THE ROLE OF FIBROBLAST GROWTH FACTORS IN EMBRYONIC

DURING LIMB DEVELOPMENT

by

Astrid Vogel

Department of Anatomy and Developmental Biology

University College London

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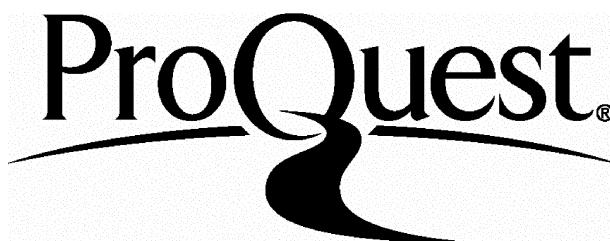Doctor of Philosophy

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ABSTRACT

This thesis examines the role of Fibroblast growth factors (FGFs) during embryonic limb development. It appears that Fibroblast Growth Factors are central to signalling in the developing limb. The apical ectodermal ridge is a specialised epithelial structure at the tip of the limb bud and is involved in a major set of epithelial-mesenchymal interactions. Local FGF-4 application to the posterior margin of the bud in the absence of the ridge maintains posterior mesenchyme. These posterior cells constitute the polarizing region that mediates patterning across the limb. A generation of a full set of proximo-distal structures is correlated with maintenance of polarizing activity. FGFs can maintain the activity of polarizing region cells *in vitro* and can also maintain responsiveness of cultured anterior cells to positional cues within the limb when grafted back to the posterior margin of a chick wing bud. FGFs also allow development of skeletal structures when either the whole limb bud of young or the distal limb tip of older embryos is removed. Expression of homeobox-containing genes in the developing limb has been linked to outgrowth and positional cues within the limb. *Msx-1*, a gene related to the *Drosophila msh* gene is expressed at the tip of the young limb bud in a zone of rapidly dividing and undifferentiated cells, under the control of the apical ectodermal ridge. *Bmp-2*, a member of the transforming growth factor family, is expressed in the apical ectodermal ridge and in posterior mesenchyme of the developing limb bud. Gene members of the HoxD complex are expressed in overlapping domains across the limb bud and these patterns can be regulated by cooperation between the polarizing region and the apical ectodermal ridge. When the apical ectodermal ridge is removed, or when posterior cells are taken from the bud and placed in culture, expression of

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Msx-1, *Hoxd-13* and *Bmp-2* is not detectable in mesenchymal cells. Local FGF-4 application maintains normal expression patterns of *Msx-1*, *Bmp-2* and *Hoxd-13* in the absence of the ridge. Furthermore, addition of FGF-2 to cultured posterior mesenchyme cells maintains expression of *Msx-1* and *Hoxd-13*.

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LIST OF ABBREVIATIONS

AER ... apical ectodermal ridge

BMP ... bone morphogenetic protein

cpm ... counts per minute

CRABP . cellular retinoic acid binding protein

CRBP .. cellular retinol binding protein

DDW ... doubled distilled water

DIG ... digoxygenin

dpp ... *decapentaplegic*

DTT ... dithiothreitol

EDTA .. ethylendiamintetraacetate

EGF ... epidermal growth factor

Evx ... *evenskipped*

FCS ... foetal calf serum

FGFR .. fibroblast growth factor receptor

FGF ... fibroblast growth factor

HCl ... hydrochloric acid

hh .... *hedgehog*

Hox ... homeobox containing gene

HPSG .. heparan sulphate proteoglycan

ISH ... *in situ* hybridisation

MEM ... minimal essential medium

mM .... milli Molar

msh ... muscle segment homeobox-containing gene

Msx ... Muscle segment homeobox containing gene

Na_2CO_3 disodium carbonate

NaCl .. Sodium chloride

$NaHCO_3$ sodium hydrogen carbonate

$NaPO_4$. sodium phosphate

PBS ... Phosphate buffered solution

PDGF .. Platlet derived growth factor

PFA ... paraformaldehyde

RAR ... retinoic acid receptor

RA .... retinoic acid

RXR ... retinoid X receptor

SDS ... sodiumdodecylsulphate

Shh ... *Sonic hedgehog*

SSC ... sodium chloride/sodium citrate

T3/T7 . transcription enzymes

ta .... *talpid*

TCA ... trichloraceticacid

TEA ... triethanolamine

TESPA . 3-aminopropyltriethoxysilane

TE .... tris-ethylendiamintetraacetate

TGF ... Transforming growth factor

t ..... time

ACKNOWLEDGEMENTS

TO VEIT

CHAPTER 1

GENERAL INTRODUCTION

1. DEVELOPMENT OF THE CHICK LIMB

1.1 Formation of the limb bud

Initially the limb bud forms through migration of mesenchymal cells from the lateral plate that give rise to connective tissue and cartilage of the limb, and migration of mesenchymal cells from the somites that give rise to all the limb muscle cells (Chevallier *et al.*, 1977; Christ *et al.*, 1977). The initial stage of limb budding appears to be influenced by the mesonephros. Removal of the mesonephros or implantation of a barrier between the mesonephros and the limb bud inhibits further proliferation of the limb bud (Geduspan and Solursh, 1992). In the chick limb outgrowth occurs due to a decrease in the rate of mitosis in the non-limb bud area, rather than to an increase in proliferation within the bud (Searls and Janners, 1971). Detwiler (1933) found that the area capable of limb formation was determined early in development and that this limb field was larger than the area which usually forms limbs. As early as stage 12 a prospective wing bud transplanted to the flank region differentiates into a limb whose dorso-ventral and antero-posterior axis correspond to those of the graft rather than those of the host (Hamburger, 1938; Saunders and Reuss, 1974). However, the self-differentiating ability of wing bud mesoderm depends upon the adjacent somitic mesoderm until the 13-somite stage in chick and mouse (Kieny, 1969, 1971; Agnish and Kochar, 1977; reviewed in Hinchliffe and Johnson, 1980).

1.2 The apical ectodermal ridge

1.2.1. Induction of the apical ectodermal ridge

The initial stage of limb budding is independ of the activity of the apical ectodermal ridge (Carrington and Fallon, 1988). Once the limb bud has formed, the mesenchyme cells induce the overlying epithelium to thicken and to form the so called apical ectodermal ridge (reviewed in Hinchliffe and Johnson, 1980). The thickening of the apical ectodermal ridge is first almost symmetrical, but subsequently the thickening becomes more prominent posteriorly (Todt and Fallon, 1984).

Prospective limb mesoderm achieves self differentiating capacity when grafted to the non-limb forming flank region and an apical ectodermal ridge is induced with its antero-posterior and dorsal-ventral axis according to the graft (Reuss and Saunders, 1965; Saunders and Reuss, 1974) and the limb mesoderm is composed predominantly out of graft cells (Dhouailly and Kieny, 1972). Prospective limb mesoderm from stage 12 until stage 17 is able to induce an apical ectodermal ridge in healing flank ectoderm (Reuss and Saunders, 1965; Saunders and Reuss, 1974), whereas the flank ectoderm can respond to form an apical ectodermal ridge until stage 19 (Carrington and Fallon, 1984a). At earlier stages than 12 only mesoderm in association with somite is able to induce an apical ectodermal ridge (reviewed in Hinchliffe and Johnson, 1980). However, by grafting flank ectoderm from stage 14-18 to dorsal limb mesoderm, mesoderm from stage 17-20 is able to induce dorsally an apical ectodermal ridge and limbs develop (Carrington and Fallon, 1986) that are similar to the mutant *eudiplopodia* (Goetnick, 1964).

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1.2.2. Role of the apical ectodermal ridge in limb development

The apical ectodermal ridge is required for continued limb outgrowth (Saunders, 1948) and maintains the progress zone, a region of undifferentiated and rapidly dividing mesenchyme cells at the tip of the bud (Summerbell *et al.*, 1973). Both the ectoderm and the apical ectodermal ridge are able to prevent differentiation of limb mesenchyme cells, but only the apical ectodermal ridge induces outgrowth (Solursh *et al.*, 1981b; Reiter and Solursh, 1982). Grafting the apical ectodermal ridge dorsally or ventrally to the limb results in the development of a second limb tip (Saunders *et al.*, 1976).

The apical ectodermal ridge is necessary for the specification of successively more distal structures. After removal of the apical ectodermal ridge, the ridge does not regenerate and truncated limbs develop. The time at which the apical ectodermal ridge is removed determines the proximo-distal level at which the truncation occurs (Saunders, 1948; Summerbell, 1974a). The apical ectodermal ridge is a self-sustaining cell population (Saunders *et al.*, 1976; Robert *et al.*, 1991; Boutin and Fallon, 1992) and is maintained by the mesenchyme (Saunders, 1948; Zwilling, 1956b, 1961, 1972, Searls and Zwilling, 1964). This observation has been confirmed in *in vitro* studies. When the apical ectodermal ridge is separated from the underlying mesenchyme and placed in culture, cell death occurs (Searls and Zwilling, 1964; Boutin and Fallon, 1984), but this can be prevented by coculturing the ectoderm with limb mesoderm (Globus and Vethamany-Globus, 1976; Solursh *et al.*, 1981b). On the other hand, removal of the apical ectodermal ridge prior to stage 21 causes cell death in the underlying mesenchyme (Rowe *et al.*, 1982). Thus, growth and differentiation of the developing limb requires epithelial-

mesenchymal interactions.

Several experiments indicate that signalling of the ridge does not specify what structure the mesenchyme cells will form. Interchanging the apical ectodermal ridges between leg and wing does not affect the development and differentiation of the underlying mesenchyme and limbs develop according to the mesenchyme (Zwilling, 1955). Reversing the antero-posterior axis of the apical ectodermal ridge on the limb bud does not affect pattern specification (Zwilling, 1956a). It appears that the apical ectodermal ridge is not directly involved in specifying the dorso-ventral axis of the limb (MacCabe et al., 1974; Saunders et al., 1976). Grafting the apical ectodermal ridge to the dorsal or ventral surface of a limb results in bidorsal or biventral symmetry of the resulting outgrowth, suggesting that the ectoderm specifies dorsal-ventral patterning of the limb (Saunders et al., 1976). When the apical ectodermal ridge of a young chick bud is placed on mesenchyme of a later stage limb bud and vice versa the proximo-distal level of the underlying mesenchyme is not affected (Rubin and Saunders, 1972). The outgrowth inducing activity of the apical ectodermal ridge decreases during stage 29 (Rubin and Saunders, 1972). Thus, the apical ectodermal ridge does not specify more distal structures and appears only to be required for continued proliferation of the underlying mesenchyme. Furthermore, grafting the ectodermal jacket of a mammal limb bud (rat) onto the mesenchyme of a chick wing bud results in the development of a normal wing bud (Jorquera and Pugin, 1971). This indicates that signalling from the apical ectodermal ridge has been conserved between vertebrates.

1.2.3. Apical ectodermal ridge maintenance factor

The ability of the limb mesoderm to maintain the morphology and activity of the apical ectodermal ridge has been attributed to the apical ectodermal ridge maintenance factor (Zwilling, 1956b; Zwilling and Harnsborough, 1956, Carrington and Fallon, 1984b). In addition, in vitro studies have shown that when apical ectodermal ridge cells are placed in culture they become necrotic, but this can be prevented by co-culturing ridge cells with limb mesoderm (Searls and Zwilling, 1964; MacCabe and Parker, 1975; Globus and Vethamany-Globus, 1976; Solursh et al., 1981; Boutin and Fallon, 1984). The apical ectodermal ridge maintenance factor appears to be diffusible (Gumpel-Pinot, 1981). The ability to maintain an apical ectodermal ridge appears to be limited to limb mesoderm because recombinants of somite or flank mesoderm with an apical ectodermal ridge is followed by flattening of the ridge and the cells die (Searls and Zwilling, 1964).

1.3 The progress zone

The developing limb bud elongates by the rapidly dividing mesenchymal cells underlying the apical ectodermal ridge at the tip of the bud, the so called progress zone (Summerbell et al., 1973). It has been suggested that the length of time that cells (or the number of cell-cycles) spend in the progress zone determines whether they form proximal or distal structures (Summerbell and Lewis, 1975). In other words, cells that spend only a short time in the progress zone will form more proximal structures than those cells that spend a longer time in the progress zone. Thus, removal of the apical ectodermal ridge at an early limb bud stage is followed by the

inhibition of cell division and only the most proximal structure the humerus forms, whereas removal of the ridge at a later stage is followed for example, by the development of humerus, radius and ulna (Saunders, 1948; Summerbell, 1974a).

1.4 The polarizing region

The polarizing region is located at the posterior margin of the limb bud and is involved in antero-posterior patterning of the developing limb (Saunders and Gasseling, 1968). Grafts of the polarizing region made to the anterior margin of a chick wing bud induce the development of a set of mirror-image duplicated digits (Saunders and Gasseling,1968). The polarizing region itself does not contribute to the extra structures formed, suggesting that the polarizing region is a signalling region (Saunders and Gasseling, 1968). Evidence for this comes from grafting experiments where polarizing regions from different species were grafted to the anterior margin of a chick limb bud, which induced additional digits of the wing (Tickle *et al.*, 1976; MacCabe and Parker, 1976; Fallon and Crosby, 1977)

Polarizing region signalling is found strongly in the posterior limb bud mesenchyme and during further limb outgrowth highest polarizing activity is found just proximal to the progress zone (MacCabe *et al.*, 1973; Honig and Summerbell, 1985; Hinchliffe and Sansom, 1985). Both, the polarizing region and the apical ectodermal ridge seem to lose activity at approximately the same stage. The apical ectodermal ridge of stage 29 limb buds can no longer induce outgrowth of distal limb parts (Rubin and Saunders, 1972) and

polarizing region activity at this stage is undetectable (MacCabe *et al.*, 1973; Honig and Summerbell, 1985). Cells of the polarizing region contribute to distal posterior tissue in the limb or die (Bowen *et al.*, 1989).

Other regions within the embryo have polarizing activity and can induce the development of additional digits when grafted to the anterior margin of a chick wing bud. This include other signalling regions like Hensen's node, notochord and floorplate, (Hornbruch and Wolpert, 1986; Wagner *et al.*, 1990; Hogan *et al.*, 1992; Izpisua-Belmonte *et al.*, 1992b). In addition, additional digits develop after grafting polarizing regions from mammals to the anterior margin of a chick wing bud (Tickle *et al.*, 1976; MacCabe and Parker, 1976; Fallon and Crosby, 1977). Thus, it appears that signalling of different signalling regions is conserved within the embryo and among vertebrates. Several other tissues have the ability to induce additional digits when grafted to the anterior margin of a limb bud. For example, somites, flank, mesonephric tissue, tail bud and genital tubercle (Saunders, 1977; Saunders and Gasseling, 1983; Hornbruch and Wolpert, 1991; Izpisua-Belmonte *et al.*, 1992b).

So far, there is no evidence that suggests that the polarizing region is required during the whole time of limb development. Removal of the polarizing region results in the development of more or less normal wings (MacCabe *et al.*, 1973; Fallon and Crosby, 1975; Tickle *et al.*, 1975).

1.4.1. Interaction between the polarizing region and the limb

The polarizing region cells act on undifferentiated and rapidly dividing cells in the progress zone (Summerbell, 1974b). Grafts of

the polarizing region placed proximally in older limb bud stages have no effect. Grafts of the polarizing region made anteriorly stay distally during further limb development, as does the endogenous polarizing region. In contrast, grafts of anterior cells stay proximally (Summerbell and Tickle, 1977). Distal movement of polarizing region cells is not required in order to allow the development of additional digits, as irradiated polarizing region cells stay proximally and additional digits develop without the induction of a polarizing region distal to the grafted cells (Smith, 1979). Thus, cells in the progress zone appear to remember exposure to the polarizing region signal and this might explain why limbs can develop in the absence of the polarizing region.

To have an effect the polarizing region has not only to be placed in the progress zone, but also adjacent to the apical ectodermal ridge (Tickle, 1981; Wilson and Hinchliffe, 1987). The polarizing region rapidly initiates the flattening of the ridge, but induces the thickening of the ridge from a distance.

1.4.2 The morphogen model

It appears that a signal from the polarizing region diffuses into the limb mesenchyme and by doing so it sets up a concentration gradient across the limb (Tickle *et al.*, 1975). Cells nearest to the polarizing region would be exposed to high concentration of the morphogen (posterior) and cells farthest away from the polarizing region would be exposed to low concentrations. This accounts for the fact that the digit next to the polarizing region has a more posterior identity than the digits further away (Tickle *et al.*,

1975). In addition, separation of anterior mesenchyme by inserting a nonpermeable barrier results in the loss of anterior structures (Summerbell, 1979). Polarizing signalling could also be reduced by decreasing the number of cells grafted (e.g. decrease of morphogen concentration) from the polarizing region to the anterior margin of a chick limb bud (Tickle, 1981).

It has been suggested that limb cells have the ability to translate differences in morphogen concentrations into a positional information system that controls limb pattern (Wolpert, 1969).

1.5. Signalling molecules in the developing limb
1.5.1. Fibroblast growth factors

The fibroblast growth factors (FGFs) constitute at present nine structurally related growth factors (see Rifkin and Moscatelli, 1989; Baird and Klagsbrun, 1991; Tanaka et al., 1992; Miyamoto et al., 1993). They include acidic FGF (FGF-1), basic FGF (FGF-2), Int-2 (FGF-3), kaposi-FGF or Hst-1 (FGF-4), FGF-5, FGF-6, keratinocyte growth factor (FGF-7), androgen-inducible growth factor (FGF-8) and glia activating factor (FGF-9). Although FGF-1, FGF-2 and FGF-9 lack a leader sequence necessary for secretion, it appears that they are released from the cell (see Mason, 1994; Vlodavsky et al., 1987; Mignatti and Rifkin, 1991; Mignatti et al., 1991). FGFs are pluri-potential, affecting the growth and function of a variety of cell types (see Thomas, 1993; Baird, 1994a).

1.5.1.1. Regulation of FGF-activity

FGF activity can be regulated by its low- and high-affinity receptors (Baird, 1994b). There are at least three types of low-affinity proteoglycan FGF receptors (syndecans, β-glycan and glypican). By binding to FGF they are thought to deliver FGF to high-affinity receptors (Klagsbrun and Baird, 1991; Yayon *et al.*, 1991; Heath *et al.*, 1991; Rapraeger *et al.*, 1991; Ornitz *et al.*, 1992), and to protect FGF from degradation, proteolysis and to increase their half lives and radius of diffusion (Gospodarowicz and Cheng, 1986; Saksela *et al.*, 1988; Flaumenhaft *et al.*, 1990; Sommer and Rifkin, 1989; Damon *et al.*, 1989; Vlodavsky *et al.*, 1991). Moreover, without binding to high-affinity FGFRs (see below), FGF-2 can be internalised into cells through an heparan sulfate proteoglycan-mediated pathway (Quarto and Amalric, 1994). Thus, the expression and/or occurrence of different proteoglycan subspecies may regulate FGF-activity. FGF-2 bound to the extracellular matrix is protected and retains activity for an extended period of time (Flaumenhaft *et al.*, 1989; Presta *et al.*, 1989; Saksela and Rifkin, 1990). For example, when cells are exposed to FGF-2 for only 10 minutes they bind the growth factor and show FGF-2 stimulated increase in DNA synthesis and protease production at least 24 hours later and this effect can be inhibited when FGF-2 is prevented from binding to heparan proteoglycans (HPSGs). Thus, the extracellular matrix functions also as a storage of FGF. FGFs also bind to other extracellular matrix molecules, like collagen IV (Thomson *et al.*, 1988), fibronectin and laminin (Feige *et al.*, 1989).

Regulation of FGF activity could also be mediated through restricted expression of high-affinity FGF receptors and it has been

shown that their distribution is cell type specific (Patstone *et al.*, 1993).

1.5.1.2. High affinity FGF-receptors

The effects of FGFs are known to be mediated by high affinity receptor tyrosine kinases. The FGF-receptor family contains at present 4 members (FGFR1-4; Pasquale, 1990; Dionne *et al.*, 1990; Partanen *et al.*, 1991). The FGF-receptors (FGFRs) have an extracellular region that consists of three Ig-like domains, a transmembrane region followed by an intracellular tyrosine kinase domain (see Johnson and Williams, 1993).

That the different FGFR do not specifically bind one FGF-family member is shown by the fact that in transfected cells, FGFR-1,-2 and -3 have similar affinities for all members tested (e.g. Dionne *et al.*, 1990; Keegan *et al.*, 1991) and splice variants of a single receptor also bind various members of the FGF-family with similar affinities (Johnson *et al.*, 1990). A splice variant of FGFR-1 that lacks one Ig-domain binds in addition to FGF-1 and FGF-2, FGF-4 with an similar affinity (Mansukhani *et al.*, 1990).

1.5.1.3. FGFs and FGFRs in the developing limb

Several FGF-family members are expressed in the developing limb bud. FGF-2 is found both in the ectoderm and in the apical ectodermal ridge as well as in the underlying mesenchyme (Savage *et al.*, 1993; Dono and Zeller, 1994). *Fgf-4* is dominantly expressed in the

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posterior part of the apical ectodermal ridge in mouse and chick
(Niswander and Martin, 1992; Suzuki *et al.*, 1992; Drucker and
Goldfarb, 1993; Laufer *et al.*, 1994; Niswander *et al.*, 1994).
Transcripts of *Fgf-4* are found as soon as the apical ectodermal ridge
forms and this expression persists until the time the ridge
regresses, after which no transcripts of *Fgf-4* are detectable
(Niswander and Martin, 1992; Suzuki *et al.*, 1992). *Fgf-5* and *Fgf-7*
are expressed in a subset of mesenchymal limb bud cells at later
stages (Haub and Goldfarb, 1991; Herbert *et al.*, 1991; Mason *et al.*,
1994), whereas *Fgf-3* does not appear to be expressed in the
developing limb bud (Wilkinson *et al.*, 1989). *Fgf-6* is expressed in
muscle cells at later stages of `limb development in the mouse and
there seems to be a proximo-distal gradient of expression with
highest expression in the proximal muscle masses (de Lapeyiere *et
al.*, 1993; Han and Martin, 1993). At early limb bud stages *Fgf-8* is
localised to the ectoderm of young mouse limb buds before the apical
ectodermal ridge forms. Once the apical ectodermal ridge has formed
Fgf-8 continues to be expressed in the apical ectodermal ridge, and
expression decreases by the time the apical ectodermal ridge
regresses (Heihinheimo *et al.*, 1994; Ohuchi *et al.*, 1994).

During mouse and chick development *FGFR-1* is diffusely
expressed in the mesenchyme of many tissues including the developing
limb bud (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1991; Patstone *et
al.*, 1993).*FGFR-2* expression is found in the epithelium of the
developing limb (Orr-Urtreger *et al.*, 1991; Patstone *et al.*, 1993).
Heparin sulfate proteoglycans are dominantly found at early stages of
limb development prior to cytodifferentiation (Kimata *et al.*, 1986;
Matsui *et al.*, 1986; Solursh *et al.*, 1990).

