (7/13) of the limbs grafted and this activity remains essentially the same at the 96 hour time point (67%, 8/12). Thus, the ability of FGF-2 to maintain ZPA signaling follows a similar pattern of decline as that previously determined for posterior microdissociate cultures. In addition to the reduced frequency of the response over time, the quality of the digits that form is also decreased (Table 3). The quality of the supernumerary response appears to be sustained longer in limbs receiving grafts of FGF-2-treated standard dissociate posterior cultures than in those receiving grafts of posterior microdissociate cultures. In other words, a direct comparison between the results from FGF-2-treated standard dissociate cultures and microdissociate cultures indicate that at every time point tested the FGF-2-treated cultures resulted in a superior level of signal maintenance. Thus, our data are consistent with the idea that all of the ZPA signaling maintenance activity associated with posterior microdissociate cultures and the supernatant from microdissociated posterior cells can be explained by the activity of FGF-2.

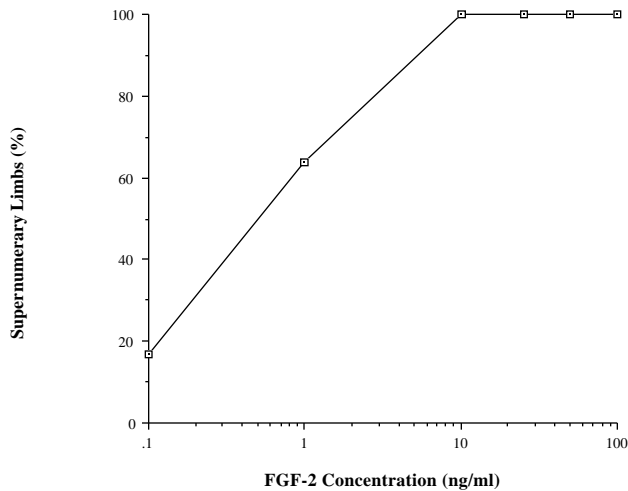


Fig. 5. Maintenance of ZPA signaling with decreasing doses of FGF-2. Standard dissociate cultures were treated with varying concentrations of FGF-2 and tested for ZPA signaling after 48 hours in culture. Cultures treated with 0.1 ng/ml FGF-2 display ZPA signaling that is near the control level (17%).

We have also tested a range of different concentrations of FGF-2 on the maintenance of ZPA activity in 48 hour standard dissociate cultures. FGF-2 concentrations of 50 ng/ml, 25 ng/ml and 10 ng/ml maintained ZPA activity in 48 hour cultures in 100% of the grafted limbs (Fig. 5). The quality of the supernumerary response was essentially equivalent at these three concentrations (see Table 3); the majority response was the formation of either two supernumerary digits or else a single supernumerary digit 3 (digit pattern 3-2-2-3-4 or 3-2-3-4) with the remaining limbs forming a supernumerary digit 2 (digit pattern 2-2-3-4). We observed no dose-related difference in the digit patterns induced from grafts of 100 ng/ml FGF-2-treated cultures or 10 ng/ml FGF-2-treated cultures. Cultures treated with 1.0 ng/ml FGF-2 resulted in a reduced supernumerary response (64%, 9/14) and a decrease in the quality of the supernumerary digits. Standard dissociate posterior cultures treated with 0.1 ng/ml FGF-2 displayed maintenance activity comparable to untreated standard dissociate cultures at 48 hours (17%, 2/12). To test for positional specificity of the FGF-2 effect standard dissociate anterior cells were cultured in the presence of 100 ng/ml FGF-2 for 24 hours and grafted into the anterior region of the chick wing bud. All grafted limbs ($n=11$) displayed a normal digit pattern. Thus, FGF-2 does not induce signaling in non-signaling cells. This result also indicates that the effect observed on limb patterning when FGF-2-treated cells are placed into the anterior of the wing bud is not the result of FGF-2 carryover.

