ROLE OF SIGNALLING MOLECULES IN THE DEVELOPING AVIAN WING

Neda Nikbakht

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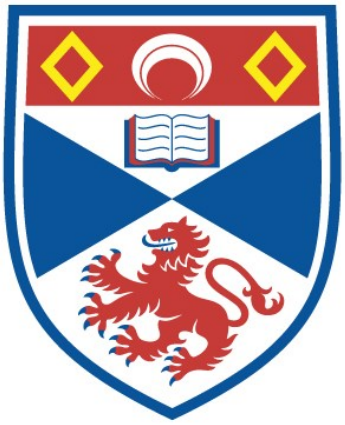

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Role of signalling molecules in the developing avian wing

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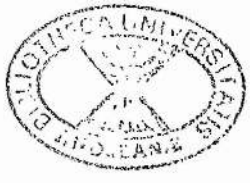

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EX OVO OMNIA

WILLIAM HARVEY (1578-1657) STUDIED THE DEVELOPMENT OF THE CHICK EMBRYO AND CONVINCED HIMSELF THAT ALL ANIMALS MUST COME FROM EGGS. "EX OVO OMNIA," HE SAID: "OUT OF EGG, ALL."

(Gonick and Wheelis, 1991)

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The mystery..... statistics,
The struggle..... making three dimensional models,
The puzzle..... soft tissue analysis,
The goal..... this thesis.,
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It is to my family that I dedicate this work.

Role of signalling molecules in the developing avian wing

Abstract

The aim of this study was to investigate the role of signalling molecules during the development of the chick limb.

First, it was demonstrated that a functional gradient of bioactive FGFs is present down the limb from distal to proximal. This functional FGF gradient decreased at stage 26, at the time of AER regression. Although morphogenetic gradients are of considerable theoretical importance in developmental biology, there are rather few practical demonstrations of their existence.

The effects of prolonging the presence of active FGF on limb pattern formation were investigated. Application of ectopic FGF-4 to the distal tip of the limb at stage 26 had a number of effects on limb development. In particular, the cartilage structure conventionally labelled element "5" increased in size and in some instances acquired a digit-like morphology. The evolutionary considerations of this finding are briefly considered. Analysis, including 3D computer reconstruction, of the musculature and vasculature of limbs after FGF implants was carried out in the hope of establishing the identity of this digit like element. This proved not to be possible. However, both muscle mass and vascularisation had increased after the procedure.

Known molecular pathways involving the proximo-distal patterning of the limb were then investigated. Whole mount in situ hybridisation studies were carried out with respect to FGF-4, sonic hedgehog, *Hoxd-11* and FGF-8. These revealed that the *shh*/FGF positive feedback loop was not involved in these changes. FGF-8 expression in late stage AERs was markedly increased. *Hoxd-11* expression was not affected by ectopic FGF implants. Together, these findings suggest that the effect of FGF implants is mediated by a novel mechanism.

The effect of FGF-4 implants on programmed cell death in the limb was examined. At stage 28 anterior necrotic zone was larger, and had shifted

distally. However, the posterior necrotic zone was absent. The implications of these findings for limb development were discussed.

Chapter 1

Introduction

Limb development

All vertebrate limbs develop in similar fashion. The limb grows out of the body wall and is composed of loose mesenchymal cells originating from the somatic region of the lateral plate. The limb is covered in an epithelial layer derived from ectoderm. The mesoderm gives rise to cartilage, tendons and connective tissues. Muscles, nerves and blood vessels invade into the mesoderm from the somites, neural tube and main vasculature respectively. All vertebrate limbs are composed of these tissues but the final arrangements are unique to particular species.
The limb provides a valuable model system of studying vertebrate pattern formation since the elements in the limb are also found in other regions of vertebrates (Slack, 1979). Hence studying limb development provides information on general developmental mechanisms.

Much of the original work carried out on vertebrate limb development consisted of grafting experiments performed on chick limbs. These grafting experiments resulted in the identification of three major morphogenetic areas within the limb bud; apical ectodermal ridge (AER), progress zone (PZ) and the zone of polarizing activity (ZPA) (Saunders, 1948; Summerbell et al., 1973).
Limb development involves cell movement, cell proliferation, differentiation and cell death. Fibroblast growth factors (FGFs), sonic hedgehog (*shh*), vertebrate homolog of *wingless* gene (*Wnt*) and Homeobox genes (*Hox* genes) are amongst signalling molecules that play important roles in all of these. These signalling molecules contribute to molecular pathways that direct proximodistal, dorsoventral and anterposterior patterning, digit formation, angiogenesis and programmed cell death in the developing limb. The molecular pathways operate

through the AER, ZPA and PZ in concert in overall patterning and development of the vertebrate limb.

Specifying the axes of the limb

Limb development involves the formation of three different axes: the anteroposterior (A-P) axis (digit 2 to 4), the dorsoventral (D-V) axis (top of the wing to the bottom) and the proximodistal (P-D) axis (girdle to the wing tip).

Various experiments have estimated the time at which the three axes are determined. Grafting prospective limb mesoderm from somite 16 at stage 13 (45-49 hours) to the flank of a host embryo gives rise to supernumerary limb (Hamburger, 1938). Reversing the A-P and D-V axis of the grafts results in ectopic limbs with a reversed A-P and D-V axis respectively (Hamburger, 1938). The P-D axis however is established as the limb starts to grow suggesting that axis determination is thought to be the characteristic of mesoderm and not ectoderm (Hamburger, 1938).

Recently several molecular mechanisms involved in axis formation of the limb have been recognised. The signalling molecules and their molecular pathways involved in limb axes formation will be now discussed.

Proximodistal axis

Apical ectodermal ridge

The AER is a distinct thickening of the ectoderm at the distal tip of the limb and it is induced by the underlying mesoderm. AER induction requires an early competent phase that includes fibroblast growth factor expression and a later differentiation phase in which fibroblast growth

factor is induced (Kuhlman and Niswander, 1997). The AER is histologically distinct from the underlying mesoderm and plays an important role in proximal/distal patterning of the developing limb bud (Saunders, 1948). If the AER is removed it fails to regenerate and the mesoderm beneath ceases to divide and the limb is truncated. The later the AER is removed, the more distal is the level of truncation (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). Removing the AER at stage 19 (3 days) or stage 23 (4 days) gives rise to limb stumps with only humerus or with radius, ulna and perhaps some wrist present respectively (Summerbell and Lewis, 1975). Grafting the AER to a developing limb causes additional limb buds to form (Zwilling and Hansborough, 1956). The mesoderm is dependent on the AER for its continuous proliferation and the AER is dependent on the mesoderm for its initial formation and maintenance (Zwilling and Hansborough, 1956). This suggests that the mesoderm produces a factor responsible for initiation, presence and position of the AER (Zwilling, and Hansborough, 1956). Replacing the mesoderm underneath the AER with non limb mesoderm causes rapid regression of the AER and therefore truncation of the limb (Zwilling, 1961). However if a small number of limb mesoderm cells are retained they will migrate to the site of the mesoderm-ectoderm junction and the limb develops normally (Zwilling, 1956a and 1972). Transplanting prospective limb mesoderm beneath flank ectoderm causes the ectopic formation of an AER and development of a supernumerary limb (Saunders and Ruess, 1974). The importance of the mesodermal-ectodermal interaction during limb development is best demonstrated in mutants *wingless* and *eudiplodia*. In the *wingless* mutant AER is observed on the third day but it soon regresses and the limb fails to develop (Zwilling, 1956b and 1974; Goetnick, 1964).

In the mutant *eudiplodia* the limb develops additional elements on its dorsal side and histological evidence shows a dorsal extension of the AER (Goetinck, 1964).

Signalling molecules play an important role in AER induction and maintenance (Altabef et al., 1997). Localized FGF from the mesoderm induces the progress zone which in turn induces the AER. Several signalling molecules from the AER are involved in patterning of the limb. These molecules and their signalling pathways will be discussed later.

Progress zone

The progress zone is the area of rapidly proliferating mesodermal cells proximal to the AER (Summerbell, 1974). The localised FGF from the flank mesoderm is thought to initiate the proliferation of mesenchyme and hence give rise to the progress zone (Niswander et al., 1993). As the limb grows the undifferentiated mesodermal cells in the progress zone are rapidly assigned to different fates. The AER maintains the progress zone in an undifferentiated state and it is thought that the longer the cells remain in the progress zone the more distal their final position will be (Summerbell, 1974).

The P-D polarity lies within the mesoderm and not the AER (Rubin and Saunders, 1972). Even though the late removal of the AER causes the formation of more distal elements and confirms that cartilaginous elements in the limb are laid down proximodistally, the AER is not involved in determining P-D polarity (Saunders, 1948; Rowe and Fallon, 1982). When AERs of different age are grafted to young host limbs, the limbs developed normally regardless of the age of the explant (Summerbell, 1974; Rubin and Saunders, 1972). In further experiments the

distal tips of young and old limb buds were exchanged. The distal tip of young limb (stage 19/20) was removed leaving the stump with material specified as humerus and replaced with a stage 24 limb tip, the resulting limbs lacked radius and ulna. The reversed experiment gave rise to limbs with duplicated humerus, radius and ulna (Summerbell and Lewis, 1975). Thus the P-D axis is determined by how long the mesodermal cells remain in the progress zone, indicating that perhaps once mesodermal cells have left the progress zone and escaped the influence of the AER their fate is irreversibly fixed (Summerbell and Lewis, 1975). However results from chick-quail chimera experiments suggests that the positional values of the cells escaping the progress zone may not be not irreversibly fixed (Kieny, 1977). Upon removing the radius and ulna competent tissues, limbs developed with portions of new radius and ulna. These structures had developed from cells that should have only given rise to a humerus. When additional tissues were added, relatively normal limbs developed from the stump, instead of duplicated humerus, radius and ulna (Kieny, 1977). This indicates that regardless of the age of the explants, the experimental limbs had shown some levels of self regulation (Kieny, 1977).

Anteroposterior axis

Zone of Polarizing activity

The zone of polarizing activity is the mesenchymal region in the posterior limb margin. The ZPA controls the anterior/posterior patterning of the developing limb as well as playing an active role in development of the proximodistal axis (Niswander et al., 1993).

Experiments have shown that grafting ZPA to the anterior region of a host limb results in axis duplication (Saunders and Ruess, 1974; Saunders and Gasseling, 1968). By grafting ZPAs of various ages and sizes to anterior part of a host chick limb, the strength and effect of ZPA has been mapped out carefully. This was achieved by evaluating the degree of digit duplication, with maximum polarizing activity being associated with the digit formation 4-3-2-2-3-4 (Honig and Summerbell, 1985).
Polarizing activity is first detected just prior to the onset of limb growth (stage 16). Initially a faint activity is detected in the flank and the activity increases reaching maximum strength in the proximal posterior margin of the limb by stage 19. As the limb grows, the polarizing activity is no longer biased to the proximal posterior region but extends along the full length of the posterior border of the limb. By stage 25 the polarizing activity is subsiding and a general shift distally is observed. The polarizing activity decreases all the time until it becomes undetectable at stage 28. Removing the ZPA past stage 22 has no impact on the normal development of the limb. This indicates that after a critical period the cells remember their exposure to the ZPA (Smith, 1980). However this may not be completely true since experiments by Fallon et al., 1995, have shown that in cases of ZPA removal some cells expressing *shh* have been left behind.
The AER, ZPA and PZ are formally distinct but functionally interdependently: the function of any one area is dependent on the activity of the other two regions (Tabin, 1991).

It was thought that a morphogen was secreted from the ZPA across the limb in a simple gradient (Tickle et al., 1975, Honig, 1981). ZPA grafts to host limbs in various positions resulted in duplication patterns compatible with the diffusing morphogen model. These duplications were thought to arise from the concentration gradient set by the graft. The result from these

experiments implied that digit 4 perhaps forms at the highest point of morphogen concentration where as digit 2 forms at the lowest concentration level (Tickle et al., 1975). Inserting barriers in the limb and separating the anterior region form the posterior caused digit formation in the posterior region suggesting that the limb morphogen originates in the posterior region (Summerbell, 1979). Application of retinoic acid was first shown to cause element duplication in regenerating salamander limbs (Maden, 1982 and 1983). Subsequently it was found that retinoic acid grafts to the anterior margin brought about the same type of digit duplication as ZPA grafts (Tickle et al., 1982; Summerbell 1983). It was shown that retinoic acid is present in a shallow gradient across the limb with the highest levels at the posterior region (Thaller and Eichele, 1987). However speculations about RA being the limb morphogen were conclusively rejected when the secreted protein sonic hedgehog (*shh*) present in the posterior region of the limb was cloned (Riddle, et al. 1993). The region of *shh* expression overlaps with the ZPA and therefore it may be involved in the mechanism through which ZPA influences the A-P patterning of the limb. Application of *shh* to the anterior region of the chick limb causes mirror duplications similar to those from ZPA transplants (Riddle et al., 1993). Therefore the molecular mechanism involving *shh* provided evidence that *shh* may indeed be the most likely candidate for the limb morphogen causing A-P patterning during development, with RA as a possible inducer of *shh*.

Dorsoventral axis

The ectoderm is capable of specifying the D-V axis prior to the formation of the AER. Combinations of ectoderm and mesoderm establish the D-V axis according to the age of the mesoderm (Geduspan and MacCabe, 1987). At

stage 16 the newly formed limb bud is already polarised along the dorsoventral axis and can be distinguished with different molecular markers such as *Wnt-7a* (Dealy et al., 1993; Riddle et al., 1995). By stage 16, reversing the dorsoventral orientation of the somatopleural ectoderm does not effect the D-V polarity of the limb bud, since it is determined by its environment and not the presumptive limb territory (Geduspan and MacCabe, 1989; Michaud, 1997). Dissociated limb bud cells or fragments of mesoderm, if repacked into the ectodermal case of a stage 23-26 limb and grafted to the flank, cause the skeleton and musculature of the distal elements to have a D-V axis pattern (MacCabe et al., 1973). Reversing the D-V axis of the ectoderm by recombining intact mesodermal cores of three to four days old limb buds with the ectodermal hulls causes reversal of musculature and the skeleton along the D-V axis (MacCabe et al., 1974). Several genes are involved in D-V patterning of the limb. In *Drosophila*, the *Wnt* homolog *wingless* (*wg*) is required for Hedgehog (*h h*) expression indicating that the interactions between *Wg* and *hh*, and *Wnt* and *shh*, are important for invertebrate and vertebrate development (Lee et al., 1992; Tabata et al., 1992). In mice homozygous for a mutation of *Wnt-7a*, dorsal structures of the limb bud adopt a ventral identity (Parr and McMahon, 1995). Also, in mice, loss of homeobox gene chicken-*En-1* (engrailed) causes dorsal transformation of ventral paw structures (Loomis et al., 1996). Chick-En (engrailed), shares homologies to the engrailed gene of *Drosophila* and is expressed during early steps of development in a restricted area of the chick embryo including cells of the developing brain, mandibular arch, spinal cord, dermatome, and ventral limb bud ectoderm (Ohkuma et al., 1990; Martinez and Alvarado-Mallart, 1990; Gardner and Barald, 1992). *En-1* mRNA and protein are distributed throughout the ventral limb bud ectoderm and *En-1* is essential for establishment and maintenance of ventral limb characterization (Logan et al., 1997).

Therefore *Wnt-7a* and *En-1* are thought to play an important role in D-V patterning of the limb.

Chick-quail chimera experiments have shown that the presumptive wing mesoderm occupies the medial half of the somatopleure at the level of somite 15-20 and the corresponding ectoderm area only gives rise to the AER (Michaud et al., 1997). *Radical fringe*(R-frn) mRNA chick gene homologous to *Drosophila fringe*) is detected in the presumptive dorsal limb ectoderm at stage 15 but it is not detected in the presumptive central ectoderm. By stage 16-18 R-frn transcripts are restricted to the dorsal side with higher concentration in the dorsoventral boundary and later in the AER (Rodrigeuz-Esteban et al., 1997). It is thought that R-frn plays an important role in formation of the AER which directly influences the dorsoventral patterning of the developing limb (Rodrigeuz-Esteban et al., 1997).

Recent evidence suggests that vertebrate limb growth and pattern formation are co-ordinated by the interaction between signals from all three axes (Yang and Niswander, 1995). It is thought that fibroblast growth factor 4 (FGF-4), sonic hedgehog (*s h h*), bone morphogenetic proteins (*BMPs*) and *Wnt-7a* are expressed throughout different stages of embryonic development indicating that they may co-ordinate many aspects of development such as the organisation of primary body axis (Fig 1) (Yang and Niswander, 1995).

Signalling molecules and their molecular pathways

Fibroblast growth factors (FGF)

Fibroblast growth factors are members of a large family of polypeptide growth factors. There are at least 17 distinct but structurally related members (Burgess and Maciag, 1989; Basilico and Moscatelli, 1992; Gosporadowicz, 1990; Szebenyi and Fallon, 1999). FGFs contain a common domain in which most of their structural homology can be found. Most FGFs have common characteristics but they also differ from each other significantly.

FGFs are expressed throughout the developing and adult body and each FGF has its own restricted spatial and temporal expression patterns. FGFs are involved in formation of many tissues and structures, for example FGF-8 is involved in mid brain formation, FGF-4 and FGF-8 are both involved in gastrulation and FGF-1 and FGF-2 are involved in neurogenesis (Mckay and Leigh, 1993; Slack, 1994; Crossley et al., 1996a; Feldman et al., 1995; Meyers et al., 1998, Vescovi et al., 1993; Qian et al., 1997). FGFs are capable of repressing differentiation, they can act as mitogens or neurotrophic factors and have angiogenic ability (Baird and Klagsbrun 1991; Mason, 1994).

FGF-2 (basic FGF) was the first member of the family to be discovered. It was found in the brain and pituitary and was shown to stimulate proliferation and differentiation in different cell types (Baird, 1994; Klagsburn and Baird, 1994; McKeehan and Khan, 1994).

Immunohistochemical studies indicate that FGF-2 shows localisation to the extra cellular matrix of numerous tissues and to the basement membrane *in vivo* (Whalen et al., 1989).

Heparin beads soaked in FGFs and implanted in the flank of chick embryos will induce formation of ectopic limbs (Cohen et al., 1995; Ohuchi et al., 1995; Vogel et al., 1996; Martin, 1998).

FGF-2, FGF-4 and FGF-8 are all expressed in the AER (Niswander and Martin, 1992, Savage et al., 1993; Savage and Fallon, 1995; Crossley and

Martin, 1995). FGF-1/2/4 and 8 are all capable of replacing the AER and promote nearly normal limb development (Niswander et al., 1993; Fallon et al., 1994; Mahmood et al., 1995, Cohn et al., 1995).

FGF-2 mRNA is located both in the ectoderm (posteriorly biased) and the mesoderm (progress zone) (Savage and Fallon, 1995; Savage et al., 1993). FGF-2 level is highest at stage 18 and stage 21 and then drops until stage 27 when it rises again (Seed et al., 1988 and Munaim et al., 1988). In limb mesenchyme cultures FGF-2 permits cell survival and stimulates cell proliferation by hyaluronan synthesis (Anderson, et al., 1993; Munaim et al., 1991). FGF-2 also prevents programmed cell death in culture (MacCabe et al., 1991). Experiments have shown that when FGF-2 is ectopically expressed by retroviral infection or a bead, it can induce supernumerary limb outgrowth *in ovo* (Riely et al., 1993).

FGF-10 is also expressed in the distal region of the limb bud mesoderm (PZ) (Savage and Fallon, 1995; Savage et al., 1993). Its expression may be involved in both limb and AER initiation (Ohuchi et al., 1997).

FGF-4 is found only in the AER and is posteriorly biased (Niswander and Martin, 1992). In both chick and mice, FGF-8 transcripts are present in the limb bud territory prior to the onset of limb bud growth and it persists in the during early development (Mahmood, et al., 1995). It has also been shown that AER induction requires an early competent phase that includes FGF-8 expression and a later differentiation phase in which FGF-4 is induced (Kuhlman and Niswander, 1997).

FGF signals are mediated by 4 transmembrane proteins with intrinsic kinase activity, known as FGF receptors (Fgfrs) (Basilico and Moscatelli, 1992; Johnson et al., 1990; Johnson and Williams, 1993; Soulet et al., 1994). These Fgfrs have a hydrophobic leader sequence, three immunoglobulin (Ig) like domains, an acidic box, a cell adhesion molecule homology domain(CAM), a transmembrane region and a divided tyrokinase domain

(Givol and Yayon, 1992). RNA in situ hybridisation shows that Fgfr1 is expressed almost entirely in the mesenchyme, Fgfr2 is expressed predominantly in the epithelium, Fgfr3 is expressed in the developing central nervous system and bone rudiment and fgfr4 is expressed in endoderm and somatic myotome (Peters et al., 1992; Yamaguchi et al., 1992; Stark et al., 1991; Orr-Urtrege et al., 1991). Fgfr mutations disrupt normal cell migration, confirming their important function during development (Amaya et al., 1991; DeVore et al., 1995; Beiman et al., 1996; Gisselbrecht et al., 1996; Peters et al., 1994, Werner et al., 1993)

FGFs are also involved in skeletal development, linking at least eight human skeletal disorders to FGF receptors (Mulvihill, 1995). Therefore FGFs are key signalling molecules involved in different aspects of development including, angiogenesis.

Since fibroblast growth factors 4 play a particularly important role in the limb bud development and patterning it is dealt with separately.

Fibroblast Growth Factor 4

Fibroblast growth factor 4 (FGF-4) is a member of the fibroblast growth factor family. It is able to substitute for the AER and therefore stimulate mesenchyme proliferation (Niswander et al., 1993; Vogel et al., 1993). The posterior region of the AER expresses FGF-4 transcripts and supports *shh* expression in the ZPA. If the AER is removed the expression of *shh* is reduced (Niswander et al., 1994b). Without FGF-4 polarizing activity is reduced and the signalling mechanism changes (Makarenkova et al., 1997). There are more gap junction in the posterior region of the limb, the ZPA, than in the anterior mesenchyme cells. These gap junction are sensitive to FGF-4. FGF-4 is shown to improve functional coupling between cultured posterior mesenchyme cells by doubling the gap junction density

(Makarenkova et al., 1997). Hence, FGF-4 is thought to closely regulate cell-cell communication and polarizing signalling (Makarenkova et al., 1997).

Sonic Hedgehog (*shh*)

Hedgehog (*h h*) is a segment polarity gene in *Drosophila*. It encodes a secreted protein produced by cells in the posterior region of each segment (Tabata et al., 1992). Cloning of *Drosophila h h* paved the way for cloning of its vertebrate homologous gene sonic hedgehog, *s h h*. *S h h* was isolated based on its expression in stage 22 chick limb. Whole mount in situ hybridisation performed by using riboprobes corresponding to the entire *s h h* cDNA clone has provided the complete expression pattern of this protein during chick limb development (Riddle et al., 1993; Johnson and Tabin, 1995).

Sonic hedgehog is expressed in several different tissues of the embryonic chick. These tissues are ZPA, Hensen's node, notochord and the floor plate of the neural tube (Riddle et al., 1993; Teillet et al., 1998). All these tissues bring about ZPA like pattern alterations when grafted into the chick limb (Saunders and Gasseling, 1983; Hornburch and Wolpert, 1986; Wagner et al., 1990).

S h h expression by stage 21 is detected in the posterior region of the developing limb and is limited to the mesenchyme. The region of the limb bud expressing *s h h* corresponds to the ZPA.

The correlation between tissue defined as ZPA and cells expressing *s h h* is first detected at stage 17. However, prior to this stage polarizing activity is seen in the flank mesenchyme posterior to the presumptive limb bud. These cells are competent to express *s h h* at a later stage and perhaps capable of activating *s h h* if transplanted to the anterior of a host limb

(Riddle, et al. 1993). This overlapping of the ZPA region and *shh* expression suggests that *shh* is involved in the complex mechanism through which the ZPA exerts its influence. Ectopic expression of *shh* in the anterior region of the chick limb causes mirror duplications similar to those from ZPA transplants, with the occasional duplication of proximal elements such as the humerus (Riddle et al., 1993). Removal of *shh* completely causes severe defects along the A-P axis of the limb indicating that removing *shh* during early stages of the limb development results either in postaxial defects, or truncation. This is perhaps due to the disruption of a positive feedback loop between FGF-4 from the AER and *shh* in the ZPA. *Shh* maintains FGF-4 which is necessary for limb bud outgrowth and FGF-4 from the AER maintains the expression of *shh* (Niswander et ., 1994b, Laufer et al., 1994). *Shh* appears to be upstream of FGF-4 expression in the AER and since AER is required to maintain polarizing activity in the posterior mesoderm, *shh* may also be downstream of the AER (Vogel and Tickle, 1993; Niswander et al., 1993; Laufer et al., 1994).

Ectopic expression of *shh* has been achieved by applying a source of RA (retinoic acid) at the anterior margin of the limb bud. This occurs distal to the source of RA in the mesenchymal cells at about 12-14 hours after RA application (Wanek, et al., 1991). This is not surprising, since RA had already shown to cause mirror imaging of the digits by inducing ectopic ZPA tissue (Tickle et al., 1982). Ectopic expression of *shh* is also detected at the site of RA application at about 24 hours after implantation in the mesenchyme distal to the bead. By 36 hours expression is strong in the anterior ectoderm, and forms a mirror image of the endogenous *shh* in the posterior margin of the limb. At 48 hours, the ectopic *shh* begins to fade. This corresponds exactly to the timing with which endogenous *shh* begins to fade(Riddle et al., 1993).