1.5.1.4. Possible roles of FGF during development

On the basis of *FGF* expression patterns it has been suggested that FGFs play a role in a wide range of developmental processes. FGFs have been shown to be potent mesoderm inducers in *Xenopus* (Kimelman and Kirschner, 1987; Slack *et al.*, 1987; Paterno *et al.*, 1989; Green et al., 1990). Evidence that FGF plays a role during development was shown in *Xenopus* by overexpressing an FGF-receptor in which the tyrosine kinase domain was deleted and this resulted in embryos that developed defective trunk and tails (Amaya *et al.*, 1991, 1993). It appears that the concentrations of mesoderm inducers (e.g. FGFs and activin) is an important factor in determining the fate of the responding cells (Ruiz i Altba and Melton, 1989; Green and Smith, 1990, 1991).

Recent studies in mouse and chick also suggest that FGFs play an important role in limb development. In an in vitro assay it has been shown that after removal of the apical ectodermal ridge of young mouse limb buds, the addition of different members of the FGF-family to the medium can replace the function of the ridge to stimulate mesenchymal proliferation and limb outgrowth (Niswander and Martin, 1993a). Limb outgrowth does not occur after removal of the ridge and application of BMP-2, BMP-4, TGF-β, PDGF, EGF, insulin or RA to the culture medium (Niswander and Martin, 1993a).

In the developing limb bud expression of *Evx-1*, the vertebrate homolog of the *Drosophila* segment polarity gene *evenskipped*, appears to be regulated by FGF-4 (Niswander and Martin, 1993b). Duplicated anterior skeletal limb elements can be formed when chick limb cells are infected with a replication-defective retrovirus expressing FGF-2

and grafted to the anterior margin of a chick wing bud (Riley *et al.*, 1993). FGF-3 appears to be required for normal development of the ear and tail (Mansour *et al.*, 1993). Disruption of the *Fgf-5* gene leads to sustained growth of their fur and thus it appears that FGF-5 functions as an inhibitor of hair elongation (Herbert *et al.*, 1994).

In vitro studies have shown that FGF-2 can stimulate limb mesenchyme proliferation (e.g. Aono and Ide, 1988; Niswander and Martin, 1993a) and to inhibit cartilage differentiation of limb mesenchyme (Anderson *et al.*, 1993).

1.5.2. Retinoids

It is known that retinoids can affect vertebrate development in that they cause developmental abnormalities. Exposure to high doses of retinoic acid (RA) during pregnancy causes defects depending on the stage of exposure. For example, exposure to RA at the time of limb bud outgrowth causes limb abnormalities in humans.

Local application of RA to the anterior margin of a chick wing bud leads to the development of mirror-image duplicated digits (Tickle *et al.*, 1982; Summerbell, 1983; Tickle *et al.*, 1985), thus mimicking the effect of grafting a polarizing region. In addition, RA can substitute for the polarizing region to allow development of a complete limb (Eichele, 1989; Tamura *et al.*, 1990). An increase in RA concentration is followed by the development of more posterior digits (Tickle *et al.*, 1985). As for the polarizing region, only mesenchymal cells in the subapical progress zone are competent to respond to RA (Tickle and Crawley, 1988). It has been suggested that RA could be the morphogen released by the polarizing region to provide positional information (Tickle *et al.*, 1985). However, grafts of mesenchyme

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located distally to the RA bead at the anterior margin induce additional digits when grafted to the anterior margin of a chick wing bud (Summerbell and Harvey, 1983; Noji *et al.*, 1991; Wanek *et al.*, 1991; Tamura *et al.*, 1993), suggesting that RA might induce a polarizing region. Moreover, application of RA to the anterior margin induces expression of RAR-β in anterior limb mesenchyme, whereas a polarizing region graft does not (Noji *et al.*, 1991). Thus, it appears that RA rather functions to induce a polarizing region than to be the morphogen released by the polarizing region (see Brockes, 1991; Noji *et al.*, 1991; Wanek *et al.*, 1991). However, there seems to be some controversy about the role of RA during limb development (reviewed in Tabin, 1991; Tickle, 1991; Tickle and Eichele, 1994). The fact that both RA and RA-receptors (RARs) are present in the developing limb suggests that retinoic acid has a function during limb development. The concentration of RA is higher in posterior part compared to the anterior part of the limb bud (Thaller and Eichele, 1987; Satre and Kochar, 1989). The second active retinoid, 3,4-didehydro RA found in chick limb tissue, is expressed at several fold higher than RA itself and appears to have equivalent polarizing activity (Thaller and Eichele, 1990), but its distribution in the developing limb has not yet been determined.

Retinoids are small hydrophobic molecules that can diffuse through the cell membrane and then, by binding to cellular receptors, can exert their effects in the nucleus. Cellular cytoplasmatic retinoic acid binding proteins (CRABP I and CRABP II) have been implicated to play a role in the retinoid signal transduction pathway, by binding RA with high affinity (reviewed in Mendelsohn *et al.*, 1992). It is possible that CRABPs are regulators of free RA

concentration, or that they are required for the transport of RA to the nucleus (reviewed in Mendelsohn *et al.*, 1992). Localised to the nucleus are retinoid receptors which by binding retinoids can act as transcription factors to control expression of several genes (see Evans, 1988). Genes that are known to contain a retinoic acid response element (RARE) are for example Hoxd-4 (Moroni *et al.*, 1993) and CRABP II (Durand *et al.*, 1992).

1.5.3. *Sonic hedgehog*

Sonic hedgehog (*Shh*), a vertebrate homolog of the *Drosophila* segment polarity gene *hedgehog* (*hh*), is expressed in the posterior mesenchyme of the developing chick limb bud that maps to the polarizing region during all stages of limb development (Riddle *et al.*, 1993). In addition, *Shh* is expressed in posterior mesenchyme of mouse and rat limb buds, as well as in zebrafish pectoral fin buds (Echelard *et al.*, 1993; Krauss *et al.*, 1993; Roelink *et al.*, 1994). In *Drosophila*, *hh* is expressed in the posterior part of the leg and wing imaginal discs. *Shh* is a secreted protein and thus a good candidate for an intercellular signalling molecule (see Smith, 1994; Pownall, 1994). In *Drosophila* it has been shown that *hh* affects pattern in a dose-dependent manner (Heemskerk and DiNardo, 1994). In addition, *Shh* is expressed in several signalling regions (Hensen's node, floorplate and notochord) known to have polarizing activity (Wagner *et al.*, 1990, Echelard *et al.*, , 1993; Riddle *et al.*, 1993). Cells transfected with *Shh* induce additional digits in a mirror-image fashion when grafted to the anterior margin of a chick wing bud (Riddle *et al.*,1993), thus mimicking the effect of grafting the polarizing region or applying RA to the anterior margin of a chick

wing bud. Ectopic expression of *hh* in the anterior of the *Drosophila*

imaginal discs results in mirror image duplications of wings and legs

(Basler and Struhl, 1994). RA, thought to induce a polarizing region

in anterior mesenchyme when applied to the anterior margin, also

induces *Shh* expression in anterior mesenchyme. *Shh* expressing cells,

like the polarizing region are able to replace the function of the

notochord to induce floorplate characteristic genes in neural tube

cells (Roelink *et al.*, 1994). Thus, *Shh* appears to be an instructive

signal used in patterning of several different embryonic structures

(see Smith, 1994; Pownall, 1994).

1.5.4. Bone morphogenic proteins

Bone morphogenic proteins (BMPs) belong to Transforming Growth

Factor-β (TGF-β) superfamily and so far seven BMPs have been

identified (reviewed in Rosen and Thies, 1992; Kingsley, 1994). *Bmps*

are expressed in specific patterns during morphogenesis and in mouse

and chick embryos at various stages of development (Lyons *et al.*,

1990; Jones *et al.*, 1991; Francis *et al.*, 1994). *Bmp-2* and *Bmp-4* are

expressed in the developing limb buds of mouse and chick. *Bmp-2* is

first expressed in stage 16/17 limb buds and in the overlying

ectoderm. At later stages transcripts of *Bmp-2* are localised to

posterior limb mesenchyme that maps the polarizing region (Francis

et al., 1994) and in the apical ectodermal ridge (Lyons *et al.*, 1990;

Francis *et al.*, 1994). *Bmp-4* is expressed throughout the limb

mesenchyme in stage 16 limb buds and in the overlying ectoderm, but

ectodermal expression is later restricted to the apical ectodermal

ridge until stage 26. Mesenchymal expression becomes progressively

restricted at stage 26 and expression is localised predominantly to anterior and posterior mesenchyme (Francis *et al.*, 1994).

In contrast to *Shh*, beads soaked in BMP-2 applied to the anterior margin of a chick wing bud do not induce additional digits (Francis *et al.*, 1994). Application of RA, ectopic expression of *Shh*, or grafts of a polarizing region to the anterior margin of the wing induces ectopic expression of *Bmp-2* (Francis *et al.*, 1994; Laufer *et al.*, 1994), suggesting that *Bmp-2* expression is involved in anterior posterior patterning of the limb.

1.5.5. Expression of *Hox* genes during limb development

Homeobox genes are good candidates to be involved in patterning the embryo. The vertebrate genome contains 4 Hox clusters (A-D), related to the *Drosophila* Bithorax and Antennapedia homeotic complexes, and genes localised to these clusters encode homeodomain proteins that function as transcription factors (reviewed in Hunt and Krumlauf, 1992; McGinnis and Krumlauf, 1992).

Expression of *Hoxa-10* to *-13* genes is initiated in the posterior mesenchyme of early limb bud stages and as development proceeds the domains extend anteriorly so that the final expression domains are across the limb bud in a proximo-distal sequence. Thus, *Hoxa-10* expression is found in the proximal region and *Hoxa-13* in the distal region of the developing limb bud (Youkouchi *et al.*, 1991). This expression pattern during limb development suggests that genes of the HoxA complex are involved in proximo-distal patterning of the limb.

Hoxd-genes are expressed in overlapping domains in the developing limb bud and show progressive restriction along the

antero-posterior axis, consistent with their position in the cluster
(Dolle *et al.*, 1989; Izpisua-Belmonte *et al.*, 1991; Nohno *et al.*,
1991). For example, *Hoxd-9* is expressed throughout the limb bud,
whereas the more 5'members of the cluster, *Hoxd-11* and *Hoxd-13* are
localised to more posterior regions of the limb bud. During limb
development, members of the HoxD complex are progressively activated
in a 3'to 5' direction which correlates with their position in the
cluster (anterior to posterior). Thus, the most 5'gene *Hoxd-13* has
the smallest, most posterior and distal pattern of expression. Based
on their expression pattern *Hoxd* genes have been proposed to regulate
antero-posterior patterning.

1.5.5.1. Effects of gene expression after manipulation of the developing limb bud

Application of RA, grafts of *Shh* expressing cells, or grafts of
the polarizing region to the anterior margin of a chick wing bud have
been shown to induce in a 3'to 5'sequence, ectopic domains of
5'members of the HoxD complex, correlating with the development of
additional digits (Izpisua-Belmonte *et al.*, 1991, 1992b, Nohno *et
al.*, 1991; Riddle *et al.*, 1993), supporting the idea that Hoxd genes
are involved in antero-posterior patterning of the limb. Ectopic
expression of *Hoxd-11* is found 20 hours after RA application, and
expression of *Hoxd-13* is induced after 24 hours (Izpisua-Belmonte,
1991). The observation that ectopic domains of *Hoxd* genes after RA
application or polarizing region grafts were always found in distal
mesenchyme nearby the ridge suggested that induction of 5'genes
requires cooperation with a signal from the ridge. In fact,

activation of ectopic domains of *Hoxd* expression was found to be dependent upon the presence of the apical ectodermal ridge (Izpisua-Belmonte *et al.*, 1992a; Koyama *et al.*, 1993).

Tissues that have been shown to induce the development of additional digits when grafted to the anterior margin of a chick wing bud, for example Hensen's node, primitive streak, genital tubercle and neural tube, also induce ectopic expression of 5'members of the HoxD-complex (Izpisua-Belmonte *et al.*, 1992b). Recently, ectopic expression of *Hoxb-8* in the anterior part of the trunk in mice has been shown to induce the development of a mirror-image duplicated digit pattern (Charite *et al.*, 1994). Concomitantly, ectopic expression of *Shh*, *Fgf-4-* and *Hoxd-11* was induced in the anterior margin of the mouse limb bud.

Transfection of the chick embryo with retrovirus containing *Hoxd-11* gene changed the combination of *Hoxd* genes in the anterior leg mesenchyme and induced the posteriorization of the most anterior leg digit (Morgan *et al.*, 1992). In the mutant *talpid* (ta^3) polydactylous limbs develop with digits that lack antero-posterior specification. In these limbs *Hoxd* genes are expressed throughout the limb and the absence of *Hoxd* polarity correlates with the lack of digit specification (Izpisua-Belmonte *et al.*, 1992c). Therefore, it has been suggested that a "Hox code" specifies the antero-posterior axis of the vertebrate limb that gives each digit its identity (see Hunt and Krumlauf, 1992). However, several studies question the "Hox-code Model". For example, the disruption of the *Hoxd-13* gene in mice is followed by the failure of the development of distal phalanges in digit 2 and 5, and there is an overall delay in limb development, whereas the Hox code model predicts the failure of the development of digit 4 (Dolle *et al.*, 1993). This suggests that Hoxd genes are

involved in the control of limb growth (Dolle *et al.*, 1993). On the

other hand, it is possible that the paralog of the HoxA-complex,

Hoxa-13, might replace the function of *Hoxd-13* during limb deve-

lopment. Disruption of the *Hoxa-11* gene results in abnormal

development in the region that normally expresses *Hoxa-11*. However,

no effect on the proximo-distal axis of the limb is observed (Small

and Potter, 1993). Furthermore, ectopic expression of *Hoxd-11* in the

anterior margin of the chick wing, did not result in a change of

digit identity and an additional digit 2 developed (Morgan *et al.*,

1992, described above).

1.5.6 Muscle segment homeobox-containing genes (*Msx*)

Vertebrates have at least three *Msx* genes (1-3) related to the

Drosophila muscle segment homeobox (*msh*) gene (mouse: Hill *et al.*,

1989; Robert *et al.*, 1989; Monaghan *et al.*, 1991; MacKenzie *et al.*,

1991, 1992; birds: Coelho *et al.*, 1991a; Takahashi and LeDouarin,

1990; Youkouchi *et al.*, 1991b; Suzuki *et al.*, 1991; Robert *et al.*,

1991).

1.5.6.1 *Msx* expression patterns during limb development

Msx-1 and *Msx-2* are widely expressed in vertebrate embryos,

dominantly in regions where epithelial-mesenchymal interactions occur

(see Davidson and Hill, 1991; Hill *et al.*, 1989; Robert *et al.*,

1989). When the limb bud starts to develop (stage 16-18) *Msx-1* is

expressed over the whole mesoderm (Robert *et al.*, 1989; 1991), while

Msx-2 is expressed by the mesoderm of the anterior border (Robert *et*

al., 1991; Youkouchi *et al.*, 1991b). In the early limb bud *Msx-1* transcripts are located to the entire mesenchyme underneath the apical ectodermal ridge. At stage 20/21 *Msx-1* appears to be more graded in an anterior to posterior expression pattern in the chick (Robert *et al.*, 1991; Suzuki *et al.*, 1991; Coelho *et al.*, 1992a) and there seems to be also a graded proximo-distal *Msx-1* expression in cells underneath the apical ectodermal ridge (Nohno *et al.*, 1992; Davidson *et al.*, 1991; Suzuki *et al.*, 1991; Brown *et al.*, 1993). *Msx-1* transcripts are slightly higher in the dorsal mesoderm than in the ventral mesoderm in limb buds of stage 19-23 (Nohno *et al.*, 1992). At stage 24/25 strong expression is found throughout the proximal anterior periphery of the limb and little or no expression is found over the proximal dorsal ventral (myogenic) regions, or the chondrogenic core (Coelho *et al.*, 1992a). In the posterior periphery expression is found in the posterior necrotic zone and at later stages, expression is found throughout the necrotic mesenchyme between the digits (Robert *et al.*, 1991; Suzuki *et al.*, 1991; Coelho *et al.*, 1991a, 1992a). Whereas *Msx-2* is strongly expressed in the apical ectodermal ridge (Robert *et al.*, 1991; Coelho *et al.* 1991a; Youkouchi *et al.*, 1991b), only weak expression of *Msx-1* is detected (Robert *et al.*, 1991; Suzuki *et al.*, 1991; Coelho *et al.*, 1992).

1.5.5.5 Regulation of *Msx-1* expression in the developing limb bud

Several observations suggest that *Msx-1* expression is under the control of the apical ectodermal ridge. For example, removal of the apical ectodermal ridge is followed by the rapid loss of *Msx-1* expression in the underlying posterior mesenchyme (Ros *et al.*, 1992), or when mouse limb bud cells are dissociated and placed in culture

(Wang and Sassoon, 1991). Grafts of non-expressing proximal limb cells placed beneath the apical ectodermal ridge of a host limb rapidly start to express *Msx-1* (Davidson *et al.*, 1991). In addition, in *limbless* mutants limb budding occurs, but an apical ectodermal ridge fails to form and concomitantly *Msx-1* expression is not maintained in mesenchymal cells, but this can be restored by grafting an apical ectodermal ridge from a normal embryo onto *limbless* mesenchyme (Robert *et al.*, 1991; Coelho *et al.*, 1991b, 1992c). In the mutant *eudiplopodia*, where a second apical ectodermal ridge forms on the dorsal surface of the developing limb bud, *Msx-1* expression is found underneath both ridges. Quail ridge cells grafted onto non-expressing proximal limb cells induce outgrowth and *Msx-1* expression in the underlying mesenchyme (Robert *et al.*, 1991). Further evidence that *Msx-1* expression is under the control of the apical ectodermal ridge comes from *in vitro* studies that have shown that the apical ectodermal ridge can induce *Msx-1* expression in cultured limb bud cells (Coelho *et al.*, 1993). However, the fact that *Msx-1* transcripts are found in the lateral mesoderm well before an apical ectodermal ridge forms and the fact that, in *limbless* mutants, *Msx-1* expression is found in the limb mesenchyme without the presence of an apical ectodermal ridge (Robert *et al.*, 1991), suggests that *Msx-1* expression in the early limb bud is apical ectodermal ridge independent. In addition to the apical ectodermal ridge, ectoderm from the anterior, but not proximal posterior border can induce strong *Msx-1* expression in cultured limb bud cells. *Msx-1* expression is localised directly subjacent to the ectoderm at the anterior border, whereas expression is not detectable in mesenchyme underneath the continuous dorsal and ventral ectoderm (Coelho *et al.*, 1993).

This is in agreement with the finding that removal of the dorsal ectoderm from stage 20-22 limb buds does not alter mesenchymal *Msx-1* expression (Ros *et al.*, 1992). Thus, it appears that the ectoderm in spatially distinct regions regulates *Msx-1* expression (Coelho *et al.*, 1993).

1.5.5.6 Possible roles of *Msx-1* and *Msx-2* during limb development

Experimental manipulations (Youkouchi *et al.*, 1991b; Coelho *et al.*, 1992b) and studies with the mutants *diplopodia-5* and *talpid^2* (Krabbenhoft and Fallon, 1992; Coelho *et al.*, 1992b) have lead to the suggestion that *Msx-1* and *Msx-2* might have a possible role in the specification of anterior positional identity in limb development. In addition, *Msx-1* and *Msx-2* are thought to be involved in programmed cell death because of their expression patterns during limb development. Both genes are expressed in regions that correspond with zones of programmed cell death, such as the posterior necrotic zone and the interdigital mesenchyme (Hill *et al.*, 1989; Robert *et al.*, 1991; Coelho *et al.*, 1991a, 1992a). However, following apical ectodermal ridge removal cell death can occur in the area of cells expressing both *Msx-1* and *Msx-2*, or in areas of cells expressing neither of those genes (Ros *et al.*, 1992). More over, the polydactylous mutant (*talpid^2*) lacks normal areas of programmed cell death in the developing limb mb (Dvorak and Fallon, 1991), but still expresses *Msx-1* in these regions (Krabbenhoft and Fallon, 1992). The fact that *Msx-1* is expressed in the progress zone of the developing limb bud, suggests that *Msx-1* is involved in stimulating mesenchymal proliferation and to maintain cells in an undifferentiated state. Transfection of a myogenic cell line with *Msx-1*, but not *Msx-2*,

inhibits their differentiation into muscle and the cells aquire a

transformed phenotype (Song *et al.*, 1992). Thus, it appears that *Msx-1* expression is involved in maintaining cells in an undifferentiated

state which correlates with the finding that grafts of the apical

ectodermal ridge onto cultured mesenchyme cells inhibits

differentiation into cartilage (Globus and Vethamany-Globus, 1976;

Solursh *et al.*, 1981b). However, although *Msx-1* deficient mice

exhibit facial defects, the limbs develop normal (Satokata and Maas,

1994).

CHAPTER 2

FGFs CAN SUBSTITUTE FOR THE APICAL ECTODERMAL RIDGE TO ALLOW

PATTERNING AND REGENERATION OF THE DEVELOPING CHICK LIMB

2.1 INTRODUCTION

The way in which cells acquire polarizing activity during limb bud outgrowth and how this activity is controlled during the period of pattern formation is unknown. Studies have indicated that the apical ectodermal ridge may play a role in maintaining polarizing region signalling during limb development. For example, polarizing region signalling in the developing chick limb bud is lost by the time the ridge regresses (Rubin and Saunders, 1972; MacCabe *et al.*, 1973; Honig and Summerbell, 1985). Grafts of polarizing region cells to the anterior margin of a chick wing bud are more efficient in inducing extra digits when placed in contact with the apical ectodermal ridge (Tickle, 1981). Furthermore, when mesenchymal cells of the polarizing region are isolated and placed in culture, polarizing activity is rapidly lost (Honig, 1983; Hayamizu and Bryant, 1992; Anderson *et al.*, 1993).

I therefore set out to test the idea that the apical ectodermal ridge plays a role in controlling polarizing region signalling by determining the effect of apical ectodermal ridge removal on polarizing activity. I have found that polarizing activity decreases dramatically in the absence of the ridge. Members of the FGF-family have been found in the entire apical ectodermal ridge (Savage *et al.*, 1993; Dono and Zeller, 1994; Heikinheimo *et al.*, 1994; Ohuchi *et al.*, 1994). *Fgf-4* is expressed in the posterior apical ectodermal ridge (Niswander and Martin, 1992; Suzuki *et al.*, 1992) and stimulates proliferation of limb bud mesenchyme in organ-culture (Niswander and Martin, 1993a). Therefore, I tested whether FGF-4 could substitute for the apical ectodermal ridge in providing a signal for maintenance of polarizing activity. In addition, I investigated whether both the

apical ectodermal ridge and FGF-4 can also function to maintain polarizing activity in limb mesenchyme cultured *in vitro*.