Differentiation of ZPA cultures

Micromass cultures of microdissociated posterior cells, untreated standard dissociated posterior cells and FGF-2-treated (100 ng/ml) standard dissociated posterior cells were stained with Alcian blue at 96 hours to determine the extent of chondrogenesis. Microdissociate and standard dissociate micromasses displayed similar levels of chondrogenesis although ectodermal fragments present in microdissociate cultures were associated with areas of reduced cartilage dif-

ferentiation (Fig. 2G,H), as previously reported (Solursh et al., 1981). We also observed that the size and frequency of ectopic cartilage formation in grafted limbs roughly correlated with the extent of differentiation observed in cultures prior to grafting (not shown). The differentiative capacity of FGF-2-treated standard dissociate micromass cultures differed dramatically from that of non-FGF-2-treated cultures. FGF-2-treated micromass cultures displayed a reduced level of chondrogenic differentiation at 96 hours of culturing (Fig. 2I). These observations support previous studies that demonstrated that FGF and the ectoderm produce conditions in culture that are characteristic of an undifferentiated state (Knudson and Toole, 1988; Munaim et al., 1991). Present findings indicate that there is a general relationship between the chondrogenic capacity of posterior cultures and their ability to produce a ZPA signal. Cultures that display the lowest level of chondrogenesis (FGF-2-treated) produce the highest level of signal, whereas cultures that display the highest level of chondrogenesis (standard dissociate cultures) produce the lowest level of signal. While this relationship is consistent with a developmental signal that is transiently expressed, the fact that microdissociation cultures are both signaling and undergoing overt chondrogenesis at the same time suggests that the two events are regulated in an autonomous manner. In other words, it seems likely that chondrogenic cells are not producing the ZPA signal, but that both signaling and chondrogenesis can occur within the same culture without directly influencing one another. This conclusion is of some consequence with regard to the nature of the ZPA signal since the primary assay for the signal involves a change in the pattern of chondrogenesis, i.e. the formation of supernumerary digits. Our evidence indicates that the relationship between the ZPA signal and overt chondrogenesis is indirect and that the necessary components required to link the two are not present in our cultures.

DISCUSSION

Previous studies of pattern formation during vertebrate limb development demonstrate that posterior limb bud cells produce a positional signal that induces anterior limb bud cells to alter their developmental fate and form supernumerary digits. Posterior cells produce this signal transiently during limb development (MacCabe et al., 1973; Honig and Summerbell, 1985), and signaling declines precociously in cultures of dissociated posterior limb bud cells of mice and chicks (Honig, 1983b; Hayamizu and Bryant, 1992). In this study, we have altered the way in which posterior cells are collected so that the loss of extracellular components released by trypsinization during dissociation is minimized. This procedure is called 'microdissociation'. Using this technique, we have been able to maintain high levels of ZPA signaling in micromass cultures of posterior mouse limb bud cells for an extended period. Our studies indicate that the presence of the ectoderm is not required for ZPA signaling although it does appear to play a supportive role. The maintenance activity does, however, appear to be intimately linked to the extracellular matrix. In addition, FGF-2 was found to maintain signaling in cells cultured under

non-maintenance (standard dissociation) conditions. Based on these data we suggest that the ectoderm, specifically the AER, produces a signal maintenance factor, possibly a member of the FGF family, that is stored in the matrix during limb outgrowth. Our evidence suggests that this maintenance factor is related to the FGF family and, based on recent *in situ* hybridization studies by Niswander and Martin (1992) and Suzuki et al. (1992), a likely candidate is FGF-4.

The role of the extracellular matrix

Our studies provide evidence that the ZPA signaling maintenance factor is present extracellularly, resistant to mild trypsin treatment, soluble following trypsin treatment and displays an affinity for either cell surfaces or extracellular matrix components. Since the level of trypsinization used to prepare microdissociate cultures has been previously determined to minimize damage to the limb bud cells (see Shi and Muneoka, 1992), we conclude that the bulk of the soluble components present in microdissociate cultures or in microdissociate supernatant is derived from proteolytic breakdown of extracellular components. Maintenance activity is soluble since we find it present in microdissociate supernatant from posterior limb bud tissue. This activity is lost by diluting microdissociate preparations 250-fold with culture medium; however, ZPA signaling is still present following a 200-fold dilution of microdissociate cultures after 2 hours of plating. Thus, although the activity is soluble, it plates out with limb cells in micromass. These results lead us to conclude that the putative maintenance activity displays an affinity for either cells or extracellular matrix components that settle out with limb cells during micromass-culturing.