By stage 21 *shh* expression overlaps the ZPA region and moves proximo-distal to occupy the anterior region of the limb. Mapping the expression of *shh* has provided regional information about the ZPA (Riddle et al., 1993). There are no specific markers for ZPA: the region itself is morphologically indistinguishable from the rest of the undifferentiated limb bud. The presence of *shh* has proven to be a reliable molecular marker for the ZPA, but not vice versa.

The question therefore arises as to whether *shh* is the ZPA signal. Several lines of argument suggest that it is not. First, areas with ZPA activity early in development need not also be expressing *shh*. Second, ZPA activity is associated with mitogenesis, and there is no evidence that *shh* is mitogenic (Cooke and Summerbell, 1980; Bell and McLachlan, 1985).

Studies have shown that *hh* encodes a secreted protein that plays multiple inductive roles during *Drosophila* development (Perrimon, 1995). In *Drosophila* *hh* regulates aspects of embryonic segmentation and patterning of adult appendages over a diameter of 1 or 2 cells via short range action. Aside from this short range activity *hh* is thought to act long range to specify the cell types in the dorsal epidermis. Whether this happens as a result of direct action of *hh* on both adjacent or distant cells or by secretion of a second long range factor is yet to be determined (Johnson and Tabin, 1995).

Following signal sequence cleavage all *hh* genes go through a second self cleaving mechanism that generates N-terminal and C-terminal fragments (Lee et al., 1994). In *Drosophila* the N-terminal fragment is associated with the extra cellular matrix and the C-terminal is released into the culture medium, indicating that perhaps the N-terminal is responsible for the short range activity and the C-terminal is responsible for the long range signal (Lee et al., 1994). However experiments have demonstrated that in assays measuring short range activity, the N-terminal was sufficient not

only for short range signalling but also for long range signalling. The C-terminal failed to show any activity in these assays (Porter et al., 1995; Fietz et al., 1995). In vertebrates the N-terminal of *shh* was also sufficient to direct both long range (motor neuron induction and sclerotome differentiation) and short range (floor plate induction) (Fan et al., 1995; Roelink et al., 1995). It is thought that the C-terminal contains proteolytic activity necessary for a *shh* precursor protein processing and perhaps responsible for restricting the diffusion of *shh* by an unknown mechanism (Lee et al., 1994). For *shh* to act long range it requires to be present a distance away from the target site: however, *shh* protein is not detected by immunostaining methods at sites distant from floor plate or notochord (Roelink et al., 1995)

Homeobox genes (*Hox* genes)

The homeobox gene cluster in vertebrates has been duplicated to give four homologous clusters. These are *Hoxa*, *Hoxb*, *Hoxc* and *Hoxd* (previously named *Hox-1*, *Hox-2*, *Hox-3* and *Hox-4*) (Tabin, 1992 and McGinnis & Krumlauf, 1992; Izpisua-Belmonte, 1992a &1992b). The four clusters of *Hox* genes have evolved from a single ancestral cluster of genes, and each have direct homologs in the other three clusters (Akam, 1989). Altogether there are 13 sets of ancestrally related homologs known as paralogue groups (Kessel and Gruss, 1990; Krumlauf, 1992; Scott, 1992; Morgan et al., 1992; Krumlauf, 1993; Nelson et. al., 1996).

The progression of *Hox* gene family expression patterns has been extensively studied from early to late limb bud stages (19 to 27) (Izpisua-Belmonte, 1992a). However, recent studies have shown that the expression of *Hox* genes in the limb is far more complicated than originally understood (Nelson et al., 1996) . As their expression patterns

evolve, their relative expression boundaries fail to fit in the nested domains originally expected by analogy with *Drosophila* studies (Burke et al., 1995).

Clones of chick *Hox* genes (*Hoxa, Hoxb, Hoxc, Hoxd*) have been isolated and their expression patterns have been studied during normal limb development by means of chicken homeobox specific probes. Whole mount in situ hybridisation of these genes indicates 3D expression domains in the limb (Kessel and Gruss, 1990; Nohno et al., 1991; Tabin 1991; Dolle et al., 1993; Morgan et al., 1994; Burke et al., 1995; Davis et al., 1995; Nelson et al., 1996).

Hoxa

During normal development *Hoxa* activation starts with the expression of *Hoxa-9, Hoxa-10, Hoxa-11* and *Hoxa-13* (Fig. 2a). At stage 17 (52- 64 hours) *Hoxa-9* is activated and its expression is uniform in the limb mesenchyme (Mackem and Mahoon, 1991). At stage 19 (68- 72 hours) there is an anterior-proximal region in the wing in which no expression of *Hoxa-9* is observed, and this persists and expands throughout development. In the remainder of the limb, *Hoxa-9* expression remains strong up to stage 23 (3.5-4 days) (Tabin, 1991; Nelson et al., 1996). By stage 25 (4 days) only very low levels of the transcript are detectable in the limb. *Hoxa-10* expression starts at stage 17, and is seen uniformly in the limb mesenchyme but not in the marginal mesenchyme. By stage 22 (3.5 days), *Hoxa-10* is expressed throughout the wing bud excluding the anterior-proximal region of the wing. As the limb grows the levels of *Hoxa-10* transcripts drops. At later stages (28-30; 51/2- 61/2 days), the transcription is only detectable at the upper wing region (Humerus) and the lower wing region (radius and ulna) but absent from the digit region of the wing. *Hoxa-11* is also first

activated at stage 17, when its expression is localised to the medial region of the wing bud, but by late stage 19, *Hoxa-11* is expressed along the distal region of the wing bud (Nelson et al., 1996). At stage 22, expression is seen in the distal half of the limb across the anterior-posterior axis and by stage 25, *Hoxa-11* expression becomes restricted to the presumptive lower wing (radius and ulna region). There is no detectable level of the transcript in the proximal wing or the digit region, and this pattern of expression persists till stage 28 when the levels begin to drop. *Hoxa-13* is activated at stage 19, when its region of expression is the posterior distal margin. By stage 23 expression is quite strong in the crescent along the distal margin of the wing bud (Laufer et al ., 1994; Nelson et al., 1996). By stage 25 the transcript is detected throughout the presumptive digit region. This pattern persists up to stage 28. At stage 23, cells are found in the distal tip of the limb bud that do not express *Hoxa-13*. These cells are thought to give rise to cells in the anterior third of the digit region which express *Hoxa-13* later at stage 27 (Nelson et ., 1996).

Hoxb

The *Hoxb* cluster expression pattern is not as elaborate as the *Hoxa* cluster. The first *Hoxb* gene activated is *Hoxb-8*, which is expressed in the posterior domain of the early limb bud, prior to the onset of the limb itself (Charite et al., 1994). It is first detected at the time of continued proliferation of lateral plate mesoderm at the presumptive limb axis (Stratford et al., 1997; Scotting et al., 1990). It is thought that *Hoxb-8* gives rise to cells that are competent to become ZPA, which in turn will be competent to express sonic hedgehog. *Hoxb-8* is not expressed in the limb during stages 19-27, when the other *Hoxb* gene expression are expressed. *Hoxb-9* is expressed at stage 19 in the anterior portion of the limb bud. It is also detected in the

limb mesoderm directly subjacent to the AER, but not observed in the ZPA region (Charite et al., 1994; Nelson et al., 1996).

Hoxc

Hoxc cluster genes, with the exception of *Hoxc-11*, are all expressed in the anterior proximal portion of the wing, starting at stage 17. The 3′ members of the cluster (*Hoxc-4*, *Hoxc-5*, *Hoxc-6* and *Hoxc-8*) are expressed in the wing. The 5′ members of the *Hoxc* (*Hoxc-9*, *Hoxc-10* and *Hoxc-11*) are expressed only in the leg (Burke et al., 1995). *Hoxc-4*, *Hoxc-5*, *Hoxc-6* and *Hoxc-8* expression is restricted to the anterior/posterior portion of the wing bud. *Hoxc* genes maintain the same anterior/ posterior expression pattern during the limb bud growth till about stage 27 (5 days) when the expression starts to taper off (Peterson et al., 1992, Nelson et al., 1996).

Hoxd

Hoxd genes are expressed at the same time as *Hoxa* genes (Dolle et al., 1989; Izpisua-Belmonte et al., 1991; Yokouchi et al., 1991). *Hoxd-9* and *Hoxd-10* are activated at the time of limb bud outgrowth anterior-posteriorly (Fig. 2b). *Hoxd-11*, *Hoxd-12* and *Hoxd-13* are activated sequentially at the posterior border of the limb bud (Laufer et al., 1994). By stage 23 the *Hoxd* gene cluster is expressed around the distal posterior region of the wing, where *Hoxd-9* and *Hoxd-10* occupy a larger surface area than *Hoxd-11*, *Hoxd-12* and *Hoxd-13*. *Hoxd-9* is first detected at stage 16 (Davis et al., 1995; Nelson et al., 1996). Its expression is uniformly spread throughout the mesoderm of the wing bud, and this expression pattern persists until stage 23/24, when it becomes excluded from the distal mesoderm (Laufer et al., 1994; Nelson et al., 1996). The expression is then only evident in the

presumptive lower wing (radius and ulna). By stage 27/28, *Hoxd-9* expression is restricted to the marginal mesoderm adjacent to the flank. *Hoxd-10* is also activated at stage 16 and the transcript is detected uniformly in the mesoderm. By stage 20 the expression becomes posteriorly biased (Nelson et al., 1996). This persists as the limb grows, and by stage 23, the expression is restricted to the proximal mesoderm. At stage 25, the expression domain is separated into the lower wing region (radius and ulna) and the digit region (Laufer et al., 1994; Nelson et al., 1996). *Hoxd-11* is activated at stage 18, and is first seen at the posterior margin (Davis et al., 1995; Nelson et al., 1996). *Hoxd-11* remains posteriorly biased, and as the limb grows the expression spreads to occupy the posterior half of the wing mesoderm. At stage 27 the expression domain separates into the radius-ulna region, where expression is strongest posterior to condensing radius, and the presumptive digit region, where expression extends from the posterior margin to the condensing cartilage of metacarpal 2 (Nelson et al., 1996).

Hoxd-12 is first detected following the activation of *Hoxd-11* in the posterior region of the wing bud. The posterior expression begins to spread anteriorly. As the expression moves anteriorly, a discrete domain in the digit region is seen, extending from digit 3 region to the condensing metacarpal. This is achieved in the developing limb by anterior divergence at the distal border (Nelson et al., 1996). By stage 23, *Hoxd-12* expression is seen only in the condensing radius region (lower wing). The last of the *Hox* genes to be activated in the limb is *Hoxd-13*. It is first observed in the posterior region of the limb at stage 19. By stage 23 the proximal expression has begun to fade while the distal expression begins to expand anteriorly. By stage 25 only the distal domain is detectable, and this domain eventually expands to occupy the presumptive digit region.

Hoxd-13 expression is not detected in the limb after stage 25 (Nelson et al., 1996).

Misexpression of *Hoxd-11* in the chick leg causes a larger than normal digit I condensation. This undergoes an additional segmentation producing a toe with two phalanges rather than one (Morgan et ., 1992). Two mechanisms may be involved in the larger initial condensation. It may result from recruitment of more mesenchyme to the condensation, or by an increase in proliferation within the early condensation, under the influence of *Hox* gene expression, indicating that *Hox* genes may act early at the time of cartilage condensation (Johnson and Tabin, 1997; Goff and Tabin, 1997)
In the case of the *Hoxa-11/Hoxd-11* double knock out, there is a complete loss of cartilage elements (Goff and Tabin, 1997).

Bone morphogenetic proteins (*BMPs*) and their role in Programmed cell death (Apoptosis)

Apoptosis

Programmed cell death (PCD) is the process whereby unnecessary tissues are eliminated during embryonic development (Loo and Rillema, 1998). The term necrosis is used to describe the dying process of a cell in response to injury and apoptosis is used to describe physiological cell death (Buja et al, 1993). Recent studies have shown the existence of a suicide program in the cell which can be activated or repressed by a variety of physiological stimuli. This suicide program is a method of self-destruction of the cell and constitutes the molecular basis for apoptosis (Hurle et al., 1995).

PCD is a normal feature of development and growth and is a prominent feature of avian limb bud development. Interdigital cell death is found throughout the Amniota, but the early avian limb bud has a specific pattern of cell death very different from that of mammals. During limb development, mesenchymal cells in restricted areas of the limb bud (anterior necrotic zone - ANZ; posterior necrotic zone - PNZ; opaque zone - OZ; and interdigital necrotic zones - INZ) are eliminated by PCD (Saunders et al., 1962). The PNZ is found only in the chick wing bud and is absent even from other avian wings. In the early stages of limb development mesenchymal cells in the ANZ and PNZ are eliminated by PCD between days 4-6 of development (Saunders et al., 1962; Hinchliffe, 1982; Saunders and Fallon, 1966). PCD also occurs in the INZ in the mesenchymal areas separating the chondrifying digits of the developing limb (Tone et al., 1997). During their evolutionary history birds have gone through a process of digital reduction and have in effect only 3 digits. It has been thought that the ANZ and PNZ might reduce the anterior and posterior axis of mesenchyme available for digit formation, in combination with the evolutionary background of pentadactyl limb development (Ganan et al., 1996a).

The PNZ first appears at stage 24. The presumptive PNZ has a commitment to cell death by stage 17, and will die on schedule if transplanted to the somite region after this stage. However, this commitment is not irreversible till stage 22. Prior to this time, the PNZ can be rescued by grafting to the dorsal region of the wing bud (Saunders, et al. 1962). At stage 22, cell division in the PNZ declines rapidly as does RNA and protein synthesis. By stage 26 the PNZ has begun to disappear (Saunders et al., 1962).

Other factors such as administration of RA at an appropriate time of development has been known to increase cell death selectively. When RA is administrated to pregnant *Hammertoe* mutant females mice, it induces interdigital cell death, perhaps indicating that RA has an specific action in inducing cell death (Zakeri and Ahuja, 1997). Conversely, Janus Green decreases PCD (Menkes and Deleanu, 1964).

Formation of digits takes place in the distal segment of the limb bud (autopod). As the digits begin to appear the undifferentiated interdigital mesenchymal cells undergo massive cell death by apoptosis creating the INZ. Early morphological studies have shown the temporal correlation between the onset of interdigital cell death and the cessation of AER activity (Pautou, 1978 and Macias et al., 1996).

The interdigital mesenchyme of the chick leg bud prior to the onset of cell death has a significant chondrogenic potential. It retains the ability to form ectopic digits in various experimental conditions (Hurle and Ganan, 1986). *In vitro*, cultures of interdigital mesenchymal cells remain highly chondrogenic up to 24 hours prior to the onset of cell death (Garcia-Martinez et al., 1993).

Development of an extra digit in the interdigital region of the chick foot can be brought about by either explantation of interdigital cells to culture and reimplantation to the limb or by removing the marginal ectoderm including the AER and a small amount of subridge mesoderm (Ros et al 1994). This is thought to occur by abolition of local antichondrogenic active in the interdigital spaces (Ganan et al., 1994b). Experiments have shown that shortly after causing injury to the third interdigital space of the leg bud at stage 28, changes in extra cellular matrix distribution associated with formation of extra digits is observed (Hurle and Colombatti, 1996). This perhaps indicates that formation of extra digits in the chick foot is due to

factors such as tenascin deposition and loss of elastin fibrillar scaffold rather than remodifying the signalling mechanisms accounting for normal digit formation/patterning during early stages of development (Hurle and Colombatti, 1996; Hurle et al., 1996). *In vitro,* tenascin deposition and loss of elastin fibrillar scaffold precedes chondrogenesis (Hurle and Colombatti, 1996).

Administrating either FGF-2 or FGF-4 at the third interdigital area of chick foot prior to the onset of cell death (stage 28-30) inhibits the regression of interdigital tissues, leading to the formation of soft tissue syndactyly (Macias et al., 1996). When extra digits are induced in the interdigital spaces of chick leg bud, the pattern of expression of the 5' located *Hoxd* genes is not modified (Ros et al., 1994). This perhaps indicates that each mesenchymal region expressing a given *Hoxd* gene has the potential to develop extra digits. Extra digit induction can occur long after the patterning mechanisms have ended, indicating that digit organisation mechanism are independent of *Hoxd* gene expression (Ros et al., 1994). These interesting observations are hard to reconcile with conventional views on limb specification.

BMPs

Many signalling molecules have been identified as being involved in mediating cell-cell communications. The secreted bone morphogenetic proteins (*BMPs*) are thought to control many aspects of vertebrate development (Hogan, 1996).

BMPs are protein extracts from bone that can initiate cartilage formation which ends in *de novo* bone formation (Wozney, 1988). The *BMP* refers to the activity derived from bone that induces the formation of cartilage and bone *in vivo.* Formation of bone and cartilage *in vivo* leads to a series of

developmental processes such as proliferation and differentiation both resulting in an transient formation of cartilage and living bone tissue complete with hematopoietic marrow (Wozney, 1989).

BMPs were originally identified by their presence in bone inductive extracts of demineralized bone. Based on the amino acid sequence homology and the conservation of 7 cysteine residues between the transforming growth factors beta (TGF- ß) and the *BMPs*, *BMP-2* through to *BMP-7* are thought to be related to each other and distantly related to the TGF-ß superfamily (Celeste et al., 1990, Wozney et al., 1990). *BMP-2* and *BMP-4* are 92% identical and *BMP-5*, *BMP-6* and *BMP-7* are about 70% identical (Wozney, 1992). The first three *BMPs* were purified from the demineralized bovine bone that induced ectopic cartilage and chondral bone when implanted in experimental animals (Wozney et al., 1988). *BMP-1* was a putative protease of the astacin family. *BMP-2* and *BMP-3* were related to human TGFß. In vertebrates *BMPs* are involved in cell fate determination, differentiation, proliferation, morphogenesis, development of all organs and tissues, basic embryonic body planning and apoptosis (Hogan, 1996).

Experiments have shown that single molecules such as *BMP-2* are capable of inducing formation of new cartilage and bone when implanted ectopically in a rodent assay system (Wozney, 1992). In situ hybridization of the developing embryonic skeleton shows that *BMP* mRNAs are spatially and temporally expressed appropriately for the proteins involved in the induction and development of cartilage and bone in the embryonic limb bud (Wozney, 1992). *BMP-2* is a molecule sufficient to induce *de novo* bone formation *in vivo* and it has been shown that *BMP-5*, *BMP-6* and *BMP-7* have similar sequence to *BMP-2* (Celeste et al., 1990).

During the late stages of limb morphogenesis, the autopodal cells leaving the progress zone will have one of two predetermined fates: apoptopic cell

death or chondrogenesis. Apoptosis occurs if the cells are to be incorporated into the interdigital region, and chondrogenesis occurs if the cells are to found in the digital rays (Lyons et al., 1995). *BMPs* are thought to be the prime mediators in deciding the fate of these autopodal cells leaving the PZ.

In the developing limb *BMP-2*, *BMP-4* and *BMP-7* are all expressed in the AER. It is thought that they might be involved in controlling cell proliferation and signalling to the progress zone (Francis et al., 1994; Laufer et al., 1994; Akita et al., 1996; Zou and Niswander, 1996). The expression pattern of these three genes is significantly different in the limb mesenchyme. ZPA grafts or ectopic sources of *shh* in the anterior mesenchyme of the limb will induce *BMP-2* expression in the adjacent cells. This however only happens in the presence of FGF from the AER. It is thought that *BMP-2* may act as a secondary signalling molecule involved in the induction of nested expression of *Hoxd-11* and *Hoxd-13* (Laufer et al., 1994). Ectopic expression of *BMP-2* has been shown to induce FGF-4 transcripts in the AER and *Hoxd-13* in the mesenchyme (Duprez et al., 1996).

The signalling pathway of the *BMPs* is not completely clear. It is thought that *BMP* signalling will not occur if there are no hetetrodimers formed between protein kinase type I and type II (Chang et al, 1997). The signalling may also occur through members of the TGF-ß super family related receptors (Chang et al, 1997). Obstruction of *BMP* signals in the limb bud results in inhibition of interdigital cell death and digit truncation {after infection with retroviruses carrying dominant negative *BMP* receptors} (Zou & Niswander, 1996)

*BMP*R-1a expression is found at low levels in the undifferentiated limb mesoderm and is thought to be involved in interdigital cell death. *BMP*R-1b expression is detected at high levels in the prechondrogenic blastemas, suggesting its involvement in chondrogenesis (Kawakami et al., 1996). *BMP*-2 and 4 are detected in all areas of cell death except the opaque zone. Over expressing a dominant negative form of *BMP* receptor in the chick leg bud causes regression of webbing at the interdigital regions and excess webbing at the anterior and posterior region of the limb bud (Kawakami et al., 1996). In ducks *BMP*s are not expressed in interdigital region, perhaps providing evidence that *BMP* signalling actively mediates cell death during the embryonic limb development (Zou & Niswander, 1996).
It is thought that the morphogenesis of digits in the avian limb is controlled by FGFs, TGFβ, and *noggin* via *BMP* signalling (Merino et al., 1998). Interaction between these signals controls the formation of digital rays. This is achieved by regulating the spatial distribution of *BMP*R-1b gene expression in the digit forming mesenchyme (Merino et al., 1998).

*BMP*R-1a is not modified by fibroblast growth factors, showing that it is not involved in formation of the digital rays (Merino et al., 1998). However, exogenous application of FGFs, TGFβ and *BMP*s causes changes in expression of *BMP*R-1b, compatible with the role of this receptor in the onset of chondrogenesis. Implants of *noggin* beads at the tips of digits causes truncation. This occurs as the cells in the progress zone fail to differentiate and eventually undergo cell death by apoptosis. *Noggin* has an anti-chondrogenic effect and once applied exogenously it causes down regulation of *BMP*R-1b expression, and inhibits the binding of *BMP*-2/4 and 7 to their receptors (Hirsinger et al., 1997; Zimmerman et al., 1996). Extra digits are formed by application of TGFβ at the interdigital mesoderm region, indicating that members of the TGFβ family may be involved in

chodrogenic proximal signals (Ganan et al., 1996a). TGFβ 2 expression in growing digits confirms TGFβ 's role in the control of digit chondrogenesis and joint and tendon formation. Implanting FGF soaked beads causes inhibition of the expression of *BMP*R-1b gene, confirming their role in keeping the progress zone in an undifferentiated state (Ganan et al., 1996a). All this is consistent with the idea that there may be a interactive feedback loop between *noggin* and *BMPs* which controls digital skeletogenesis (Merino et al., 1998). Inhibition of cell death following the application of *noggin* beads in the interdigital regions also supports the suspected role of *BMPs* during apoptosis (Zou & Niswander, 1996).
It is thought that the morphogenesis of digits in the avian limb is controlled by FGFs, TGFβ, and *noggin* via *BMP* signalling (Merino et al., 1998). Interaction between these signals controls the formation of digital rays. This is achieved by regulating the spatial distribution of *BMP*R-1b gene expression in the digit forming mesenchyme.

Epithelial FGF has been shown to prevent the *BMP* mediated apoptosis in the mesenchyme, suggesting that there is an epithelial-mesenchyme interactions (Buckland et al., 1997). FGF-4 implants between stages 20-22 prevent the *BMP* induced apoptosis where as application of *BMP* during the same stages causes apoptosis to occur. The balance between the effects of FGFs and *BMPs* has been shown to control the size of chondrocyte precursor cell pool (Buckland et al., 1997). Implanting *BMP*-4 to the core of a stage 36 limb bud 24 hours after removal of the AER causes chondrogenesis rather than the expected apoptosis, application of FGF-4 has been shown to nullify this effect. This suggests that rate of chondrogenesis is controlled by the a balance between the effects of FGFs and *BMPs*, indicating that FGFs may control the size of appendages when they are initially formed (Buckland et al .,1997)

BMP-4 may also play an important role in mediating apoptosis during chick limb development. Its expression is detected in the PNZ, ANZ and INZ. All this evidence shows that *BMP-4* may be involved in programmed cell death in different regions of the embryo at different times during development.

Digit Morphology

Many different disciplines of biology including paleontology, embryology, comparative anatomy and molecular genetics have provided invaluable information about embryonic patterning. However, molecular genetics is perhaps the most accurate method of examining phylogenetic relatedness by clarifying the genomic sequence homologies. It has provided tools which allow researchers to understand the molecular events during development and embryonic pattern formation.

The developing avian limb has been a main model system for studying vertebrate pattern formation. By investigating the molecular regulatory events underlying vertebrate pattern formation, explanations for the mechanisms of evolutionary changes in morphology may be finally understood. One area of great interest lies in answering the question of the origin of the pendactyl limbs. All tetrapods with the exception of a few fossils have limbs with five or fewer digits (Tabin, 1992). A true digit pattern of greater than five has not been observed. Mutations in mice, chicks and even humans have produced extra digits, however these "novel" digits upon inspection have failed to be identified as true digits but rather a duplication of an already existing digit, usually a mirror image of the digit it sits closest to (Tabin, 1992). 'New' fingers have been seen in frogs and pandas, but in all cases the digit has not been a true finger but rather a modification of an already existing wrist bone (Gould, 1980). The

development of such sham digits is thought to occur when developmental constraints respond by trying to maintain the morphology of five real digits (Gould, 1980). Alberch (1985) proposed that the ability to select for an extra digit is directly related to the size of the embryonic limb, thus St. Bernards are often seen to have an extra digit, but poodles never do. Investigating the molecular basis involved in digit pattern formation has been the only way to explore such claims. When polydactyly arises, the two digits defined as having the same identity are of similar size, morphology and have the same number of phalanges (Tabin, 1992). Since the duplicated digit may have very limited evolutionary use, it has never been selected for a distinct function, which is perhaps the explanation why tetrapods rarely maintain a polydactylous limb (Tabin, 1992).
Homeobox containing genes are thought to be the best candidate for specifying the positional information in the developing limb.