Niswander *et al.*,(1993) have shown that posterior application of FGF-4 following entire ridge removal of young chick wing buds is required in order to allow the development of distal skeletal structures. Application of FGF-4 to either the apical or anterior mesenchyme is not sufficient and truncated limbs develop. To investigate the correlation between polarizing region signalling and the formation of a full set of proximo-distal skeletal elements, I assayed posterior mesenchyme after various time points for polarizing activity following entire ridge removal and application of FGF-4 soaked beads to either the apical or posterior mesenchyme.

From the results above and those to be described, FGF emerges as the ridge signal required for outgrowth. Although it has been reported that mammals have got the ability to regenerate amputated limbs at either very early or late stages of limb development (Deuchar, 1976; Wanek *et al.*, 1989; Chan *et al.*, 1991; Lee, 1992), birds are not able to regenerate amputated limbs at any stage of development (see Maden, 1981; Muneoka and Sasoon, 1992). The failure of developing chick limb buds to regenerate distal parts after amputation has been attributed to their inability to regenerate the excised apical ectodermal ridge). However, when an apical ectodermal ridge is transplanted onto an amputated limb stump outgrowth resumes (Zwilling, 1956c; Saunders *et al.*, 1957, 1959; Rubin and Saunders, 1972). Furthermore, removal of limb mesenchyme of stage 18 limb buds (Barasa, 1964), or removal of distal mesenchyme of later limb buds leaving the apical ectodermal ridge intact is followed by the development of a normal limb (Hayamizu *et al.*, 1994). The ability of

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proximal cells to respond to signals of the ridge has been also shown by the fact that these cells, when grafted beneath the apical ectodermal ridge, start to express genes that are usually expressed in distal limb mesenchyme (Davidson *et al.*, 1991). To investigate whether FGFs can substitute for the apical ectodermal ridge in order to allow development and/or regeneration of skeletal limb structures, I removed either whole limb buds of young, or distal limb parts of older embryos and applied FGF-soaked beads to the mesenchyme of the remaining stump.

2.2 MATERIALS AND METHODS

2.2.1. 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 24h and 30h, posterior mesenchyme was assayed for polarizing activity, as described below. To investigate the skeletal wing structures, some embryos were left to develop for a further 6 days. The embryos were then fixed in 5% Trichloracetic-acid (TCA), stained in alcian green, dehydrated and cleared in methyl salicylate to evaluate the cartilage stucture of the wing.

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 250µm were soaked in 2µl of 1 mg/ml FGF-4 for at least 1h, before transferring

into the limb. To implant the FGF-4 soaked beads, a small cube of mesenchyme was removed, or the FGF-4 bead was placed at the margin of limb mesenchyme. To keep the bead in place, I used sterile staples formed out of platinum wire (0.025mm, Goodfellow). In a small series of experiments, FGF-4 was not applied immediately after the apical ectodermal ridge had been removed, but 16 or 24h later. In these cases the bead was 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 the FGF-4 bead had been implanted, posterior mesenchyme was assayed as below, at 24h and 30-32h after ridge removal.

In a third set of experiments, the entire ridge was removed as described above. In some cases a FGF-4 soaked bead was either placed apically, or posteriorly without removal of limb mesenchyme. After 24hours, 32 hours and 48 hours following the operation posterior mesenchyme of both the operated and unoperated limb was assayed for polarizing activity as described below.

2.2.2. 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 min 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% TCA and stained for cartilage as descibed above. To measure polarizing activity, the development of a wing with no additional

digits was scored as 0 (no polarizing activity, Fig. 2.1A). The appearance of a small knob of extra cartilage next to digit 2, that was not sufficiently well developed to be counted as a duplicated digit 2 was scored 0.5 (12.5% polarizing activity, Fig. 2.1B). The development of digit 2 was scored 1 (25% polarizing activity, Fig. 2.1C), the development of digit 3 was scored 2 (50% polarizing activity, Fig. 2.1D) and the development of an additional digit 4 was scored 4 (100% polarizing activity, Fig. 2.1E and 2.1F). 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 quantiative data that relate the strength of the polarizing region signal and the number of polarizing region cells (Tickle, 1981). The percentage of limbs with pattern alterations was also calculated to give an indication of how many limbs had a changed digit pattern.

2.2.3. Removal of whole limb buds or distal parts of various limb bud stages and application of FGF

Whole limb buds of stage 19 to 23 were removed by cutting along the body wall using fine forceps. In some cases distal parts of the limb bud were excised from various stages (stage 22 to 26) using fine forceps. Before and after the operation the length of limb buds was measured using a graticule to estimate the size of the removed distal limb part (usually between 250μm and 625μm length by measuring the right angle to the body wall). Because posterior outgrowth is more advanced compared to anterior outgrowth, removal of distal limb parts usually contained larger amounts of posterior mesenchyme. In some cases heparin acrylic beads were soaked in either FGF-4 (700μg/ml) or

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FGF-2 (1mg/ml) and applied to different positions (anterior,

posterior and apical, as described above) of the limb mesenchyme,

where distal parts or whole limb buds had been removed. The embryos

were left to develop for further 6 days. To analyse the skeletal

pattern of the operated limbs, the embryos were fixed and stained for

cartilage, as described above.

2.2.4. Cell cultures and composition of media

Micromass cultures were prepared from the posterior 1/3 of limb

buds of 9.5-11d mouse embryos (strain C57/BL/H-a^t and H-2K^b-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 minimal essential medium

(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 2mM L-glutamine

(GIBCO). The hind- 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 min 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 10^7 cells/ml

with serum-containing CMRL-medium. One 10µl drop of the cell

suspension containing 10^5 cells was plated per well, using 4 well
multidishes (Nunclon Delta), and the cells allowed to attach for 1-
1.5h at 37°C. The high density (micromass) cultures were then flooded
with 300µl CMRL-medium (GIBCO) containing 10% FCS (GIBCO), 2mM L-
Glutamine (GIBCO) and 1% antibiotic/antimycotic (GIBCO). Cultures
were incubated at 37°C with 5% CO_2 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 5-10µl drops (containing 1
-3 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
1.5h and then flooded with 300µl serum-containing CMRL-medium.
Usually not all the ectoderm-jackets plated attached to the
substratum. Ectoderm-jackets which did attach, started to flatten
after about 24h and in some experiments there were between one to
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 multidish. 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.

2.2.5. Growth factors

Fibroblast growth factors (FGF-4), heparan sulphate (Sigma) and
acetylated bovine serum albumin (New England Biolabs) were kindly
provided by Dr. Lee Niswander (University of California, San
Francisco) and Dr. John Heath (University of Oxford). FGF-2 was
added at a concentration of 1, 10 and 100ng/ml to the serum-
containing culture medium. When FGF-4, or FGF-2 was added to the
culture medium, heparan sulfate was also added for stabilization of
the FGF-protein. 30µl of heparan ,sulphate stock (1µg/ml) and 30µl of
FGF-4 protein (1µg/ml) was added to 300µl serum-containing CMRL-
medium, which gives a final concentration of approximately 80ng/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 diluted with MEM containing 1% BSA to 1µg/ml in siliconized
eppendorf.

2.2.6. 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 19-21 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 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. Six
days after performing the grafts, the thorax with limbs of the embryo
was fixed and stained for cartilage as described above.

2.3. RESULTS

2.3.1. Effects of the apical ectodermal ridge and FGF-4 on polarizing activity in chick limb buds

Removal of posterior apical ectodermal ridge is followed by a decline in polarizing activity of the limb bud. 24h after removal of the posterior part of the ridge of early wing buds, posterior outgrowth was clearly inhibited in most cases (13/17; Fig. 2.2a). Buds with a skewed shape resulted because some outgrowth continued anteriorly. Polarizing activity in all regions of these skewed buds was reduced compared of that of the mesenchyme from the posterior margin of the contralateral limb (stage 24/25) which was 100% (8 cases; Fig. 2.2b). The region from the posterior tip (B) showed highest polarizing activity (52%) and 69% of the grafted wings had pattern alterations, whereas piece D taken most anteriorly had the lowest activity, 2% (Fig. 2.2a and Table 2.1A). In a few cases (4/17), some outgrowth occured 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 30h 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 wings had pattern alterations; 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 1mg/ml FGF-4 inserted posteriorly, bud outgrowth at the posterior margin continued and

polarizing activity did not decrease. At 24h, polarizing activity of posterior cells (piece A) was 95% (100% limbs with pattern alterations and grafts of piece B, more distal posterior mesenchyme, had a polarizing activity of 82% (100% limbs with pattern alterations; Fig. 2.2c and Table 2.1B).

FGF-4 had to be applied by 16h after ridge removal to rescue polarizing activity of the posterior mesenchyme of 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 15-16h after ridge removal, outgrowth of the posterior part of the chicken limb bud appeared to resume and 6h later grafts of posterior mesenchyme (piece A) still had 100% polarizing activity (2 cases). Grafts of piece B, the more distal part of the newly formed posterior outgrowth resulted in 50% polarizing activity (2 cases; one gave an additional digit 4, the other gave no additional digits). When beads soaked in FGF-4 were implanted a little later, 24h after ridge removal, no posterior outgrowth was observed 5-6h after bead insertion. Even though the contralateral left wing bud (stage 25/26) still had full polarizing activity (100% ; 4 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 pattern alterations; 6 cases each). Thus, application of FGF-4 24h after ridge removal, had no effect on maintaining polarizing activity.

2.3.2. Effect of FGF-4 on development of skeletal pattern after posterior ridge removal

Following posterior ridge removal wings developed humerus and radius (9/13 cases; Fig. 2.3A). In the remaining 4 cases wings developed humerus, radius and ulna presumably due to incomplete removal of the posterior apical ectodermal ridge. Application of FGF-4 to posterior mesenchyme after posterior ridge removel was followed by the development of humerus, radius, ulna and a bunch of posterior like digits (4/7 cases; Fig. 2.3B). The remaining three wings failed to develop digits. In those wings the bead was integrated into the mesenchyme and did not stay distally and posterior outgrowth was not observed. Thus, FGF-4 can substitute for the posterior ridge to allow the development of posterior structures.

2.3.3. Effect on polarizing activity after entire ridge removal and application of FGF-4 to different positions of limb mesenchyme

Previous work showed that removal of the entire ridge at stage 19/20 leads to development of only the humerus but when FGF-4 is applied simultaneously to both posterior and apical mesenchyme, a virtually complete limb develops (Niswander *et al.*, 1993). After entire ridge removal, application of a single FGF-4 bead to the posterior margin allowed development of all posterior structures; apical FGF-4 application gave humerus, radius and ulna but no digits, whereas anterior FGF-4 application resulted in a thickened or split humerus (Niswander *et al.*, 1993).

To investigate the correlation between polarizing region signalling and the development of distal skeletal structures

following entire ridge removal and application of FGF-4 to different positions of mesenchyme (Niswander *et al.*, 1993), I assayed polarizing activity from contralateral limb bud stages where the ridge had been left in place. Posterior mesenchyme isolated from contralateral limbs of stage 25/26 (32 hours after stage 19/20) limb had 100% polarizing activity and 100% of the wings were duplicated (6 cases, Fig. 2.4). Posterior mesenchyme from the contralateral limb bud of stage 27/28 (48 hours after stage 19/20) had 40% polarizing activity (5 cases; Fig. 2.4) and 60% of the wings were duplicated. These results are consistent with previous studies that have shown that polarizing activity decreases at about stage 28 in the developing wing bud (MacCabe *et al.*, 1973; Honig and Summerbell, 1985).

Entire ridge removal was followed by the loss of polarizing activity in posterior mesenchyme. 24 hours after entire ridge removal limb outgrowth and polarizing activity of posterior mesenchyme was greatly reduced compared to the contralateral limb (Fig. 2.2b; Fig. 2.4). Grafts of the posterior tip (piece B) had polarizing activity of 37.5% and 100% of the wings duplicated (2 cases). Posterior mesenchyme proximally to the posterior tip (piece A) had highest polarizing activity (62.5%) and 100% of the wings had a duplicated digit pattern (2 cases, Fig. 2.4; Tab. 2.2). When posterior mesenchyme was assyed after 32 hours following entire ridge removal, polarizing activity was lost in pieces of either region A (3 cases) or B (2 cases; Fig. 2.4; Tab. 2.2). 48 hours following the operation posterior mesenchyme of the distal tip (piece B) had no polarizing activity (2 cases: Fig. 2.4; Tab. 2.2). Thus, following entire ridge removal of young chick limb buds polarizing activity is rapidly lost.

To see, whether following entire ridge removal local application of FGF-4 can maintain polarizing region signalling, I applied FGF-4 soaked beads to either the posterior or apical mesenchyme. When posterior mesenchyme was assayed 24 hours after entire ridge removal and apical application of FGF-4, posterior mesenchyme (piece A) had polarizing activity of 75% and 100% of the wings duplicated (5 cases), whereas posterior mesenchyme of the distal tip (piece B) had 50% polarizing activity and 75% of the wings duplicated (4 cases; Fig. 2.4; Tab. 2.2).

32 hours after entire ridge removal and apical application of FGF-4 to the mesenchyme, posterior mesenchyme from region A had 33% polarizing activity and 67% of the wings were duplicated (3 cases), whereas posterior mesenchyme of region B had 12.5% polarizing activity and 25% of the wings were duplicated (4 cases; Fig. 2.4; Tab. 2.2). When grafts of posterior mesenchyme was assayed for polarizing activity after 48 hours following entire ridge removal and apical application of FGF-4 to the mesenchyme both, mesenchyme from region A (3 cases) and region B (4 cases) had no polarizing activity (Fig. 2.4; Tab. 2.2). Thus, apical application of FGF-4 to limb mesenchyme following entire ridge removal is not sufficient to maintain polarizing region signalling of posterior mesenchyme.

32 hours after entire ridge removal and FGF-4 application to the posterior margin, posterior mesenchyme of region A had 100% polarizing activity and 100% of the wings were duplicated (3 cases; Fig. 2.4; Tab. 2.2), whereas posterior mesenchyme at the tip of the bud (B) had 83% polarizing activity and 100% of the wings were duplicated (3 cases). 48 hours after entire ridge removal and application of FGF-4 to the posterior margin polarizing activity of piece A was decreased to 50% (2 cases; Tab. 2.2) and of piece B was

decreased to 31% (4 cases; Fig. 2.4; Tab. 2.2). Taken together, in the absence of the ridge FGF-4 acts locally to maintain polarizing region signalling. FGF-4 has to be applied to posterior mesenchyme in order to maintain polarizing region signalling and this correlates with the development of distal skeletal structures (see Niswander *et al.*, 1993).

2.3.4 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 posterior cells were 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 96h. The relationship between polarizing activity and time in micromass culture is shown in Fig. 2.5A. Even after 24h, polarizing activity of cells in micromass cultures was much reduced. The polarizing activity of cells cultured for 24h was 20% (Fig. 2.5A) although 71% of grafted wings were duplicated (Fig. 2.5B). This compares with a polarizing activity of 50% (87% wings with pattern alterations) which was obtained when pieces of posterior mesenchyme from mouse limb buds were grafted directly to the anterior margin of the chick limb buds (Fig. 2.5A). With longer times in culture, the polarizing activity was reduced still further. The difference in extent of the polarizing activity of the cells at 24h and 72h is shown by comparing the character of the additional digits produced;

at 72h only 1 out of 6 wings showed marked changes in pattern and an additional digit 2 formed, whereas at 24h, 4 out of 7 wings had either an additional digit 2 or an additional digit 3 (Tab. 2.3A).

2.3.5 Co-culture with ectoderm maintained polarizing activity of cultured posterior limb bud 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 96h; 2 out of 12 grafts gave duplications. With micromass cultures there was also maintenance of polarizing activity after 96h: 38% of the limbs were duplicated after receiving a graft of micromass cocultured with ectoderm (Fig. 2.1C; Fig. 2.5A,B and Tab. 2.3B), whereas when mesenchyme was cultured in the absence of ectoderm, no duplications were obtained after grafting at this time point. However, at 72h of co-culture, the mesenchyme had 4% polarizing activity (18% of wings with pattern alterations).

2.3.6 FGF-4 maintains polarizing activity of cultured posterior limb bud cells

To see, whether FGF-4 can maintain polarizing activity *in vitro* just as it did in intact buds, FGF-4 was added to micromass cultures of posterior mesenchyme cells. After 24h, polarizing activity was 53% and 100% of wings had pattern alterations, compared

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with 20% polarizing activity (71% wings with pattern alterations) 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 4 (Fig. 2.1E; Tab. 2.3C), a result which was never obtained in the absence of the growth factor. Even after 72h, the activity with FGF-4 was 19% and three wings with an additional digit 3 developed (Tab. 2.3C), whereas without FGF, cultured cells had only 4% polarizing activity. However, by 96h no polarizing activity could be detected even with FGF-4 (Fig. 2.5A and 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 24h, polarizing activity after a further 24h was 20% (40% of the wings had pattern alterations) and after 72h 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 24h, 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 24h had a polarizing activity of 50% (100% of the wings had pattern alterations, 5 cases). In the presence of 10ng/ml and 100ng/ml, grafts had 43% and 46% polarizing activity (100% of wings with pattern alterations at both concentrations: 9 and 14 cases respectively).

2.3.7 FGF application to limb mesenchyme following amputation of whole limb buds of young embryos allows development of skeletal wing structures

The extent of development of skeletal structures following amputation of young chick wing buds and application of FGF-4 to the mesenchyme depends on the position of FGF application and on the stage at which the bud has been removed. When whole limb buds from stage 19 to 23 were removed, wings that developed were completely truncated and only scapula and coracoid formed (Fig. 2.6A; Tab. 2.4). When FGF-4 was applied posteriorly after removal of the whole limb bud at stage 19/20 substantial regeneration of structures occured. Humerus, radius, ulna and a bunch of posterior-like digits formed in one out of two cases (Fig. 2.6B; Tab. 2.4). In the other case only humerus and cartilage structures distally to the humerus developed Tab. 2.4). Application of two FGF-4 beads to the posterior and apical mesenchyme following removal of a stage 19/20 limb bud also allowed the development of humerus, ulna radius and posterior like digits (Fig. 2.6C; Tab. 2.4).

Removal of stage 20/21 wing buds and anterior FGF-4 application was followed by development of both, a duplicated humerus (partially developed) and coracoid (Tab. 2.4; Fig. 2.6D). Application of FGF-4 to posterior mesenchyme following bud removal allowed development of a complete humerus and cartilage structures distally to it (Tab. 2.4; Fig. 2.6E). Two FGF-4 beads applied to posterior and apical mesenchyme following removal of a stage 20/21 limb bud had the greatest effect and humerus, radius, ulna and digit-like structures formed (Fig. 2.6F; Tab. 2.4). However, they appeared to be not as well developed as the structures generated when the operation was

carried out at stage 19/20 limb buds. Removal of stage 21/22 limb

buds and anterior FGF-4 application was followed by partial

development of a thickened humerus (Tab. 2.4; Fig. 2.6G) and FGF-4

application to posterior mesenchyme resulted in partial development

of humerus and cartilage nodules distally to it (Tab. 2.4; Fig.

2.6H). Generally, operated limbs were much reduced in size compared

to contralateral limbs. Thus, the extent of development of skeletal

strucutres following removal of limb buds and posterior FGf-4

application appears to decrease with older limb bud stages.

2.3.8 Effect of FGF following removal of distal limb bud parts of older embryos

Removal of increasing amounts of distal tissue from older limb

buds resulted in limbs that were truncated at progressively more

proximal levels. To see whether FGF can allow

development/regeneration of distal skeletal limb parts in the

developing limb, I applied FGF-soaked beads to the posterior

mesenchyme of the stump following excision of distal limb parts.

Following removal of approximately 250μm of the distal tip of limb

buds between stage 22 to 24, outgrowth was reduced and the resulting

limbs developed humerus, radius and ulna, but digits failed to

develop (4 cases, Fig. 2.7A; Tab. 2.5A). In contrast, removal of 250-

375μm of the distal part of stage 23 to 25 limb buds and FGF

application to posterior mesenchyme of the remaining stump lead to

the development of digits (8/9 cases, Tab. 2.5B; Fig. 2.7 B). In the

other case digits failed to develop. Removal of 375-625μm of distal

limb parts of stage 22 to 23/24 resulted in development of the

humerus respectively (7/8 cases; Tab. 2.5A; Fig. 2.7C). In one case

the proximal part of one forearm bone developed in addition to the

humerus. When approximately 400μm was removed from a stage 23 limb

bud and 2 FGF soaked beads were applied to posterior and apical

mesenchyme a humerus, radius and parts of the ulna developed (1 case,

Tab.2.5B). In one case approximately 400μm of the distal tip from a

stage 23/24 limb bud was removed and FGF applied to the anterior

margin of the remaining stump. In this case humerus, radius, ulna and

digits developed (Tab. 2.5B; Fig. 2.7D). At stage 25/26 removal of

250-500μm resulted in the development of humerus, radius and ulna,

but digits failed to develop (6/7 cases; Tab. 2.5A

whereas in the other case digits developed. In two cases FGF beads

were applied to apical mesenchyme following removal of 250μm of the

distal tip of stage 25/26 limb buds and in these cases digits did

develop (Tab. 2.5B). Thus, it appears that FGF application allows the

development/regeneration of skeletal structures following amputation

of distal limb parts.

2.4 DISCUSSION

Polarizing region signalling of posterior limb mesenchyme requires a local signal from the apical ectodermal ridge, which can be provided by FGF-4. In addition, local maintenance of polarizing region activity in posterior mesenchyme after entire ridge removal correlates with the development of a full set of proximo-distal limb structures. Moreover, FGF application following either amputation of young chick limb buds, or excision of distal parts of later limb buds is followed by the development of skeletal limb structures.

2.4.1 The apical ectodermal ridge is required to maintain polarizing region signalling in posterior limb mesenchyme *in vivo* and *in vitro*

The maintenance of polarizing activity in mesenchyme cells at the posterior margin of the limb bud is dependent on a local signal from the apical ectodermal ridge. When either the entire or 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 antero-posterior axis with bud outgrowth. Thus as successive structures along the proximo-distal axis are specified in the progress zone, their antero-posterior 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 develop in the apparent absence of the polarizing region (MacCabe *et al.*,1973, Fallon and Crosby, 1975, Smith, 1979).