The efficacy of microdissociate cultures in maintaining ZPA signaling appears to be the result of the preservation of the extracellular matrix and its components. Microdissociated and diluted cells plated in the presence of posterior microdissociate supernatant maintain ZPA signaling while standard dissociated cells treated with similar supernatant do not. This suggests that a factor(s) necessary for ZPA signal maintenance is found extracellularly and is preserved during microdissociation despite the cells being diluted. In addition, preliminary data indicate that microdissociated cells plated in the presence of a polyclonal antibody against bovine cartilage link protein (Cateron et al., 1979; Weislander and Heinegard, 1979; Poole et al., 1980) substantially reduced the level of ZPA signaling, suggesting that matrix stabilization plays an important role in signal maintenance (R. Anderson, unpublished). The ability of FGF-2 to maintain ZPA signaling in standard dissociate cultures would suggest that the matrix itself is not the maintenance factor, but that a matrix bound signal plays a direct role in signal maintenance.

The role of FGF

The results from our initial characterization of this ZPA signaling maintenance factor is consistent with the idea that a matrix-bound growth factor may be playing a role in maintaining ZPA signaling. One possible candidate for this role is a member of the FGF family of growth factors, which

currently includes seven members (Goldfarb, 1990): FGF-1 (aFGF), FGF-2 (bFGF), FGF-3 (int-2), FGF-4 (kFGF/hst), FGF-5, FGF-6 and FGF-7 (KGF). Since FGF-1 and FGF-2 were the first in this family to be isolated and purified, these two factors have been most extensively studied and many of the known characteristics of this family of growth factors stem from studies of these two proteins. Some of these characteristics parallel those that we have deduced from our studies on the ZPA signaling maintenance factor and, in addition to our finding that FGF-2 maintains ZPA signaling *in vitro*, represent evidence that support the existence of an endogenous FGF-like maintenance factor. First, the FGFs are characterized by their strong binding affinity for heparin/heparan sulfate (for review, Burgess and Maciag, 1989), a ubiquitous component of extracellular matrices (Hook et al., 1984) and, as we have previously noted, ZPA maintenance activity appears to be bound to extracellular matrix components present in microdissociate cultures. Second, the importance of FGF-heparin interactions has been demonstrated by the observation that heparin or heparan sulfate chains of proteoglycans protect FGF-2 from enzymatic degradation, such as trypsinization (Sommer and Rifkin, 1989). In our experiments, microdissociated posterior cells, as well as microdissociation supernatant, retain maintenance activity after trypsinization. Third, several groups have shown that FGF-2 is found in the extracellular matrix (Baird and Ling, 1987; Folkman et al., 1988; Vlodavsky et al., 1987), suggesting that the extracellular matrix may serve as a reservoir for FGF-2. Our studies indicate that ZPA signaling maintenance activity is present in the extracellular compartment and that this activity can be replaced by FGF-2.

In addition to sharing characteristics with the ZPA signaling maintenance factor, the presence of various members of the FGF family in the developing limb has been well-documented, although a definitive picture of the production and storage of FGF is still unclear. Extracts from chick limb buds at different stages of development demonstrate significant levels of FGF-2 (Munaim et al., 1988) and other heparin-binding activity (Seed et al., 1988) associated with early limb bud stages when cell proliferation in the developing limb is at its peak. FGF-2 has also been shown to be present in myoblasts of later stage limbs using immunohistochemical staining with a polyclonal antibody raised against human FGF-2 (Joseph-Silverstein et al., 1989). *In situ* hybridization studies indicate that RNA transcripts of FGF-4 and FGF-5 are localized to the early limb bud; FGF-4 mRNAs are present only in the posterior half of the AER (Niswander and Martin, 1992; Suzuki et al., 1992) and FGF-5 mRNA expression is found localized to a small patch of cells near the base of the developing limb (Haub and Goldfarb, 1991). The localization of FGF-4 transcripts to the posterior limb bud along with the finding that FGF-2 and FGF-4 both bind to and activate a common FGF receptor (Mansukhani et al., 1990) suggest an *in vivo* role for FGF-4 in maintaining ZPA signaling. In addition to the presence of FGFs during limb development, mRNA transcripts of two murine FGF receptors, FGFR1 and FGFR2, are also present in the limb bud, and these transcripts are localized to the mesenchyme and the ectoderm, respectively (Orr-Urtreger et al., 1991; Peters et al., 1992).