Angiogenesis

The vascular system in the limb is not only important in terms of providing nutrition but has also been proposed as an important contributor in establishing the skeletal pattern (Feinburg and Saunders, 1982; Fienberg and Beebe, 1983).
The vascular pattern of the developing limb bud seems to be related to the position of the AER (Feinberg and Saunders, 1982). The AER borders the entire limb bud prior to appearance of marginal veins. The AER becomes more distally restricted and asymmetrical as the limb elongates, being thicker postaxially than preaxially. Removing the AER at a stage prior appearance of the marginal veins results in failure of marginal veins to form whereas the proximal parts of the vascular pattern develop normally provided they had been established prior to removal of the AER (Feinberg

and Saunders, 1982). Therefore development of a characteristic vascular pattern is influenced by the AER and the remainder of the overlying limb ectoderm (Feinberg et al., 1983).

At early stages of development the blood vessels are homogeneously distributed throughout the distal portion of the limb. Avascular areas arise posterior-anteriorly, corresponding in position to the distal skeletal elements (Feinberg et al., 1986). Cartilage differentiation occurs after vascular regression begins in the areas with few or no perfused capillaries (Feinberg et al., 1986). In any case by stage 24 the vascular network is isolated in two separate areas - an avascular area which corresponds to prechondrogenic area and a well vascularized region corresponding to the premyogenic area (Gould et al., 1972). Studies have shown that the establishment of the differential vascular pattern is concurrent with establishment of the primary subclavian artery at stages 20 to 21 (Caplan and Koutroupas, 1973; Goodwin and Cohen, 1969; Wolpert, 1969).

It has been shown that vascular changes precede morphological and molecular changes in the limb (Caplan and Koutroupas, 1973). Studies of chick circulatory pattern show the limb bud is supplied by many small lateral offshoots of the aortas until stage 20 (Evans, 1909). These vessels begin to broaden at late stage 20 and spread to the wing by stage 21. A recognisable central artery (primary subclavian artery) can be seen by late stage 21 or early stage 22. The central and the marginal vessels form by the widening of existing vessels. As the vessels broaden the prospective cartilage forming areas of the limb bud become less vascularized as compared to the prospective muscle forming areas (Caplan and Koutroupas, 1973). By stage 22 fewer capillary sized vessels are present in the core region than the peripheral regions of the limb bud. At stage 24 the core of the limb is essentially avascular while the prospective muscle

forming tissues are well vascularized. It has been suggested that the prospective chondrogenic regions are poorly vascularized where as the prospective myogenic regions are richly vascularized, and this differential vascularization is of developmental significance (Caplan and Koutroupas, 1973). It has also been proposed that this differential vascularization brings about a gradient of oxygen and low molecular weight metabolic substances. One such substance is nicotinamide, which has been proposed as being responsible for patterning of inner core of cartilage and outer dorsal and ventral blocks of muscle (Caplan and Koutroupas, 1973). This theory was thought viable since *in vitro* high oxygen tension was believed to cause limb mesodermal cells to express a myogenic phenotype while low oxygen levels seemed to induce chondrogenic expression (Caplan and Koutroupas, 1973). However it was found that 3-acetylpyridine induced muscle hypoplasia is preceded by complete peripheral nerve degeneration which is perhaps the reason for muscle degeneration (McLachlan et al, 1976).

The mesenchymal cells of the limb give rise to cartilage and connective tissues. Relevant to the theories of an angiogenic control of muscle formation is the observation that after replacing the somites of a chick at the limb level with quail somites the connective tissues and the cartilage are of chick origin whereas the muscles of the wing are all composed of quail cells (Christ et al., 1977; Chevallier et al., 1977a; Kieny et al., 1986). Replacing the somatopleure of the chick with that of a quail shows that all the tissues were of quail origin except the muscles (Christ et al., 1977). Removing the somites by irradiation gives rise to limbs without muscles (Chevallier et al., 1978). These experiments suggest that the skeletal muscle cells are derived solely from the somites whereas the connective tissues and cartilage cells are derived from the somatopleure. Evidence also suggests that in the absence of somites, other cells of the limb may

give rise to muscles. When chick somites were grafted into quails, the resulting limbs contained both chick and quail muscle cells (Chevallier et al., 1977b). Also irradiation of somites results in muscleless limbs in only two thirds of the limb (Chevallier et al., 1977b). However, McLachlan and Hornbruch (1982) suggested that somatopleural cells could give rise to muscle cells under some circumstances for instance cells normally labile can give rise to muscle in the absence of somatically derived muscle cells (McLachlan and Hornburch, 1979).

The models for limb development and pattern formation originally proposed by Saunders and Zwilling has been greatly modified in recent years. Molecular biology has provided methods of isolating molecules involved in limb development. Certain signalling molecules are known to play substantial roles in limb patterning (Johnson and Tabin, 1997). Fibroblast growth factors, *Hox* genes, sonic hedgehog and bone morphogenetic factors are amongst the signalling molecules involved in patterning of the limb.

Aims of this thesis

FGFs play an important role in limb. The importance of the *shh*/FGF-4 feed back loop has been investigated extensively. However these experiments have primarily been carried out on young chick limb buds stages 17-21. In culture posterior cells with FGF-4 were unable to maintain *shh* expression but when grafted to a host limb bud *shh* expression was recovered, suggesting that FGF-4 does not maintain *shh* expression itself but it is competent of *shh* expression (Kimura and Ide, 1998).

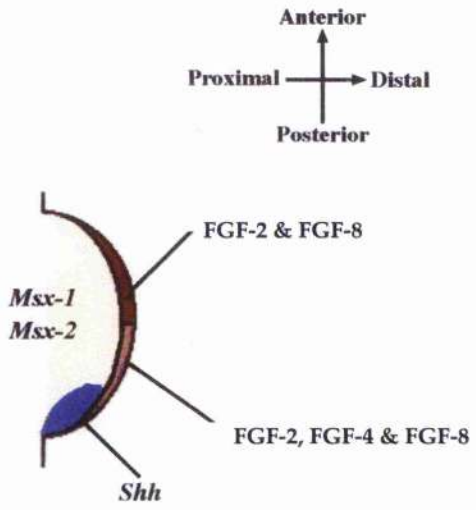
In the following chapters, the role of FGF on a stage 26 chick limb will be investigated. In this thesis the following will be discussed; the presence of

a proximodistal gradient of FGF in the limb; the potential for extending the active life of the AER; the consequence of extending the active life of the AER; soft tissue analysis, digit morphology; the molecular mechanisms operating in the posterior region and programmed cell death through apoptosis.

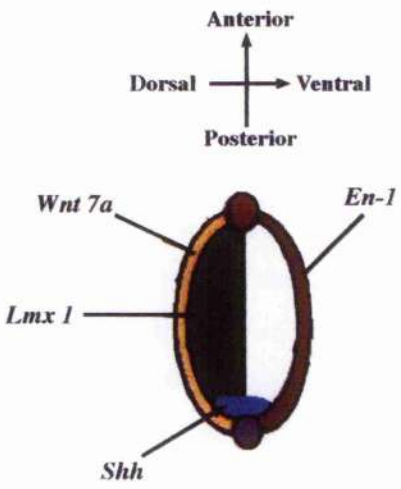
Figure 1(a and b).

1a. Molecules expressed at the tip of the limb bud. Transcripts of FGF-2, 4 and 8 are expressed in the AER. Sonic hedgehog transcripts are expressed in the posterior distal limb region.

1b. Transverse section through the wing bud. *Wnt-7a* is expressed in the dorsal ectoderm and *Lmx-1* is restricted in the dorsal mesenchyme. *En-1* is expressed in the ventral mesenchyme .


Anterior
Proximal
Distal
Posterior
FGF-2 & FGF-8
Msx-1
Msx-2
FGF-2, FGF-4 & FGF-8
Shh

a)

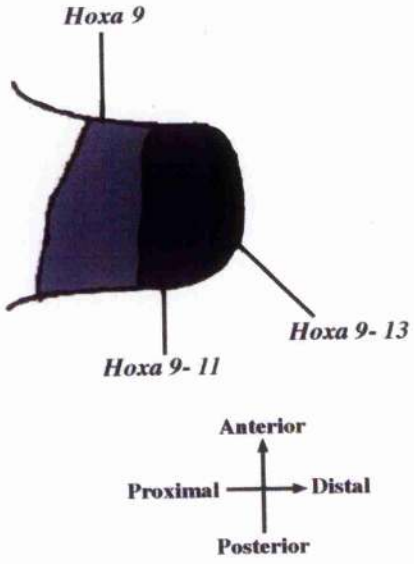

Anterior
Dorsal
Ventral
Posterior
Wnt 7a
En-1
Lmx 1
Shh

b)

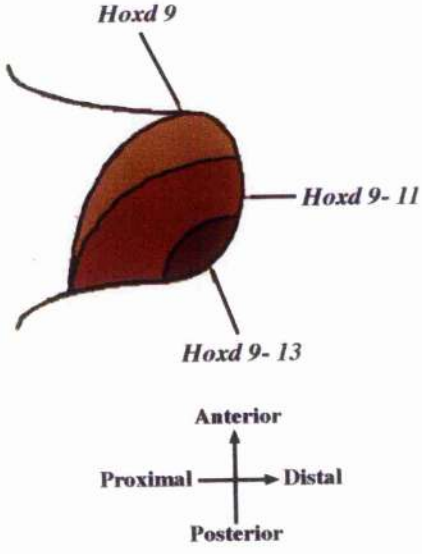
Figure 2 (a and b). Illustration of expression pattern of *Hoxa* and *Hoxd* genes in the developing limb bud.

2a. Hoxa-9 through to 13 show nested expression pattern domains along the proximodistal axis.

2b. Expression pattern of Hoxd-9 through to 13 in a nested domain along the anteroposterior axis.


Hoxa 9
Hoxa 9- 13
Hoxa 9- 11
Anterior
Proximal
Distal
Posterior

a)


Hoxd 9
Hoxd 9- 11
Hoxd 9- 13
Anterior
Proximal
Distal
Posterior

b)

Chapter 2

General Methods and Materials

Methods and materials

Egg Incubation

The eggs used in all experiments were White Leghorn free range, purchased from J K Needle & Co, Poyndon Farm in Hertfordshire. Each egg was dated and incubated blunt side up at 38°C. Since low humidity causes dehydration and subsequent death of the embryo, the moisture levels were checked. The incubation period prior to the start of the experiments was 3-5 days depending on the stage required. Usually the eggs were left at room temperature about 60 minutes before the experiments were carried out. Leaving the embryos at room temperature for an hour caused a decline in the embryo's heart rate and movements. The embryos were returned to the incubator within 75 minutes since over cooling could decrease the survival rate.

All experimental and control embryos were incubated up to 10 days maximum.

Windowing the Eggs

At the appropriate stage the eggs were removed from the incubator and wiped clean with 70% alcohol. The opening was made at the air sac is therefore eliminating the need for puncturing the egg prior to windowing.

The shell was cracked carefully and removed in small pieces until the opening was wide enough to work through. The thin Vitelline membrane was teased away gently until the embryo was exposed. Once the procedures were carried out the eggs were sellotaped and placed back in the incubator.

Histological procedures

The limbs used were either implanted with FGF-4 beads, with PBS beads, untreated controls or shams. Untreated limbs were sectioned as comparisons.

The limbs were separated from the flank and fixed in Formol saline (40% formaldehyde diluted 1:10 in PBS) for 3 hours. The limbs were then dehydrated in ascending concentrations of ethanol (75%, 96% and 100%). For each concentration of ethanol two washes were carried out, 1 hour per wash.

The dehydrated limbs were treated in 50:50 ethanol: chloroform for 30 minutes and then placed in pure chloroform for 60 minutes. The limbs were then ready to be embedded in paraffin wax.

The limbs were immersed in paraffin wax at 40°C for a total period of 4 hours. The paraffin was changed every hour to ensure that the chloroform was completely removed from the limbs (failure to remove the chloroform from the samples prevents the paraffin from infiltrating the limb completely and once sectioned the tissue will break). After 4 paraffin changes the limb was oriented correctly, embedded and placed in a 4°C refrigerator to set.

Each limb was embedded in an individual block of paraffin wax, the blocks were then trimmed down to an area immediately surrounding the limbs and sectioned transversely at 7 μm using a microtome. The sections were cut in series and placed on a 40°C water bath. The water bath contained gelatin (1g) and potassium dichromate (1g) to increase the adhesiveness of the sections once placed on slides.

The sections were mounted on slides and left in a 40°C oven overnight. The heat ensured the paraffin expansion and flattening of the tissues sectioned. The slides were then stained for nuclei, cartilage and mucin (Purple/ blue) and muscle, connective tissue and red blood cells (Pink / red) in Hemotoxylin and Eosin.

Hemotoxylin and Eosin staining process

The sections were placed in a rack and immersed in Xylol for 4 minutes. The slides were rehydrated in a descending concentration of alcohol (100%, 96%, and 75%), 4 minutes each wash. The slides were then immersed in distilled water for 2 minutes.

The sections were stained in Harris's Hemotoxylin for 10 minutes exactly and rinsed in distilled water. To remove the excess stain the sections were placed in a an acid/alcohol solution (0.5% HCL in 96% alcohol) for 1 minute(differentiation process).

The sectioned were then transferred in to running tap water for 10 minutes, the alkali in the tap water turned the sections blue (bluing in tap water process).

The sections were counterstained in 0.5% Eosin for 2 minutes exactly, and then rinsed rapidly in tap water followed by 75% alcohol. Once the staining process was completed the sections were dehydrated again in alcohol (75%, 96% and 100%) for a period of 2 minutes per each concentration.
Finally the sections were placed in Xylol for further clearing and mounted in DePex.

Chapter 3

A Proximo-distal gradient of FGF-like activity in the embryonic chick limb bud

Abstract

In a microassay for anchorage independent growth in soft agar, NR6 cells form colonies in a dose dependent manner in the presence of fibroblast growth factor (FGF). Using this assay system, the ability of thin sequential slices of embryonic chick limb bud to promote colony formation was investigated. A functional gradient of colony promoting ability along the proximo-distal axis of the developing chick limb bud (stages 22-26) was observed. The highest number of colonies was observed in the presence of the most distal slices, and colony number decreased progressively at proximal levels. This gradient was specifically eliminated by the addition of anti FGF antibody to the assay, indicating that it was caused by a functional gradient of an FGF-like molecule.
Limbs of stages 21 to 26 were assayed: before this time limb buds are too small to slice in the proximo-distal axis in the required manner. The FGF-like gradient was observed at stages 22 to 26.

Introduction

There are a number of pieces of evidence which implicate fibroblast growth factors (FGFs) as a controlling factor in limb distalisation. mRNAs for at least three FGFs have been found in varying locations within the limb bud. FGF-2 is located throughout the bud, in both ectoderm and mesoderm (Savage, et al., 1993). It has already been shown by immunostaining of the chick limb bud that FGF-2 is present in the limb ectoderm and sub-epithelial mesoderm from stages 16-25 (Savage, et al., 1993). FGF-4 transcripts are found in the posterior AER, while FGF-8 is found throughout the AER (Ohuchi, et al., 1994). FGF-8 transcripts have been found to be present in the ectoderm of the limb bud territories prior

to the onset of limb bud growth in both chick and mouse embryos (Mahmood, et al., 1995).

Purified FGF-8 protein has been shown to rescue limb bud outgrowth in mouse limbs lacking an Apical Ectodermal Ridge (AER), but has failed to maintain the expression of sonic hedgehog gene (*shh*) (Mahmood et al, 1995). In the chick it has been shown that FGF-8 can replace the AER to maintain expression and outgrowth and patterning of the limb bud (Vogel et al, 1996). The effect of the AER in chick limbs, can also be substituted by application of FGF-4 (Niswander et al, 1993, Niswander et al, 1994). FGF-4 also maintains the signal for polarising activity produced by the posterior mesenchyme in the normal limb (Niswander, et al., 1993). *In vitro*, FGF-4 has been shown to stimulate the proliferation of late limb mesenchymal cells (Niswander and Martin, 1993).

Even though FGF-4 mRNA is expressed in the AER and FGF-4 stimulates limb mesenchyme cells to divide (Cohn et al., 1995), this still does not prove that the growth factor is present in functional form in the limb extracellular compartment, since the molecule must be translated, exported and often extensively modified before becoming active (Morris, 1996).

The aim of this investigation is therefore to ascertain if a functional gradient of exported, processed and active FGF exists along the proximal/distal axis of the developing chick limb, using a sensitive microassay. This employs NR6 cells, which are derived from NRK cells, and lack functional EGF and TGF αreceptors. They respond to the presence of members of FGF family in a dose dependent manner by showing transformed behaviour in soft agar anchorage independence

assays. No other single growth factors are known to have this effect (Morris, 1996).

Methods and Materials

Cell Culture

NR6 cells were cultured in Alpha-Eagles Medium containing 10% Fetal Calf Serum (FCS) and passaged every 3-4 day intervals. Equal volumes of 0.6% agar and double strength medium, containing 10^5 NR6 cells/ml, were mixed gently by inversion. The 0.6% agar was boiled for 15 minutes and then transferred to a 40°C water bath for 30 minutes. The media supplements were such that at final concentration the agar/medium mixture contained 10% FCS, 50 IU/ml penicillin, 50 mg/ml streptomycin and 20 mM HEPES buffer. The mixture was dispensed in 0.1 ml volumes to each of the 60 centre wells of a sterile 96 well plate. The outer wells were filled with phosphate buffered saline (PBS) to prevent drying out. The microwell plates were cooled at 4°C for 4 minutes to ensure that the agar mixture had solidified prior to incubation.

Preparation of Embryonic Material

Fertilised hen eggs were incubated blunt end up in a humidified environment at 38°C for approximately 3-5 days, depending on the stage required. Entry was made via the air space, and embryos were removed to dishes of Alpha-Eagles Medium for cleaning (removing remaining attached membranes, cranial region) and examination. Each embryo was staged (stages 16-26) according to Hamburger and Hamilton, 1951 (Hamburger and Hamilton, 1951). Using electronically sharpened

tungsten needles, 200μm slices were made through embryonic chick limbs from distal to proximal, transverse to the long axis of the limb, at a range of stages (Fig. 1). This was achieved by placing a grid of 200μm underneath the limb prior to cutting to ensure accuracy. The flank slices were cut following the same method. 2 slices at each level and stage were added to the culture cells.

Scoring the Assays

Once the tissue samples had been added to the wells of the assay, the plates were placed in a humidified incubator at 37°C and 5% CO_2 for 7 days before being fixed by the addition of a drop of formal saline to each well.
Colonies were examined unstained using an inverted microscope and only colonies containing four or more cells were scored. The chick limb cells can not invade the semi-solid medium.
Extracted bovine pituitary FGF-2 (Sigma) was added to wells of the soft agar assay in varying concentrations as a positive dose response control.
In experiments to investigate the ability of anti-FGF antibodies (Rabbit polyclonal, sigma F3393) to inhibit colony forming ability of limb bud explants, anti-FGF antibody was added to the wells in either 1:800 or 1:400 dilutions (Alpha-Eagles Medium was used to make up the dilutions). Blank solutions of equivalent volume but containing no additives were added to control wells.
Experiments were run to investigate the effects of a non-specific antibody (Rabbit polyclonal anti-fd bacterophage, sigma B7786) upon the colony forming ability of NR6 cells. The non-specific antibody was added to the wells in either 1:800 or 1:400 dilution (Alpha-Eagles Medium was used to make up the dilutions).

Measuring the Volume of the Limb Slice

It is important in principle to correct for the differing volumes of the slices, though in practice the shape of the limb means that volume increases from distal to proximal, thus acting against the spurious observation of a gradient with high distal values.
In order to measure the volume of the slices, a stage 23 limb was sectioned transversely to the proximo-distal axis at 5μm intervals and then stained with Haematoxylin and Eosin (Appendices). The sections were traced using an Olympus microscope with a drawing tube at 100x magnification and scanned into a Macintosh Performa 620 PowerPC. The area of the scans were measured using NIH Image freeware, and the volume of each section was obtained. The volume of all the sections making up a 200μm slice was summed to give the total volume of the slice.

Statistical analysis

Statistical analyses were carried out using Minitab. In all cases two way Analysis of Variance (ANOVA) was performed to determine whether the experimental sub groups were from the same or different populations. If the assay categories were found not to belong to the same population, Tukey post-hoc analyses were carried out on the data sets to determine which data sets were significantly different from their neighbours.

Results

The results demonstrate that
(i) Increasing concentrations of FGF-2 give a dose dependent response in terms of NR6 colony formation (Fig. 2). ANOVA indicated that there were

significant differences between samples (P<0.0001). Tukey pairwise comparison test (family error rate =0.05) showed that there were significant differences between 0 ng/ml and 1ng/ml, and between 1ng/ml and 10ng/ml FGF-2 treatments. No significant difference was observed between 10ng/ml and 50ng/ml.

(ii) Proximo-distal slices down the chick limb evoke a graded response in terms of NR6 colony formation, with the most distal tissue giving the highest number of colonies, at all stages tested from 22 to 26 (Fig. 3a, b). At stage 21 there were significant difference between the samples by ANOVA (F=8.49, DF=9, 40, P<0.001), but the Tukey's pairwise comparison showed that these differences existed between the limb slices and the flank and not between proximo-distal levels along the limb. At stages 22-26 there were significant differences between levels by ANOVA (F=126.50, DF=9, 40, P<0.001) and Tukey pairwise comparison showed that these difference were significant between each sequential slice (P≤0.001).

(iii) This graded response was removed by addition of polyclonal anti-FGF-2 at all stages tested (Fig. 4: stage 23 is shown as an example. An identical response was observed in all experiments.) Anti FGF was added to the wells at two different dilutions; 1:800 and 1:400. There was significant difference between the samples by ANOVA (P< 0.0001): Tukey's pairwise comparison showed that these differences existed between the limb slices treated with anti FGF and the positive controls. There was no significant difference between the colony forming of the limb slices at 1:800 and 1:400 anti FGF dilutions. At stages 21-26 there was significant difference by ANOVA (F=203.46, DF=5, 24, P<0.0001) and Tukey's pairwise comparison showed that these differences were significant between the slices treated with anti FGF and the slices treated with non-specific anti body. The non-specific antibody did not have any effect on the formation of colonies by limb explant slices (Fig. 5). In all experiments 200μm transverse slices of

limb buds were added to the assay wells. However, proximal limb bud slices have a greater diameter, and were therefore larger than distal slices. Volume measurements in a representative limb showed that the volume for the 200μm distal tip slice was 0.04275 mm^3 and that for the first proximal slice was 0.07838 mm^3. Correcting for this change in size markedly enhances the gradient effect, and I believe this justifies neglecting it in the first instance.

Slight variations was observed from day to day in both experimental and control values. However, in general the results were sufficiently similar to be directly compared without the necessity of converting to a common base line.

Previous experiments at stages 16-19 where the limb bud is 200μm or less have failed to show a difference between whole limb buds and flank (McLachlan, unpublished observations).

Discussion

NR6 cells respond in a dose dependent manner to the presence of FGF by forming colonies: they also respond to limb slices by forming colonies, with colony number increasing from proximal to distal. This colony forming ability is removed by anti-FGF antibodies (they respond to all FGF family), but not by non-specific antibodies. It therefore seems reasonable to conclude that the gradient effect is mediated by an FGF like molecule. Identification of which FGF is involved awaits the development of suitable specific antibodies.

FGF is regulated by being exported from cells, where it appears to be cell associated with heparan sulfate-related proteoglycans (Baird, 1994). These observations are consistent with the hypothesis that a functional gradient of an active FGF-like molecule is secreted by the AER at stages 22-26.

The results are consistent with the idea that a chemical gradient of growth factor may exist *in vivo*, acting over approximately a range of 600μm. However this is not the only possible explanation, FGF and FGF receptors are present in the underlying mesenchyme, thus a functional gradient of FGF could be produced in the mesenchyme itself. This may therefore prove to be a suitable test system to distinguish between long range diffusible morphogens and cell-cell relays as a signalling system (Smith, 1996).

At stage 21 no significant difference between the distal tip and the zone immediately proximal to it was observed, and this may reflect an absence of a gradient before stage 22. Alternatively the microassay used may not have been sensitive enough to detect a gradient.

In these experiments no distinction was made between the wing and the leg buds. However, in previous investigations it has been observed that whole leg buds induce slightly fewer colonies than wing buds (McLachlan, 1988).

No correlation was observed between the number of colonies formed and the distance from the explant, suggesting that the FGF like molecule diffuses evenly throughout the agar.

In conclusion, these results are consistent with the theory that FGFs are locally produced by the AER and exert a graded influence over the underlying mesoderm, and extend it by demonstrating for the first time that there is indeed a functional gradient of FGF from distal to proximal in the limb, as required by the standard theory of limb development.

Figure 1. 200 μm transverse slices of the chick limb bud were made by a sharp tungsten needle against a 200 μm grid. The red lines show the level of the cuts made; distal tip, first proximal slice and the second proximal slice.