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2.4.2 FGF-4 replaces the ridge to maintain polarizing region signalling of posterior limb mesenchyme *in vivo* and *in vitro*

FGF-4 could to be the signal from the apical ridge that locally maintains polarizing activity of posterior limb bud mesenchyme. I 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. *FGFR-1* is expressed in limb mesenchyme of mouse and chick (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1991; Patstone *et. al.*,1993) and an isoform of FGFR-1 binds FGF-4 (Mansukhani *et al.*, 1990). Anderson *et al.*,(1993) and I have shown that FGF-2 can also maintain polarizing activity *in vitro*. FGF-2 is found in limb bud ectoderm including the entire apical ectodermal ridge and in the underlying mesenchyme (Savage *et al.*, 1993; Dono and Zeller, 1994) and binds to FGFR-1 (Dionne *et al.*, 1990). However, antibodies against FGF-2 did not inhibit maintenance of polarizing activity of cultureddissociated posterior limb bud cellssuggesting that another FGF-family member might be the endogenous ridge factor that maintains polarizing region signalling in the developing limb bud (Anderson *et al.*, 1993). Transcripts of *Fgf-5* and *Fgf-7* are only found in a subset of cells in the developing limb at later stages (Haub and Goldfarb, 1991; Mason *et al.*, 1994)) and *Fgf-3* is not expressed in the developing limb (Wilkinson *et al.*, 1989), suggesting that these FGF-family members are not involved in maintaining polarizing region signalling in posterior limb mesenchyme. Possible

candidates to be involved in maintaining polarizing region signalling are FGF-4 and FGF-8. *Fgf-8* expression is found throughout the entire apical ectodermal ridge (Heikinheimo *et al.*, 1994; Ohuchi *et al.*, 1994) and *Fgf-4* transcripts are localised to the posterior part of the apical ectodermal ridge (Niswander and Martin, 1992; Suzuki *et al.*, 1992). Expression of both genes is lost by the time the ridge regresses (Niswander and Martin, 1992; Ohuchi *et al.*, 1994), which correlates with the loss of polarizing activity in posterior limb mesenchyme (MacCabe *et al.*, 1973; Honig and Summerbell, 1985).

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 simply promotes 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) and I propose that FGF-4 acts on posterior mesenchyme at the tip of the limb to maintain the progress zone. This could stimulate proliferation of precursors of the polarizing region, which

would take on polarizing activity as they leave the progress zone. 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.

2.4.3 Local maintenance of polarizing region signalling by FGF-4 in the absence of the ridge correlates with the development of distal skeletal structures

Local application of FGF-4 to posterior mesenchyme maintains polarizing region signalling in the absence of the ridge. Application of FGF-4 to apical mesenchyme after entire ridge removal is not sufficient to maintain polarizing region signalling in posterior mesenchyme. FGF-4 has to be applied to posterior mesenchyme in the absence of the ridge in order to maintain the development of distal skeletal structures (e.g. digits), which otherwise would fail to develop (Niswander *et al.*, 1993). This suggests that a signal from the polarizing region in addition to FGF in the absence of the ridge is required to maintain an active progress zone. This is supported by the observation that following entire ridge removal, application of RA and FGF-4 to either apical or anterior mesenchyme is followed by the development of the most distal skeletal structures, e.g. digits (Niswander *et al.*, 1993; Niswander *et al.*, 1994). Taken together, these observations suggest that FGF-4, by maintaining polarizing region signalling and proliferation of distal mesenchyme, links growth with patterning in the developing limb bud.

2.4.4 FGF application following amputation of wing buds of young embryos leads to the development of skeletal wing structures

When whole limb buds of early limb bud stages were removed, no skeletal wing structures developed. Application of FGF to mesenchyme at the body wall level after removal of the limb bud was followed by the development of skeletal wing structures and the extent of skeletal development depended on the position of the applied bead. Fewer skeletal structures are formed of FGF with increasing limb bud stage and application of FGF. This is consistent with the finding that grafts of proximal tissue from older stages placed at the tip of young limb buds progressively lose the ability to participate in the formation of distal structures as the limb bud develops (Saunders *et al.*, 1959; Searls and Janners, 1969). Thus, it appears that the failure to regenerate skeletal limb structures in the developing chick limb is not only because of the inability to regenerate an apical ectodermal ridge but also because of the inability of older limb mesenchyme to respond to signals of the apical ectodermal ridge (e.g. FGF).

The results obtained following removal of whole limb buds and application of FGF to different positions of the mesenchyme are comparable to results of Niswander *et al.*,(1993), where only the apical ectodermal ridge had been removed. For example, here it was found that application of FGF to anterior mesenchyme following bud removal resulted in development of parts of a thickened or split humerus, whereas application of FGF to both apical and posterior mesenchyme resulted in the development of virtually all skeletal wing structures, although reduced in size. Thus, these results support our former suggestion that, a signal from the polarizing region/posterior

mesenchyme is required to allow the development of distal skeletal wing structures. During limb development highest polarizing region signalling is found just proximal to the progress zone at the tip of the bud (MacCabe *et al.*, 1973; Honig and Summerbell, 1985). Absence of polarizing activity in the stump following amputation of older limb buds might explain the decrease in the ability to form distal skeletal structures following FGF application. Therefore, it would be interesting to see whether, for example RA in addition to FGF could improve the development of distal skeletal wing structures following amputation. Moreover, it would be interesting to assay polarizing region signalling of posterior mesenchyme following amputation and FGF application to see whether a polarizing region signal is required for development of distal skeletal structures.

2.4.5 FGF allows regeneration of distal skeletal elements after excision of distal parts of late limb bud stages

Application of FGF to posterior mesenchyme following excision of distal wing parts leads to development of distal skeletal elements that would otherwise not have formed. Previously, it has been reported that when distal parts of late limb bud stages are excised and the apical ridge is left in place, regeneration of distal skeletal structures in the chick limb can occur (Hayamizu *et al.*, 1994). Thus, our results and the recent results of Taylor *et al.*, (1994) appear to show that FGF can replace the apical ectodermal ridge to allow regeneration of the chick limb. A variety of neural tissue synthesize FGFs (see Baird, 1994a). Implantation of the neural tube into the amputated chick limb stump can allow regeneration

(Fowler and Sisken, 1982) and FGF-2 has been found in the neural tube
of the developing chick (Savage *et al.*, 1993; Dono and Zeller, 1994).
Moreover, FGFs injected into denervated early regenerates of adult
newt forelimbs stimulated proliferation in dedifferentiated
mesenchymal cells (Mescher and Gospodarowicz, 1979). Thus, it is
possible that, by releasing FGF, implants of neural tissue can allow
regeneration in species that normally do not regenerate. However,
whether the development of skeletal limb structures by FGF following
removal of distal limb parts is followed by dedifferentiation,
proliferation and redifferentation (e.g. regeneration) has still to
be clarified. Although, differentiation into cartilage and muscle
appears in proximal cells of stage 23 to 25 limb buds (Gould *et. al.*,
1972; Thorogood and Hinchliffe, 1975), cells in about 400-500μm
distance from the apical ectodermal ridge retain the characteristics
of unspecialised mesenchymal cells (Searls, 1965; Searls *et.
al.*,1972). The size of this undifferentiated subridge region remains
constant during subsequent stages of limb development (Searls, 1965;
1973; Stark and Searls, 1973; Summerbell, 1976). Thus, regeneration
of skeletal structures by FGF would only occur when appoximately
500μm of undifferentiated mesenchyme from the distal tip is removed.
Several studies indicate that proximal mesenchyme isolated from
younger limb bud stages (up to stage 24) grafted beneath the apical
ectodermal ridge is able to differentiate in accordance with its
localisation, whereas proximal cartilage forming mesenchyme from
older stages appears to become stabilised as cartilage by stage 24/25
(Saunders *et al.*,1957; Cairns, 1965a; Searls and Janners, 1969). This
is consistent with the finding that proximal mesenchyme cells of
older mouse limb buds grafted beneath the apical ectodermal ridge
lose the ability to respond to the apical ectodermal ridge and do not

express *Msx-1* (Kostakopoulou, personal communication). Therefore, it has to be investigated whether removal larger slices of the distal limb bud and FGF application is followed by the development of skeletal limb structures and whether these cells start to express *Msx-1*. Moreover, it would be interesting to investigate, whether the extent of regeneration after removal of different sizes of distal limb parts is correlated with expression domains of homeobox-containing genes, such as *Msx-1* or *Shh*.

Figure 2.1 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 24h (score 0.5); C)

duplicated wing, digit pattern 223 4, resulting from a graft of

micromass cocultured with ectoderm for 96h (score 1). Note nobs 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 24h (score 2); E)

duplicated wing, digit pattern 43334, resulting from a graft of a

piece of micromass of posterior cells cultured for 24h 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).
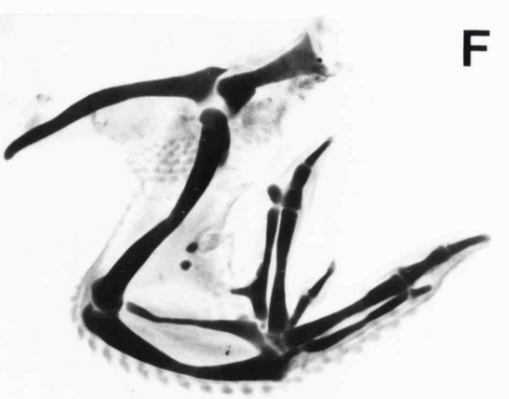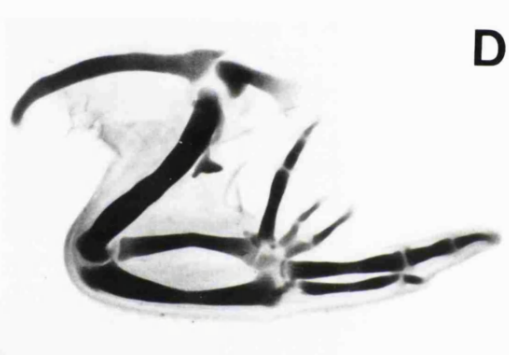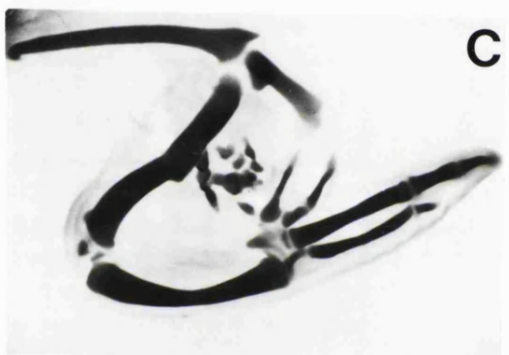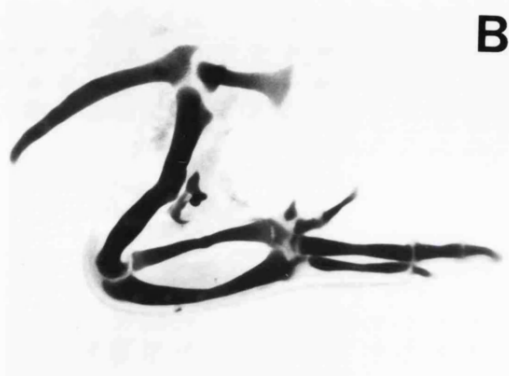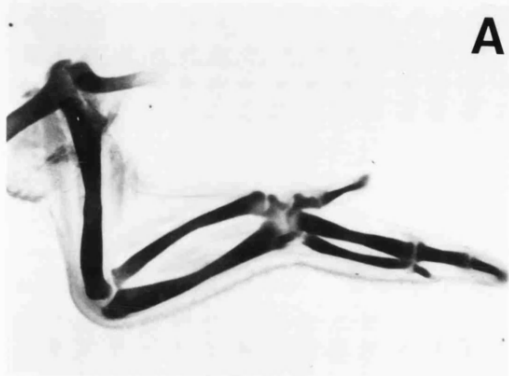
Figure 2.2 Maps of polarizing activity of limb buds 24h after
posterior ridge removal with and without application of FGF-4 to
posterior mesenchyme. 24h after removal of the posterior ridge and
showing the effects of adding FGF-4. a) removal of the posterior
ridge; b) controls showing polarizing activity of unoperated limb
buds; c) removal of the posterior ridge and adding FGF-4 beads. After
24h 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.

a) limb buds after posterior ridge removal:
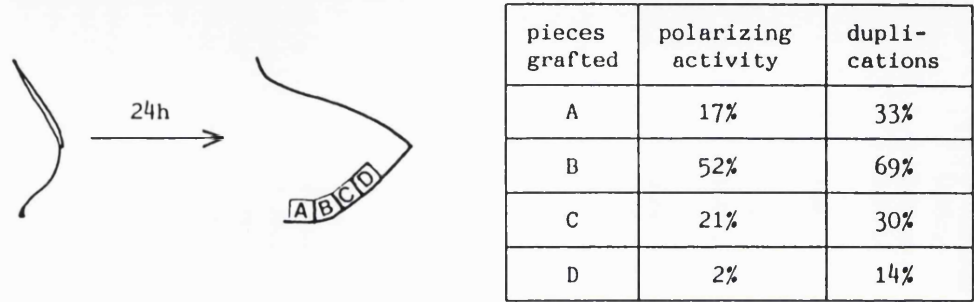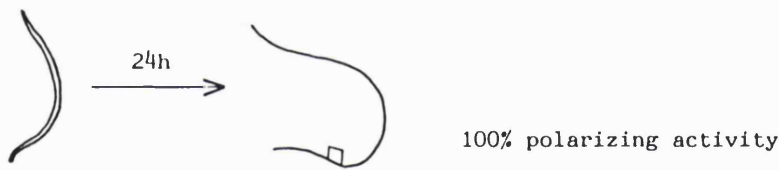
pieces grafted	polarizing activity	dupli-cations
A	17%	33%
B	52%	69%
C	21%	30%
D	2%	14%

b) controls (contralateral limb):

100% polarizing activity

c) limb buds after posterior ridge removal and addition of FGF-4 soaked bead:

pieces grafted	polarizing activity	dupli-cations
A	95%	100%
B	82%	100%
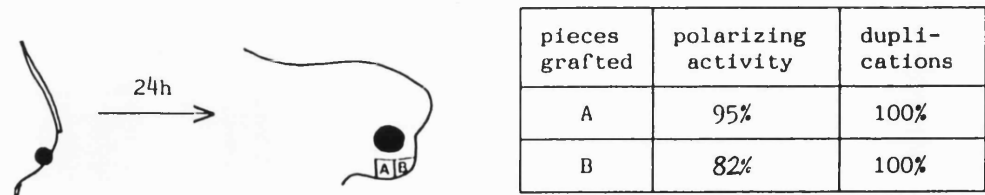
Figure 2.3 Effect of FGF-4 on the development of skeletal structures following posterior ridge removal. (A) Posterior ridge removal was followed by the development of humerus and radius. (B) Application of FGF-4 to posterior mesenchyme following posterior ridge removal was followed by the development of humerus, radius, ulna and a bunch of posterior-like digits.

A
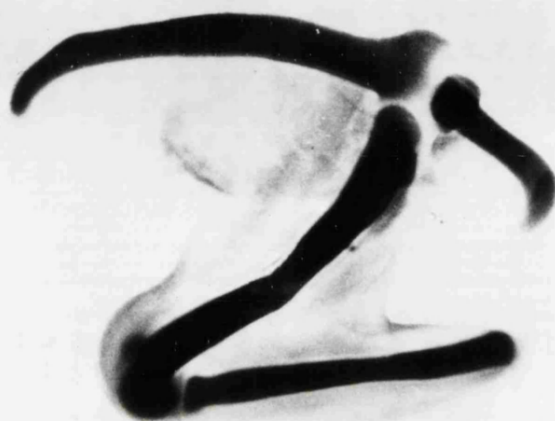
B
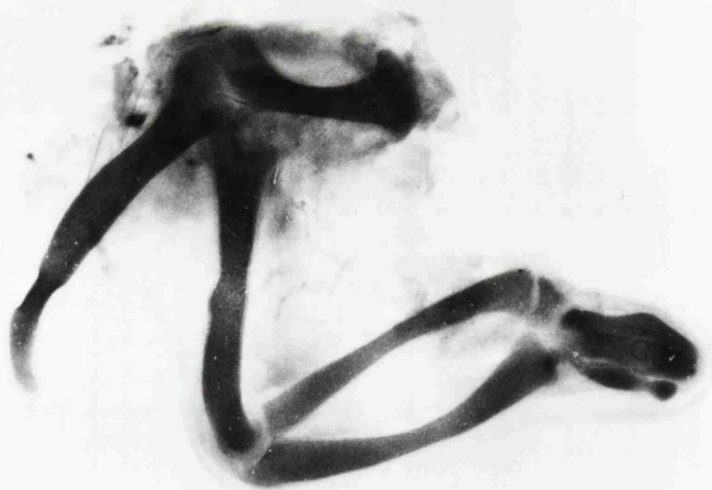
Figure 2.4 Local effects of FGF-4 on the maintenance of polarizing

activity after removal of the entire ridge. The graph shows

polarizing activity that remains after various times in unmanipulated

limbs, in limbs from which the ridge has been removed, and those in

which FGF-4 has been applied to either apical or posterior mesenchyme

after ridge removal. Specimens of the latter assayed at 24 hours

after manipulation were subjected to only posterior ridge removal.

The number in parenthesis indicate the number of limbs assayed.

Time of assay (hours after ridge removal)
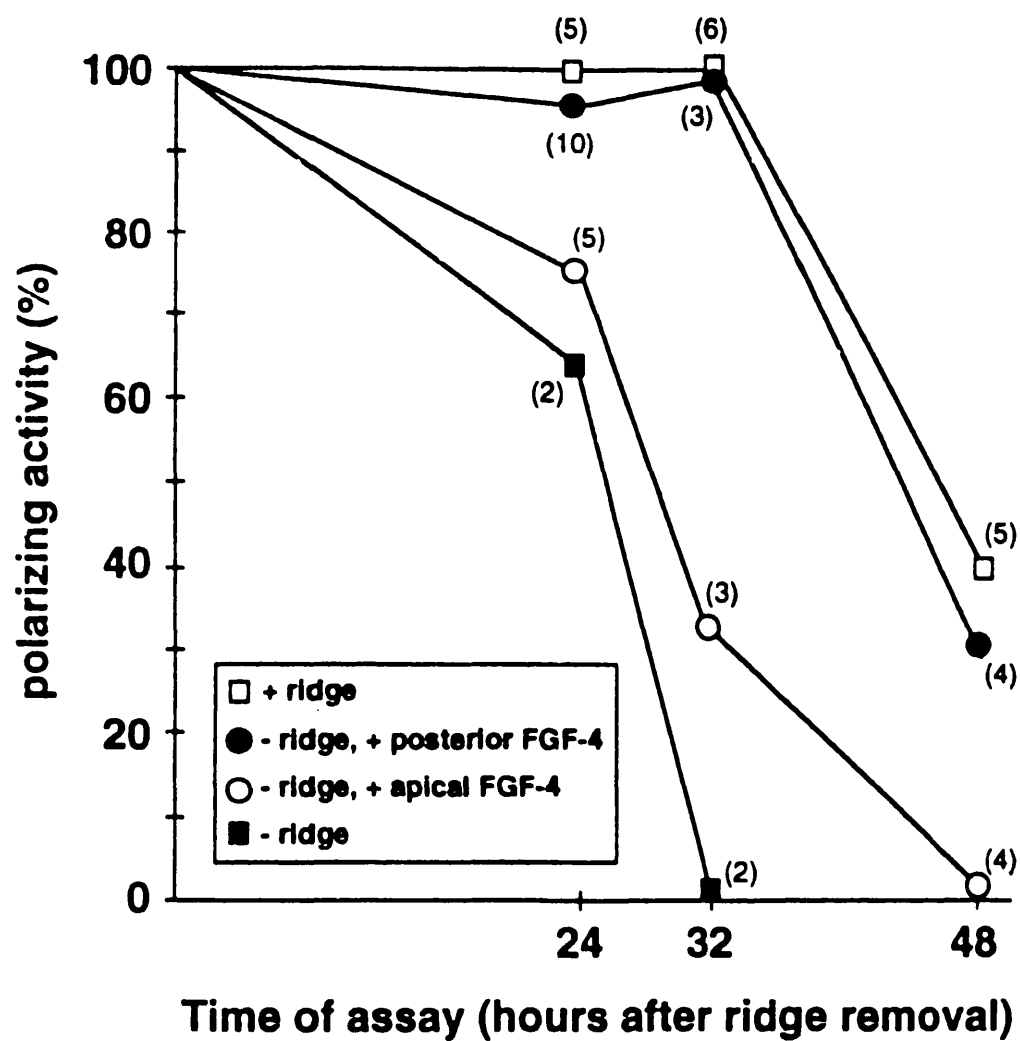
Figure 2.5 Polarizing activity (A) and percentage limbs with pattern

alterations (B) of grafts of pieces of micromass cultures of

posterior mesenchyme of mouse limb buds, assayed by placing at the

anterior margin of a chick wing bud. The micromasses were cultured in

serum-containing medium (■); with FGF-4 protein and heparan

sulphate at a concentration of approximately 80ng/ml (▨), or

cocultured with ectoderm (☐). Posterior mesenchyme of mouse limb

buds grafted directly to anterior limb mesenchyme (▦).