The activity of FGFs on limb bud cells, however, is at present unclear. In addition to the evidence that FGFs act as mitogens in *in vitro* studies, there is substantial evidence that members of the FGF family function as morphogens to regulate pattern formation during early vertebrate embryogenesis (for review, Jessell and Melton, 1992). In the chick, FGF stimulates the growth of mesodermal limb cells (Munaim et al., 1987) whereas, in the mouse, FGF-2 alone is not mitogenic for limb bud cells cultured either at high density or as a monolayer (Kaplowitz et al., 1982). Aono and Ide (1988) report that FGF is a position-specific mitogen for dissociated chick limb bud cells; proliferation of anterior cells is stimulated, whereas posterior cell proliferation is not. Since ZPA tissue fragments cause a similar position-specific mitogenic effect in coculture experiments, FGF-like activity has been implicated in the morphogenetic potential associated with the ZPA (Aono and Ide, 1988). The evidence that FGF may possess morphogenetic potential and also acts variably as a mitogen for limb cells can be understood in part based on a ZPA signal maintenance activity and recognizing that the ZPA signal itself is associated with a position-specific mitogenic activity. Since limb bud cells display clear position-specific growth requirements *in vitro*, the stabilization of the production of position-specific mitogens by developing cells would be important in understanding the results of growth studies. The idea that growth regulation results from positional interactions during limb development is consistent with the intercalation hypothesis proposed in the polar coordinate model (French et al., 1976; Bryant et al., 1981).

A trivial explanation for the maintenance of ZPA signaling by FGF-2 is that FGF-2 exerts its effect on posterior cells by preventing programmed cell death that autonomously occurs in our cultures. This idea stems from recent work by MacCabe et al. (1991) who found that explant cultures of the chick posterior necrotic zone (PNZ), a region that normally undergoes programmed cell death and displays ZPA signaling activity, are prevented from dying following treatment with FGF. A number of reasons make this interpretation untenable for murine cells. First, there is no evidence for the existence of a PNZ in the developing mammalian limb (Wise and Scott, 1986). Therefore, we do not anticipate programmed cell death in our posterior cultures. Second, MacCabe et al. (1991) shows that cell death still occurs following disaggregation and culturing in monolayer; however, FGF-2 does not prevent cell death in such cultures. Third, we have used trypan blue exclusion as an assay for cell death in our cultures and we find no evidence to support the idea that programmed cell death occurs in either monolayer culture (Shi and Muneoka, 1992) or micromass (R. Anderson, unpublished).

The idea that FGF-4 is acting to maintain ZPA signaling during limb development is further supported by a comparison of the spatial-temporal pattern of expression of FGF-4 during limb development with maps of ZPA signaling in subjacent mesenchyme. Maps of ZPA signaling during the development of the chick wing (MacCabe et al., 1973; Honig and Summerbell, 1985) and mouse hindlimb (Wanek and Bryant, 1991) indicate that signaling is submaximal and declining from the equivalent mouse limb stages 6 to 8 (Wanek et al., 1989). The onset of the decline

in ZPA signaling correlates roughly with the reduction of FGF-4 transcripts in the mouse AER; FGF-4 transcripts are reported to be undetectable in the stage 6 limb bud (embryonic day 12.0, Niswander and Martin, 1992). Similar comparisons between ZPA signaling maps and the onset of FGF-4 expression suggest that FGF-4 is not involved in inducing the signal.

While there is general interest in characterizing the FGF signaling pathway with respect to vertebrate pattern formation, the effect that FGFs have on the regulation of developmental patterns of transcription are largely unknown. Ruiz i Altaba and Melton (1989) have found that the expression of the homeobox-containing gene *Xhox-3* is rapidly induced in animal pole cells exposed to FGF-2. In addition, exposure to FGF-2 in conjunction with TGF β -1 (transforming growth factor beta) or TGF β -2 induces the expression of another homeobox-containing gene, *Mix.1* (Rosa, 1989). This link between the activity of growth factors and the regulation of homeobox-containing genes is of some interest with respect to limb pattern formation. The spatial and temporal expression pattern of the *Hoxd* (formerly *Hox 4*) complex in limb development is suggestive that the cumulative expression of these genes plays an important role in the specification of positional information (see Duboule, 1991). The expression domains of these genes overlap with the 5 genes displaying a progressively more restricted expression pattern; the most 5 gene, *Hoxd-13*, co-localizes with regions of the limb bud expressing ZPA activity (Dolle et al., 1989). Thus, associated with the signaling properties of the posterior limb bud is the combinatorial expression of all of the genes in the *Hoxd* complex. The observation that FGF-2 alone or in combination with other growth factors induces expression of homeobox-containing genes in early development raises the possibility that one or more of the *Hoxd* genes may be regulated by FGF-2 and associated with ZPA signaling.