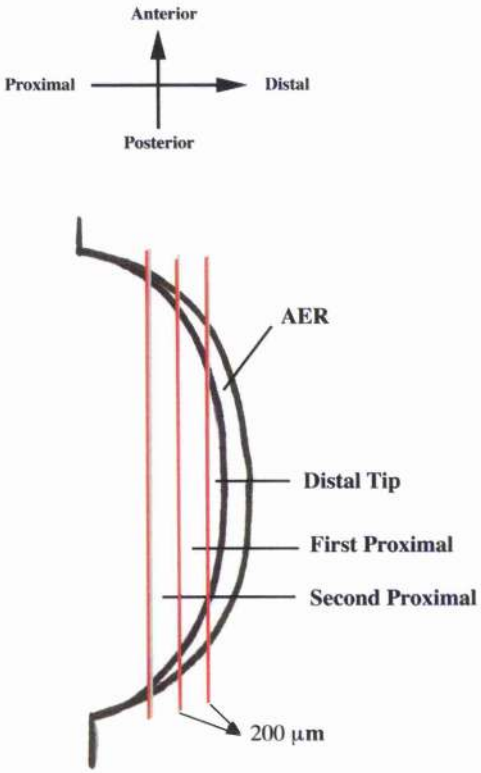

Anterior
Proximal
Distal
Posterior
AER
Distal Tip
First Proximal
Second Proximal
200 μm

Figure 2. Dose-dependent transformation of NR6 cell line by b-FGF. The transforming ability of b-FGF appears to plateau at 10 ng/ml. Transformation was measured as the ability to form colonies of four or more cells. The number of those colonies was counted in each well, which at the start of the assay contained on average 500 cells. Error bars represent standard deviations.

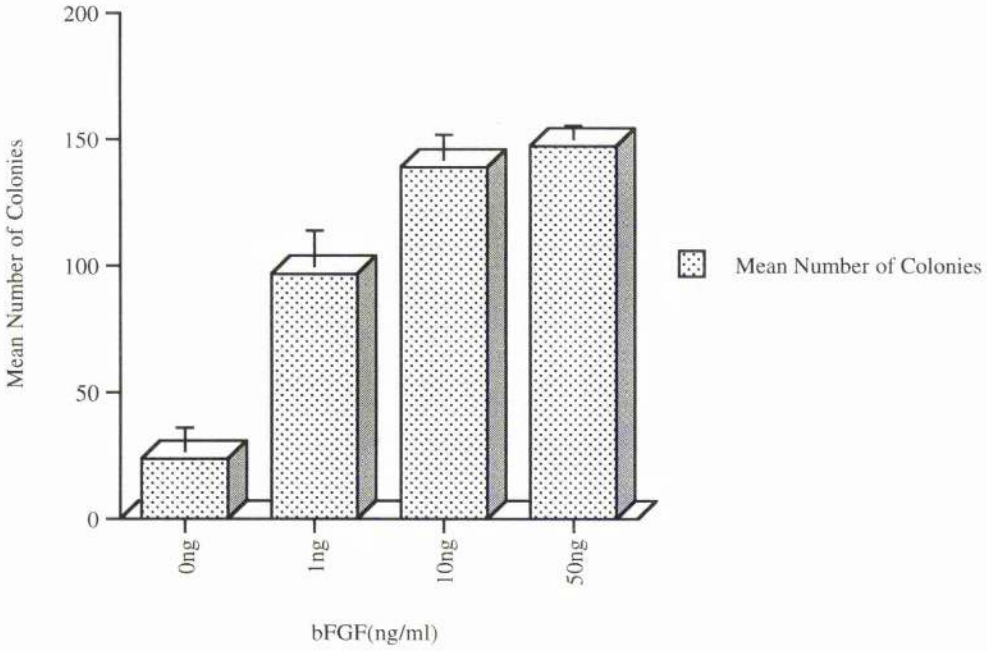

200
150
100
50
0
Mean Number of Colonies
0ng
1ng
10ng
50ng
bFGF(ng/ml)
Mean Number of Colonies

Figure 3 a. Transformation of the NR6 cell line by embryonic chick limb slices (200μm) at stages 21-23, compared with the controls in the absence of limb explants. By Tukey post-hoc test, there is a significant difference between wells containing limb slices with wells containing flank or no explants ($p < 0.001$).

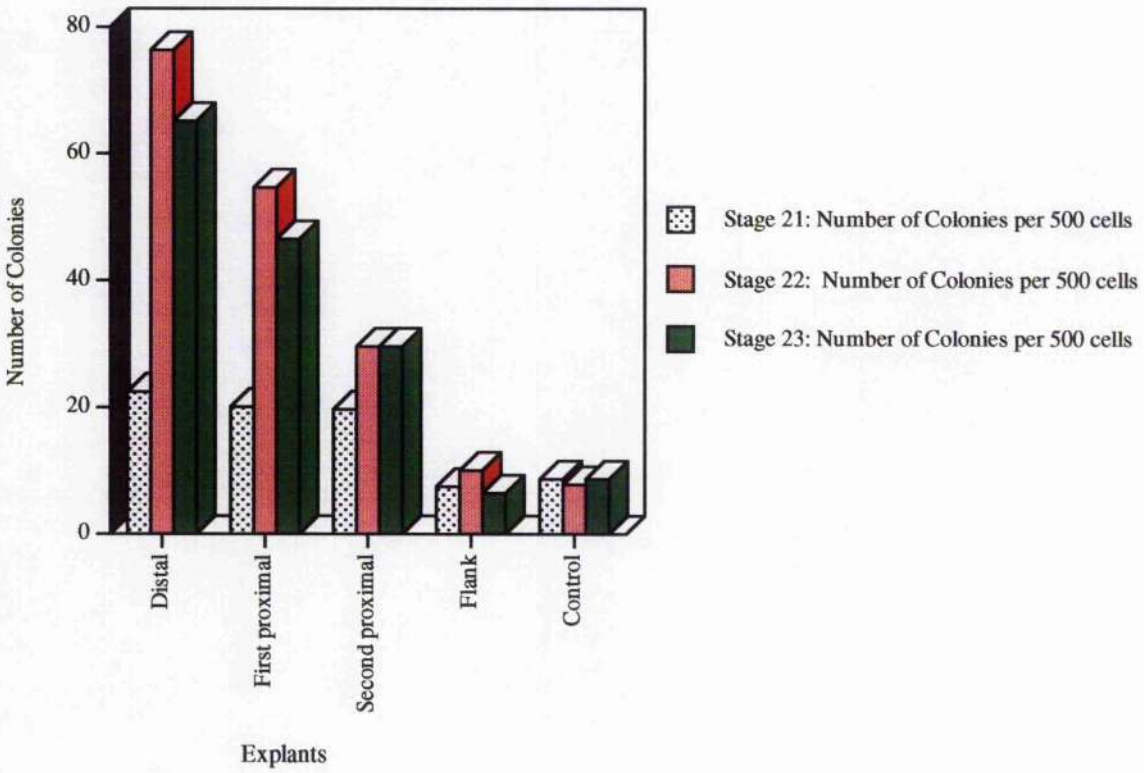

80
60
40
20
0
Number of Colonies
Distal
First proximal
Second proximal
Flank
Control
Explants
Stage 21: Number of Colonies per 500 cells
Stage 22: Number of Colonies per 500 cells
Stage 23: Number of Colonies per 500 cells

Figure 3 b. Transformation of the NR6 cell line by embryonic chick limb slices (200μm) at stages 24-26, compared with the controls in the absence of limb explants. By Tukey post-hoc test, there is a significant difference between wells containing limb slices with wells containing flank or no explants ($p < 0.001$).

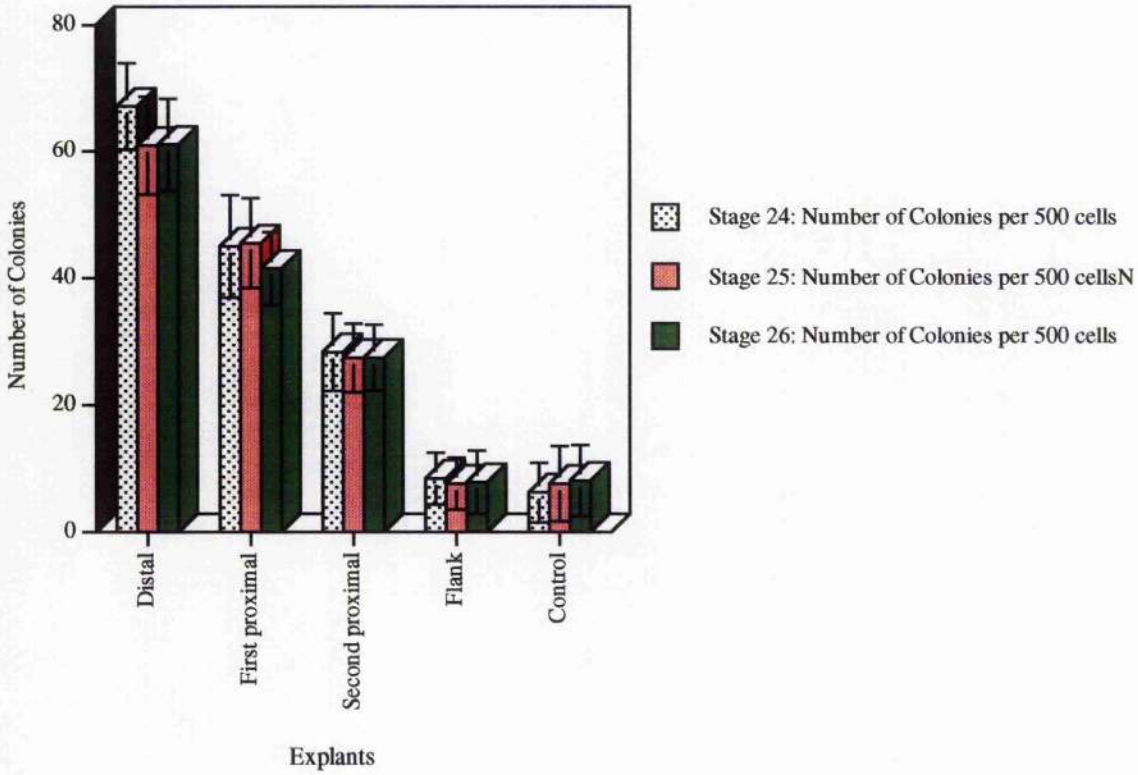

80
60
40
20
0
Number of Colonies
Distal
First proximal
Second proximal
Flank
Control
Explants
Stage 24: Number of Colonies per 500 cells
Stage 25: Number of Colonies per 500 cellsN
Stage 26: Number of Colonies per 500 cells

Figure 4. Transformation of NR6 cell line by embryonic chick slices (200μm) at stage 23, in the presence of anti-FGF. Wells contained blank solution, 1:800 and 1:400 dilutes of anti-FGF. By Tukey post-hoc test, there are significant differences between wells containing limbs only and wells containing 1:800 and 1:400 dilutions of anti-FGF ($p < 0.0001$).

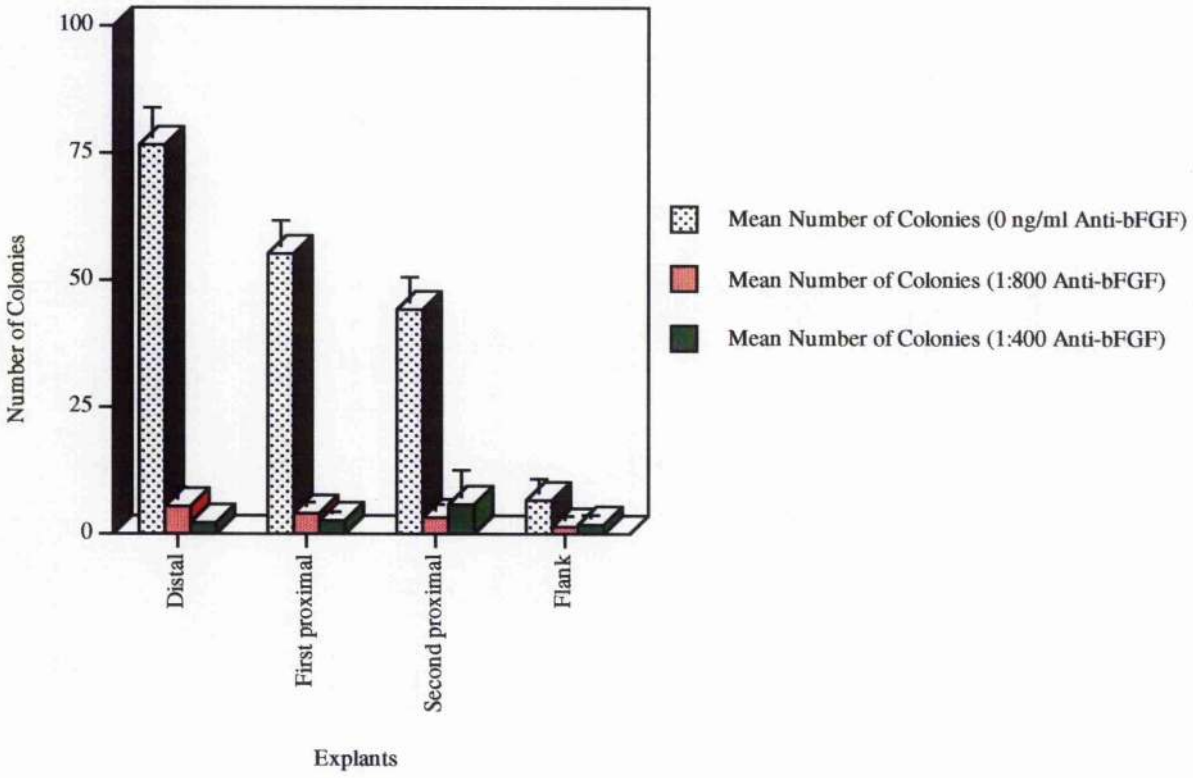
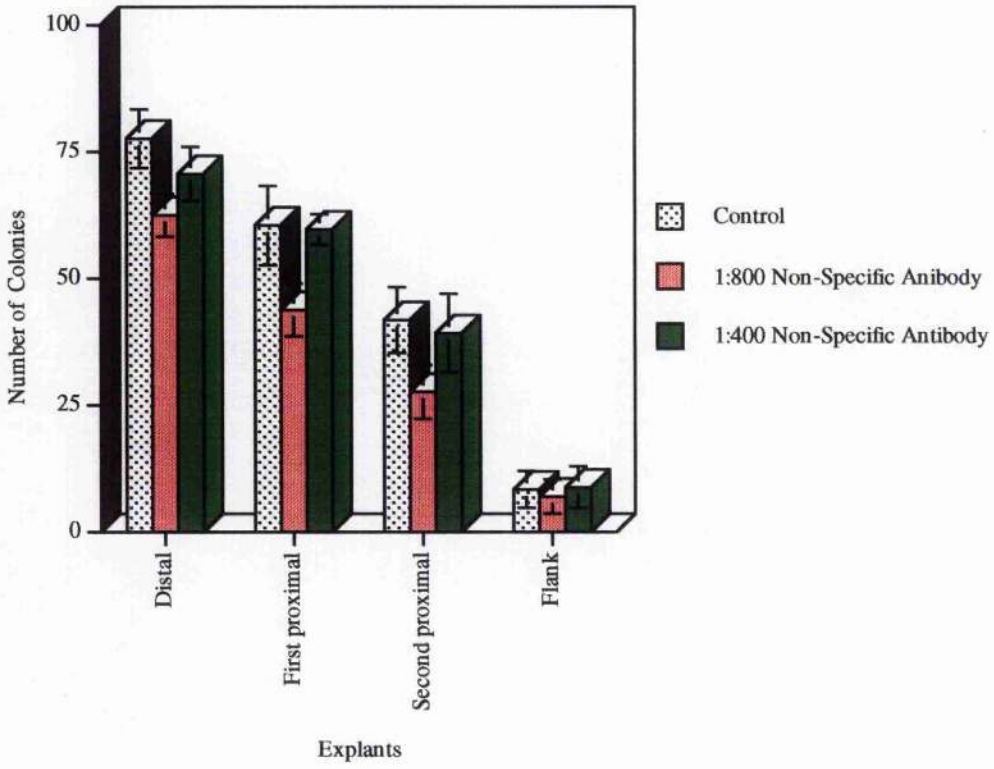

100
75
50
25
0
Number of Colonies
Distal
First proximal
Second proximal
Flank
Explants
Mean Number of Colonies (0 ng/ml Anti-bFGF)
Mean Number of Colonies (1:800 Anti-bFGF)
Mean Number of Colonies (1:400 Anti-bFGF)

Figure 5. Transformation of NR6 cell line by embryonic chick slices (200μm) at stage 23, in the presence of non-specific antibody. Wells contained blank solution, 1:800 and 1:400 dilutes of non-specific antibody By Tukey post-hoc test, there is no significant differences between wells containing limbs only and wells containing 1:800 and 1:400 dilutions of non-specific antibody.


100
75
50
25
0
Number of Colonies
Distal
First proximal
Second proximal
Flank
Explants
Control
1:800 Non-Specific Anibody
1:400 Non-Specific Antibody

Chapter 4

Restoring avian wing digits

Abstract

The precise identification of the digits of the avian wing is of importance in evolutionary studies. If the digits are numbered 2, 3 and 4, this has been taken to suggest that birds are not descended directly from dinosaurs. If the digits are numbered 1, 2 and 3, dinosaur origins become more plausible. Studies of the development of the avian wing have failed to resolve this dilemma. However, in some instances, it is possible to deduce information about evolutionary morphologies by manipulating development experimentally. Beads loaded with fibroblast growth factor 4 were grafted into the distal tip of chick wing buds at times when the apical ectodermal ridge is regressing. The consequence was that the cartilage structure conventionally labelled element "5" increased dramatically in size and acquired a digit-like morphology in some instances. Corresponding changes in soft tissue morphology were also observed. It may be possible to resolve the issue of avian digit homology by the induction of experimental atavisms of this kind.

Introduction

The Apical Ectodermal Ridge (AER) is essential for vertebrate limb outgrowth and distalisation (Summerbell, 1974). Its action can be replaced by the implantation of heparin arcylic sulphate beads previously soaked in FGF-4 (Niswander et al., 1993). Purified FGF-8 protein has been shown to rescue limb bud outgrowth in mouse limbs lacking an Apical Ectodermal Ridge (AER), but has failed to maintain the expression of sonic hedgehog gene (*shh*) (Mahmood et al, 1995). In the chick it has been shown that FGF-8 can replace the AER to maintain *shh* expression and outgrowth

and patterning of the limb bud (Vogel et al, 1996). FGF-4 beads can also replace limb initiation by the AER, in that they can induce the outgrowth of additional limbs from the flank (Cohn, 1995). Normally, the AER regresses at stage 26 (Summerbell, 1974) (staging according to Hamburger and Hamilton, 1992). My intention was to observe the consequences of prolonging AER activity in development, with particular regard to the evolutionary implications. This approach of inducing experimental atavisms has previously been successfully employed in studies of the re-appearance of the distal articulation of the fibula in the avian leg (Muller, 1989). Spontaneous limb atavisms, such as the reappearance of elements of whale hind limbs (Hall, 1984), or the reappearance of claws in the wings of the domestic hen (Cole, 1967), demonstrate that the information for limb morphologies may become suppressed but remain present during the course of evolution.

The development of the cartilaginous elements of the avian wing has been extensively studied in the hope of resolving the problem of the homology of the digits. However, these studies have not produced a universally accepted answer; indeed workers have drawn opposite conclusions from their studies, perhaps because limb morphology has often been viewed in the light of existing prejudices on evolutionary history. To define avian digit homology, developmental biologists assume the conservation of embryonic patterning, while the paleontologist use phylogenetic systematics thus grouping birds with theropod dinosaurs, identifying the digits as 1, 2 and 3 (Sereno and Novas, 1992). Recently, it has been suggested, by comparison with crocodilians and reptiles, that the cartilaginous patterns is consistent with the identification of the elements as 2, 3 and 4 (Burke and Feduccia, 1997). Studies of forelimb development in turtles, alligators have shown that the primary axis unequivocally gives rise distally to digit 4, this serves as a potential marker for digit identity

and would assign the digit of the bird wing as 2, 3 and 4 (Burke and Feduccia, 1997). The reality is, however, that studies of the existing elements will probably never resolve this argument, since the information present is too scanty.

In addition to the digits conventionally numbered 2, 3 and 4, there is another element in the digital array, variously identified as the metacarpal of digit 5, or a rudimentary digit 5 (Hinchliffe and Griffiths, 1983). Obviously, exact identification of this element (which to avoid unwarranted assumptions will be called 'element 5') would be of value in resolving avian digit homologies.

Methods

Experimental manipulation of chick limbs.

Fertilised white hen eggs were incubated at 38°C until they reached stages 25-26. The membranes were teased away to expose the wing bud. One or two heparin acrylic beads (Sigma H5263) soaked in FGF-4 as described below were inserted into the tip of the wing bud just beneath the regressing AER. The beads were held in place with sterile platinum wire (diameter 0.025 μm), bent to form a staple (Fig, 1). The limbs were examined the next day, and the position of the bead(s) within the limb bud recorded. Embryos in which the bead had moved from the original place or had been lost all together were discarded. Eggs were incubated till 10 days of incubation, and the embryos were generally fixed in 5% trichloroacetic acid, stained with Alcian green, and cleared in Methyl Salicylate. After examination in whole mount, selected limbs were embedded in wax, sectioned and stained with Hematoxylin-Eosin according to standard procedures (Chapter 2). Since tissue morphology is

adversely affected by the whole mount procedure, experimental and control limbs were also sectioned blind, without a whole mount stage. Control experiments took the form of mock operations without bead implants, implant of beads washed in Phosphate Buffered Saline (PBS), and implant of beads soaked in PBS as below.
Limbs were photographed using a Wild dissecting microscope with a tube mounted Olympus OM2 camera. Elements were measured directly from the negatives using a graticule scale.

Preparation of heparin beads

A 2-3 ml drop containing 1 μg/ml recombinant FGF-4 (Sigma) was placed in the centre of a 35 mm plastic petri dish, and surrounded with 20 drops of PBS each of 8 ml, to ensure humidification.
Heparin Beads of diameter 200-250 μm were selected, washed in PBS and soaked for at least an hour at room temperature in the FGF-4 solution prior to use within three hours. Control beads were prepared in the same way, and soaked in PBS or used after washing.

Results

FGF beads were implanted in 150 chick limbs. 99 embryos survived and of these, 63 survived the incubation period with beads in place. 300 embryos were used for controls. 200 survived and of these, 138 survived the incubation period with beads in place.
None of the control procedures affected size or the morphology of element 5. Results from all control limbs were therefore pooled. The FGF beads could disturb normal development in their immediate locality, in that

elements were sometimes distorted or broadened and no real qualitative changes in the length of the three normal digits was observed.

The most consistent non local consequence effect was a marked elongation of element 5 in FGF treated limbs (Fig. 2a, b & c; Fig. 3a, b) ($p<0.001$ by t-test). The element was not markedly increased in width. The ulnare was generally increased in size in experimental limbs also. In some cases, element 5 appeared to show signs of further subdivision, suggesting that the element had given rise to phalangeal components (Fig. 3c, d). On rare occasions, an additional small cartilaginous structure was present posterior to element 5 (Fig. 3e). However, this is not conclusive as evidence that two elements can be provoked to develop from the caudal margin. This would require further development of the structures to a point where they are clearly identifiable as digits. No additional structures were identified anterior to digit 2.

In the soft tissue of sectioned FGF-treated limbs, a number of changes had occurred. The FGF-treated limbs were compared with human limbs with regard to muscle and blood vessel morphology, since these provide an example of pentadactyl limb structure, and are exceptionally well documented. In the chicks muscle mass was increased distal to the wrist region: extra muscular and tendinous structures were visible but could not be identified by comparison with humans. For details on the vascular system see chapter 5.

Discussion

The most important observation was that element 5 consistently elongated from proximal to distal in the form of a digit. This indicates that it is not a rudimentary carpal element as has been suggested. Several possible mechanisms may be responsible; for instance the FGF implants may bring

about persistence of AER beyond its natural life span, the increased vascularisation to the wrist region resulted in extended growth of element 5, or cell death may be inhibited.

In conclusion, it is possible to induce experimental atavisms in chick digit morphology by prolonging the action of the apical ectodermal ridge by means of FGF implants under the ridge prior to regression. By these means 'element 5' is shown to be a rudimentary digit. Its identity is further considered in Chapter 5.

Figure 1. Heparin acrylic beads soaked in FGF-4 or PBS was inserted into the tip of the wing bud just beneath the regressing AER.

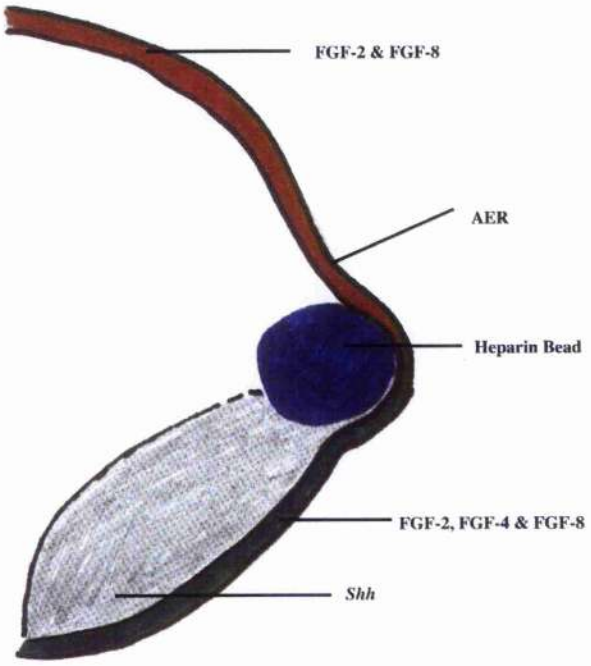

FGF-2 & FGF-8
AER
Heparin Bead
FGF-2, FGF-4 & FGF-8
Shh

Figure 2a. Comparison of length of element 5 in FGF implanted limbs and controls. Error bars indicate standard errors.