A

polarizing activity [%]

time in culture [hrs]

B

digit duplications [%]

time in culture [hrs]
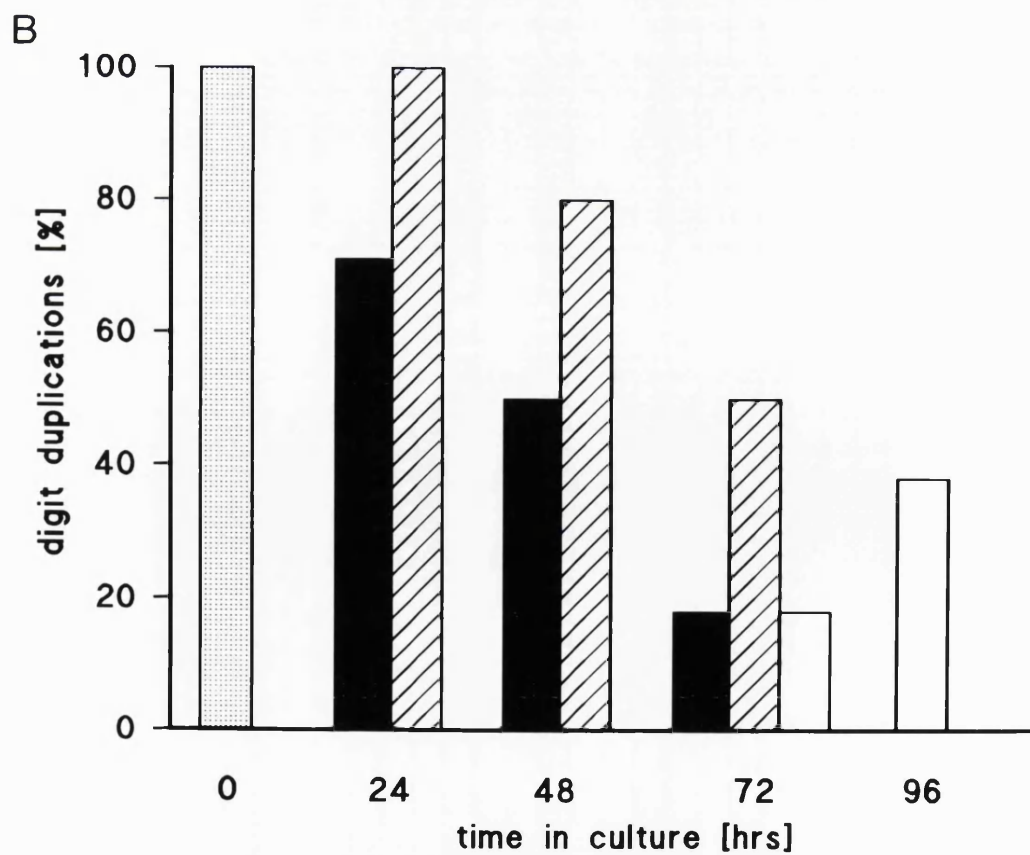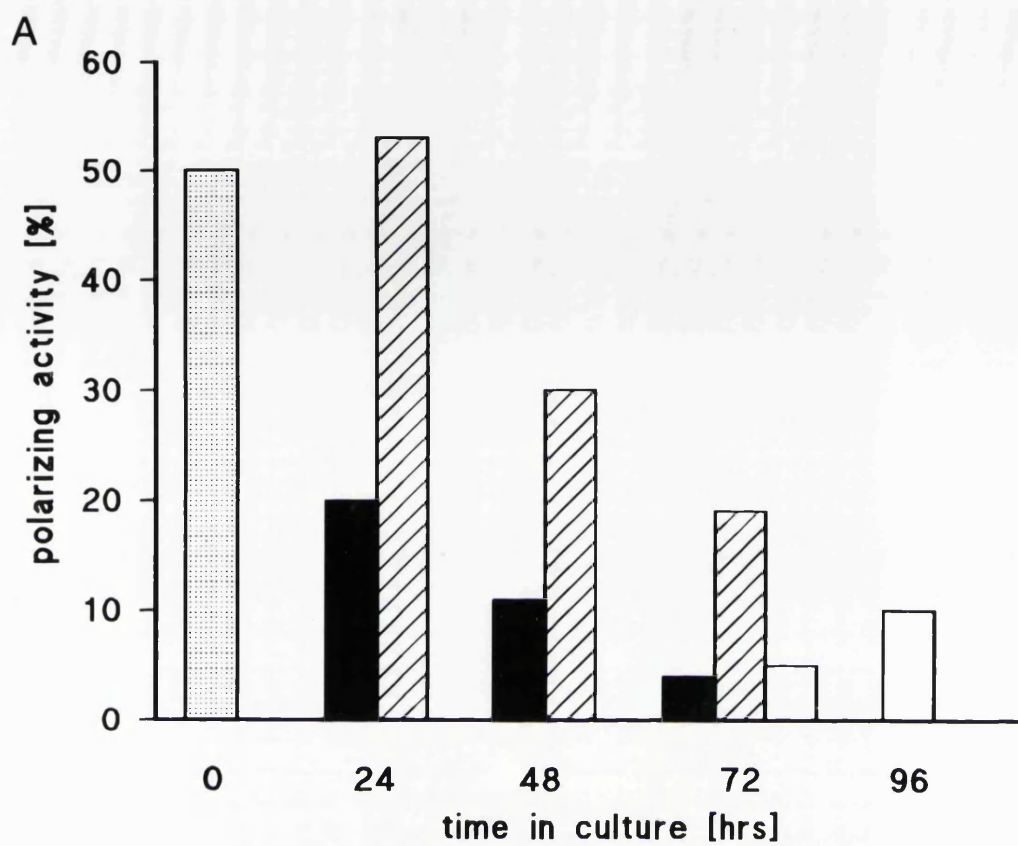
Figure 2.6 Effect of FGF application on the development of skeletal
structures following removal of whole limb buds at different stages.
(A) Skeletal pattern following removal of stage 19-23 limb buds.
(B,C) Skeletal pattern following removal of stage 19/20 limb bud and
application of FGF to (B) posterior, or (C) both apical and posterior
mesenchyme. (D-F) Skeletal pattern following removal of stage 20/21
limb bud and application of FGF to (D) anterior, (E) posterior, of
(F) both apical and posterior mesenchyme. Skeletal pattern following
removal of (G,H) stage 21/22 limb buds and application of FGF to (G)
anterior, or (H) posterior mesenchyme.
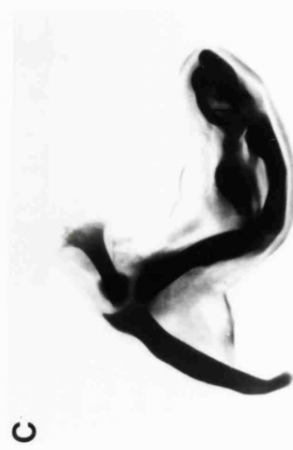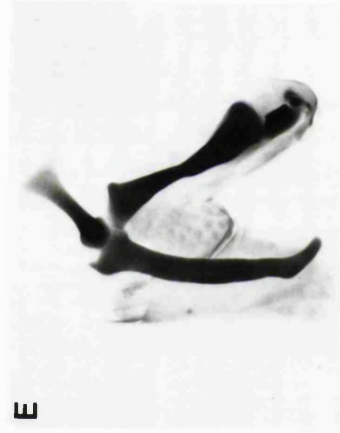
Figure 2.7 Skeletal pattern of wings following removal of (A) 250µm

of a stage 24 limb bud. Humerus, radius and ulna develop, whereas

digits fail to develop. (B) Posterior application of FGF to the

mesenchyme of the remaining stump after removal of 250µm of the

distal tip of a stage 24 limb bud is followed by the development of a

virtually complete limb. (C) Following removal of 400µm of the distal

tip of a stage 23/24 limb bud only the humerus developed. (D)

Anterior FGF-application to posterior mesenchyme following removal of

500µm of the distal tip of a stage 23/24 limb bud.

A

stage of oper-ation	piece grafted	n	normal 2 3 4	? 2 3 4	2 2 3 4	3 3 4 (etc)	4 3 3 4 (etc)
19	A	1	1	-	-	-	-
	B	2	2	-	-	-	-
	C	2	2	-	-	-	-
	A	5	3	-	-	2	-
	B	11	2	-	1	3	5
20	C	11	7	-	1	1	2
	D	7	6	1	-	-	-

B

stage of oper-ation	piece grafted	n	normal 2 3 4	? 2 3 4	2 2 3 4	3 2 2 3 4	4 3 3 4
19	A	1	-	-	-	-	1
	B	2	-	-	-	-	2
20	A	10	-	-	-	1	9
	B	9	-	-	-	4	5

Table 2.1 Digit pattern of wings following grafts of posterior mesenchyme (A) 24h after removal of the posterior apical ectodermal ridge, and (B) 24h after removal of the posterior apical ectodermal ridge and placing an FGF-4 soaked bead at the posterior margin of the wing bud.

FGF-4 application	time assayed mesenchyme	piece grafted	n	digit pattern of limbs normal 2 3 4	2 2 3 4	3 3 4	4 3 3 4
none	24h	A	2	-	1	-	1
		B	2	-	1	1	-
	32h	A	3	3	-	-	-
		B	2	2	-	-	-
	48h	B	2	2	-	-	-
apical	24h	A	·5	-	1	1	3
		B	4	1	-	2	1
	32h	A	3	1	-	2	-
		B	4	3	-	1	-
	48h	A	3	3	-	-	-
		B	4	4	-	-	-
posterior	32h	A	3	-	-	-	3
		B	3	-	-	1	2
	48h	A	2	1	-	-	1
		B	4	2	-	1	1

Table 2.2 Digit patterns of wings following grafts of posterior mesenchyme 24h, 32h and 48 hours following either entire ridge removal with or without application of FGF-4 to apical or posterior mesenchyme.

A) posterior mesenchyme cells cultured in micromass.

time in culture	number of grafts	digit pattern of limbs				
		normal 2 3 4	? 2 3 4	2 2 3 4	3 2 2 3 4	4 3 3 4
24h	7	2	1	3	1	-
48h	8	4	1	3	-	-
72h	6	5	-	1	-	-
96h	18	18	-	-	-	-

B) posterior mesenchyme cells in micromass co-cultured with 4-6 ectodermal jackets.

time in culture	number of grafts	digit pattern of limbs				
		normal 2 3 4	? 2 3 4	2 2 3 4	3 3 4	4 3 3 4
72h	17	14	1	1	1	-
96h	13	8	-	5	-	-

C) posterior mesenchyme cells in micromass cultured with FGF-4

time in culture	number of grafts	digit pattern of limbs				
		normal 2 3 4	? 2 3 4	2 2 3 4	3 3 4	4 3 3 4
24h	7	-	-	1	5	1
48h	5	1	-	2	2	-
72h	12	6	-	3	3	-
96h	18	18	-	-	-	-

Table 2.3 Digit pattern of chick wings following grafts of micromass cultures of posterior mesenchyme cells of mouse limb buds.

stage of limb bud removal	FGF-application	humerus	radius	ulna	digits	cases
19-23	no	-	-	-	-	6
19/20	p	2^+	1^*	1	1	2
19/20	p + a	1	1	1	1	1
20/21	p	3	-	3^{*+}	-	3
20/21	an	1	-	-	-	1
20/21	p + a	2	2	2	2	2
21/22	p	2^+	-	-	-	2
21/22	a	1	-	-	-	1
21/22	an	1	-	-	-	1

Table 2.4. Skeletal pattern of wings following removal of whole limb buds of different stages with and without application of FGF soaked beads to different positions of the remaining mesenchyme (- : skeletal element did not develop; p = posterior application; p + a = posterior and apical application; an = anterior application; * = skeletal structure developed partially; + = unidentifiable cartilage structures developed distally to the element)

A

limb bud stage	size of removed part	humerus	radius	ulna	digits	cases
22-24	250μm	4	4	4	-	4
22-23	375-625μm	8	1*	-	-	8
25,26	250-500μm	7	7	7	1	7

Table 2.5A. Skeletal pattern of wings following excision of different sizes of distal limb parts of older limb bud stages without application of FGF soaked beads to the remaining limb stump (* = unidentifiable forearm bone).

B

limb bud stage	size of removed part	humerus	radius	ulna	digits	cases
23-25	250-375μm	9	9	9	8	9
23	400μm	1	1	1	-	1#
24	400μm	1	1	1	1	1+
25/26	250μm	2	2	2	2	2*

Table 2.5B Skeletal pattern of wings following excision of different sizes of distal limb parts and application of FGF soaked beads to the remaining limb stump (+ = anterior; # = posterior and apical; * = apical application of FGF beads to mesenchyme of the remaining stump).

CHAPTER 3

EFFECT OF FGF ON GENE EXPRESSION IN LIMB BUD CELLS

IN VIVO AND *IN VITRO*

3.1 INTRODUCTION

Genes whose expression in the mesenchyme is likely to be regulated at least in part by a signal released from the apical ectodermal ridge include transcription factors and growth factors. For example, as described in Chapter 1, expression of *Msx-1* is found in the progress zone of early limb buds and it appears *Msx-1* expression is regulated by the apical ectodermal ridge (e.g Coelho *et al.*, 1991; Robert *et al.*, 1991; Davidson *et al.*, 1991). Activation of *Hoxd-11* and *Hoxd-13*, which are expressed in overlapping domains within the posterior limb bud, appears to require in addition to a polarizing region signal, a signal released from the ridge (Izpisua-Belmonte *et al.*, 1992a; Koyama *et al.*, 1993). Another possible gene to be regulated by the ridge is *Bmp-2*. *Bmp-2* transcripts are found in posterior mesenchyme (Francis *et al.*, 1994) and throughout the entire apical ectodermal ridge (Lyons *et al.*, 1990; Francis *et al.*, 1994).

I used whole-mount *in situ* hybridisation to investigate to what extent members of the FGF-family are able to substitute for the ridge to maintain expression of *Msx-1*, 5' members of the HoxD-complex, and *Bmp-2* in the developing limb and in cultured posterior mouse limb bud cells. Furthermore, I investigated whether FGF can maintain responsiveness of cultured anterior mesenchyme cells to positional cues in the posterior mesenchyme of the developing limb bud.

3.2 MATERIAL AND METHODS

3.2.1 Removal of the apical ectodermal ridge and application of FGF-4 to young chick wing buds.

The apical ectodermal ridge of stage 19-21 chicken wing buds (Hamburger and Hamilton, 1951) was removed by cutting along the base of the apical ectodermal ridge with tungsten needles. The ridge was lifted and cut off using fine forceps. For FGF-4 application heparin acrylic beads (H5263, Sigma) of a size of 200-250µm were soaked in 2µl of 1mg/ml FGF-4 for at least 1 hour at room temperature, before applying to the limb. To keep the beads in place I used staples formed out of platinum wire (0.025mm^2, Goodfellow). Various time points after the operation, the embryos were removed from the eggs and fixed overnight in 4% paraformaldehyde (PFA) at 4°C. The extraembryonic membranes were removed and the head and hindlimb region was removed. The trunks were washed twice in PBS for 5 minutes, dehydrated in 25%, 50%, 75% Methanol/PBT and twice in 100% methanol for 5 minutes each and then stored at -20°C before using for whole-mount *in situ* hybridisation (see 3.2.5.2).

3.2.2 Micromass cultures of mouse limb bud cells

Micromass cultures were prepared from either the posterior one-third, or anterior one-third of limb buds of 10-10.5 day mouse embryos and cultured following the techniques described in chapter 2. In some experiments, FGF-2 (British Biotechnology Ltd, Oxford) at a concentration of 100 ng/ml, together with Heparan sulphate (Sigma) at

a concentration of 100 ng/ml were added to the serum-containing

culture medium.

3.2.3 Assaying gene expression of cultured limb bud cells after grafting to the anterior or posterior margin of a chick wing bud

24 hours after culturing either posterior or anterior

mesenchyme in the presence or absence of FGF-2, the culture was

scraped off the substratum and cut into pieces. A piece of cultured

posterior mesenchyme cells was then grafted to the anterior margin

of a chick wing in a slit beneath the apical ectodermal ridge.

Cultured anterior cells were grafted to the posterior margin of the

chick wing bud beneath the apical ectodermal ridge. 24-26 hours

following the operation the embryos were fixed in 4% paraformaldehyde

and then either processed for whole-mount *in situ* hybridisation, or

wax embedded and sectioned for radioactive *in situ* hybridisation.

Whole-mount *in situ* hybridisation was carried out as described

(Wilkinson, 1993) and the embryos were probed with chick (S. Wedden),

or mouse *Msx-1* (R. Hill), chick or mouse *Hoxd-11*, or *Hoxd-13* (D.

Duboule), or chick *Bmp-2* (P. Francis-West) derived from plasmids.

Sections were hybridized as previously described (Davidson *et al.*,

1988) with species specific radioactive labelled probes for mouse and

chick *Hoxd-11* and *Hoxd-13*.

Whole-mounts or sections of mouse limb buds from normal embryos were

assayed for their expression pattern to ensure specifity of the mouse

probes.

3.2.4 Radioactive *in situ* hybridisation (ISH)

3.2.4.1. Preparation of ^{35}S-labelled RNA probe

Templates of the riboprobes were linearised with the appropriate restriction enzymes at 37°C for at least 2 hours. The reaction was stopped by adding 10mM EDTA, and the enzyme removed by phenol/chloroform extraction. The template was then preticipitated in 1/10th volume sodium acetate and 2.5 volumes absolute ethanol at -70°C, and then spun down for 25 minutes at 13000 rpm. The template was washed in 70% ethanol, spun down for 4 minutes, before removing the supernatant to let the pellet dry. The dried pellet was resuspended in DDW to make a final concentration of 0.2μg/ml of linearised plasmid and stored at 4°C.

A complementary riboprobe was then synthesized by transcription from the cDNA template. The transcription reaction solution contained 6μl 5x transcription buffer (T3/T7), 1μl 1M dithiothreitol (DTT), 5μl DNA (0.2μg/ml), 5μl DDW, 1μl 10mM thymidine-, 1μl 10mM adenosine-, 1μl 10mM uridine triphosphate, 1μl 50μM cytosin triphosphate and 7μl of ^{35}S-labelled cytosine triphosphate. At last 1.5μl RNAse inhibitor, 1.5μl T3 or T7 RNA polymerase. The added DTT in this reaction protects ^{35}S from oxidation and inhibits RNAse. The labelling reaction was carried out at 37°C for 40 minutes. Further 1μl T3 or T7 RNA polymerase was added and the reaction was continued for further 40 minutes.

To remove the cDNA template 1μl RNAse-free DNAse, 1μl t-RNA (10mg/ml) and 0.5μl RNAse inhibitor was added and incubated for 10 minutes at 37°C. The reaction was stopped with 1μl 0.2 EDTA. The volume was then increased to 200μl with Tris-EDTA buffer containing

50mM DTT for easier handling for the following phenol/chloroform extraction. The probe was precipitated in 1/10 volume of 3M sodium acetate pH 5.2 and 3 volumes of absolute ethanol at -70°C for at least 30 minutes. The pellet was then spund down for 25 minutes at 4°C (13000 rpm), washed twice in 500µl 80% ethanol containing 50mM DTT. After centrifugation for 4 minutes the supernatant was removed and the probe was allowed to dry. The dried probe was resuspended in 100µl DDW containing 50mM DTT. A sample of 1µl probe was then added to 5 ml scintillation solution for counting in a scintillation counter. The probe was stored at -70°C.

3.2.4.2 Probe hydrolysis

The probe was hydrolysed into pieces approximately of 150-300 base pairs in length in order to penetrate the section adequately and thus gain access to the mRNA. The duration of hydrolysis for each probe was calculated. Hydrolysis was achieved by incubating the probe in 95µl sodium bicarbonate (80mM $NaHCO_3$, 120mM Na_2CO_3, pH 10.2) and 5µl 1M DTT at 60°C for the calculated hydrolysis time (15-60 minutes). The reaction was stopped with 100µl 6M ammonium acetate, pH 5.2. The probe was precipitated by adding 1µl 10mg/ml tRNA and 1200µl absolute ethanol at -70°C. The pellet was spun for 25 minutes at 4°C (13000 rpm), washed twice with 80% ethanol containing 50mM DTT. After centrifugation for 5 minutes at 4°C (13000 rpm) the supernatant was carefully removed and the pellet let to dry. The dried pellet was then resuspended in 50µl DDW containing 10mM DTT. The probe was recounted in a scintillation counter and stored at -70°C and used within 4 weeks.

3.2.4.3 Pretreatment of slides for ISH

To prevent that sections get washed off, slides were first cleaned by dipping them for 10 seconds each into acid alcohol (10% HCl in 70% alcohol), DDW and 95% absolute ethanol. The slides were then dried at 150°C overnight. At this stage the slides can be stored or can straight be coated. To coat the slides with 3-aminopropryltriethoxysilane (TESPA) the slides were dipped for 10 seconds in 2% TESPA in acetone, and twice 10 seconds in 100% acetone before drying them at 42°C overnight. The TESPA coated slides were used within 2 days. During the whole treatment gloves were used to prevent RNAse-contamination.

3.2.4.4 Wax processing of specimens

The embryos were fixed in filtered 4% paraformaldehyde in PBS for 24-48 hours at 4°C. The trunk was isolated by cutting off head- and hindlimb region from the embryo. The embryos were then processed after the following schedule:

30 min PBS	(on ice)
30 min 150mM NaCl	(on ice)
15 min 1:1 150mM NaCl: absolute alcohol	(on ice)
15 min 1:1 150mM NaCl: absolute alcohol	(on ice)
15 min 70% alcohol	
15 min 70% alcohol	
30 min 85% alcohol	
30 min 95% alcohol	
30 min 100% alcohol	

30 min 100% alcohol

30 min touluene

30 min toluene

60 min filtered wax (at 60°C)

60 min filtered wax (at 60°C)

After embedding the embryos the wax blocks were stored at 4°C.

3.2.4.5 Wax sectioning

Ribbons of 8μm were cut using a microtome and mounted in DDW
onto a slide. Every 10 section was collected and placed on a slide.
This sections were stained with Biebrich-Scarlet to localise the
mouse grafts in the sections. The sections containing the graft were
then placed on TESPA-coated slides in this way that adjacent sections
were placed on different slides to probe with different probes. The
slides were dried overnight on a warm plate and stored at room
temperature in sealed boxes containing silicial gel.

3.2.4.6 Prehybridisation washes for ISH

Pretreatment of the sections is necessary, a) in order to
increase the permeability of the tissue to the riboprobe, using
proteinase K and b) to reduce non-specific binding of the riboprobe
to basic residues, by acetylating basic residues with acetic
anhydride.

Slides were first dewaxed in xylene for 10 minutes, then rehy-
drated for 2 minutes each in 100%, 90%, 70%, 50% and 30% ethanol.

Slides were then washed for 5 minutes each in 0.85% NaCl and PBS before fixation for 20 minutes in fresh and filtered 4% PFA. Fresh PFA is required, because it degrades quickly to formaldehyde which will increase the cross-linking. After the fixation the slides were twice washed in PBS for 5 minutes and the section were then incubated in 20μg/ml proteinase K in 50 mM Tris, 5mM EDTA pH 7.2 for 7.5 minutes. Following two 5 minutes washes in PBS the sections were refixed with 4% paraformaldehyde for 5 minutes. After rinsing the slides for 10 seconds in DDW the sections were acetylated by incubation for 10 minutes in 0.1M triethanolamine (TEA) with 1/400 volume acetic anhydride. This reaction was carried out on a stirrer in the fumehood. Because of the short halflife of only 1 minute, acetic anhydride was added just prior the sections were added to the TEA. Sections were then washed 5 minutes each in PBS and 0.85% NaCl, dehydrated through alcohols for 1 minute each in 30%, 50%, 70%, 90% and 5 minutes in 100% ethanol. The slides were then air dried and protected from dust. Except of Proteinase K and TEA solutions, all solutions can be reused for up to 6 times.

3.2.4.7 Hybridisation

The probe was diluted with 50mM DTT/TE to 1×10^6 cpm/ml to prevent pipetting faults in a total volume of 250μl. The probe was then further diluted with hybridisation buffer to a final concentration of 1×10^5 cpm/ml. 2 ml of hybrisation buffer contained 1 ml formamide, 100μl 0.5 mg/ml t-RNA, 400μl 50% dextran sulphate, 20μl 100x denhardts, 40μl 1M Tris (pH 8), 120μl 5M NaCl, 20μl 0.5M EDTA, 20μl 1M NaPO$_4$ (pH 6.8), 20μl DTT and 260μl DDW. The hybridisation buffer was vortexed well, spun down and stored at -70°C for not

longer than 6 month.

After adding the probe to the hybridisation buffer the mixture was vortexed and spun for 3 minutes. The probe was then denatured at 80°C for 2 minutes and rapidly cooled on ice. A volume of 70µl probe (10^5 cpm/ml) was placed on each slide and covered with a coverslip and bubbles were carefully removed. The slides were then placed into hybridisation chambers containing whatman paper soaked in 10 ml 50% formamide and 2x SSC (sodium chlorid/ sodium citrate) to maintain humidity. The sealed box was incubated for 15-18 hours at 55°C.