Though the evidence that we present is suggestive of a role for FGF-2 or a related factor in maintaining ZPA signaling *in vivo*, neutralizing antibodies against FGF-2 are unsuccessful in blocking ZPA signaling (R. Anderson, unpublished). The inability of FGF-2 antibodies to impede signaling in microdissociation cultures may reflect the possibility that other members of the FGF family could be responsible for the observed signal maintenance. The known cross reactivity that occurs between members of the FGF family and their receptors (Johnson et al., 1990; Bortaro et al., 1990; Keegan et al., 1991) could explain the ability of FGF-2 to maintain ZPA signaling in standard dissociation cultures while antibodies against FGF-2 in microdissociation cultures fail to prevent ZPA signaling. It is also possible that FGF-2 found in the matrix is prevented from being bound by the neutralizing antibody (see Dennis and Rifkin, 1990). Thus, in light of this result, we cannot definitively assign an *in vivo* role to FGF-2 in the maintenance of ZPA signaling.

Conclusions

We have found that a ZPA signaling maintenance factor is present in the developing limb bud. The preservation of extracellular matrix components in conjunction with the maintenance of ZPA signaling in microdissociate cultures

suggests that the maintenance factor is matrix bound. In addition, ectoderm can augment the level of maintenance activity when cocultured transfilter with ectoderm-free microdissociated posterior cells indicating that the matrix-bound ZPA maintenance factor is diffusible and produced by the limb bud ectoderm and stored bound to the matrix surrounding the mesenchymal cells. We have demonstrated that FGF-2 maintains ZPA signaling of non-signaling standard dissociate posterior cells for an extended period, thus suggesting that a member of the FGF family is responsible for maintenance of ZPA signaling *in vivo*. The level of signal maintenance that FGF-2 imparts far exceeds that determined from experiments using supernatant derived from microdissociated limb bud cells and can account for all of the maintenance activity associated with microdissociate cultures. We propose that an FGF-like maintenance factor, possibly FGF-4, is produced by the AER, immobilized by the extracellular matrix and acts by stabilizing positional signaling, like that from the ZPA, of limb bud cells during normal development.

We are grateful to Susan Bryant, Angela DiStefano, David Gardiner, Terry Hayamizu, Shoanguang Li, Gail Taylor and Chris Trevino for comments on the manuscript and for helpful discussions during the course of this work. This work was supported by HD 20662, HD 23921 and the Monsanto Company.