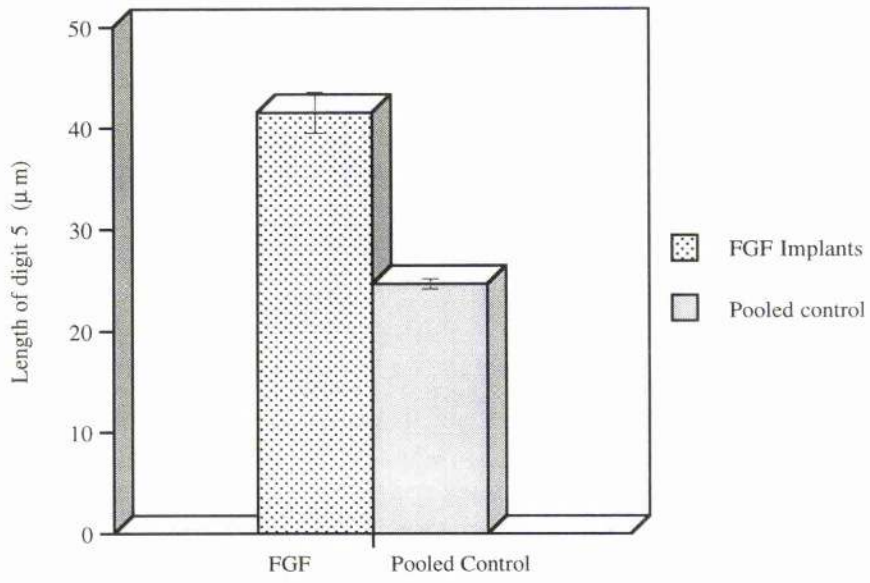

50
40
30
20
10
0
Length of digit 5 (μm)
FGF
Pooled Control
FGF Implants
Pooled control

Figure 2b. Frequency distribution of lengths of element 5 in control limbs.

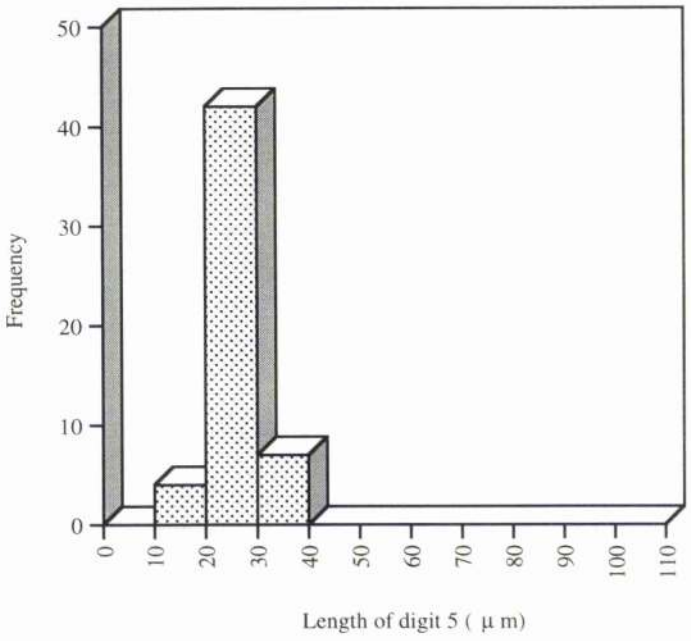

Frequency
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
110
Length of digit 5 (μ m)

Figure 2c. Frequency distribution of lengths of element 5 in FGF implanted limbs.

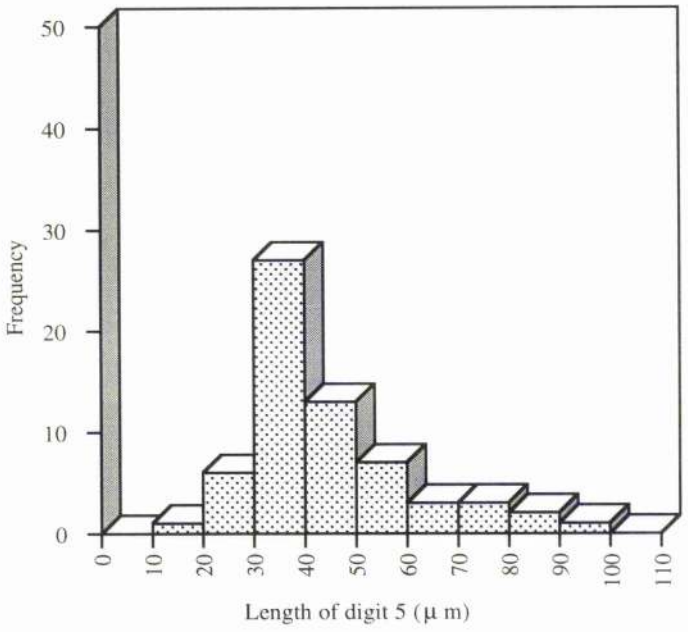

50
40
30
20
10
0
Frequency
0
10
20
30
40
50
60
70
80
90
100
110
Length of digit 5 (μ m)

Figure 3a. Wrist region of control right limb. In all these images, proximal is to the left and distal to the right. Element 5 is indicated: digits 2, 3 and 4 are ranged immediately above this. The wrist element immediately proximal to element 5 is the ulnare.
Scale bar: 1cm = 20 μm

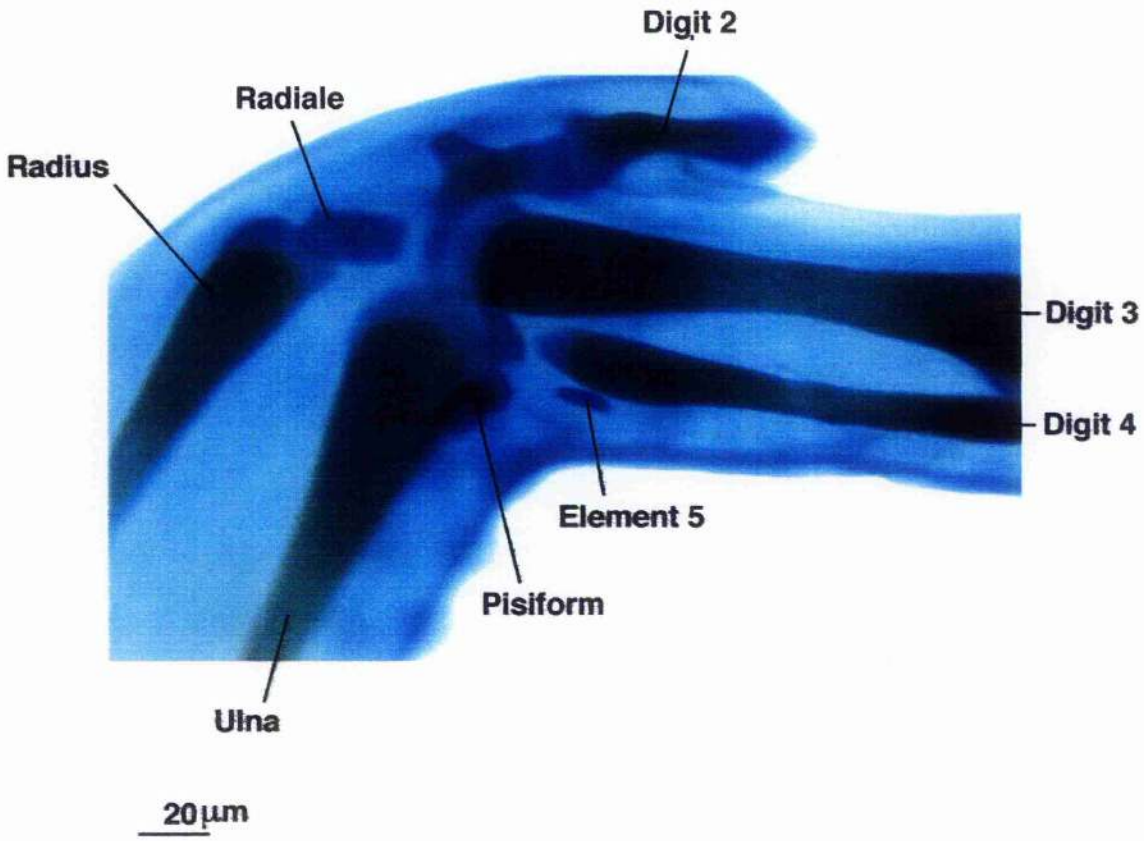

Digit 2
Radiale
Radius
Digit 3
Digit 4
Element 5
Pisiform
Ulna
20 µm

Figure 3b. Wrist region of FGF implanted limb. The bead is visible between digits 3 and 4.

Scale: 1cm = 20 μm

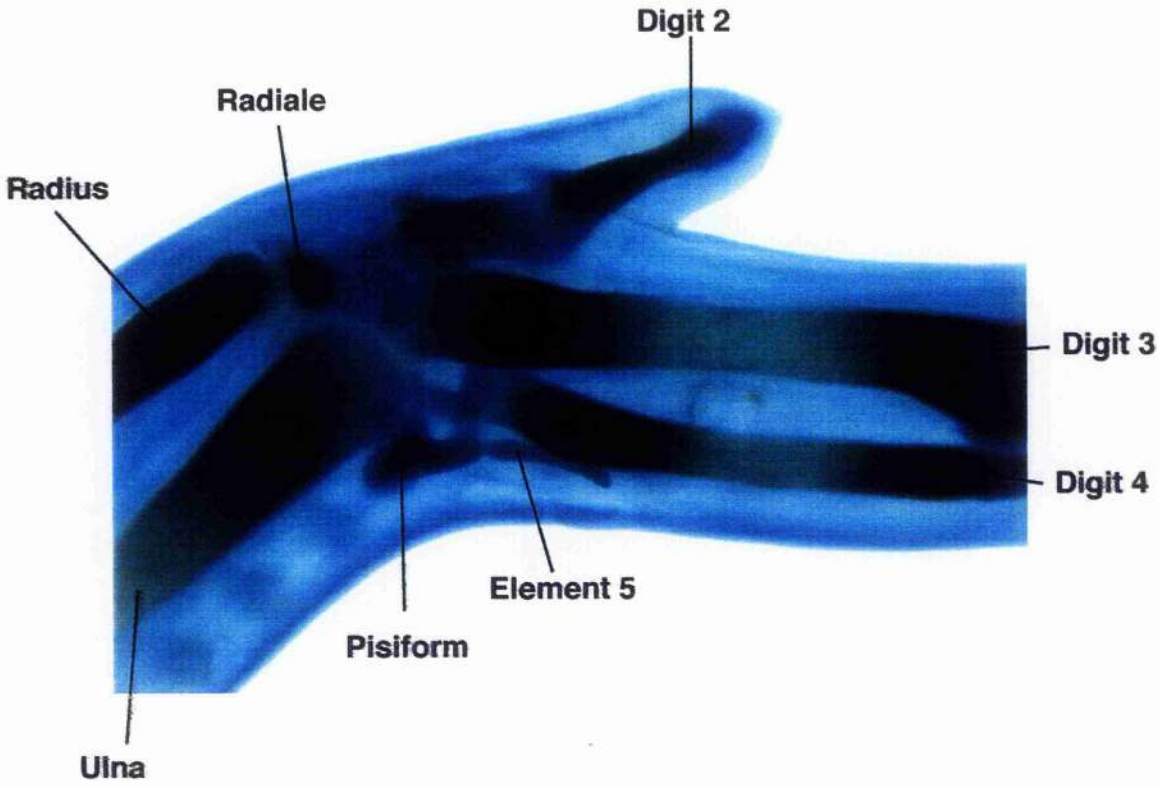

Digit 2
Radiale
Radius
Digit 3
Digit 4
Element 5
Pisiform
Ulna

Figure 3c. Wrist region of FGF implanted limb.

Scale: 1cm = 20 μm

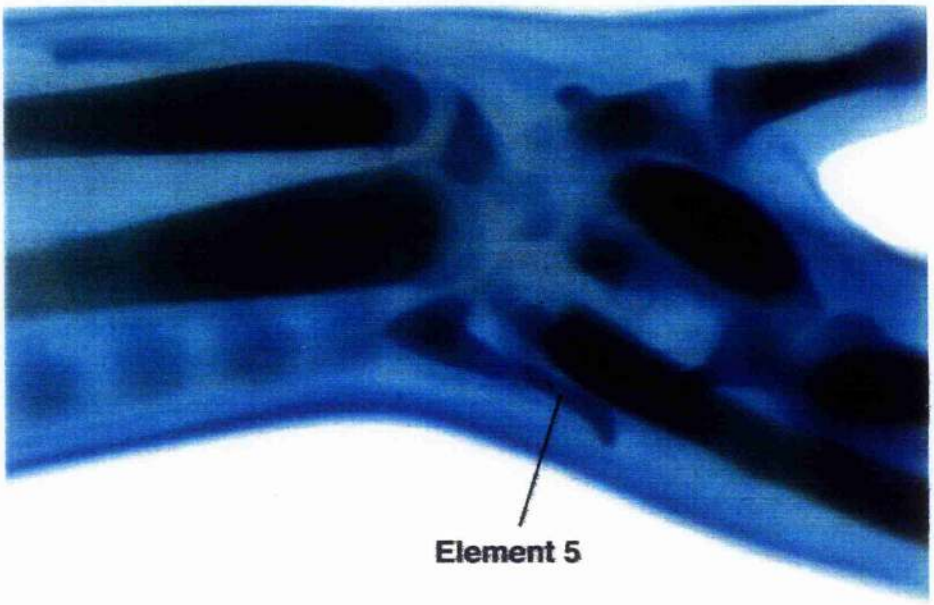

Element 5

Figure 3d. Wrist region of FGF implanted limb.

Scale: 1cm = 20 μm

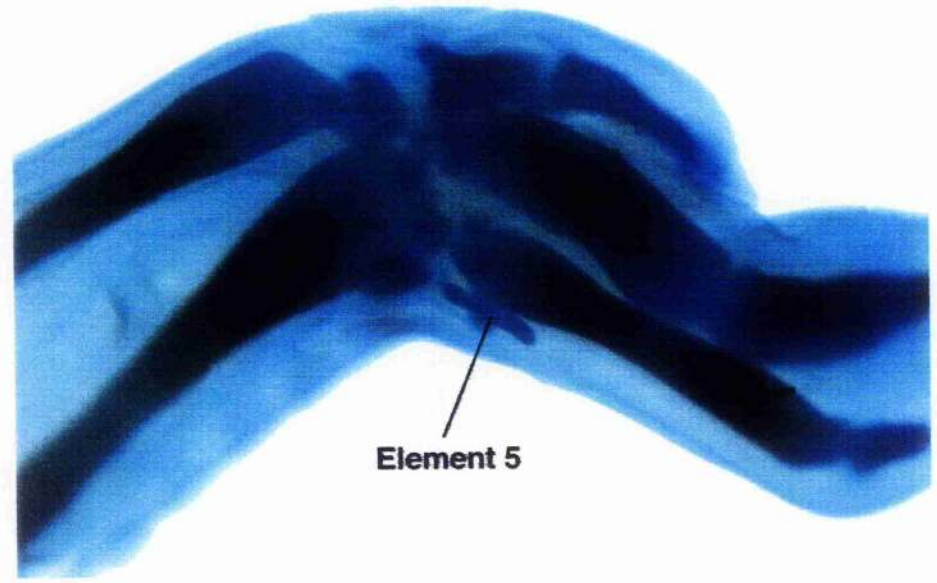
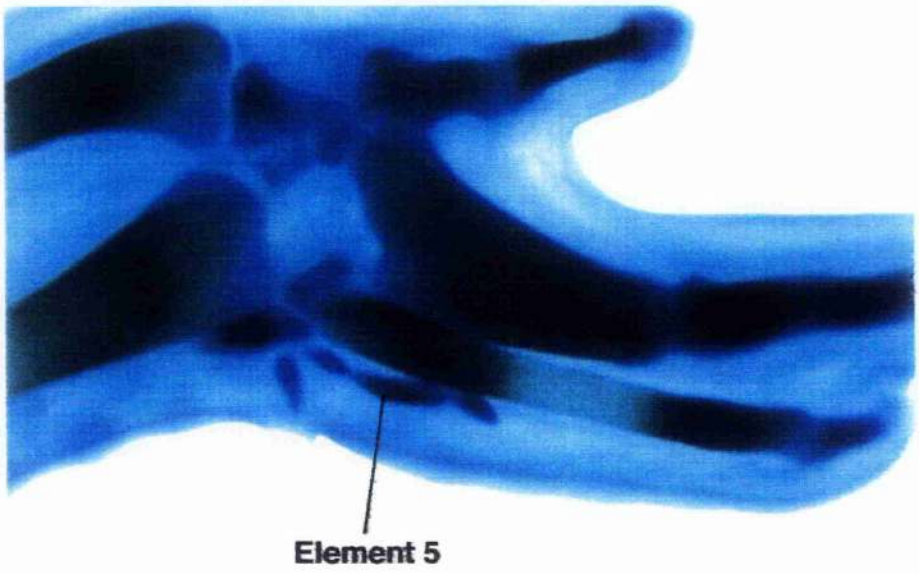

Element 5

Figure 3e. Wrist region of FGF implanted limb. Element 5 is subdivided, and an additional structure is present posterior to element 5.
Scale: 1cm = 20 μm


Element 5

Chapter 5

Soft tissue analysis of avian wing

Abstract

Limbs implanted with FGF-4 were sectioned, stained and analyzed. The sections revealed that ectopic FGF-4 had caused a marked extension in element 5 in the posterior region. The UMD muscle had also extended and angiogenesis had occurred in the region of the extended element 5. The anterior region of the experimental limb had not been influenced by the presence of ectopic FGF-4. This perhaps indicates that the molecular mechanism involved in digit formation only exists in the posterior region of the developing chick limb bud. Three dimensional surface models of the limbs showed the extension pattern of UMD and element 5. The soft tissue analysis failed to provide conclusive evidence as to the identity of element 5, but showed that this element is not a rudimentary carpal as previously suggested.

Introduction

Angiogenesis is the development of vessels from pre-existing vessels and is vital for normal embryonic development and growth (Blood and Zetter, 1990). Vasculogenesis is the development of blood vessels *de novo*. Studies of angiogenesis and the factors regulating it date back to the late eighteenth century (Evans, 1909; Loeb, 1893). Recently researchers have investigated chemical factors involved in the development of the embryonic vasculature (Millauer, 1993; Krah et al., 1994; Flamme et al., 1995). In the limb the development of the vessels is thought to involve both angiogenesis and vasculogenesis. Grafting avascular quail limb explants to host quail limb showed that the newly formed vessels had originated from the host wing vasculature (Wilson, 1983).

As described in chapter 1, when the limb bud first appears it contains a simple capillary network derived from aorta (Evans, 1909). As the limb grows the capillary network also enlarges to a three dimensional network separated from the overlying ectoderm by an avascular system (Caplan and Koutroupas, 1973). The central components of the capillary network give rise to the subclavian artery, peripherally the capillaries give rise to the anterior and posterior components of the marginal vein (Feinberg and Saunders, 1982). Extension of the marginal veins is more rapid posteriorly and anterior and at the distal tip of the limb bud the marginal vasculature tends to remain in a form of a terminal plexus rather than a single marginal vein (Seichert and Rychter, 1971). The posterior marginal vein is larger and provides the major portion of limb drainage and it also gives rise to basilic and humeral veins (Feinberg and Saunders, 1982). The preaxial marginal vein gives rise to the brachial and radial veins (Stark and Searls, 1973).

The true identity of element 5 has been under constant dispute for over a hundred years. The bird digits were first identified as 2-3-4 (Owen, 1836). Later the same digits were renumbered as 1-2-3 (Parker, 1888). Developmental biologists have adopted the 2-3-4 numbering while paleontologists number the digits as 1-2-3. Evidence from experimental embryology suggests that the bird digits and that of Archaeopteryx are 2-3-4 (the three middle digits) which is in accord with digital reduction pattern of the primitive amniotes (Hinchliffe, 1985; Shubin, 1991). In early theropods digits 1-2-3 are retained which is a postaxial side digit reduction (digits 4, 5 and fifth metacarpal were reduced) not similar to primitive amniote digit reduction (Sereno and Novas, 1992). For the time being the digital relation of birds and dinosaurs remains elusive.

In the experiments carried out in chapter 4, the length of element 5 had increased upon administration of ectopic FGF-4. It is possible that the life of the AER was prolonged by the ectopic source of FGF-4. The relationship between the AER and the formation of marginal and proximal veins is well documented as well as lack of vasculization at the presumptive cartilage forming areas. Thus perhaps studying the vascular and muscular patterns of the FGF-4 implanted limbs will provide some insight as to what has occurred in the limb after FGF-4 had been administered.
In addition since cartilage morphology alone is inadequate for identification of element 5, it was hoped that the study of soft tissues would shed light on the identity of the elongated element 5. The human hand is by far the best studied and described pentadactyl limb. It was therefore used as a key comparison in attempting to identify the elongated element 5 as digit 4 or 5.

Methods and Materials

The chick limbs implanted with FGF-4 and PBS beads (see methods and materials in chapter 4) were sectioned and stained. Histological methods are described in chapter 2. Sections were viewed with a video camera dissecting microscope set up, and traced on transparent acetate. Using Shellswell's studies of the development of the pattern of muscles and tendons (Shellswell, 1977) the limb elements in both the experimental and the controls were identified.

The traces were then scanned in to Adobe Photoshop 4.0. Digits 2, 3, 4, element 5 and the muscle UMD were all registered using the Surfdriver 3

software program. Using Surfdriver 3.0, three dimensional surface models of the registered elements were constructed, viewed and photographed.

Results

Control

The sections were examined and the radial artery was identified as the artery closest to the radius and the ulnar artery as the artery closest to the ulna. This was validated by comparing the sections with the existing arterial maps of human and chick limbs (Levinsohn et al., 1984, Bardeen and Lewis, 1901). Both arteries lie between the radius and ulna. At the wrist region, the arteries approach each other and the radial artery runs underneath the wrist elements and therefore becomes very hard to follow. The ulnar artery remains in view. Once past the wrist elements the radial artery comes into view again, seen close to digit 3 with a smaller branch close to digit 2.

Figure 1a is a transverse section of a control chick limb stage 36 (10 days old) at the level element 5 begins (distal tip).

A branch of the ulnar artery is seen close to element 5 (Fig. 2a). Element 5, being 30μm in length on average is only visible in about 4 or 5 sections (each at 7μm thick). Also close to element 5 is the UMD muscle. The UMD muscle is proximal to IOD, associating it with the region of element 5.

Figure 3a shows a model of the limb indicating the location of element 5 and the UMD muscle close to it.

Limbs implanted with FGF soaked Bead

In the FGF-4 implanted chick limbs an overall proximo-distal increase in the muscle mass was observed, extra muscles and tendinous-like structures were present in the wrist region (Fig 1b).

The blood vessel pattern had also altered. The radial artery, which in chicks normally becomes insignificant at about the level of the tip of digit 2 (Levinsohn et al, 1984), and in humans supplies digits 1 and 2, extended to the distal tip of digit 3 in FGF-treated limbs. At the wrist region the ulnar artery had given rise to many branches not seen in the control limbs. Figure 2b is a section of an FGF-4 treated limb, corresponds to the same level as the control section in Figure 1a. In Figure 2b element 5 is visible, indicating that it is located more distally. By examining serial sections of experimental limbs, the approximate length of element 5 ws estimated. The average length of element 5 was 78μm.
Apart from the extensive vascular network observed throughout the limb the most noticeable expansion had occurred in the muscle ulnametacarpalis dorsalis (UMD). UMD's origin is distal to the ulna with the tendon close to element 5. In the experimentals UMD had extended in proportion to the elongation of element 5. Figure 3b shows the 3D model of a FGF implanted limb. Elongation of element 5 can be see as well as the increase in the size of UMD muscle.

Both the radial and ulnar arteries branched off to smaller arteries around the phalanges i.e. the digit region. A branch of ulnar artery was seen close to the extended element 5.

In general the noticeable differences between the controls and the FGF studies were; the visible increase in the number of blood vessels around

the area of FGF bead implants, an even increase in vascularization in general and an increase in the length of UMD muscle.