3.2.4.8 Posthybridisation washes

Posthybridisation washes are used to remove as much unhybridised probe as possible to decrease the background. Therefore the slides are washed at high stringency to break weaker interactions that ate involved in non-specific binding of the probe. In addition RNAse is used to degrade single stranded RNA.

Coverslips were first removed by incubating the slides for 15 minutes in pre-warmed 5x SSC containing 10mM DTT at 55°C. Following two stringent washes in 50% formamide, 10mM DTT and 2x SSC at 65°C the slides were then washed twice in NTE (0.5M NaCl, 10mM Tris (pH 7.5), 5mM EDTA) at 37°C for 15 minutes. RNAse treatment was carried out in coplin jars by adding 40µg/ml RNAse to NTE buffer. The slides were then washed in NTE buffer at 37°C for 30 minutes. This was followed by a second stringent wash in 50% formamide, 10mM DTT, and 2x SSC at 65°C for 25 minutes. The slides were then washed in decreasing salt concentrations, twice in 2x SSC and twice in 0.1x SSC for 10 minutes at room temperature. Sections were then dehydrated

through 30%, 50%, 70%, 90% ethanol containing 0.3 M ammonium acetate
for 1 minute each followed by 5 minutes in absolute alcohol. Slides
were then dried.

3.2.4.9 Autoradiography, development and staining

The slides were placed into an autoradiography box and fixed
with tape. A Kodak fast film was then placed onto the slides, the box
sealed and stored at room temperature. After 2-3 days exposer the
film was developed to see if the *in situ* hybridisation had worked and
to calculate the exposure time.

The slides were carefully dipped in prewarmed (40°C) emulsion
containing a 1:1 emulsion and DDW. All appearing bubbles were
removed. The slides were dried and stored to develop in sealed box at
4°C for 5 days to 5 weeks.

Slides were developed in Kodak D19 developer for 5 minutes,
rinsed in tap water and fixed in Kodak Unifix for 5 minutes. After
rinsing the slides for 20 minutes under tap water and rinsing in DDW
the sections were stained in 0.5% Malachitgreen not longer than 5
minutes. The slides were again rinsed with tap water until the right
staining was achieved and then dried overnight. The sections were
coverslipped and dried. The slides were then carefully cleaned from
the emulsion using a rasor blade and 70% alcohol.

3.2.5 Whole mount *in situ* hybridisation

3.2.5.1. Preparation of the DIG-labelled RNA probe

The template of the riboprobe was linearised with an
appropriate enzyme, cleaned by phenol/chloroform extraction, ethanol

100

precipitated and resuspended in DDW at 0.2μg/ml (as descried above) and stored at 4°C.

A complementary riboprobe was then synthesized by transcription from the cDNA template. To synthesize the probe a transcription reaction was set up consisting of 4μl 5x T3/T7 transcription buffer, 1 μl 0.2M DTT, 5μl DDW, 2.5 x Nucleotidtriphosphate-mix (2.5mM GTP; 25mM ATP; 25mM CTP; 1.625mM UTP, 0.875mM DIG-UTP (Boehringer Mannheim GmbH), 5μl 0.2μg/μl linearised plasmid, 1μl (50 units) RNase inhibitor and 1μl (10 units) transcription enzyme (T3 or T7). The reaction was then incubated at 37°C for 2 hours. The eppendorf was vortexed and 1μl of the reaction was then run on a 1% agarose/TBE minigel to ensure that the probe had been transcribed properly and to estimate the amount synthesized. To remove the DNA template 2μl of ribonuclease-free DNaseI was added and the reaction was incubated for 15 minutes at 37°C. To precipitate the probe 100μl 1M TE, 10μl (4M) lithium chloride and 300μl absolute ethanol was added to the reaction, mixed and incubated at -20°C for at least 1 hour. The eppendorf was vortexed and microfuged at 12 000 rpm for 10 min at 4°C. The pellet was then washed with 70% ethanol and respun for 2 minutes at 12 000 rpm at 4°C. The 70% ethanol was removed and the pellet air dried. The DIG-labelled probe was redissolved in TE at approximatly 0.1μg/μl and stored at -20°C.

3.2.5.2 Pretreatments

(After Wilkinson, 1993)

The embryos (see 3.2.1) were rehydrated in 75%, 50% and 25% methanol/PBT before washing them twice in PBT (5 minutes each). The

embryos were then bleached in 6% hydrogen peroxide/PBT for 1 hour to inhibit the alkaline phosphatase. This was followed by 3 washes in PBT and treatment with 10μg/ml proteinase K in PBT for 15 minutes. To inactivate the proteinase K the embryos were washed in 2mg/ml glycine in PBT for 5 minutes, followed by two washes in PBT. The embryos were refixed in fresh 0.2% glutaraldehyde/4% PFA in PBT for 20 minutes and washed twice in PBT. The PBT was replaced with 1.5ml prehybridisation mix (50% deionized formamide, 5xSSC pH 4.5, 50μg/ml phenol/chloroform treated yeast total RNA, 50μ/ml heparin, 1% SDS) for 5 minutes. The prehybridisation mix was replaced with fresh prehybridisation mix and incubated at 70°C for 1 hour (embryos can be stored at -20°C at this time point). The hybridisation mix including approximately 1μg/ml DIG-labelled RNA probe was added and the embryos incubated at 70°C overnight.

3.2.5.3. Posthybridisation washes

The next day the hybridisation mix was removed and stored at -20°C to reuse for up to 3 times. The embryos were then washed twice with prewarmed solution 1 (50% deionised formamide, 5x SSC pH 7.5, 0.1% SDS) at 70°C for 20 minutes. This was followed by a 10 minutes wash at 70°C in an equal volume of solution 1 : solution 2 (solution 2 - 0.5M NaCl, 10mM TrisHCl, pH 7.5, 0.1% Tween-20). Three 5 minute washes were carried out in solution 2 at room temperature, followed by two washes in 100μg/ml RNase A in solution 2 for 30 minutes at 37°C to remove unbound RNA and DIG-labelled probe. The embryos were then washed twice in solution 2 for 5 minutes before washing them 5 minutes in solution 3 (50% formamide, 2x SSC, pH 4.5) at room temperature. Two washes were then carried out in solution 3 for 30

minutes at 65°C, followed by three 5 minute washes in TBST (stock

solution of 10xTBS: 100ml: 8g NaCl, 0.2g KCl, 25ml 1M TrisHCl, pH

7.5; This was diluted to 1x TBS and 1% Tween-20 and 2mM levimasole

was added on the day of use). The embryos were then preblocked for 90

minutes with 10% sheep serum in TBST. The sheep serum was heat

treated at 70°C 30 minutes before use. During this, the anti-

digoxygenin antibody was preabsorbed as followed. 3 mg of chick or

mouse embryo powder (see below) was weighed out, 0.5 ml TBST and 5μl

sheep serum per embryo was added and heat treated at 70°C for 30

minutes. After cooling on ice 1μl of anti-digoxygenin antibody was

added and shaken gently at 4°C for 1 hour. This was then microfuged

for 10 minutes. The supernatant was taken and put into a fresh tube

and diluted with 2ml 1% heat-treated sheep serum in TBST. The 10%

blocking serum was then removed from the embryos and replaced with

preabsorbed antibody solution and rocked overnight at 4°C.

Mouse or chick embryo powder was prepared as followed: 12.5-

14.5d old mouse or 10d old chick embryos were homogenised in a

minimum volume of PBS. Four volumes of ice-cold acetone was added and

this was mixed well and placed on ice for 30 minutes. This was

centrifuged at 10 000 rpm for 10 minutes, the supernatant discarded

and the pellet washed in ice-cold acetone, before recentrifuging. The

pellet was grind into fine powder and allowed to air-dry. The embryo

powder was stored in an air-tight tube at 4°C.

3.2.5.4 Post-antibody washes

The next day the antibody was removed and the embryos washed 3 times in TBST for 5 minutes, followed by 5 washes in TBST for 30 minutes. The embryos were then washed 3 times for 10 minutes in NTMT (100mM NaCl. 100mM Tris-HCl, pH 9.5, 50mM $MgCl^2$, 0.1% Tween-20, 2mM levimasole - made from stocks on day of use). The embryos were then incubated in NTMT including 4.5μl NBT (nitroblue tetrazolium salt - 75mg/ml in 70% dimethylformamide), and 3.5μl BCIP (5-bromo-4-chloro-3-indolyl phosphate - 50mg/ml in dimethylformamide) per ml for 20 minutes and kept in the dark until the colour had developed to the desired extent. To stop the colour reaction the embryos were then washed twice in PBT and stored at 4°C, ready to be photographed.

3.3 RESULTS

3.3.1 Effects of FGF-4 on gene expression in the developing limb bud *in vivo*

3.3.1.1 *Msx-1* expression

In the normal limb bud at stage 24, *Msx-1* is strongly expressed in the posterior and anterior mesenchyme and weakly in mesenchyme at the apex of the bud (Fig. 3.2A). Previous work has shown that after removal of the ridge, *Msx-1* expression in posterior mesenchyme is rapidly lost but anterior expression is relatively stable (Ros *et al.*, 1992). To investigate whether local application of FGF-4 can maintain *Msx-1* expression in posterior mesenchyme after entire ridge removal, a bead soaked in FGF-4 was applied to posterior mesenchyme. I found that *Msx-1* expression was maintained around the part of the bead that integrated into the posterior mesenchyme (Fig. 3.1A; Tab. 3.1A). However, I also noted that *Msx-1* expression in the anterior mesenchyme was not maintained in the absence of the ridge (Fig. 3.1A). Therefore I tested whether FGF application to apical mesenchyme after entire ridge removal could maintain *Msx-1* expression. Strong expression was found in anterior mesenchyme between bead and nearby ectoderm, but transcripts of *Msx-1* were less abundant in the apical mesenchyme (Fig. 3.1B; Tab. 3.1A). Thus, just as in a normal wing bud at stage 24 (Fig. 3.2A), *Msx-1* was expressed strongly anteriorly and weakly apically. When two FGF-4 beads were simultanously applied to apical and posterior mesenchyme after removal of the entire ridge *Msx-1* expression was maintained locally by both beads. *Msx-1* was maintained strongly in anterior and

posterior mesenchyme, whereas *Msx-1* transcripts were less abundant in the mesenchyme at the apex of the bud (Fig. 3.1C; Tab. 3.1A). Thus, after ridge removal, local application of FGF-4 can reproduce an almost normal pattern of *Msx-1* expression (compare with Fig. 3.2A).

To compare local effects of both FGF-4 and the apical ectodermal ridge on *Msx-1* gene expression during limb development, I removed either the anterior or posterior half of the ridge and, in some cases, applied FGF-4 soaked beads to the limb mesenchyme. 24 hours following posterior ridge removal without application of FGF-4, outgrowth ceased posteriorly resulting in a limb with skewed shape (Fig. 3.2B,E,H). *Msx-1* expression was lost in posterior mesenchyme but was still locally maintained in mesenchyme beneath both the anterior apical ectodermal ridge and anterior ectoderm (3 cases, Fig. 3.2B; Tab. 3.1B). Application of FGF-4 to posterior limb mesenchyme after posterior ridge removal maintained posterior outgrowth and *Msx-1* expression around the part of the bead that integrated into the mesenchyme (4 cases, Fig. 3.2C; Tab. 3.1B).

After anterior ridge removal without application of FGF-4 *Msx-1* expression was not detectable in anterior limb mesenchyme at 24 hours, although low levels of *Msx-1* transcripts were found in the anterior part of the shoulder region and expression was locally maintained in mesenchyme beneath the posterior apical ectodermal ridge (3 cases, Fig. 3.3A; Tab. 3.1B). Application of FGF-4 to anterior mesenchyme after anterior ridge removal lead to local maintenance of *Msx-1* expresssion and transcripts of *Msx-1* were found between bead and anterior ectoderm (2 cases, Fig. 3.3B; Tab. 3.1B). Generally I found that when the FGF-4 bead completely integrated into the limb mesenchyme, *Msx-1* expression was never found around the

whole bead, but instead expression was localised to nearby mesenchyme
between bead and ectoderm. When the bead was stapled to the outside
of the limb, *Msx-1* expression was found around that part of the bead
that integrated into the mesenchyme. Thus, FGF-4 can maintain *Msx-1*
expression in mesenchymal cells at the rim of the limb.

3.3.1.2 *Hoxd-13* expression

Removal of the posterior ridge lead to downregulation of *Hoxd-13* expression. 24 hours after removal of the posterior half of the
ridge, transcripts of *Hoxd-13* were not detectable in whole-mounts (3
cases, Fig. 3.2E; Tab. 3.1B), but application of FGF-4 maintained
Hoxd-13 expression in posterior distal mesenchyme. At 24 hours after
FGF-4 application a small patch of expression of *Hoxd-13* in the
distal apical mesenchyme was detectable in 1 out of 3 cases, and
Hoxd-13 transcripts were found in distal posterior mesenchyme between
the integrated bead and ectoderm even at 48 hours in 2 out of 3 cases
(Fig. 3.2F; Tab. 3.1B). In the other case in which the bead ended up
proximally at 48 hours, *Hoxd-13* was not detectable and posterior
outgrowth was not observed. Thus, *Hoxd-13* expression is dependent
upon a signal released from the apical ectodermal ridge, which can be
provided by FGF-4.

3.3.1.3 *Bmp-2*-expression

In the developing chick limb bud, *Bmp-2* expression is localised
to the posterior mesenchyme and the apical ectodermal ridge (Fig.
3.2G, see also Francis *et al.*, 1994). When the posterior ridge was
removed, *Bmp-2* expression was not detectable in posterior mesenchyme

24h following the operation (3 cases, Fig. 3.2H; Tab. 3.1B).

Application of FGF-4 to posterior mesenchyme maintained mesenchymal

Bmp-2 expression. *Bmp-2* tanscripts were not found evenly around the

bead and transcripts were more abundant between bead and posterior

ectoderm. In addition, it appeared that *Bmp-2* transcripts were more

abundant in the FGF-4 treated limb bud compared to the contralateral

limb (3 cases, compare Fig. 3.2G,I; Tab. 3.1B). Thus, FGF-4 can

subsitute for the ridge to maintain *Bmp-2* expression in posterior

mesenchyme.

After both sets of experiments, posterior ridge removal with

and without addition of FGF-4 to posterior mesenchyme, *Bmp-2*

expression was found in the anterior ridge which was left intact,

although it appeared that expression of *Bmp-2* in the anterior ridge

was more intense when FGF-4 was applied to posterior mesenchyme. With

posterior FGF-4 application there was no epithelial expression of

Bmp-2 detectable in the posterior part of the bud showing that the

healed ectoderm did not express ridge specific molecules.

3.3.2 Effects of FGF-2 on gene expresssion on cultured posterior limb bud cells

I further investigated, whether FGF could maintain gene

expression in cultured limb bud cells. Mouse posterior limb bud cells

were placed in high density culture for 24 hours either in the

absence or presence of FGF-2. I then grafted the cultured cells to

the anterior margin of a host chick limb bud and 24 hours later

assayed for expression of *Msx-1*, *Hoxd-11* and *Hoxd-13* using probes

specific for the mouse genes.

3.3.2.1 *Msx-1* expression

In grafts made from posterior cells cultured in the absence of
FGF-2, there was no detectable expression of *Msx-1* (2 cases, Fig.
3.4A; Tab. 3.2).
Msx-1 expression was detected in grafted cells which had been
cultured in the presence of FGF-2. In these cases *Msx-1* expression
was found in a small stripe at the periphery of the graft near the
apical ectodermal ridge (2 cases; Fig. 3.4B; Tab. 3.2).

3.3.2.2 *Hoxd gene* expression

Hoxd-11 transcripts were in grafted cells which had been
cultured both in the presence and absence of FGF-2. In both, whole
mounts and in sections using radioactively labelled probes,
expression of *Hoxd-11* was found throughout the graft, but higher
signal appeared to be located in more distal parts of the graft (Fig.
3.4C,D; Fig. 3.5A,D; Tab. 3.2). *Hoxd-11* transcripts in grafts of
cells cultured in the absence of FGF-2 appeared to be less abundant
than in grafts of cells cultured in the presence of FGF-2 (6 cases,
compare Fig. 3.4C,D). In contrast, *Hoxd-13* transcripts were only
observed in those grafts which comprised cells cultured in the
presence of FGF-2. In those cases, expression was found in patches in
the graft (5 cases, Fig. 3.4F; 3.5B; Tab. 3.2). Expression of *Hoxd-13*
was not detectable in grafted cells that had been cultured in the
absence of FGF-2 (Fig. 3.4E, 3.5E; Tab. 3.2). Thus, it appeared that
FGF is required for continued expression of *Hoxd-13* in cultured
cells, whereas *Hoxd-11* expression appears to be more stable. In
addition, 26 hours after grafting FGF-2 treated posterior cells to

the anterior margin of a chick wing bud, ectopic expression of *Hoxd-13* in host anterior limb mesenchyme was observed (1/3 cases; Fig. 3.5C). No ectopic expression of either *Hoxd-11* or *Hoxd-13* was detectable 26 hours after grafting cells that had been cultured in the absence of FGF-2 (3 cases; Fig. 3.5F).

3.3.3 FGF-2 maintains the responsiveness of cultured anterior mesenchyme cells to positional cues within the developing limb

Previous work has shown that when anterior cells are grafted directly to the posterior margin of a host chick wing bud, they start to express *Hoxd-11* and *Hoxd-13* (Izpisua-Belmonte *et al.*, 1992b). To investigate whether FGF-2 can maintain responsiveness of cultured anterior cells to positional cues within the limb, I cultured anterior mesenchyme cells either in the absence or presence of FGF-2. 24 hours after cells cultured in the absence of FGF-2 had been grafted to the posterior margin of a chick wing bud, neither *Hoxd-11* nor *Hoxd-13* transcripts were detectable in grafted cells (Fig. 3.4G; Tab. 3.2). However, *Hoxd-11* and *Hoxd-13* transcripts were detected in grafts of cells that had been cultured in the presence of FGF-2. Transcripts of *Hoxd-11* (Tab. 3.2 and data not shown) as well as *Hoxd-13* (Fig. 3.4H; Tab. 3.2) were found distally in the graft.

3.4 DISCUSSION

Expression of *Msx-1*, *Hoxd-13* and *Bmp-2* in posterior mesenchyme of the limb bud is dependent on a local signal from the apical ectodermal ridge. When the apical ectodermal ridge of young chick wing buds is removed, or when posterior cells are taken from the bud and placed in culture, gene expression of *Msx-1*, *Hoxd-13*, and *Bmp-2* is no longer detected. In contrast, *Hoxd-11* expression in cultured posterior mesenchyme appears to be more stable. Local application of FGF-4 soaked beads to posterior.limb mesenchyme following ridge removal, or addition of FGF-2 to cultured posterior cells maintains expression of *Msx-1*, *Hoxd-13* and *Bmp-2*. Expression of *Msx-1* in anterior and apical cells is also locally maintained by the apical ectodermal ridge and this effect can be reproduced by local application of FGF-4. Furthermore, addition of FGF-2 to cultured anterior limb bud cells maintains their ability to respond to positional cues when grafted back to chick limb buds.

3.4.1 Expression of *Msx-1*, *Hoxd-13* and *Bmp-2* in posterior limb mesenchyme is dependent on a local signal from the apical ectodermal ridge.

Following removal of the posterior ridge, expression of *Msx-1*, *Hoxd-13* and *Bmp-2* in posterior mesenchyme is not detectable. In addition, posterior mesenchyme cells isolated from the ridge, cultured, and grafted back into limb buds do not express *Msx-1* or *Hoxd-13*. These results are consistent with previous work that indicated that *Msx-1* expression is largely dependent upon a signal released by the apical ectodermal ridge (Davidson *et al.*, 1991;

Robert *et al.*, 1991; Ros *et al.*, 1992; Coelho *et al.*, 1991b; 1993).

In contrast to *Msx-1* expression, previous work suggested that

expression of posterior Hoxd genes is relatively stable in posterior

cells when the apical ectodermal ridge is removed (see for example

Izpisua-Belmonte *et al.*, 1992b). Here I could not detect transcripts

of *Hoxd-13* in the absence of the ridge, although *Hoxd-11* expression

was found both after ridge removal *in vivo* (Hayamizu *et al.*, 1994;

Niswander, personal communication) and in cultured posterior

mesenchyme cells. Thus, it appears that *Hoxd-11* expression is

relatively stable in the absence of the ridge, whereas expression of

Hoxd-13 and *Msx-1* appears to be dependent on a factor(s) released by

the apical ectodermal ridge. The present results show that *Bmp-2*

expression in posterior limb mesenchyme is also maintained by the

posterior ridge and that anterior ridge is not sufficient to maintain

Bmp-2 expression. Thus, it appears that the anterior ridge cannot act

at a distance to maintain *Bmp-2* expression in posterior limb

mesenchyme and that a local acting factor maintains *Bmp-2* expression.

3.4.2 FGF-4 maintains *Msx-1*, *Hoxd-13* and *Bmp-2* expression in posterior mesenchyme cells

FGF-2 has already been shown to substitute for the ridge and to

maintain *Msx-1*, *Hoxd-11* and *Hoxd-13* expression in the developing limb

bud (Niswander and Martin, 1993a; Fallon *et al.*, 1994). Here I show

(in addition to our previous *in vitro* studies, Niswander and Martin,

1993a) that FGF-4 too can locally maintain expression of these genes

as the bud continues to grow out *in vivo*. Moreover, I show that *Bmp-2*

expression is dependent on the ridge and that FGF-4 can maintain *Bmp-*

2 expression in posterior limb mesenchyme.

The *Msx-1* expression pattern that is maintained by two FGF-4 beads placed appropriately in the operated limb bud is similar to the expression pattern observed at this stage during normal limb development. Thus, *Msx-1*-expression is maintained strongly in anterior and posterior mesenchyme, but only weakly in apical mesenchyme. In posterior cells cultured in the presence of FGF-2 and then grafted to the anterior limb bud margin, *Msx-1* expression in the graft is located in cells nearest the ridge. Previous studies suggested that *Msx-1* expression is maintained in cultured limb mesenchyme cells by FGF-2 (Watanbe and Ide, 1993). It is possible that the pattern of *Msx-1* expression in the graft is regulated by signals released from the host ridge when the cells are placed in the limb (see also Davidson *et al.*, 1991). This would explain why cells in the graft furthest from the ridge do not express *Msx-1*.

FGF-4 application maintains *Hoxd-13* expression in the distal posterior mesenchyme after either posterior or entire ridge removal. The continued expression of *Hoxd-13* at the tip of the elongated bud is consistent with the idea that an FGF family member is one of the signals that is required for *Hoxd* expression. Maintenance of *Hoxd-13* expression in posterior mesenchyme of the limb bud is correlated with posterior outgrowth. If posterior outgrowth does not occur, *Hoxd-13* expression in the developing limb is not maintained and digits do not develop. However, digits can develop in the absence of *Hoxd-13* transcripts (Dolle *et al.*, 1993).