REFERENCES

- Aono, H. and Ide, H. (1988). A gradient of responsiveness to the growth-promoting activity of ZPA (zone of polarizing activity) in the chick limb bud. *Dev. Biol.* **128**, 136-141.
- Baird, A. and Ling, N. (1987). Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells *in vitro*: implications for a role of heparinase-like enzymes in the neovascular response. *Biochem. Biophys. Res. Comm.* **142**, 428-435.
- Bottaro, D. P., Rubin, J. S., Ron, D., Finch, P. W. and Aaronson, S. A. (1991). Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. *J. Biol. Chem.* **265**, 12767-12777.
- Brulet, P., Babinet, C., Kemler, R. and Jacob, F. (1980). Monoclonal antibodies against trophoderm-specific markers during mouse blastocyst formation. *Proc. Natl. Acad. Sci. USA* **77**, 4113-4117.
- Bryant, S. V., French, V. and Bryant, P. J. (1981). Distal regeneration and symmetry. *Science* **212**, 993-1002.
- Bryant, S. V. and Iten, L. (1974). The regulative ability of the limb regeneration blastema of *Notophthalmus viridescens*: experiments *in situ*. *Wilhelm Roux Arch. Entw. Mech. Org.* **174**, 90-101.
- Burgess, W. H. and Maciag, T. (1989). The heparin-binding (fibroblast) growth factor family of proteins. *Annu. Rev. Biochem.* **58**, 575-606.
- Carlson, B. M. (1984). The preservation of the ability of cultured quail wing bud mesoderm to elicit a position-related differentiative response. *Dev. Biol.* **101**, 106-115.
- Carlson, B. M. (1988). A test of positional properties of avian wing bud mesoderm. *Am. J. Anat.* **182**, 96-105.
- Caterson, B., Baker, J. R., Levitt, D. and Paslay, J. W. (1979). Radioimmunoassay of the link proteins associated with bovine nasal cartilage proteoglycan. *J. Biol. Chem.* **254**, 9369-9372.
- Dennis, P. A. and Rifkin, D. B. (1990). Studies on the role of basic fibroblast growth factor *in vivo*: Inability of neutralizing antibodies to block tumor growth. *J. Cell. Physiol.* **144**, 84-98.
- Dolle, P., Izpisua-Belmonte, J.-C., Brown, J. M., Tickle, C. and Duboule, D. (1991). Hox-4 genes and the morphogenesis of vertebrate genitalia. *Genes Dev.* **5**, 1767-1776.
- Dolle, P., Izpisua-Belmonte, J.-C., Falkenstein, H., Renucci, A. and Duboule, D. (1989). Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature* **342**, 767-772.
- Duboule, D. (1991). Patterning in the vertebrate limb. *Current Opinion in Genetics and Development* **1**, 211-216.
- Fallon, J. F. and Crosby, G. M. (1977). Polarizing zone activity in limb buds of amniotes. In *Vertebrate Limb and Somite Morphogenesis* (eds. D. A. Ede, J. R. Hinchliffe and M. Balls), pp. 55-69. Cambridge: Cambridge University Press.
- Folkman, J., Klagsbrun, M., Sasse, J., Wadzinski, M., Ingber, D. and Vlodavsky, I. (1988). A heparin-binding angiogenic protein-basic fibroblast growth factor is stored within basement membrane. *Am. J. Pathol.* **103**, 393-400.
- French, V., Bryant, P. J. and Bryant, S. V. (1976). Pattern regulation in epimorphic fields. *Science* **193**, 969-981.
- Goldfarb, M. (1990). The fibroblast growth factor family. *Cell Growth Differ.* **1**, 439-445.
- Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49-92.
- Haub, O. and Goldfarb, M. (1991). Expression of the fibroblast growth factor-5 gene in the mouse embryo. *Development* **112**, 397-406.