Discussion

The limbs implanted with FGF-4 bead were examined very closely and compared with the controls. The arterial system was carefully observed from the mid ulna and radial region to the very tip the limbs, i.e. proximo-distally (Levinsohn et al., 1984, Bardeen and Lewis, 1901). FGF is known to be an angiogenic factor, and therefore may have induced blood vessel development directly. However, angiogenesis observed was not just at the level of the implant, but extended over some distance. More blood vessels were observed around the area of FGF-4 bead implants but extensive vascularization was also observed in the region of the elongated element 5 (Fig 1b and 2b). The vascularization of this region was identified as branches of the ulnar artery. The ulnar artery did not given rise to any novel branches in the region of element 5 in the control limbs (Fig 2a).
In the FGF treated limbs more soft tissue was present in the posterior region. However, this increase was not observed in the anterior region of the limb. Perhaps information for development was present at the posterior and not the anterior zone.
In FGF treated sections an overall increase in the length of the digits was observed. The first phalanges of digits 2,3 and 4 had increased in length, consistent with the limb's general increase in length. The over all elongation of the digits did not give rise to further branching of the arteries. The angiogenesis observed at the distal tip of the limb was in the immediate area surrounding the bead, confirming that arterial branching observed was due to the presence of ectopic FGF-4 and not due to the increased limb length. Also the muscles and tendons appeared unaffected

which was consistent with the controls. The ectopic source of FGF-4 had increased the active life of the AER and therefore caused more proximodistal growth. However in the posterior region the ectopic FGF-4 had caused the elongation and in some cases the subdivision of element 5. In some limbs, structures were also present posterior to element 5.

The soft tissue morphology did not provide conclusive evidence for identifying the true identity of element 5. There are no specific tendons or muscles associated with element 5 thus the unidentified structures in the posterior region of the limb did not provide further evidence.

Since the limbs implanted with FGF-4 had displayed a marked growth of cartilage and soft tissue in the posterior region then it seemed necessary to investigate the molecular mechanisms present and active in the posterior region of the limb. The mechanisms of interest were those involving the AER and FGF-4 directly. Also Hoxd genes, *shh* and programmed cell death play crucial roles in development and digit formation of all vertebrate limbs. Therefore the effects of ectopic FGF-4 on *shh* /FGF-4 positive feedback loop and Hoxd-11 as well as PCD will be discussed in the following chapters.

Figure 1a. Transverse section of a control limb.

M 4 (metacarpal 4), M 3 (metacarpal 3), M 2 (metacarpal 2), IOD (interosseus dorsalis), MFI (muscle, flexor indicis).

Scale: 1cm = 10μm

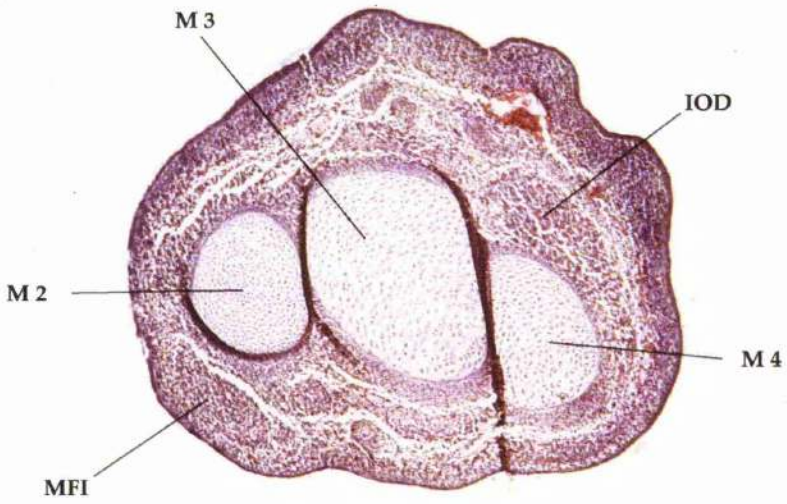

M 3
IOD
M 2
M 4
MFI

Figure 1b. Transverse section of FGF treated limb, corresponding to the same level as the control limb section (Fig 1a). Blood vessels in the posterior region of the limb (element 5 region) are branches of the ulnar artery. Also angiogenesis is observed in the distal tip of the limb close to the FGF-4 bead.

M 4 (metacarpal 4), M 3 (metacarpal 3), M 2 (metacarpal 2), UMD (ulnametacarpalis dorsalis), MFI (muscle flexor indicis).

Scale: 1cm = 10μm

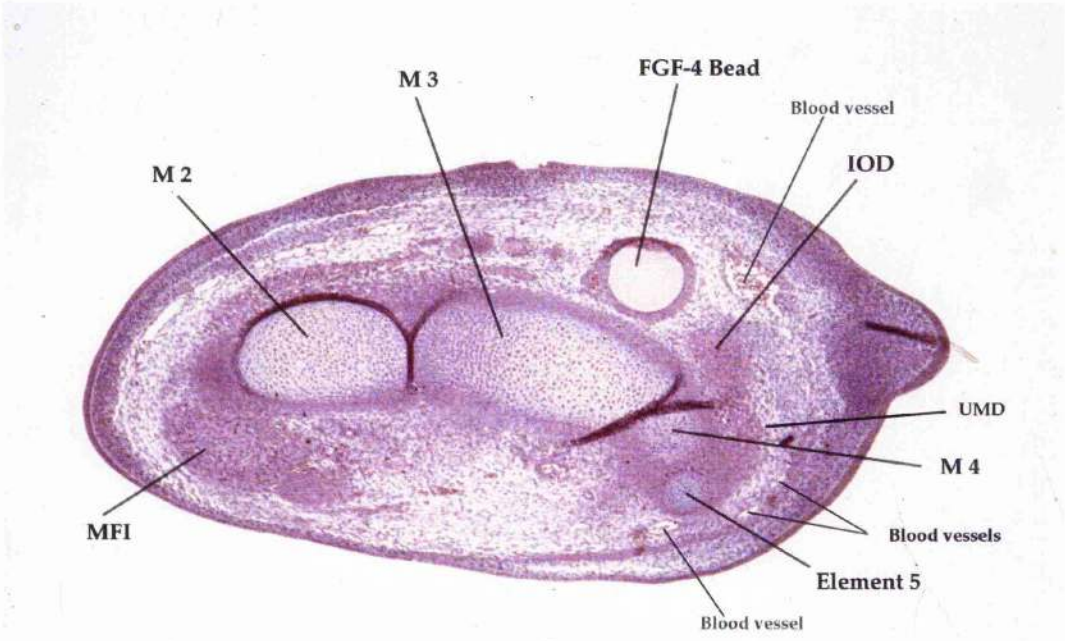

M 3
FGF-4 Bead
Blood vessel
M 2
IOD
UMD
M 4
MFI
Blood vessels
Element 5
Blood vessel

Figure 2a. Transverse section of a control limb at the most distal tip of element 5. The blood vessel in the posterior region of the limb (element 5 region) is the branch of the ulnar artery.
M 4 (metacarpal 4), M 3 (metacarpal 3), M 2 (metacarpal 2), UMD (ulnametacrpalis dorsalis), MFI (muscle, flexor indicis).
Scale: 1cm = 10μm

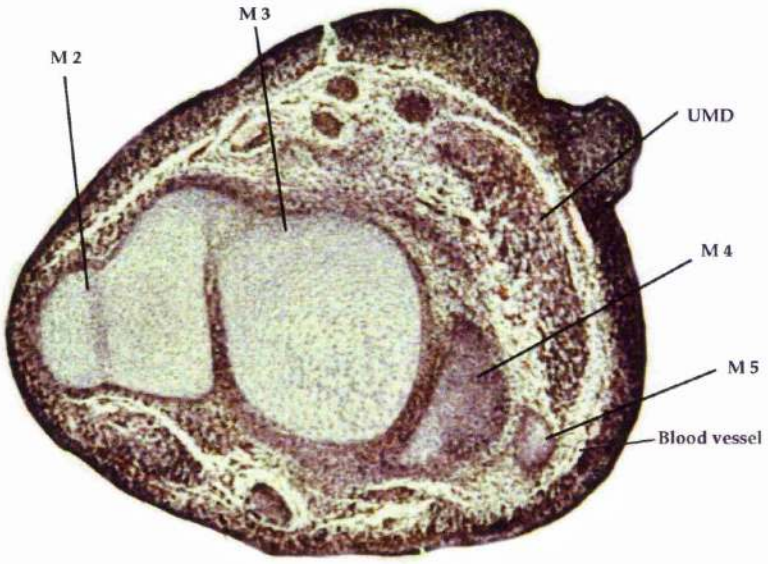

M 3
M 2
UMD
M 4
M 5
Blood vessel

Figure 2 b. Transverse section of FGF treated limb, corresponding to the same level as Fig. 2a. Blood vessels in the posterior region of the limb are branches of the ulnar artery. More blood vessels are also visible in the distal region of the limb.

M 4 (metacarpal 4), M 3 (metacarpal 3), M 2 (metacarpal 2), UMD (ulnametacarpalis dorsalis), MFI (muscle flexor indicis).

Scale: 1cm = 10μm

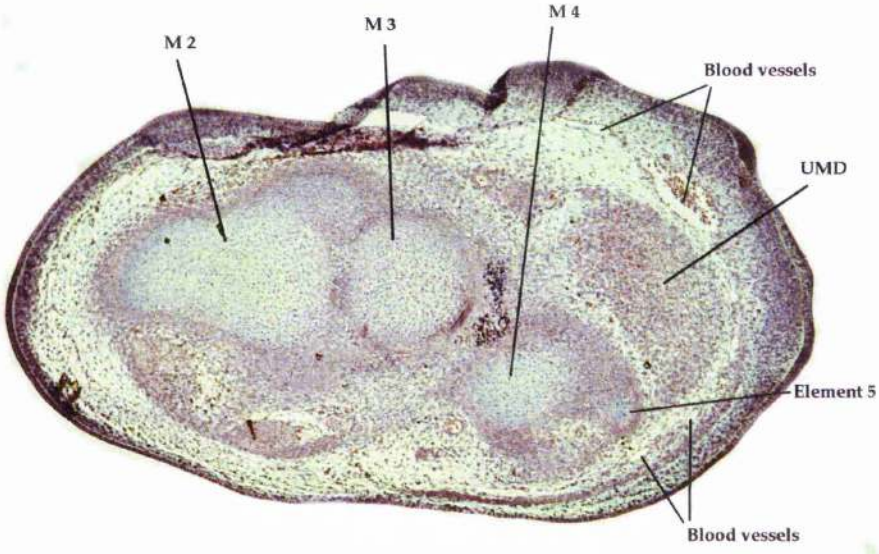

M 2
M 3
M 4
Blood vessels
UMD
Element 5
Blood vessels

Figure 3 (a, b). Three dimensional models of 10 days limbs. Digits 2, 3, 4, element 5 and the muscle ulnametacarpalis dorsalis have been reconstructed.

Figure 3a. Dorsal view a control limb. Length of element 5 was measured at 30μm.

Figure 3b. Dorsal view of a FGF-4 implanted limb. Element 5 was measured at 78μm. UMD extends to the distal phalanx of digit 4.

Scale: 1cm = 20μm

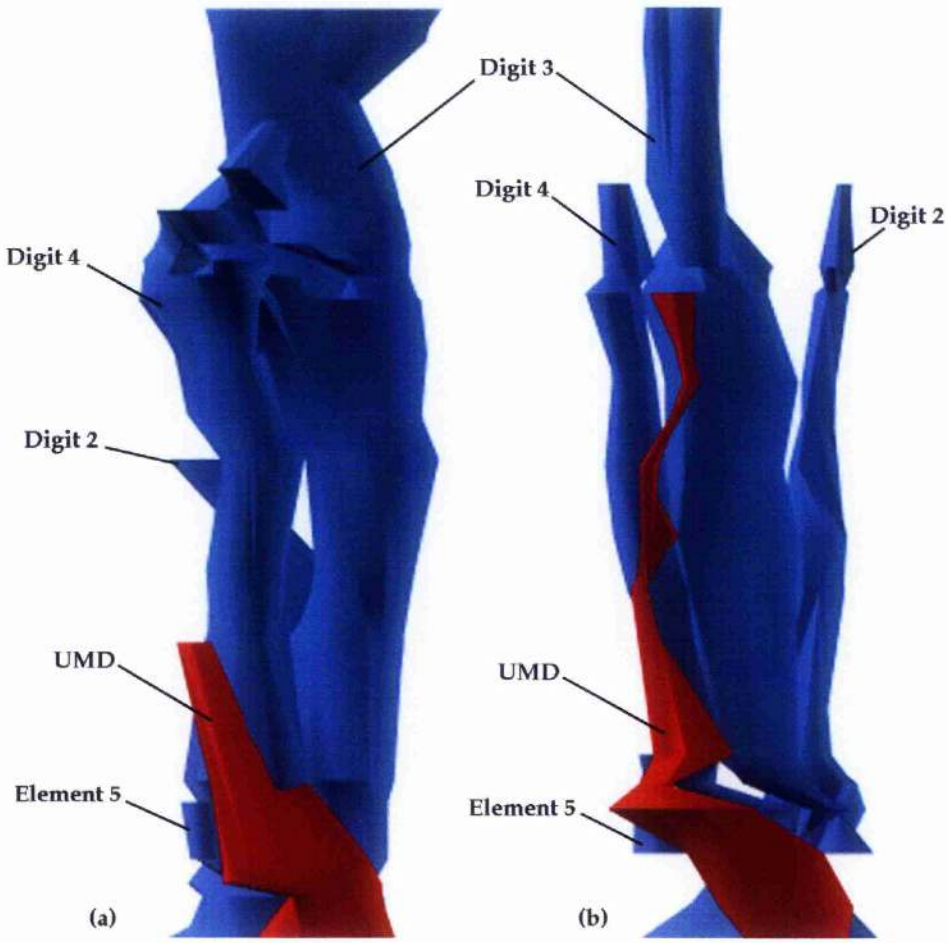

Digit 3
Digit 4
Digit 2
UMD
Element 5
(a)
Digit 3
Digit 4
Digit 2
UMD
Element 5
(b)

Figure 5a. Ulna border view of 10 day control limb. Digits 2, 3, 4, element 5 muscle ulnametacarpalis dorsalis (UMD) have been reconstructed. Scale: 1cm = 20μm

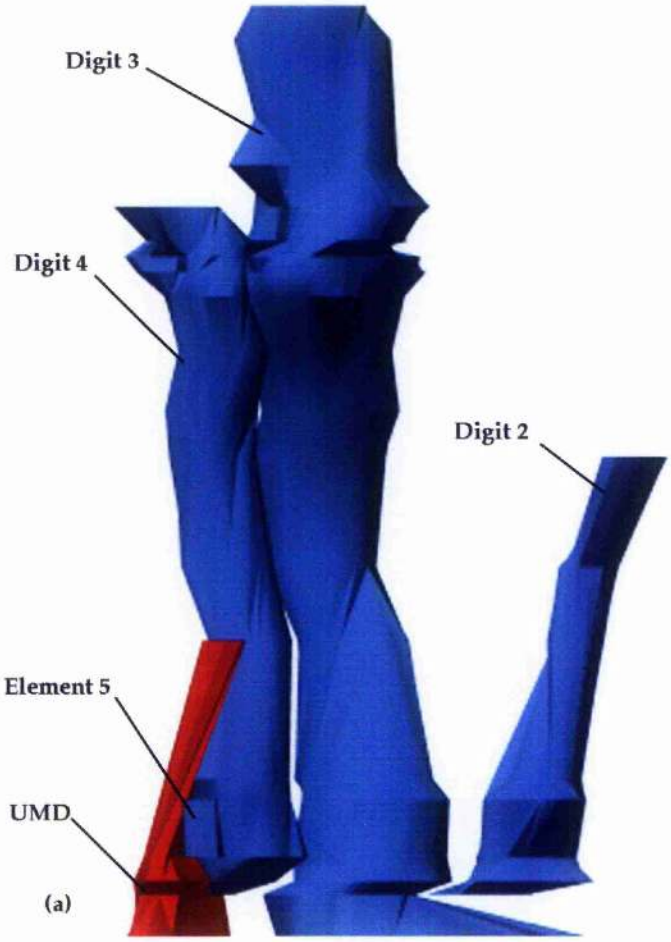
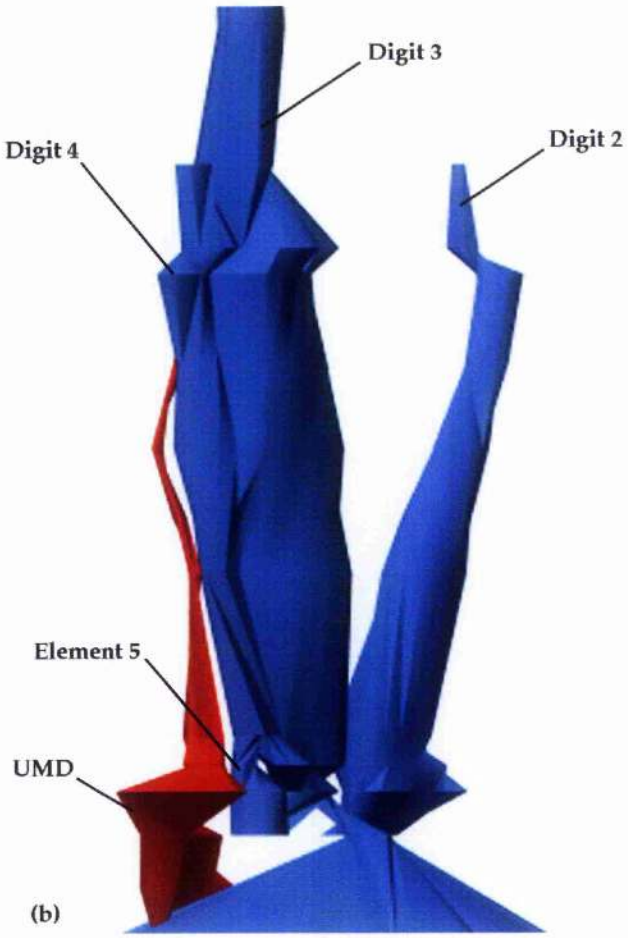

Digit 3
Digit 4
Digit 2
Element 5
UMD
(a)

Figure 5b. Ulna border view of a 10 days FGF-4 implanted limb. Digits 2, 3, 4, element 5 and the muscle ulnametacarpalis dorsalis (UMD) have been reconstructed.

Scale: 1cm = 20μm


Digit 3
Digit 4
Digit 2
Element 5
UMD
(b)

Chapter 6

Gene expression in the avian wing

Abstract

Chick limbs implanted with Heparin sulphate beads soaked in FGF-4 showed a marked increase in the length of element 5 demonstrating that the fate of the limb cells had been altered by FGF-4 implants. Several mechanisms may be involved in A-P patterning of the limb. However, my results show that element 5 elongation did not occur via alterations in the FGF-4/*shh* feedback loop, or through alterations in *Hoxd-11* expression. Therefore another as yet unknown mechanism must be involved. This mechanism is possibly not a component of the A-P specification mechanisms.

Introduction

Prior to the onset of limb development there is specification of molecular precursors which control the development of the limb (Chapter 1). The limb rudiment will start to grow, and the component cells will be organized into limb structures. Transcription factors encoded by homeobox containing genes are known to be involved in all these steps (Morgan and Tabin, 1993).

Experiments have shown that the limb develops proximal-distally (Saunders, 1948). The developmental stages may be separated into three distinct phases, and these may correspond to the three phases of *Hoxd* gene activation described in Chapter 1. The initial phase of *Hoxd* gene expression in the chick limb occurs prior to limb onset at stage 16 (Laufer et al., 1994). At this stage *Hoxd-9* and *Hoxd-10* are expressed without any anterior posterior bias in the limb mesoderm. Phase 2 begins at stage 18. *Hoxd-9* through to *Hoxd-13* are activated sequentially in the posterior-

distal margin of the limb. At stage 23, the third phase of Hoxd gene expression begins with the sequential transcription of *Hoxd-13* through to *Hoxd-10* in an inverted temporal sequence.

The upper wing forms in Phase 1, where there is a non-polar expression of *Hoxd-9, Hoxd-10, Hoxa-9* and *Hoxa-10*. The lower wing (radius and ulna) develops in Phase 2, when there is a sequential activation and posteriorly polarized expression of *Hoxd-9* through to *Hoxd-13*. There is also a uniform expression of *Hoxa-11*. Phase 3 in limb development is the digit forming stage, which occurs after stage 23 and coincides with the onset of *Hoxa-13* expression at the posterior border of the limb bud. This is followed by the expression of *Hoxd-13, Hoxd-12, Hoxd-11, Hoxd-10* and *Hoxd-9*.

This phased expression of the *Hoxd* cluster is thought firstly to mark boundaries in the proximal-distal development of the limb and secondly to explain the fact that the 3 limb segments can be modified independently during evolution. The second and third phase of *Hox* gene expression may be associated with the innovation that separated tetrapod limbs from their fin precursors. *Hoxd* gene expression in the limb bud provides five distinct regions in the presumptive digit region, allowing for specification of up to 5 unique digits. It is thought that a duplication in any of the *Hoxd* genes can cause an expanded capacity for encoding position along the anterior-posterior axis. However since the *Hoxd* genes are also expressed in the central nervous system, a misexpression in the limb could be lethal or cause deleterious alterations elsewhere. Thus even though polydactyly is observed in humans and chicks, the extra digit is generally a reduplication of an already existing structure.

Changing the morphology of an already existing digit or wrist element is distinct from specifying its identity. *Hox* genes themselves do not specify

the digit morphology, since they are expressed in the wing as well as the leg.

Shh is believed to be the gene responsible for induction of Hox genes in the chick limb (Riddle et al., 1993). *Shh* signal is thought to be involved in initiating phase 2 and phase 3 expression of Hoxd genes by activating *Hoxd-9* through to *Hoxd-13*.
Shh expression by stage 21 is detected in the posterior region of the developing limb and is limited to the mesenchyme. The region of the limb bud expressing *shh* corresponds to the ZPA.

Removal of *shh* completely causes severe defects along the A-P axis of the limb indicating that removing *shh* during early stages of limb development results either in postaxial defects or truncation. This is believed to be due to the disruption of a positive feedback loop between FGF-4 from the AER and *shh* in the ZPA. *Shh* maintains FGF-4 necessary for limb bud outgrowth and FGF-4 from the AER maintains the expression of *shh* (Niswander et al., 1994, Laufer et al., 1993). *Shh* appears to be upstream of FGF-4 expression in the AER and since AER is required to maintain polarizing activity in the posterior mesoderm, *shh* may also be downstream of the AER (Vogel and Tickle, 1993; Niswander et al., 1993; Laufer et al., 1994).

Studies have shown that both ZPA grafts and RA implants induce Hoxd gene cluster expression also involved in the polarizing process (Nohno et al., 1991). *Shh* implants anteriorly have also shown to induce ectopic expression of the Hoxd genes.
FGF-8 is also present in the AER and previous experiments have shown that FGF-8 has the ability to sustain limb outgrowth in the absence of AER

(Mahmood et al., 1995). It is also present in the limb at this late stage and is expressed anteroposteriorly.

Experiments were carried out to investigate the molecular mechanisms involved after FGF-4 implantation at stage 26. It seemed reasonable to investigate molecular mechanisms involved with or dependent on FGF-4.

Since *Hoxd-11* is expressed posteriorly, persists in the presumptive digit region post stage 25 and does not fade or move towards the flank by stage 27, it seems likely that the Hoxd cluster is the group most involved in the digit region of the developing wing bud. Considering the timing, position and the effect of the FGF-4 implants resulting in the elongating of element 5, (Chapter 3) and the timing and expression patterns of the Hoxd cluster, *Hoxd-11* was the most likely candidate for further study by whole mount in situ hybridisation.

Methods and Materials

Preparing the embryos: FGF-4 and PBS implants

Stage 26 chick limbs were implanted with heparin sulphate beads soaked in FGF-4 or PBS for an hour at room temperature. The beads were placed underneath the AER in the proliferating mesenchymal layer (for full protocol see chapter 4). The implants were checked for the first 9 hours and if the bead had moved or fallen out the embryos was discarded. The beads were left in the limbs and the embryos were fixed 24 hours after implantation and prepared for whole mount in situ hybridisation process. The FGF-4/*shh* feedback loop is the only molecular pathway involving both the ZPA and the AER and therefore the effect of the FGF-4 implant

on this particular pathway was chosen for investigation. The following whole mount in situ hybridization experiments were carried out:

1. Limbs implanted with FGF-4 were labelled for:

i) FGF-4 expression
ii) *Shh* expression
iii) *Hoxd-11* expression
iv) FGF-8 expression

2. Controls and limbs implanted with PBS were labelled for:

i) FGF-4 expression
ii) *Shh* expression
iii) *Hoxd-11* expression
iv) FGF-8 expression
(The untreated limb in each embryo was also stained for normal gene expression, used as part of the control group).

FGF-4 expression:

Stage 26 embryos were implanted with heparin beads soaked in FGF-4 (Fig 1). The embryos were fixed at 24, 48 and 120 hours after implantation (stages 28, 31 and 36 respectively) .

In Situ hybridisation

The objective of In Situ hybridisation is to fix the tissue in question so that all the mRNA being transcribed at the time is retained within the cells.

The mRNA transcribed by a specific gene is then detected in situ by hybridisation with a probe labelled so that it can be detected either by auto radiography, chromogenic stains, or fluorescence. All that is required is a supply of the appropriate stages of the organism on which the gene of interest is expressed and a cloned fragment of this gene.
The procedure of in situ hybridisation can be divided into the following stages (Shamim et al., 1998):
(Gloves were worn throughout the entire procedure)

Linearisation of DNA.