Posterior cells cultured in the presence of FGF-2 continue to express *Hoxd-11* and *Hoxd-13* when grafted back into the limb. Although *Hoxd-11* expression is maintained in posterior cells that have been cultured in the absence and presence of FGF-2, it appears that FGF-2

maintains higher levels of *Hoxd-11* expression in the graft. Expression of *Hoxd-11* is found throughout the graft, whereas expression of *Hoxd-13* is localised to patches within the graft. The patchy pattern of *Hoxd-13* expresssion could be due to sorting of limb bud cells in culture (Watanabe and Ide, 1993; Ide *et al.*, 1994). The maintenance of *Hoxd-13* expression by FGF both *in vivo* and *in vitro* suggests that an FGF-family member, possibly FGF-4, is required for expression of *Hoxd-13* in posterior mesenchyme, whereas expression of *Hoxd-11* appears to be less dependent of FGF. This is consistent with the observed expression pattern of *Hoxd-11* and *Hoxd-13* in the developing limb. Expression of *Hoxd-11* is localised to regions in mesenchyme that do not appear to be influenced by the ridge, whereas *Hoxd-13* expression remains at the tip of the outgrowing bud near the ridge.

Bmp-2 expression in posterior limb mesenchyme is also maintained by FGF-4. *Bmp-2*, which is a vertebrate homolog of the Drosophila *decapentaplegic* (*dpp*) gene, has been suggested to function downstream of *Shh*. This is based on genetic studies in Drosophila which indicate that *hedgehog* induces *dpp* expression (Baseler and Struhl, 1994). In the vertebrate limb, *Bmp-2* is expressed in posterior mesenchyme in a domain that overlaps and extends slightly beyond the *Shh* expression domain (Francis *et al.*, 1994; Riddle *et al.*, 1993). *Shh* expression in posterior mesenchyme is also dependent on a ridge signal and FGF-4 can provide this signal (Niswander *et al.*, 1994). Therefore, following ridge removal, it could be that *Bmp-2* expression is lost because *Shh* is not maintained in the posterior mesenchyme. Following this reasoning, when the ridge is removed and FGF-4 applied, *Bmp-2* may be maintained because *Shh* expression is maintained.

Bmp-2 is expressed in the apical ridge in addition to posterior limb mesenchyme (Lyons *et al.*, 1990; Francis *et al.*, 1994). Following posterior ridge removal, *Bmp-2* is still detectable in the anterior ridge. Therefore, *Bmp-2* expression in the anterior ridge does not appear to be dependent on signals from the posterior ridge. This correlates with previous work, suggesting that the apical ectodermal ridge may be a self-autonomous cell-population (Saunders *et al.*, 1976). However, our studies do not indicate whether anterior *Bmp-2* expression is dependent on signals from either the underlying anterior mesenchyme or posterior.mesenchyme. Thus, I have shown that FGF can maintain *Msx-1*, *Hoxd-13* and *Bmp-2* expression in posterior limb mesenchyme. The maintenance of gene expression in posterior mesenchyme by FGF might explain why polarizing activity is maintained by FGF in the absence of the ridge either *in vivo* or in *in vitro* (Anderson *et al.*, 1993; Niswander *et al.*, 1993; Chapter 2).

3.4.3 Regulation of gene expression in anterior mesenchyme cells

Msx-1 expression in anterior cells is dependent upon signals from the anterior apical ectodermal ridge and these can be provided by FGF-4. However, *Fgf-4* is only expressed in the posterior part of the apical ectodermal ridge, and it is possible that FGF-4 mimics the function of another FGF-family member that is expressed throughout the ridge, such as FGF-2 or FGF-8 (Savage *et al.*, 1993; Dono and Zeller, 1994; Heikinheimo *et al.*, 1994; Ohuchi *et al.*, 1994). *Msx-1* expression may keep cells in a proliferating and undifferentiated cell state (Song *et al.*, 1992) and may be associated with progress zone function. Our previous studies suggest that in the absence of the ridge, a signal from the polarizing region together with FGF-4 is

required in order to maintain an active progress zone (Niswander *et al.*, 1993; Niswander *et al.*, 1994). In this context it is surprising that apical application of FGF maintains *Msx-1* expression, although the progress zone is not maintained. This suggests that *Msx-1* expression may not be directly related to progress zone function.

Anterior mesenchyme cells are able to respond to endogenous signals in the limb such that they begin to express *Hoxd-11* and *Hoxd-13* when grafted directly to posterior margin of a chick limb (Izpisua-Belmonte *et al.*, 1992b). Here, I show that anterior cells maintained in culture require FGF to retain the ability to respond to positional signals when grafted to the posterior limb mesenchyme. This is consistent with previous results which showed that *Hoxd* gene activation in anterior cells requires cooperation between the ridge and a polarizing signal (Izpisua-Belmonte *et al.*, 1992a; Koyama *et al.*, 1993).

Fig. 3.1 Distribution of *Msx-1* transcripts in whole mount prepara-
tions of wing buds at stage 23/24, 24 hours after entire ridge
removal and application of FGF-4 soaked beads to (A) posterior
mesenchyme, (B) apical mesenchyme, or (C) both posterior and apical
mesenchyme. Regions to which probe has hybridized stained purple.
After entire ridge removal and FGF-4 application to posterior
mesenchyme the humerus, ulna and posterior digits develop
(schematically diagrammed in (A), bottom left), apical FGF-4
application allows development of humerus, radius and ulna (B, bottom
left) whereas application of two FGF-4 beads to apical and posterior
mesenchyme allows development of a virtually complete limb (C, bottom
left).
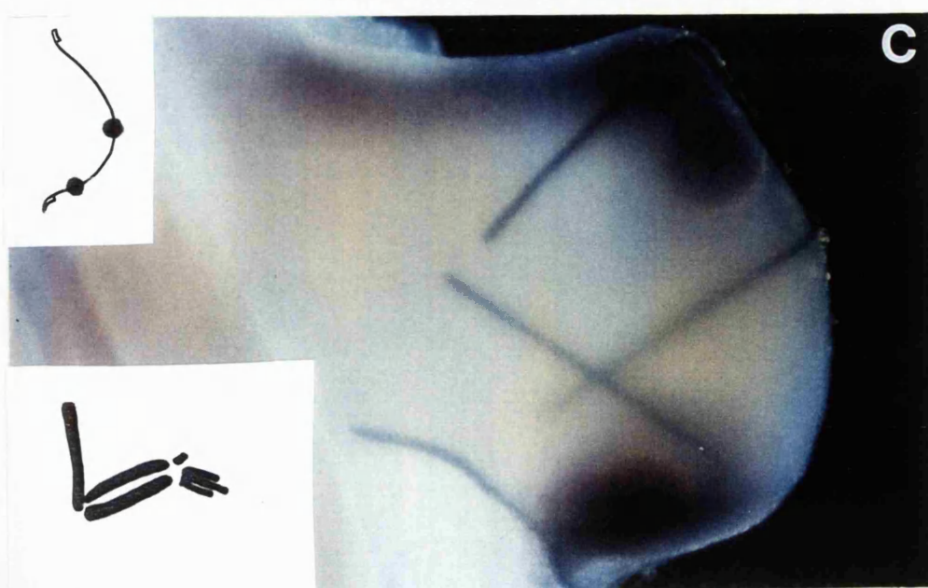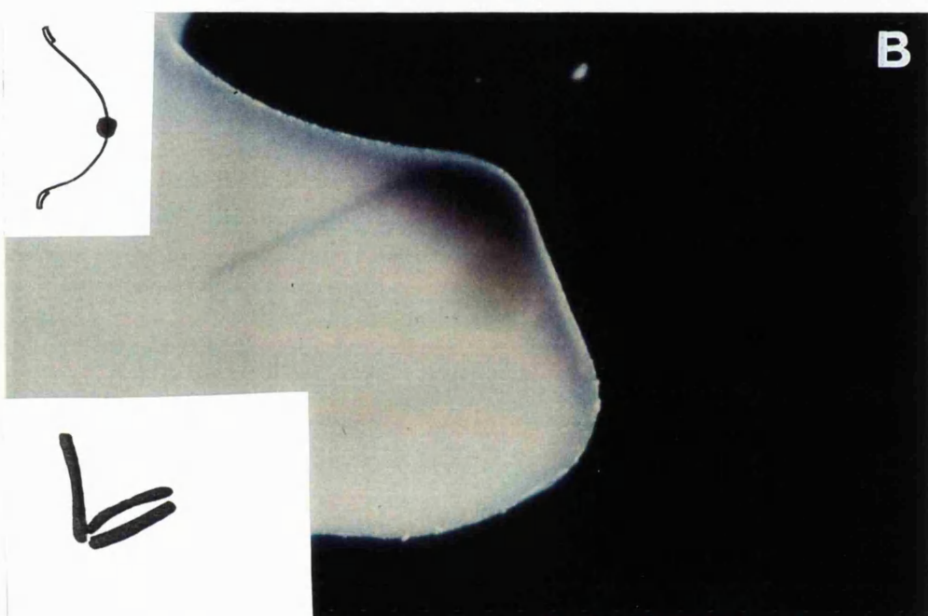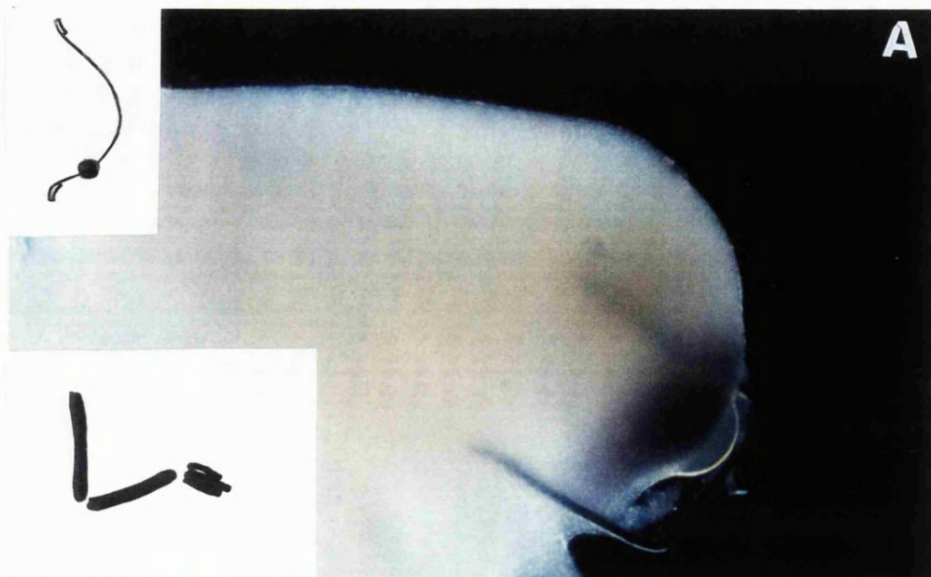
Fig. 3.2 Gene expression of (A) *Msx-1*, (D) *Hoxd-13* and (G) *Bmp-2* in whole mounts of developing limb buds (stage 24). *Msx-1* transcripts were abundant in anterior mesenchyme beneath the anterior ridge/ectoderm and posterior ridge, whereas transcripts were less abundant in the apical mesenchyme beneath the ridge (A). *Hoxd-13* transcripts were localized to distal posterior mesenchyme beneath the ridge (D). *Bmp-2* transcripts were found in posterior limb mesenchyme and in the entire apical ectodermal ridge (G). 24 hours after posterior ridge removal expression of (B) *Msx-1* was not detectable in posterior mesenchyme but expression is locally maintained underneath the anterior ridge. Following posterior ridge removal, expression of (E) *Hoxd-13* and (H) *Bmp-2* was not detectable in posterior mesenchyme. Application of FGF-4 to posterior mesenchyme after posterior ridge removal (C,F,I), locally maintained (C) *Msx-1* expression around the part of the bead that integrated into the mesenchyme at 24 hours. (F) *Hoxd-13* transcripts were localized to the distal mesenchyme 48 hours after the operation and (I)*Bmp-2* transcripts were found around the FGF-4 bead in posterior mesenchyme at 24 hours. Posterior ridge removal was followed by the development of humerus and radius only (B, bottom left). Application of FGF-4 soaked beads to the posterior mesenchyme following posterior ridge removal allowed the development of a virtually complete limb (C, bottom left).
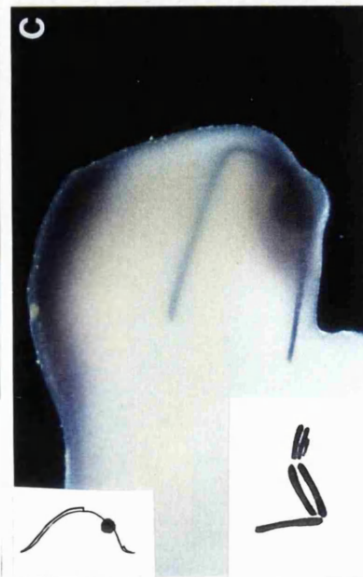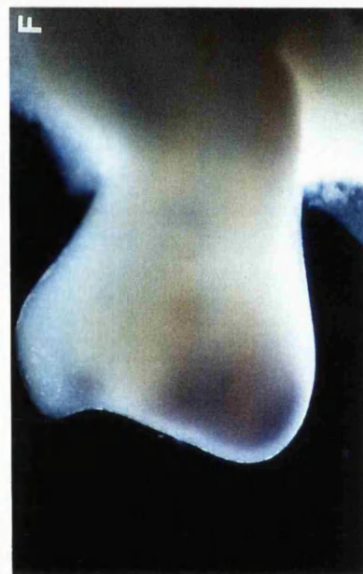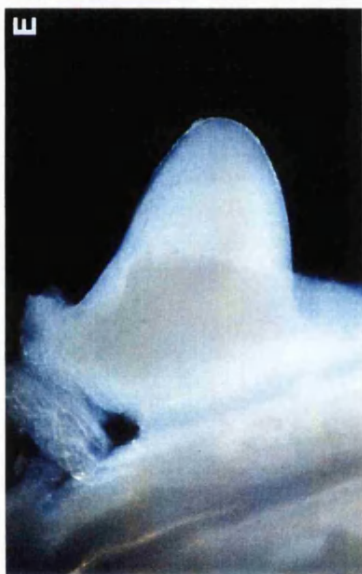
Fig. 3.3 *Msx-1* expression in whole mounts 24 hours after anterior

ridge removal. (A) *Msx-1* expression was maintained beneath the

posterior ridge, whereas expression in anterior mesenchyme was lost.

(B) Application of a FGF-4 soaked bead to the anterior mesenchyme

after anterior ridge removal maintained locally expression of *Msx-1*.

Note that *Msx-1* transcripts were localized between the FGF-4 bead and

the ectoderm. Following anterior ridge removal, posterior skeletal

structures were formed, but radius and digit 2 failed to develop (A,

bottom left). Application of FGF-4 soaked beads to anterior

mesenchyme after anterior ridge removal allowed in some cases the

development of part of the radius (B, bottom left).
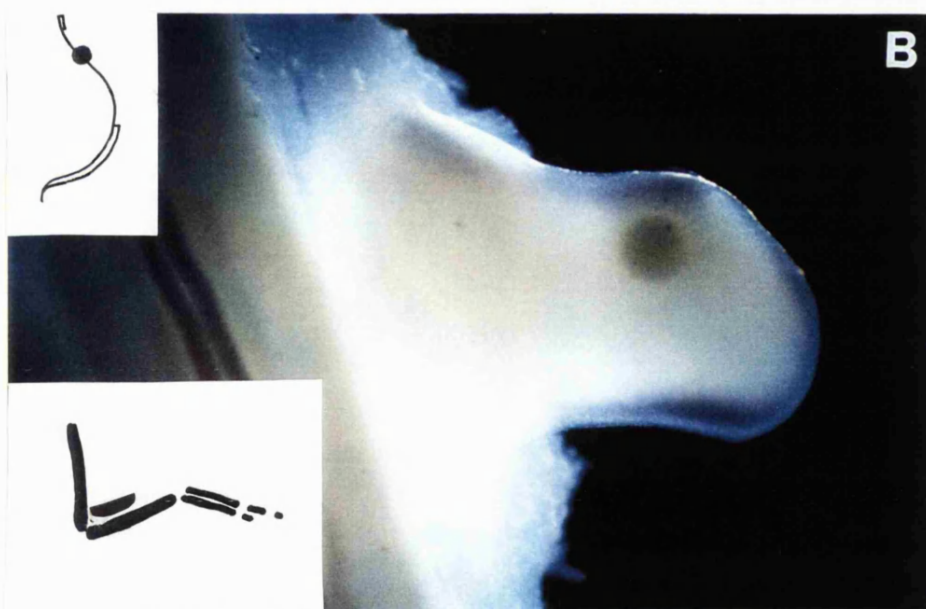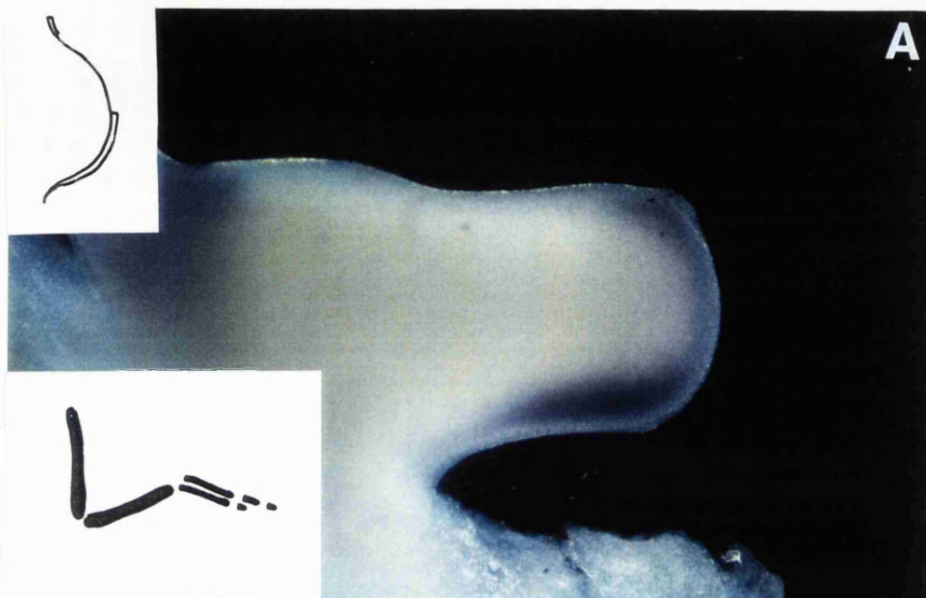
Fig. 3.4 Distribution of mouse *Msx-1* (A,B) mouse *Hoxd-11* (C,D), mouse *Hoxd-13* transcripts (E,F,G,H) 24 hours after grafting cultured mouse limb bud cells to a host limb bud. The mouse limb bud cells were isolated from the posterior (A-F) or anterior (G,H) mesenchyme and cultured for 24 hours in the absence (A,C,D,G) or presence of FGF-2 (B,D,F,H). When posterior cells were cultured in the absence of FGF-2, no expression of (A) *Msx-1* and (E) *Hoxd-xd-13* was detectable in the graft, whereas (C) *Hoxd-11* expression was found throughout the graft. When posterior cells were cultured in the presence of FGF-2, (B) *Msx-1* transcripts were found in small stripes at the periphery of the graft nearest the ridge and (D) *Hoxd-11* expression was detectable throughout the graft. Transcripts of *Hoxd-11* were more abundant in the distal part of the graft and expression appeared to be stronger compared to grafts of cells that had been cultured in the absence of FGF-2 (compare C with D). In grafts of posterior cells that had been cultured in the presence of FGF-2,*Hoxd-13* transcripts were localized in patches within the graft (F). In grafts of anterior cells that had been cultured in the absence of FGF-2, *Hoxd-13* transcripts were not detectable (G). In contrast, *Hoxd-13* transcripts were found in the distal part of grafted anterior cells that had been cultured in the presence of FGF-2 (H).