- Hayamizu, T. F. and Bryant, S. V. (1992). Retinoic acid respecifies limb bud cells *in vitro*. *J. Exp. Zool.* **263**, 423-429.
- Honig, L. S. (1983a). Does anterior (non-polarizing region) tissue signal in the developing chick limb? *Dev. Biol.* **97**, 424-432.
- Honig, L. S. (1983b). Polarizing activity of the avian limb examined on a cellular basis. In *Limb Development and Regeneration* (eds. J. F. Fallon and A. I. Caplan), pp. 99-108. New York: Alan R. Liss, Inc.
- Honig, L. S. (1984). Pattern formation during development of the amniote limb. *Symp. Zool. Soc. Lond.* **52**, 197-221.
- Honig, L. S., Smith, J. C., Hornbruch, A. and Wolpert, L. (1981). Effects of biochemical inhibitors on positional signalling in the chick limb bud. *J. Embryol. Exp. Morph.* **62**, 203-216.
- Honig, L. S. and Summerbell, D. (1985). Maps of strength of positional signalling activity in the developing chick wing bud. *J. Embryol. Exp. Morph.* **87**, 163-174.
- Hook, M., Kjellen, L., Johansson, S. and Robinson, J. (1984). Cell-surface glycosaminoglycans. *Annu. Rev. Biochem.* **53**, 847-869.
- Hornbruch, A. and Wolpert, L. (1986). Positional signaling by Hensen's node when grafted to the chick limb bud. *J. Embryol. Exp. Morph.* **94**, 257-265.
- Izpisua-Belmonte, J.-C., Brown, J. M., Crawley, A., Duboule, D. and Tickle, C. (1992). Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. *Development* **115**, 553-560.
- Javois, L. C. and Iten, L. E. (1981). Position of origin of donor posterior chick wing bud tissue transplanted to an anterior host site determines the extra structures formed. *Dev. Biol.* **82**, 329-342.
- Javois, L. C. and Iten, L. E. (1986). The handedness and origin of supernumerary limb structure following 180° rotation of the chick wing bud on its stump. *J. Embryol. Exp. Morph.* **91**, 135-152.
- Jessel, T. M. and Melton, D. A. (1992). Diffusible factors in vertebrate embryonic induction. *Cell* **68**, 257-270.
- Johnson, D. E., Lee, P. L., Lu, J. and Williams, L. T. (1990). Diverse forms of a receptor for acidic and basic fibroblast growth factors. *Molec. Cell. Biol.* **10**, 4728-4736.
- Joseph-Silverstein, J., Consigli, S. A., Lyser, K. M. and Ver Pault, C. (1989). Basic fibroblast growth factor in the chick embryo: immunolocalization to striated muscle cells and their precursors. *J. Cell Biol.* **108**, 2459-2466.
- Kaplowitz, P. B., D'Ercole, A. J. and Underwood, L. E. (1982). Stimulation of embryonic mouse limb bud mesenchymal cell growth by peptide growth factors. *J. Cell Physiol.* **112**, 353-359.
- Keegan, K., Johnson, D. E., Williams, L. T. and Hayman, M. J. (1991). Isolation of an additional member of the fibroblast growth factor receptor family, FGFR-3. *Proc. Natl. Acad. Sci. USA* **88**, 1095-1099.
- Kemler, R., Brulet, P., Schnebelen, M.-T., Gaillard, J. and Jacob, F. (1981). Reactivity of monoclonal antibodies against intermediate filament proteins during embryonic development. *J. Embryol. Exp. Morph.* **64**, 45-60.
- Knudson, C. B. and Toole, B. P. (1988). Epithelial-mesenchymal interaction in the regulation of hyaluronate production during limb development. *Biochem. Int.* **17**, 735-745.
- MacCabe, A. B., Gasseling, M. T. and Saunders, J. W., Jr. (1973). Spatiotemporal distribution of mechanisms that control outgrowth and anteroposterior polarization of the limb bud in the chick embryo. *Mechanisms of Aging and Development* **2**, 1-12.