The following were added in order
22 µl of DEPC water
3 µl of Buffer
2 µl of DNA, mixed by pipetting
1 µl of the enzyme (Most enzymes are 10 U/ml concentration, thus 0.1 ml is enough to cut I µg of DNA)
(The final volume of the digest was twice the volume of DNA and 10 times the volume of the enzyme.)
The digest was incubated at 37°C for 2 hours (1 unit of enzyme cuts 2 µg of DNA in 1 hour at 37°C.)
After the incubation period the tube was spun down. At this point the digest could be frozen at -20°C to be used later.
(FGF-4, *Shh* and *Hoxd-11* constraints were cut with ECOR 1, and the buffer used was buffer H)

Phenol/chloroform Purification of the DNA

To the digest the following was added:

100 µl of DEPC water,

100 µl phenol/chloroform (pH 7.8)

Vortexed for 1 minute and the spun at 13000g at room temperature for 3 minutes. The top aqueous layer was removed and transferred to a new tube (a bubble formed every time when aspirating the top layer)

Precipitation of DNA

0.1 volume (10 µl) of 3M NaAc (pH 5.2) was added,

2.2 volumes (220 µl) of ethanol were added, mixed by inverting the tube and the DNA was allowed to precipitate over night at -20°C (or 1 hour at -70°C) and then centrifuged at room temperature for 30 minutes.

The supernatant was removed leaving a white pellet.

The pellet was washed in 100 µl of 70% ethanol and spun for 1 minute.

The ethanol was then removed and the pellet was left to dry. The DNA was re-suspended in 10 µl TE (DEPC. pH 8). 1 ml was removed and added to 4 ml of TE water and ran on an agarose gel.

Preparation of DIG-Labelled Riboprobes

The following were added in the appropriate order

2 µl of 10X DIG nucleotide,

2 µl of transcription buffer,

2 µl of 200 mM DTT,

1 µl of RNase inhibitor,

1 µl of (T7) RNA Polymerase (kept on ice at all time)

2 µl of the Plasmid (linearised earlier)

The volume was taken up to 20 µl by adding 10 µl of DEPC water and mixed gently by pipetting the content up and down, centrifuged for 5 seconds and then incubated at 37°C for 2 hours.

Precipitation of RNA

After the incubation in 37°C for two hours. 1ml of the product was taken and diluted in 5 µl TE-buffer (pH 8) and ran on the agarose gel.
To the rest of the product the following were added,
2 µl RNase free DNase was added and incubated at 37°C for 15 minutes exactly.
100 µl of TE-buffer (pH 8, made with DEPC treated water was added),
10 µl of 4M Lithium Chloride (LiCl) DEPC treated,
300 µl of ethanol, mixed with pipetting up and down and incubated on ice for 10 minutes.
The product was then centrifuged at room temperature for 10 minutes.
The supernatant was removed, leaving a white pellet behind.
The pellet was washed with 70% ethanol and centrifuged at 13000 rmp for 1 minute. Ethanol was removed and the pellet was allowed to air dry.
Once it was completely dry the pellet was re-suspended in 100ml of TE (made with DEPC treated water). Finally 2 µl of Rnasin (2500U Promega N2111) was added and 5ml was removed to be taken to the agarose gel.
The probes were usually made ahead of time and kept at -20°C until the embryos were ready to be incubated with it.

Agarose gel Electrophoresis

0.8g of high temperature Agarose (electrophoresis grade) was added to 100 ml of 10% TE -buffer (made from 10X stock) and microwaved for 2

minutes. Once the mixture had cooled down, 1 µl of Ethidium bromide was added. The gel was then poured and allowed to set. The running buffer used was the same 10% TE-buffer. (Ethidium Bromide is a mutagen and was used in a fume hood).

Whole mount RNA in situ hybridisation protocol

Embryos were dissected in Howard's Ringer and the extra embryonic tissues were removed. The embryos were then fixed in 4% w/v Paraformaldehyde in PBS overnight at 4°C (Embryos could be stored at this stage with for 3-4 weeks with out any signal loss).

The embryos were then washed in PBT twice, 5 minutes each wash and placed on a rocker.

To prevent gas bubbles forming during subsequent bleaching step the embryos were dehydrated:

Dehydration:

25% methanol/ 75% PBT, 10 minutes on the rocker

50% methanol/ 50% PBT, 10 minutes on the rocker

75% methanol/ 25% PBT, 10 minutes on the rocker

100% methanol, first wash 10 minutes, second wash 1 hour,

Once the embryos are dehydrated they sink to the bottom (at this stage the embryos can be stored at -20 °C).

The embryos were then rehydrated :

Rehydration:

100% Methanol, 10 minutes on the rocker

75% methanol/ 25% PBT, 10 minutes on the rocker

25% methanol/ 75% PBT, 10 minutes on the rocker

100% PBT, two washes, 10 minutes each on the rocker.

The embryos were bleached in 6% hydrogen peroxide in PBT for at least 1 hour. The peroxide was removed completely by washing the embryos in PBT at least 3 times.

The embryos were then treated with 10 mg/ml of Proteinase K, the length of time depending on the embryo's stage. For embryos later than stage 22 a minimum of 20 minutes was required (the embryos were treated for at least than 30 minutes). The embryos were washed in PBT twice, 10 minutes each wash and then post fixed in 0.2% gluteraldehyde/ 4% paraformaldehyde in PBT for 20 minutes (the embryos were carefully handled since they were extremely fragile until the post-fixed step).

The embryos were rinsed in PBT three times and then placed in pre-hybridisation solution at 70°C for at least 1 hour (at this stage embryos could have been stored at -20°C in pre-hybridisation solution pre or post 70°C incubation step).

The pre-hybridisation solution was then replaced with hybridisation solution containing 1mg/ml of the riboprobe, and incubated over night at 70°C.

Post Hybridisation Washes

Hybridisation solution was removed and the embryos were washed twice with solution 1 (30 minutes each wash) at 70°C.

Solution 1 was then replaced with solution 3 (2 washes, 30 minutes each) at 70°C.

Solution 3 was discarded and the embryos were washed three times in TBST. The embryos were left in TBST and incubated at 70°C for 40 minutes, this step reduced the background. The embryos were then pre-blocked with 10% Sheep serum in TBST for 2 hours. The 10% serum was

replace with anti-digoxygenin AP antibody and the embryos were left on the rocker over night at 4°C.

Post antibody washes and histochemistry

The antibody was removed and the embryos were washed in TBST. The first three washes were 15 minutes each and then hourly washes in TBST, throughout the day and night.
TBST was replaced with freshly prepared NTMT (pH 9.5). The embryos were then incubated in NTMT containing 3.5 mg/ml of each NBT and BCIP (Boehringer Manneheim, 1383213) wrapped in aluminium foil and left at room temperature until desired reaction was achieved. Once the reaction was completed the embryos were washed in TBST and then prefix in 4% w/v paraformaldehyde. The embryos could be stored in Paraformaldehyde indefinitely.
For full recipes to all the solutions see appendices

Results

FGF-4 beads were implanted in 260 chick limbs. 210 embryos survived and of these, 180 survived the incubation period with beads in place. 200 embryos were used for controls. 170 survived and of these, 135 survived the incubation period with beads in place.

Limbs implanted with heparin beads, soaked in either FGF-4 or PBS were labelled for the expression of FGF-4, *shh*, *Hoxd-11* and FGF-8. The expression patterns in the experimentals were compared with the controls and the following observations were made.

FGF-4 expression:

Stage 26 embryos were implanted with heparin beads soaked in FGF-4 (Fig 1, Chapter 4).

FGF-4 beads were implanted in 60 chick limbs. 46 embryos survived and of these, 40 survived the 24 hours incubation period with beads in place. 50 embryos were used for controls. 35 survived and of these, 30 survived the incubation period with beads in place.

The embryos were fixed at 24, 48 and 120 hours after bead implantation (stages 28, 31 and 36 respectively) .

Figure 1a, b and c show the typical expression of FGF-4 in normal limbs.

Figure 2a, b and c show the typical expression of FGF-4 in the limbs implanted

shh expression

Heparin beads soaked in FGF-4 were implanted in stage 26 chick embryos, the embryos were fixed 24 hours after implantation.

FGF-4 beads were implanted in 60 chick limbs. 39 embryos survived and of these, 31 survived the 24 hours incubation period with beads in place. 50 embryos were used for controls. 38 survived and of these, 31 survived the incubation period with beads in place.

Figure 3a shows the expression observed in the control limb.

Figure 3b shows ectopic expression of *shh* in the exprimentals.

Hoxd-11 expression

Heparin beads soaked in FGF-4 were implanted in stage 26 chick embryos, the embryos were fixed 24 hours after implantation.

FGF-4 beads were implanted in 60 chick limbs. 50 embryos survived and of these, 33 survived the 24 hours incubation period with beads in place. 50 embryos were used for controls. 41 survived and of these, 36 survived the incubation period with beads in place.

Figure 4 shows the expression of *Hoxd-11* in the limb at stage 28.
4a shows *Hoxd-11* expression in the untreated limb.
4b shows *Hoxd-11* expression in the implanted limb fixed 24 hours after implantation.

FGF-8 expression

Heparin beads soaked in FGF-4 were implanted in stage 26 chick embryos, the embryos were fixed 24 hours after implantation.
FGF-4 beads were implanted in 60 chick limbs. 40 embryos survived and of these, 29 survived the 24 hours incubation period with beads in place. 50 embryos were used for controls. 42 survived and of these, 37 survived the incubation period with beads in place.

FGF-4 implanted limbs were also stained for FGF-8 Expression. Figure 5 shows the expression of FGF-8.
5a shows expression of FGF-8 in the control.
5b shows the expression pattern of FGF-8 in the experimental limbs, fixed 24 hours after FGF-4 implantation.

Discussion

Since limbs implanted with FGF-4 at stage 26 showed a general increase in the length of element 5, these experiments were carried out to investigate

the molecular mechanisms involved. It seemed reasonable to investigate any molecular mechanism which involved FGF-4. FGF-4 expression in the AER is posteriorly biased and *shh* is expressed in the posterior region of the limb indicating that perhaps *shh* influences the FGF-4 expression in the AER.

A plausible initial hypothesis to explain the results described in Chapter 4 was that the positive FGF-4/*shh* feedback loop might be sustained or re-established in the presence of ectopic FGF-4. The results however indicate differently.

24 hours after FGF-4 implants (stage 28) embryos were fixed and stained for FGF-4 expression. Figure 2a shows ectopic expression of FGF-4 in the AER at stage 28, in contrast to the control (Fig. 1a). Expression is extended all around the AER and is not biased posteriorly. FGF-4 expression is not detected in embryos fixed 48 and 120 hours after implantation (Fig. 2b and c). The ectopic FGF-4 expression in the AER region at stage 28 perhaps indicates that the active life of the AER has been extended.

This observation raises the possibility that ectopic FGF-4 causes ectopic expression of *shh* in the posterior region of the limb.

Shh expression is not detected in control limbs after stage 28 (Fig. 3a). Whole mount in situ hybridisation of experimental limbs shows that ectopic expression of *shh* is not detected 24 hours after FGF-4 implantation (Fig, 3b).

Clearly, then, FGF-4 implantation does not alter expression of *shh*.

In other words, FGF-4 implants failed to sustain or re-establish the FGF-4/*shh* positive feedback loop.

Since *shh* is expressed in the ZPA, perhaps the feedback loop is only essential in the early stages. Once the ZPA has established the fate of the cells with regards to A-P positioning, the ZPA activity decreases, and the feedback loop cannot be re-established.

RA implants and ZPA grafts to the anterior region of chick limb at stage 21 or earlier always bring about *shh* expression and cause digit duplication. The ectopic *shh* expression always begins to taper off in concert with endogenous message (Riddle et al., 1993).

If the FGF-4/*shh* is not involved, another possible candidate for involvement in digit 5 lengthening is FGF-8. Purified FGF-8 protein has been shown to rescue limb bud outgrowth in mouse limbs lacking an Apical Ectodermal Ridge (AER), but has failed to maintain the expression of sonic hedgehog gene (*shh*) (Mahmood et al, 1995). Whole mount in situs for FGF-8 showed that in the FGF-4 implanted limb, expression of FGF-8 is markedly stronger and more extensive (Fig. 5). As FGF-8 is a useful marker for the AER, this may indicate that the AER has not regressed in the way that it normally would.

Whole mount in situs for *Hoxd-11* indicate that the *Hoxd-11* expression pattern is identical in both experimental and control limbs (Fig. 4a, b). These results demonstrate that an ectopic source of FGF-4 does not alter the expression pattern of *Hoxd-11*.

The experiments in Chapter 4 demonstrate that the fate of the limb cells had been altered by FGF-4 implants. However, the results in this Chapter show that this did not occur via alterations in the FGF-4/*shh* feedback loop, or through alterations in *Hoxd-11* expression. Therefore another as yet unknown mechanism must be involved. This mechanism is possibly not a component of the A-P specification mechanisms.

Previously it has been shown that FGF-4 and *BMPs* have antagonistic activities, where *BMP-2* was shown to inhibit the effect of FGF-4 on proliferation of mesenchyme cells in mouse limb bud assay (Niswander and Martin, 1993). Therefore *BMPs* may be another candidate through which FGF-4 extended the life of the AER. *BMPs* involvment during apoptosis and chondrogenesis in the developing chick limb is well

documented. In chapter 7 the affects of FGF-4 implants on apoptosis at stage 28 (24 hours after implantation) will be examined in detail.

Figure 1 (a, b and c) show the expression of FGF-4 in control limbs.

Figure 1a. Stage 28 limb labelled for the expression of FGF-4. The result shows that at this stage FGF-4 is not expressed in the AER.

Figure 1b. Stage 30 limb labelled for the expression of FGF-4. The result shows that at this stage FGF-4 is not expressed in the AER.

Figure 1c. Stage 35 limb labelled for the expression of FGF-4. The result shows that at this stage FGF-4 is not expressed in the AER

Scale: 1cm = 20μm

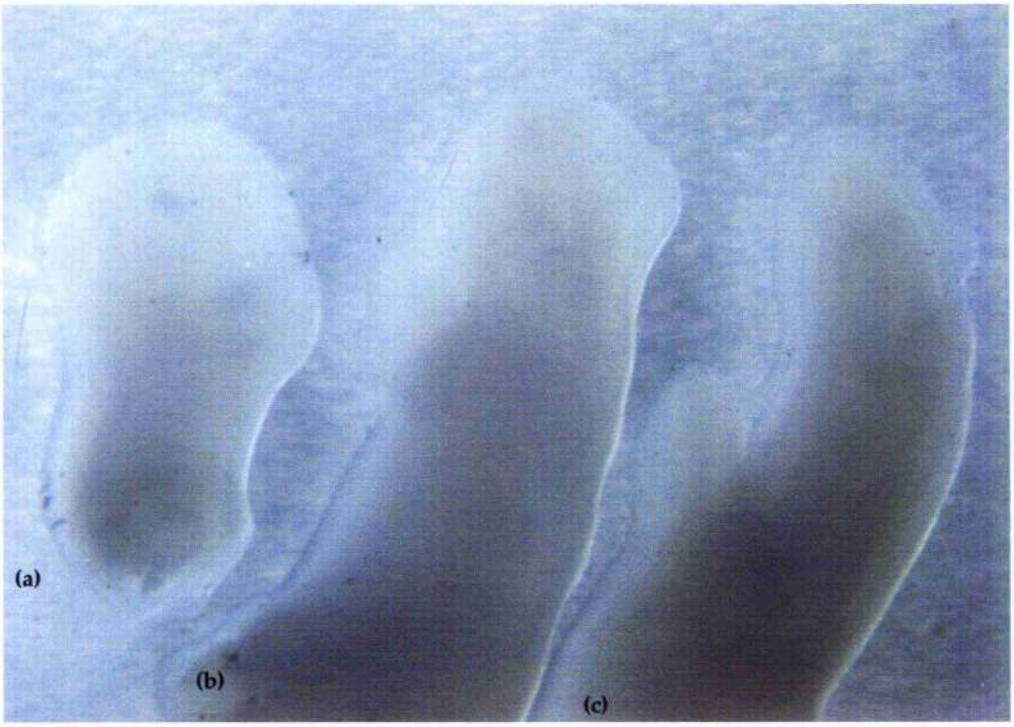

(a)
(b)
(c)

Figure 2 (a-c). Expression of FGF-4 in the AER of FGF-4 implanted limbs

Figure 2a. Stage 28 limb labelled for the expression of FGF-4. The result shows an ectopic expression of FGF-4 in the AER. Ectopic FGF-4 is expressed anterposteriorly.

Figure 2b. Stage 30 limb labelled for the expression of FGF-4, 48 hours after FGF-4 implantation. The result shows that FGF-4 is not expressed ectopically in the AER.

Figure 2c. Stage 35 limb labelled for the expression of FGF-4. The result shows that FGF-4 is not expressed ectopically in the AER.

Scale: 1cm = 20μm

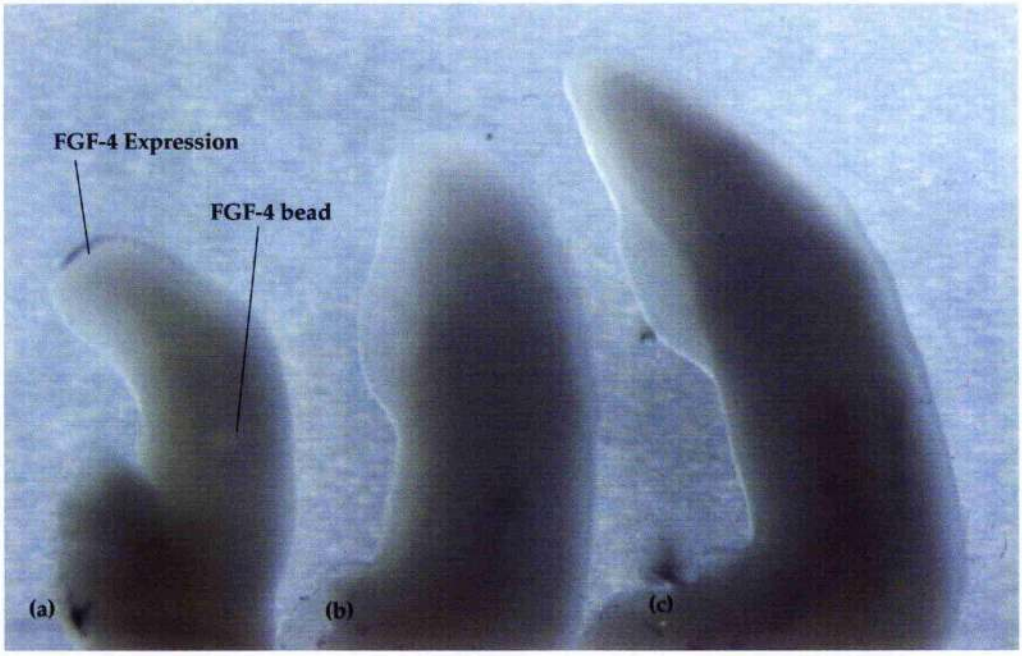

FGF-4 Expression
FGF-4 bead
(a)
(b)
(c)

Figure 3a. Stage 28 control limb labelled for the expression of *shh*. The result shows that at this stage *shh* is not expressed in the posterior region of the limb.

Scale: 1cm = 10μm

Figure 3b. Stage 28 limb labelled for the expression of *shh*, 24 hours after FGF-4 implantation. The result shows that *shh* is not expressed ectopically.

Scale: 1cm = 10μm

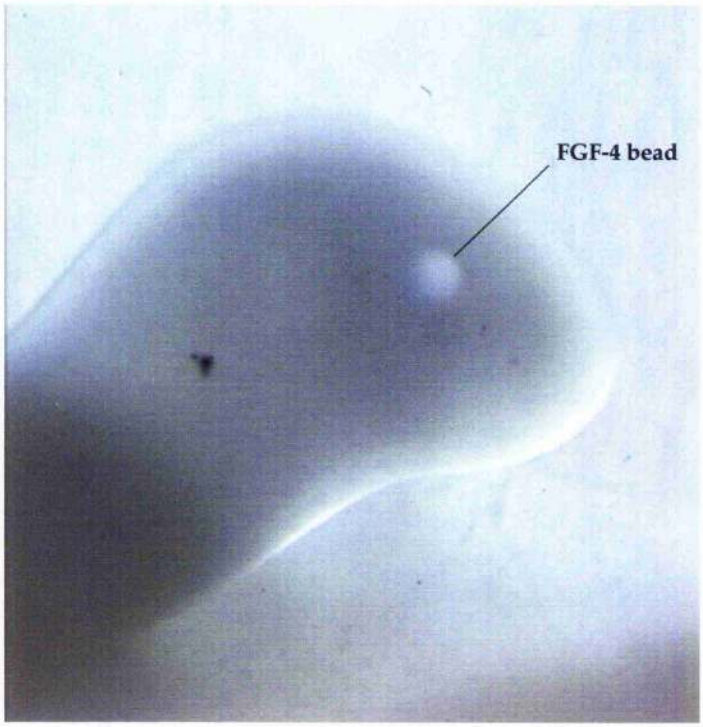

FGF-4 bead

Figure 4 (a-b). Expression of *Hoxd-11* in the limb at stage 28.

Figure 4a. Stage 28 control limb labelled for the expression of *Hoxd-11*. The result shows the normal expression pattern of *Hoxd-11*.

Figure 4b. Stage 28 limb labelled for ectopic expression of *Hoxd-11*, 24 hours after FGF-4 implantation. FGF-4 implant has not altered the expression pattern of *Hoxd-11*.

Scale: 1cm = 10μm

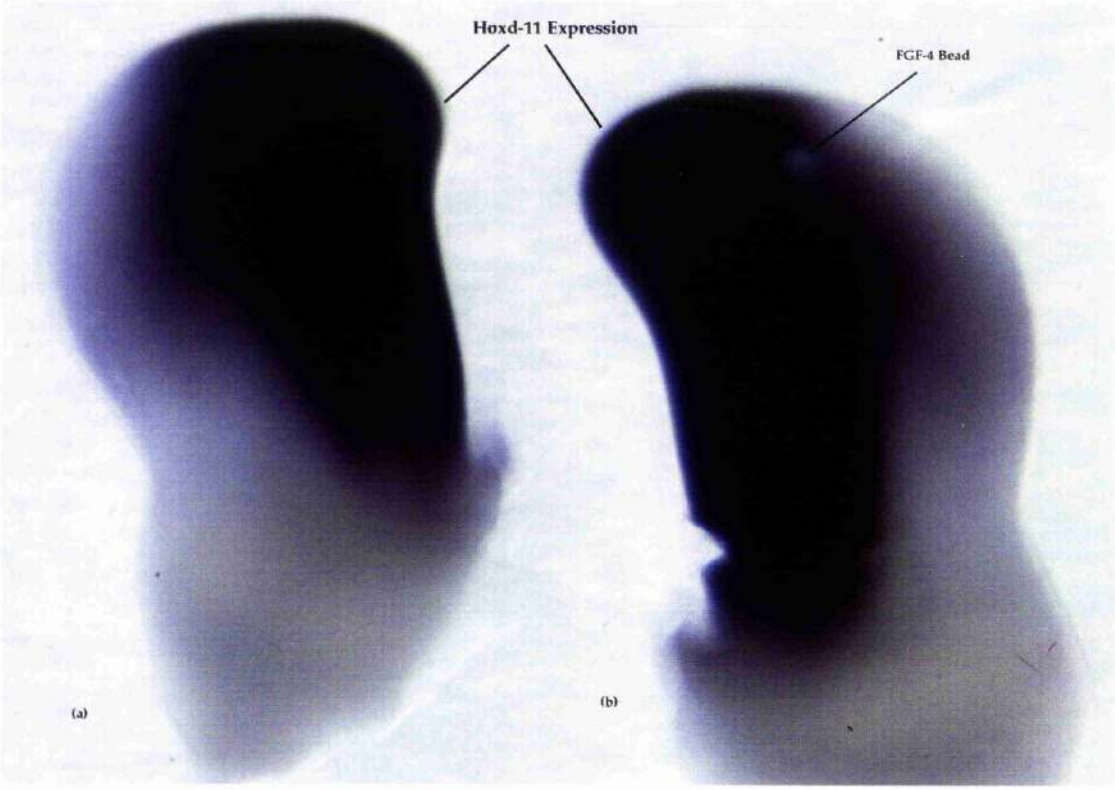
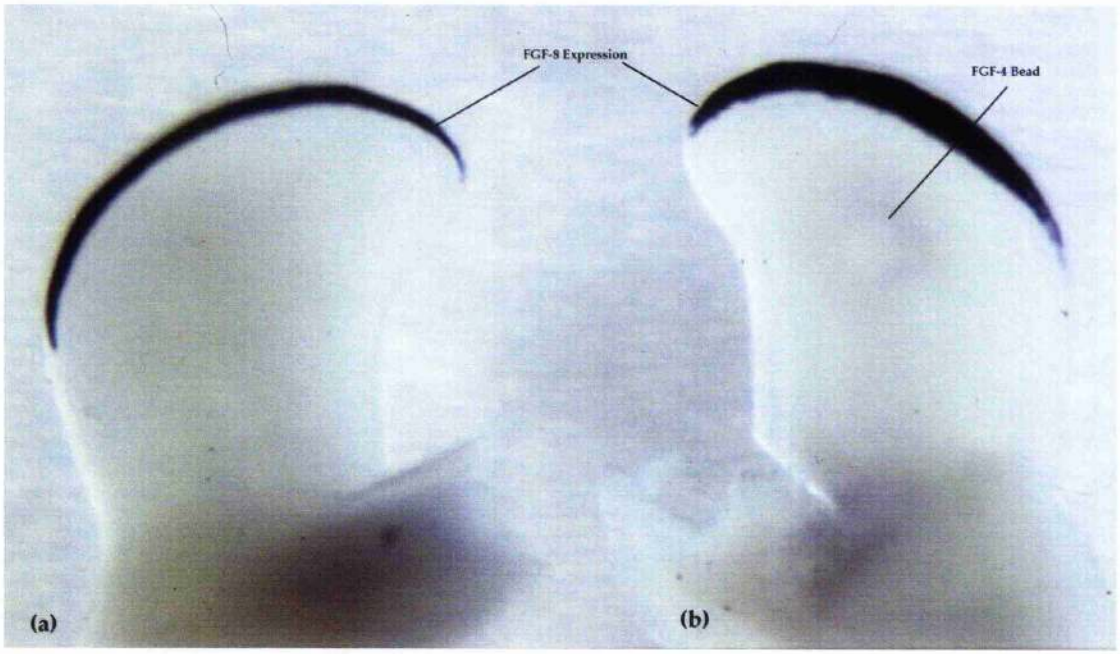

Hoxd-11 Expression
FGF-4 Bead
(a)
(b)

Figure 5 (a-b). Expression pattern of FGF-8.