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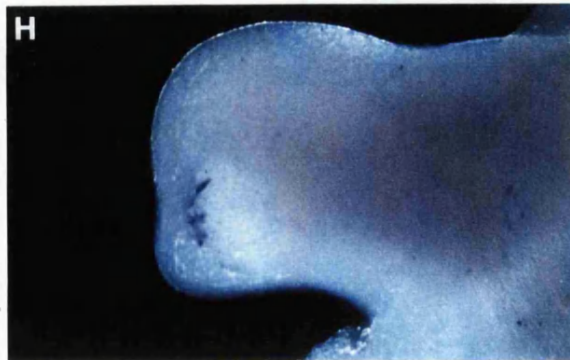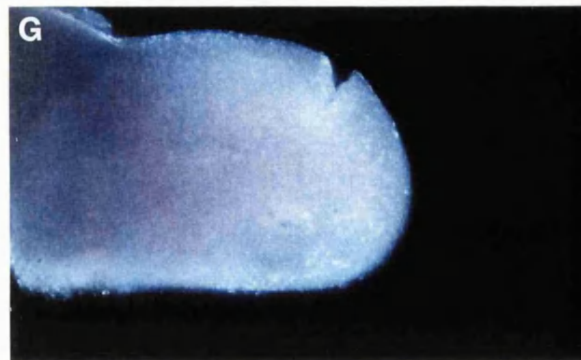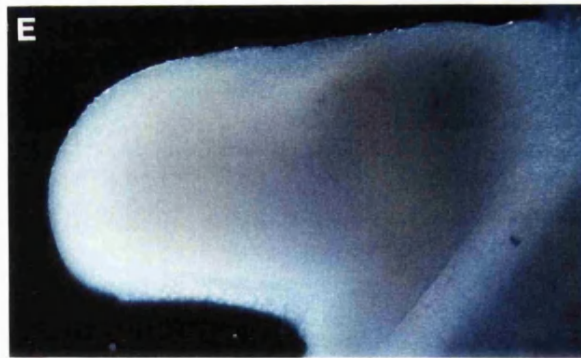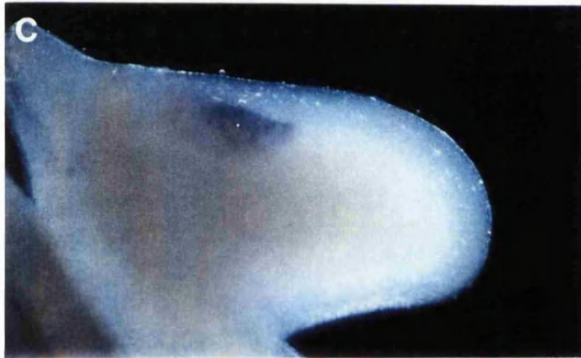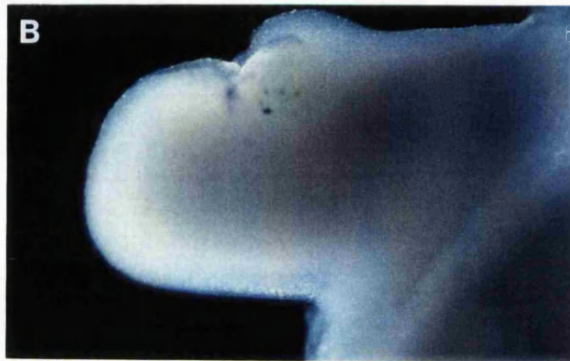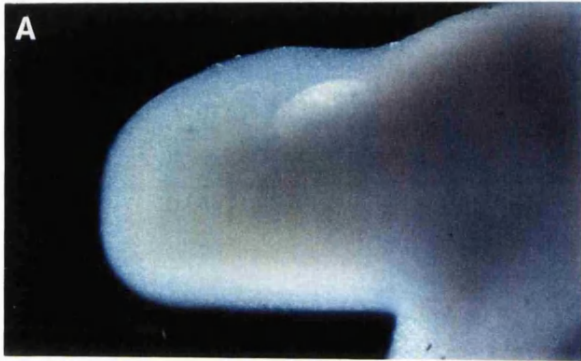
Fig. 3.5 Distribution of mouse *Hoxd-11* (A,D), mouse *Hoxd-13* (B,E)
and chick *Hoxd-13* (C,F) transcripts in adjacent sections of chick
wing buds, 26 hours after grafting posterior mouse limb bud cells
that had been cultured in the presence (A,B,C) or absence of FGF-2
(D,E,F) to the anterior margin of the chick wing bud.
Autoradiographic signal is visible as white grains under dark-field
illumination. In the presence of FGF-2, cultured posterior cells (A)
express high amounts of *Hoxd-11* throughout the graft, whereas (B)
transcripts of *Hoxd-13* are located in patches. (C) The ectopic
domain of chick *Hoxd-13* transcripts (arrow) in anterior mesenchyme
induced by grafted cells that had been cultured in the presence of
FGF-2 is indicated with an arrow. Grafts of cells that had been
cultured in the absence of FGF-2 (D) still express *Hoxd-11* throughout
the graft, whereas (E) transcripts of *Hoxd-13* are not detectable and
(F) no ectopic domain of chick *Hoxd-13* is found in anterior
mesenchyme of the chick wing bud.
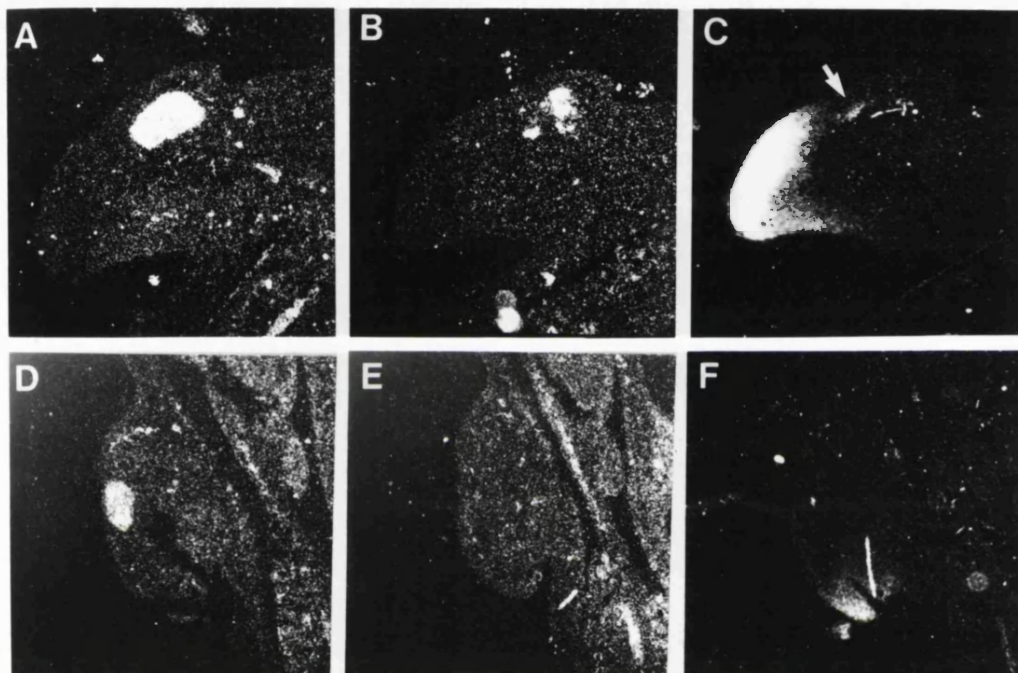
FGF-4-application	Msx-1	Hoxd-11	Hoxd-13
none	nd	$3^{*}/3$	-/3
posterior	$2^{*}/2$	2/2	2/2
apical	$2^{+}/2$	nd	nd
anterior	nd	$8^{*}/8$	-/4
posterior+apical	1/1	2/2	5/5

Table 3.1A Gene expression of *Msx-1*, *Hoxd 11* and *Hoxd 13* following

entire ridge removal and application of FGF-4 to either the

posterior, apical or anterior mesenchyme, or to both the apical and

posteriorlimb mesenchyme (- = no expression detected; * = expression

in posterior limb mesenchyme; + = expression in anterior/apical

mesenchyme; nd = not determined).

partial ridge removal	time assayed	FGF-4-application	Msx-1	Bmp-2	Hoxd-13
posterior	24h	none	$-^*/3$	-/3	-/3
posterior	24h	posterior	$4^*/4$	3/3	1/3
posterior	48h	posterior	$1^*/1$	nd	2/3
anterior	24h	none	$-^+/3$	nd	nd
anterior	24h	anterior	$2^+/2$	nd	nd

Table 3.1B Gene expression of *Msx-1*, *Bmp-2* and *Hoxd-13* after partial ridge removal of young chick wing buds with and without addition of FGF-4 beads to the mesenchyme (- = no expression detected; * = expression in posterior mesenchyme; + = expression in anterior mesenchyme; nd = not determined).

cultured limb bud cells	FGF-4- application	*Msx-1*	*Hoxd-11*	*Hoxd-13*
posterior	none	-/2	6/6	-/4
posterior	+ FGF-4	2/2	5/5	3/3
anterior	none	-/2	-/2	-/2
anterior	+ FGF-4	·nd	2/2	3/3

Table 3.2: Gene expression of mouse *Hoxd-11*, *Hoxd-13* and *Msx-1* in grafts of mouse limb bud cells that had been cultured in the presence or absence of FGF-2 using whole-mount and radioactive in situ hybridisation. Cultured posterior cells were grafted to the anterior margin and cultured anterior cells were grafted to the posterior margin of young chick wing buds (nd = not determined; - = no expression detected)

CHAPTER 4

GENERAL DISCUSSION

The aim of the thesis was to investigate the role of fibroblast growth factors during embryonic development. My main findings were:

1. Local FGF-4 application to the posterior margin of the bud in the absence of the ridge, or addition of FGFs to cultured posterior mesenchyme cells, maintains polarizing region signalling.

2. A generation of a full set of proximo-distal skeletal structures by FGF-4 in the absence of the ridge is correlated with maintenance of polarizing activity.

3. FGFs allow development of skeletal structures when either the whole limb bud of a young embryo or the limb bud tip of older embryos is removed.

4. When the apical ectodermal ridge of young chick wing buds is removed, or when posterior cells are taken from the bud and placed in culture, expression of *Msx-1*, *Hoxd-13* and *Bmp-2* is not detectable in posterior mesenchymal cells.

5. Local application of FGF soaked beads to posterior limb mesenchyme following ridge removal maintains *Msx-1*, *Hoxd-13* and *Bmp-2* expression. Following addition of FGF to cultured posterior limb bud cells *Msx-1-* and *Hoxd-13* expression is detectable after the cells have been grafted to the anterior margin of the chick wing bud.

6. Addition of FGF to cultured anterior limb bud cells maintains their ability to respond to positional cues when grafted back into the developing limb bud.

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In this thesis it was shown that application of FGF-4 to posterior mesenchyme can substitute for the posterior ridge in order to maintain polarizing region signalling. *Sonic hedgehog (Shh)*, the vertebrate homolog of the segment polarity gene hedgehog in *Drosophila* appears to mediate the action of the polarizing region (Riddle *et al.*, 1993). Consistent with my finding that FGF-4 can replace the function of the ridge to maintain polarizing region signalling, other workers have since shown that *Shh* expression is rapidly lost following entire and posterior ridge removal, but expression can be maintained by application of FGF-4 to posterior mesenchyme (Laufer *et al.*, 1994; Niswander *et al.*, 1994).

As shown for the first time (see Chapter 2), FGF-4 appears to link limb outgrowth with patterning. Application of FGF-4 to posterior, but not apical or anterior mesenchyme following entire ridge removal, maintains polarizing region signalling and this correlates with the development of a full set of proximo-distal structures. Niswander *et al.* (1994) have since shown that *Shh* expression is not maintained after ridge removal and application of FGF-4 to apical mesenchyme. However, whether *Shh* is the sole signal of polarizing activity has still to be determined. For example, following posterior ridge removal polarizing activity is still detectable (see Chapter 2) long after the time *Shh* expression has been lost in posterior mesenchyme (Laufer *et al.*, 1994).

There is a reciprocal dependence between the apical ectodermal ridge and the underlying mesenchyme. Posterior mesenchyme underlying the apical ectodermal ridge is required in order to maintain a functional ridge and the ridge is required in order to maintain polarizing region signalling. Recent work shows that there is a reciprocal interaction between the ridge and the posterior mesenchyme at the

molecular level. When *Shh* expressing cells are grafted to the anterior margin of a chick wing bud ectopic expression of Fgf-4 is induced in the anterior part of the apical ectodermal ridge (Laufer *et al.*, 1994; Niswander *et al.*, 1994). On the other hand, when the entire or posterior ridge is removed *Shh* expression is rapidly lost in posterior mesenchyme (Laufer *et al.*, 1994; Niswander *et al.*, 1994). Moreover, *Fgf-4* expression in the posterior ridge is likely to be controlled by posterior limb mesenchyme, possibly *Shh*. Thus, it appears that a positive feedback loop between *Shh* and *Fgf-4* at the tip of the developing bud coordinates limb outgrowth with patterning (Laufer *et al.*, 1994; Niswander *et al.*, 1994).

Several other genes known to be expressed in the developing limb bud appear to be regulated by the apical ectodermal ridge. These genes encode transcription factors or growth factors. Application of FGF following ridge removal induces or maintains expression of *Msx-1*, *Hoxd-13*, *Bmp-2* and *Evx-1* (Niswander and Martin, 1993a, 1993b; Laufer *et al.*, 1994; Niswander *et al.*, 1994; Chapter 3). It appears that *Msx-1-* and *Evx-1* expression might be correlated with mesenchymal proliferation and limb outgrowth. Following ridge removal expression of these genes is induced or maintained by FGF independent of the position of the applied bead. This mirrors the stimulation of mesenchymal proliferation by FGF which is also position independent (Niswander and Martin, 1993a, 1993b). In contrast, maintenance of *Hoxd-13*, *Bmp-2* and *Shh* for example, appears to require a signal from the posterior mesenchyme in addition to FGF-4. These genes are thought to be involved in antero-posterior patterning of the limb and by maintaining expression of *Shh*, *Bmp-2* and *Hoxd-13* and possibly other genes expressed in posterior mesenchyme, in additdition to *Msx-*

1 and *Evx-1* expression, FGF-4 allows both patterning and limb outgrowth.

FGF also substitutes for the ridge to allow development of skeletal structures following excision of limb mesenchyme. It appears that FGF can stimulate proliferation and *Msx-1* expression in proximal non-*Msx-1* expressing mesenchyme (Kostakopoulou, personal communication) and thereby appears to reestablish a progress zone. It is possible that *Msx-1* expression by FGF in proximal tissue can only be induced before terminal differentiation has taken place.

As shown in this thesis, addition of FGF maintains responsiveness of cultured cells to positional cues within the limb. Only cells in the progress zone respond to the polarizing region signal (Summerbell, 1974b), or *Shh* expressing cells (Laufer *et al.*, 1994). Thus, it appears that FGF also substitutes for the ridge to maintain mesenchyme cells in a responsive state required to allow patterning of the developing limb.

4.1 Conclusions

The vertebrate limb is a good model to study developmental mechanisms of growth, differentiation, pattern formation and morphogenesis. Extensive embryological manipulations have identified two major signalling regions, the apical ectodermal ridge and the polarizing region, that are required for proper development of the limb. Recent work and work in this thesis have identified FGFs and *Shh* as molecular mediators of signalling of the apical ectodermal ridge and the polarizing region and this will greatly contributed to understand limb development on a molecular basis. Embryological manipulations and the existence of molecular techniques will in

future help to elucidate how for example, FGF expression in the apical ectodermal ridge is controlled and help to identify genes who's expression is directly controlled by FGF. Understanding the molecular mechanisms of limb development, e.g. how gene expression is controlled, could also be important to find ways that will help to treat deseases such as cancer.

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FGF-4 maintains polarizing activity of posterior iimb bud celis in vivo and in vitro

Astrid Vogel* and Cheryll Tickle

Department of Anatomy and Developmental Biology, University College and Middlesex School of Medicine, Windeyer Building, Cleveland Street, London W1P 6DB, UK

*Author for correspondence

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 quantiative 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^b-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 10^7 cells/ml with serum-containing CMRL medium. One 10 μl drop of the cell suspension containing 10^5 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_2 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 multidish. 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
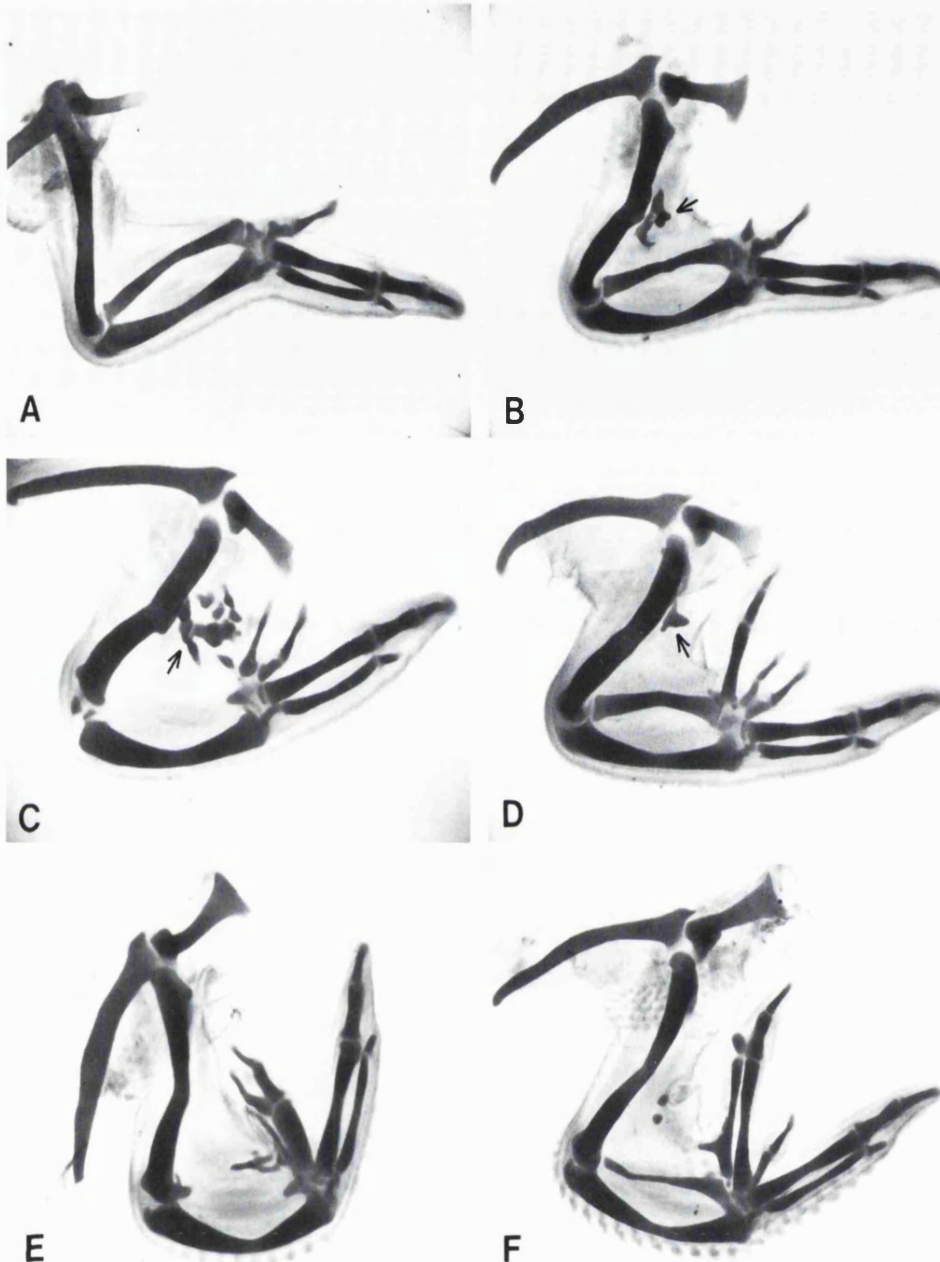
Fig. 1. Whole mounts of embryonic chick wings stained with alcian green to show digit patterns. (A) Normal wing (score 0); (B) wing with small anterior blip resulting from a graft of a piece of micromass of posterior mouse limb bud cells cultured for 24 hours (score 0.5); (C) duplicated wing, digit pattern **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).

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

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:
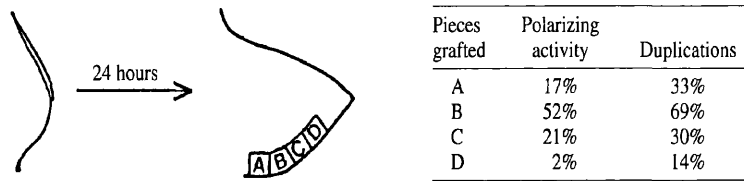
Pieces grafted	Polarizing activity	Duplications
A	17%	33%
B	52%	69%
C	21%	30%
D	2%	14%

(B) Controls (contralateral limb):

100% polarizing activity

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

Pieces grafted	Polarizing activity	Duplications
A	95%	100%
B	82%	100%
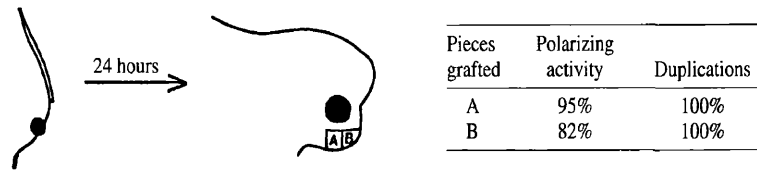
Fig. 2. Maps of polarizing activity of limb buds 24 hours after posterior ridge removal. 24 hours after removal of the posterior ridge and showing the effects of adding FGF-4. (A) removal of the posterior ridge; (B) controls showing polarizing activity of unoperated limb buds (stage 24/25); (C) removal 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.

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

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

Stage of operation	Piece grafted	n	Digit pattern of limbs				
			Normal 234	?234	2234	334 (2334 etc)	4334 (4434 etc)
19	A	1	1	–	–	–	–
	B	2	2	–	–	–	–
	C	2	2	–	–	–	–
20	A	5	3	–	–	2	–
	B	11	2	–	1	3	5
	C	11	7	–	1	1	2
	D	7	6	1	–	–	–

(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

Stage of operation	Piece grafted	n	Digit pattern of limbs				
			Normal 234	?234	2234	32234	4334
19	A	1	–	–	–	–	1
	B	2	–	–	–	–	2
20	A	10	–	–	–	1	9
	B	9	–	–	–	4	5

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 and 6 hours later grafts of posterior mesenchyme (piece A) still had 100% polarizing activity (2 cases). Grafts of piece B, the more distal part of the newly formed posterior outgrowth resulted in 50% polarizing activity (2 cases; one gave an additional digit 4, the other gave no additional digits). 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% ; 4 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 grafted 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
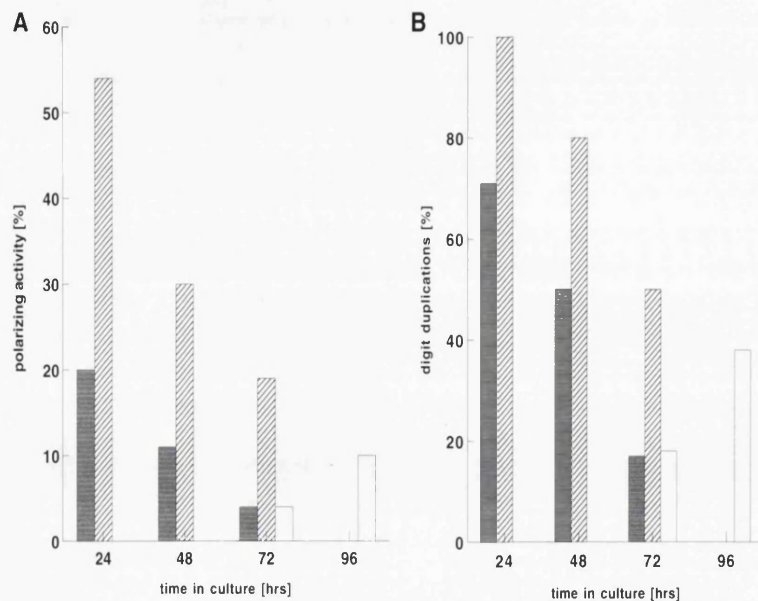
Fig. 3. Polarizing activity (A) and percentage of limbs with pattern alterations involving digit duplications (B) of grafts of pieces of micromass cultures of posterior mesenchyme of mouse limb buds, assayed by placing at the anterior margin of a chick wing bud. The micromasses were cultured in serum-containing medium (■); with FGF-4 protein and heparan sulphate at a concentration of approximately 80 ng/ml (▨), or cocultured with ectoderm (□).

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 **4** (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 **3** 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

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

Time in culture	Number of grafts	Digit pattern of limbs				
		Normal 2 3 4	? 2 3 4	2 2 3 4	3 2 2 3 4	4 3 3 4
24 h	7	2	1	3	1	–
48 h	8	4	1	3	–	–
72 h	6	5	–	1	–	–
96 h	18	18	–	–	–	–

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

Time in culture	Number of grafts	Digit pattern of limbs				
		Normal 2 3 4	? 2 3 4	2 2 3 4	3 3 4	4 3 3 4
72 h	17	14	1	1	1	–
96 h	13	8	–	5	–	–

(C) Posterior mesenchyme cells in micromass cultured with FGF-4

Time in culture	Number of grafts	Digit pattern of limbs				
		Normal 2 3 4	? 2 3 4	2 2 3 4	3 3 4	4 3 3 4
24 h	7	–	–	1	5	1
48 h	5	1	–	2	2	–
72 h	12	6	–	3	3	–
96 h	18	18	–	–	–	–

develop in the apparent absence of the polarizing region (MacCabe et al., 1973; Fallon and Crosby, 1975; Smith, 1979).

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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