- MacCabe, A. B. and Parker, B. W.** (1976). Polarizing activity in the developing limb of the Syrian hamster. *J. Exp. Zool.* **195**, 311-317.
- MacCabe, J. A., Blaylock, R. L., Jr., Latimer, J. L. and Pharris, L. J.** (1991). Fibroblast growth factor and culture in monolayer rescue mesoderm cells destined to die in the developing avian wing. *J. Exp. Zool.* **257**, 208-213.
- Mansukhani, A., Moscatelli, D., Tallarico, D., Levytska, V. and Basilico, C.** (1990). A murine fibroblast growth factor (FGF) receptor expressed in CHO cells is activated by basic FGF and Kaposi FGF. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4378-4382.
- Munaim, S. I., Klagsbrun, M. and Toole, B. P.** (1987). Fibroblast growth factor activity in the early stages of development of chick embryo limb buds. *J. Cell Biol.* **105**, 275a (abstract).
- Munaim, S. I., Klagsbrun, M. and Toole, B. P.** (1988). Developmental changes in fibroblast growth factor in the chicken embryo limb bud. *Proc. Natl. Acad. Sci. USA* **85**, 8091-8093.
- Munaim, S. I., Klagsbrun, M. and Toole, B. P.** (1991). Hyaluron-dependent pericellular coats of chick embryo limb mesodermal cells: induction by basic fibroblast growth factor. *Dev. Biol.* **143**, 297-302.
- Muneoka, K. and Sassoon, D.** (1992). Molecular aspects of regeneration in developing vertebrate limbs. *Dev. Biol.* **152**, 37-49.
- Niswander, L. and Martin, G. R.** (1992). Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114**, 755-768.
- Orr-Urtreger, A., Givol, D., Yayon, A., Yarden, Y. and Lonai, P.** (1991). Developmental expression of two murine fibroblast growth factor receptors, *flg* and *bek*. *Development* **113**, 1419-1434.
- Paulsen, D. F. and Solursh, M.** (1988). Microtiter micromass cultures of limb-bud mesenchymal cells. *In Vitro Cell. and Dev. Biol.* **24**, 138-147.
- Peters, K. G., Werner, S., Chen, G. and Williams, L. T.** (1992). Two FGF receptor genes are differentially expressed in epithelial and mesenchymal tissue during limb formation and organogenesis in the mouse. *Development* **114**, 233-243.
- Poole, A. R., Reiner, A., Tang, L. H. and Rosenberg, L.** (1980). Proteoglycans from bovine nasal cartilage. Immunochemical studies of link protein. *J. Biol. Chem.* **255**, 9295-9305.
- Rosa, F. M.** (1989). Mix. 1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. *Cell* **57**, 965-974.
- Ruiz i Altbaba, A. and Melton, D. A.** (1989). Interaction between peptide growth factors and homeobox genes in the establishment of antero-posterior polarity in frog embryos. *Nature* **341**, 33-38.
- Saunders, J. W., Jr.** (1977). The experimental analysis of chick limb and development. In *Vertebrate Limb and Somite Morphogenesis* (eds. D. A. Ede, J. R. Hinchliffe and M. Balls), pp. 1-24. Cambridge: Cambridge University Press.
- Saunders, J. W., Jr. and Gasseling, M. T.** (1983). New insights into the problem of pattern regulation in the limb bud of the chick embryo. In *Limb Development and Regeneration*, part A (eds. J. F. Fallon & A. E. Caplan), pp. 67-76. New York: Alan R. Liss, Inc.
- Seed, J., Olwin, B. B. and Hauschka, S. D.** (1988). Fibroblast growth factor levels in the whole embryo and limb bud during chick development. *Dev. Biol.* **128**, 50-57.
- Shi, C.** (1991). 'Studies of Mitogenic and Morphogenic Signals during Mouse Limb Development.' Ph. D. dissertation, Tulane University.
- Shi, C. and Muneoka, K.** (1992). Position-specific growth of mouse limb bud cells *in vitro*. *Dev. Biol.* **151**, 9-17.
- Solursh, M., Singley, C. T. and Reiter, R. S.** (1981). The influence of epithelia on cartilage and loose connective tissue formation by limb mesenchyme cultures. *Dev. Biol.* **86**, 471-482.
- Sommer, A. and Rifkin, D. B.** 1989. Interaction of heparin with human basic fibroblast growth factors: protection of the angiogenic protein from degeneration by a glycosaminoglycan. *J. Cell Physiol.* **138**, 215-220.
- Suzuki, H. R., Sakamoto, H., Yoshida, T., Sugimura, T., Terada, M. and Solursh, M.** (1992). Localization of Hst1 transcripts to the apical ectodermal ridge in the mouse embryo. *Dev. Biol.* **150**, 219-222.
- Tickle, C.** (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289**, 295-298.
- Tickle, C., Shellswell, G., Crawley, A. and Wolpert, L.** (1976). Positional signalling by mouse limb polarising region in the chick wing bud. *Nature* **259**, 396-397.
- Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J. and Klagsbrun, M.** (1987). Endothelial cell-derived basic fibroblast growth factors: synthesis and deposition into subendothelial extracellular matrix. *Proc. Natl. Acad. Sci. USA* **84**, 2292-2296.
- Wagner, M., Thaller, C., Jessell, T. and Eichele, G.** (1990). Polarizing activity and retinoid synthesis in the floor plate of the neural tube. *Nature* **345**, 819-822.
- Wanek, N. and Bryant, S.V.** (1991). Temporal pattern of posterior positional identity in the mouse limb buds. *Dev. Biol.* **147**, 480-484.
- Wanek, N., Gardiner, D. M., Muneoka, K. and Bryant, S.V.** (1991). Conversion by retinoic acid of anterior cells into ZPA cells in the chick limb bud. *Nature* **350**, 81-83.
- Wanek, N., Muneoka, K., Burton, R., Holler-Dinsmore, G. and Bryant, S.V.** (1989). A staging system for mouse limb development. *J. Exp. Zool.* **249**, 41-49.
- Wieslander, J. and Heinegard, D.** (1979). Immunochemical analysis of cartilage proteoglycans. Antigenic determinants of substrates. *Biochem. J.* **179**, 35-45.
- Wise, L. and Scott, W. J.** (1986). Teratogenesis: Abnormalities of morphogenesis and growth. In *Principles of Drug and Chemical Action in Pregnancy* (eds. S. Fabro and A. R. Scialli), pp. 251-291. New York: Marcel Dekker, Inc.

(Accepted 5 February 1993)