Figure 5a. Stage 28 control limb labelled for the expression of FGF-8. The result shows the normal expression pattern of FGF-8 in the regressing AER.

Figure 5b. Stage 28 limb labelled for the expression of FGF-8, 24 hours after FGF-4 implantation. The result shows the ectopic expression of FGF-8 in the AER.

Scale: 1cm = 10μm


FGF-8 Expression
FGF-4 Bead
(a)
(b)

Chapter 7

Apoptosis in the avian wing

Abstract

Cell death is a fundamental part of embryonic morphogenesis, differentiation and teratogenesis. During normal development cell death contributes to the patterning of the organism. It is known that FGF-4 inhibits *BMP* activity and in turn causes elimination of cell death (Zou and Niswander, 1966). In this set of experiments the pattern of Programmed cell death (PCD) in FGF-4 implanted chick limbs was investigated. Originally it was thought that ectopic FGF-4 inhibits PCD in both anterior and posterior necrotic zones. Anterior and posterior necrotic zones are thought to reduce the anterior and posterior axis of mesenchyme available for digit formation. Thus ectopic FGF-4 might have inhibited cell death at the appropriate time (4-6 days of incubation) and contributed to the elongation of element 5. Cell death pattern in the ANZ was larger and more distally located, but the ectopic source of FGF-4 eliminated cell death in the PNZ. Ectopic FGF-4 might have delayed the onset of *BMP* activity in the posterior region of the limb and as a result delayed or eliminated the PCD in that region. The molecular information for digit formation may be present in the posterior region of the limb and elimination or delaying PCD might have encouraged elongation of element 5.

Introduction

Programmed cell death (PCD) is the process whereby unnecessary tissues are eliminated during embryonic development. Cell death in multicellular organisms is either physiological (PCD) or is a response to physical/chemical insult (necrosis) (Loo and Rillema, 1998; Hurle et al., 1995). PCD is a normal feature of development and growth and is

prominent in avian limb bud development. The early avian limb bud has a specific pattern of cell death very different from that of mammals (Chapter 1).

In limb development, the importance of AER and its interactions with the underlying mesenchyme is well documented (Chapter 1). FGF-4 is expressed in the AER at the time of proliferation of the underlying mesenchyme (Niswander and Martin, 1993). FGF-4 and morphogenetic protein-2 (*BMP-2*) both encode secreted signalling molecules and studies have shown that bone *BMP*s are also expressed in the AER. *In vitro* FGF-4 protein stimulates proliferation of mesenchyme in the early mouse limb bud, while in contrast *BMP-2* inhibits limb growth (Niswander and Martin, 1993).

It is thought that the morphogenesis of digits in the avian limb is controlled by FGFs, TGFβ, and *noggin* via *BMP* signalling (Merino et al., 1998). Interaction between these signals controls the formation of digital rays. This is achieved by regulating the spatial distribution of *BMP*R-1b gene expression in the digit forming mesenchyme.

BMP-4 may also play an important role in mediating apoptosis during chick limb development (Yokouchi, et al., 1996). Its expression is detected in the PNZ, ANZ and INZ (Yokouchi et al., 1996). All this evidence shows that *BMP-4* may be involved in programmed cell death in different regions of the embryo at different times during development (Graham et al., 1994; White et al., 1994).

It was shown in Chapter 4 that ectopic sources of FGF-4 at stage 26 cause elongation of element 5. However, in Chapter 6, it was shown that this did not occur via a modification of the *shh*/FGF-4 positive feedback loop.

Since experiments have shown that extra digits in the chick foot can be induced without altering the molecular mechanisms involved in pattern specification (Hurle and Colombatti, 1996) then alternative theories are required to explain both these observations and the elongation of element 5.

Programmed cell death by apoptosis is plainly an important aspect of the developing limb. The effect of FGF-4 on PCD was therefore investigated.

Methods and Materials

The chick limb were implanted with Heparin sulphate beads either soaked in FGF-4 or PBS at stage 25-26 (Chapter 4).

The embryos were fixed at exactly 24 hours after FGF implantation and stained in 1% neutral red for cell death.

Limbs were also stained with Alcian green and cleared for cartilage at stages 28 and 31 (Chapter 4).

Staining with vital dyes

The limbs were stained in 1% neutral red for 30 minutes at 37°C (and checked every few minutes to prevent over staining). The limbs were then fixed in neutral formalin at 4°C for 16-24 hours: the timing here is crucial since over fixing could cause loss of staining. The limbs were then dehydrated in pure 2-propanol, cleared in Xylene and photographed immediately (Hurle, 1999).

When vital dyes are used the living cell with intact cell membranes exclude the specific dye and the dead cells with compromised cell

membranes show positive staining. The drawback of vital dyes is that the cells undergoing apoptosis retain cells membrane integrity until late in the apoptotic programme when secondary necrosis begins and therefore there is always an underestimation of cell death (Loo and Rillema, 1998).

Results:

FGF beads were implanted in 200 chick limbs. 166 embryos survived and of these, 140 survived the incubation period with beads in place. 300 embryos were used for controls. 259 survived and of these, 230 survived the incubation period with beads in place.
Limbs implanted with heparin beads, soaked in either FGF-4 or PBS were stained and cell death patterns in the experimentals were compared with the controls and the following observations were made. FGF-4 implanted and control limbs were also stained with Alcian green and cleared for cartilage.

Control and FGF-4 implanted limbs:

Stage 26 embryos were implanted with heparin beads soaked in FGF-4 (see Fig 1, chapter 4). The embryos were fixed 24 hours after bead implantation (stages 28).

Figure 1a and b show the cell death pattern in a typical FGF-4 implanted limb and corresponding controlateral control. In the control limb (Fig. 1a) cell death is detected in both PNZ and ANZ. In contrast the FGF beads eliminated normal cell death pattern in the PNZ, while the ANZ had broadened and was located more distally (Fig. 1b). None of the control procedures affected size or the location of cell death (Fig 1a). Results from all control limbs were therefore pooled.

Figure 1c and d show further examples of cell death in both control (1c) and experimental limbs (1d).
Figure 2a shows cartilage formation in a normal limb. Figure 2b shows cartilage formation of FGF-4 implanted limb. Close examination of the cartilage at stage 28 shows no difference between the controls (Fig. 2a) and the experimentals (Fig. 2b).

Figure 3a shows cartilage formation in the normal limb at stage 31. Figure 3b shows the cartilage formation in an FGF-4 implanted limb at stage 31. Element 5 is present in the FGF-4 implanted limb

Discussion

Limbs implanted with FGF-4 at stage 26 showed a general increase in the length of element five (Chapter 4). Investigating the molecular mechanisms involved showed that ectopic source of FGF-4 had not altered the FGF-4/*shh* feedback loop. Involvement of *BMP*s during apoptosis and chondrogenesis is also well documented (Kawakami et al., 1996; Zou & Niswander, 1996). It has been shown that *BMP-2* inhibits the effect of FGF-4 on proliferation and mesenchyme cells in a mouse limb bud assay (Niswander and Martin, 1993).

In this set of experiments the cell death pattern after the application of ectopic FGF-4 was investigated. The limbs were stained with neutral red 24 hours after FGF-4 implantation. Staining with vital dyes is a commonly used approach to quantifying cell death. The embryos were stained at stage 28 since the mesenchymal cells in the ANZ and PNZ are eliminated by PCD between days 4-6 of development.

Vital dye staining of the limbs with neutral red provided some information about the level of cell death in the limb after application of ectopic FGF-4.

It is known that FGF-4 inhibits the activity of *BMPs* and obstruction of *BMP* signals in the limb bud results in inhibition of interdigital cell death and digit truncation (Zou & Niswander, 1996). It would therefore be expected that FGF-4 implants would decrease or eliminate PCD in the PNZ and ANZ. The PNZ cell death pattern observed in this set of experiments was as predicted. The PNZ shows no evidence of cell death as expected. The ANZ cell death pattern seen in the experimental limb is in fact larger and more distally located.

Examining stage 28 limbs stained for cartilage did not provide further explanations for the elongation of element 5, since Figure 2a and b shows no difference between the experimentals and the controls.

By stage 31 PCD is no longer observed in the posterior and anterior necrotic zones. However, limbs were stained for cartilage formation and the result showed presence of element 5 in the experimentals only.

It is known that signals from *BMP-2* are capable of inducing formation of new cartilage (Wozney, 1992) and are thought to be the prime mediators in deciding the fate of these autopodal cells leaving the PZ (Lyons et al., 1995). In the limb mesenchyme ectopic *BMP-2* expression is only observed in the presence of FGF from the AER (Zou & Niswander, 1996). The signalling pathway of the *BMPs* is not completely clear and obstruction of *BMP* signals in the limb bud results in inhibited interdigital cell death and caused digit truncation (Zou & Niswander, 1996). In these set of experiments FGF-4 implants did not cause digit truncation. At stage 28 the cartilage formation in the control and the experimental limbs was identical. By stage 31 element 5 was only observed in the FGF-4 implanted

limbs. The increased size of element 5 may be due to the FGFs and BMP balance in the limb mesenchyme since members of the FGF families are known to control the size of the appendages when they initially form (Buckland, 1997). In the experimental limbs digit formation and interdigital cell death was not disturbed indicating that presence of ectopic FGF-4 had not affected the *BMP* signals in the INZ. Since the *BMP* expression pattern in the mesenchyme and the PZ is different then it is possible that FGF-4 implants had influence the *BMP* signals in the mesenchyme and not the PZ.

The ectopic source of FGF-4, having extended the active life of the AER, had perhaps delayed the onset of *BMP*s in the limb mesenchyme. This possibly explains the larger and more distally located ANZ and the lack of PNZ cell death in the FGF-4 implanted limb. Whether lack of PNZ presence 24 hours after FGF-4 implantaion indicates complete elimination of PCD or a later onset of cell death in the region is unknown, however the process might have increased the posterior axis of mesenchyme available for digit formation. The true mechanisms involved are not clear and need further investigation.

Figure 1(a, b). Cell death pattern in typical FGF-4 implanted limb and corresponding controlateral control.

Figure 1a. Cell death pattern in the untreated limb at stage 28. Cell death is detected in both PNZ and ANZ but, the PCD pattern is faint in both regions.

Figure 1b. Cell death pattern in the controlateral FGF-4 implanted limb at stage 28, 24 hours after implantation. Cell death in the PNZ is not detected. Cell death in the ANZ is more distally situated.

Scale: 1cm = 20μm

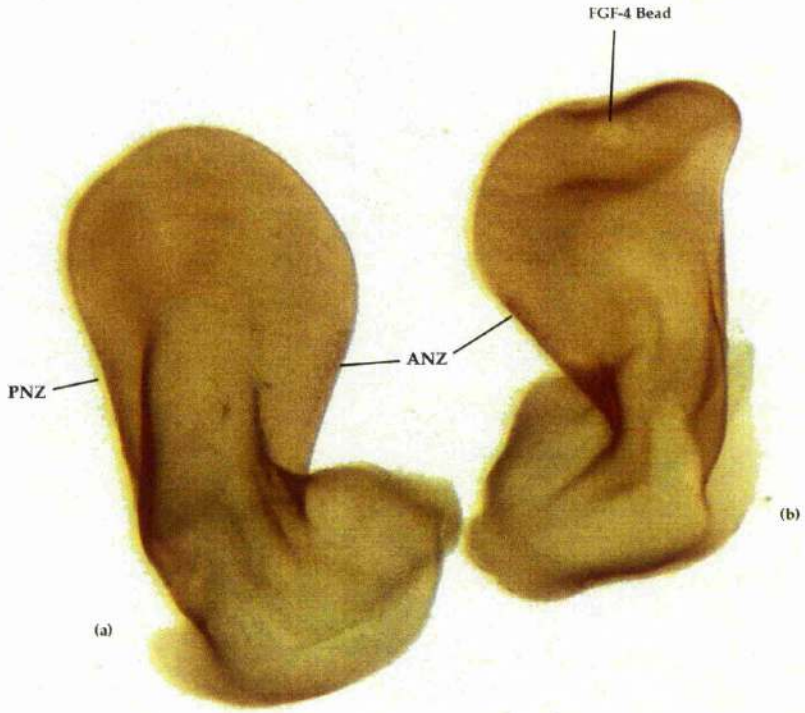

FGF-4 Bead
PNZ
ANZ
(a)
(b)

Figure 1c. Further demonstration of cell death pattern in untreated stage 28 limb. Cell death detected in both PNZ and ANZ is faint since at stage 28 cell death in both regions is near completion.

Scale: 1cm = 10μm

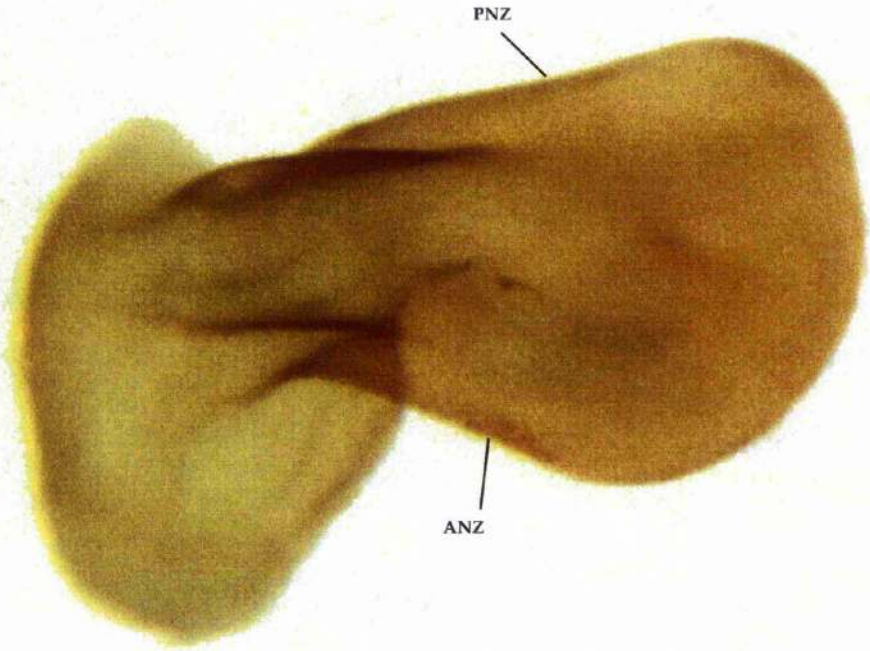

PNZ
ANZ

Figure 1d. Further demonstration of typical cell death pattern in FGF-4 implanted limb at stage 28, 24 hours after implantation. Cell death in the PNZ is not detected. Cell death in the ANZ is stronger and more distally located.

Scale: 1cm = 15μm

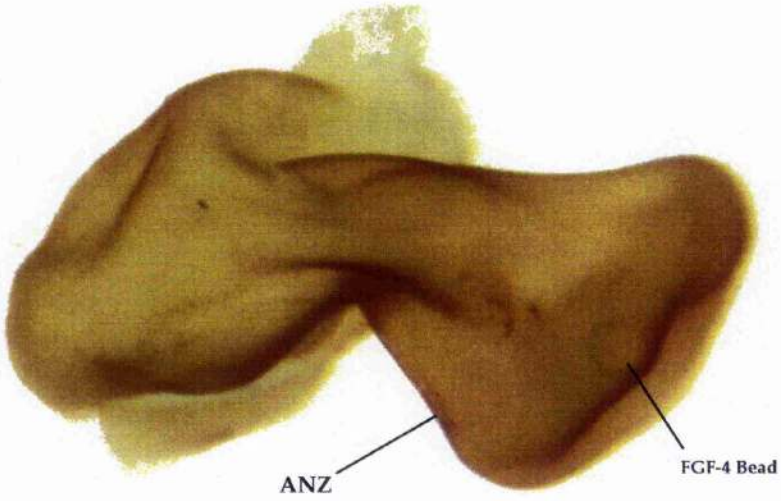

ANZ
FGF-4 Bead

Figure 2 (a-b). Cartilage formation in untreated and FGF-4 implanted limbs.

Figure 2a. Cartilage formation in a typical untreated limb at stage 28.

Figure 2b. Cartilage formation in a typical FGF-4 implanted limb at stage 28, 24 hours after implantation. Close examination of the cartilage at stage 28 shows no difference between the experimentals and the controls.

Scale: 1cm = 20μm

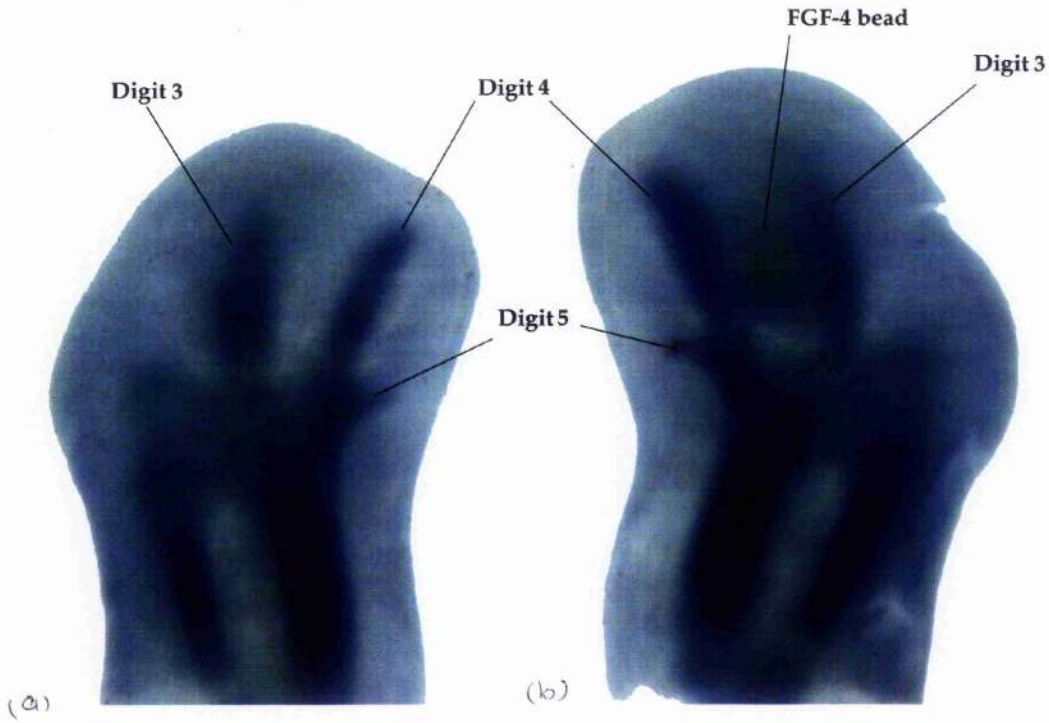

Digit 3
Digit 4
Digit 5
FGF-4 bead
Digit 3
(a)
(b)

Figure 3a. Cartilage formation in a typical untreated limb at stage 31.

Digits 2, 3 and 4 are present.

Scale: 1cm = 20μm

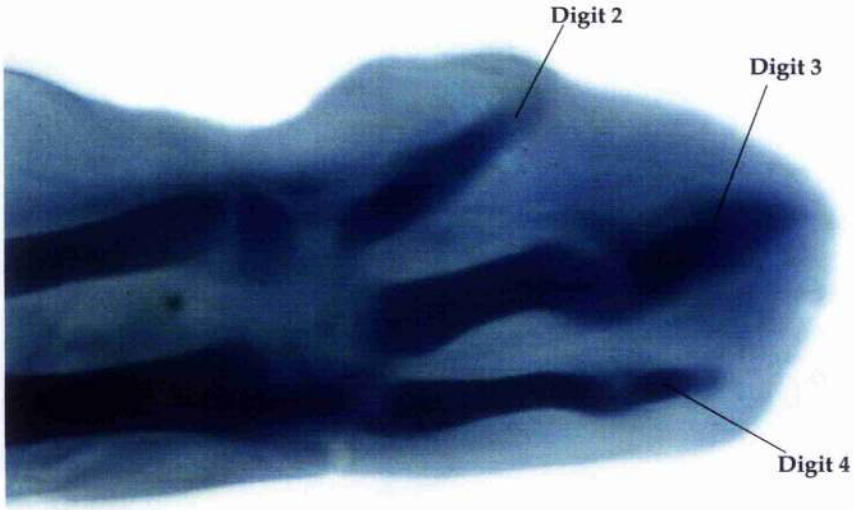
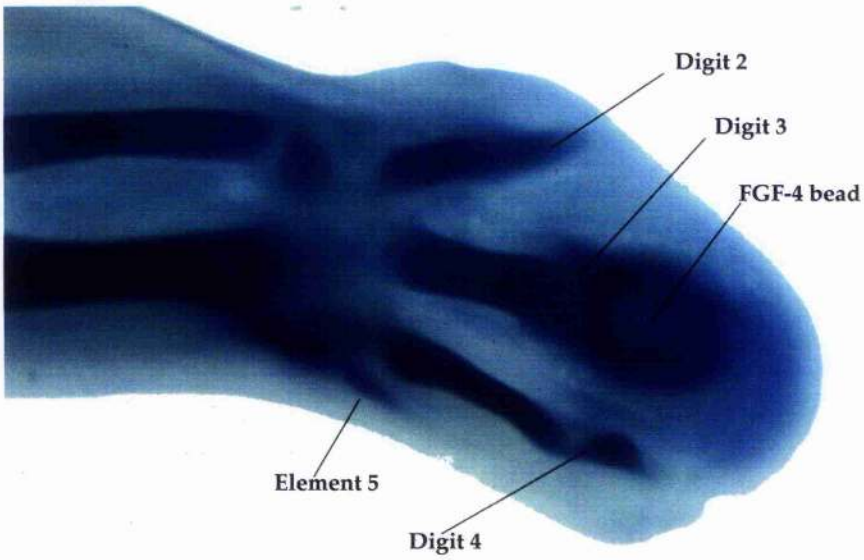

Digit 2
Digit 3
Digit 4

Figure 3b. Cartilage formation in a typical FGF-4 implanted limb at stage 31, 48 hours after implantation. Digits 2, 3, 4 and element 5 are present. Scale: 1cm = 20μm


Digit 2
Digit 3
FGF-4 bead
Element 5
Digit 4

Chapter 8

General Conclusion

The chick limb has been a favoured model system used for studying vertebrate pattern formation. In recent years research on the avian wing has been focused on determining the function and role of signalling molecules in the hope of identifying a limb morphogen. Certain molecules or families of molecules are thought to be involved in all stages of vertebrate patterning and development. The formation of structures at the appropriate time and place is thought to be controlled by the signals of these molecules specifying the target cell's position. Transcripts of fibroblast growth factors (FGFs), sonic hedgehog (*s h h*) and homeobox genes (Hox) are amongst these signalling molecules.

The presence of FGF RNA during limb development has been well documented (Niswander et al., 1993; Vogel and Tickle, 1993). However this is not the same as demonstrating that a functional gradient of FGF exists in the developing limb since gene transcripts must be translated, exported and extensively processed before they become active (Morris, 1996). This is controversial for FGFs since they lack a signal sequence and it has been argued that they might be secreted by an alternative secretion pathway (Mignatti et al., 1992). Therefore, the range of FGF activity in the limb and the possibility of the existence of a functioning FGF gradient was first investigated in a microassay for anchorage independent growth in soft agar using NR6 cells. These cells formed colonies in a dose dependent manner in the presence of FGF. The response of NR6 cells confirmed the existence of a proximosdistal functional gradient in the limb between stages 22-26. These observations were consistent with the hypothesis that a functional gradient of an active FGF-like molecule was secreted by the AER between stages 22-26. These results suggested that perhaps FGFs were being produced locally by the AER and exerted a graded influence over the underlying mesoderm from proximal to distal in the limb. However, presence of FGF and FGF receptors in the underlying mesenchyme also

indicates that the functional gradient of FGF could have been produced in the mesenchyme itself. This functional gradient was observed to be active over a distance of about 600 μm. There are rather few actual examples of functional gradients of morphogenetic substances despite their theoretical importance. A morphogen may be defined as a molecule that forms a functional gradient whose concentration directly specifies position (Turing 1952; Wolpert, 1989).

In the chick limb the AER activity begins to decrease after stage 22 which corresponds with the decrease in ZPA activity. By stage 26 the functional FGF gradient decreased. The correlation between the decline in the functional gradient of FGFs and the regression of the AER indicated that the FGF in question might have been produced locally in the AER.

By the time the AER begins to regress, the positioning information of the cells has been determined and the molecular pathways involved in the patterning of the limb are in the process of down streaming. Thus, stage 26 seem to be the appropriate time during limb development where the active life of the AER could have been extended. Thus the consequence of prolonging the active life of the AER was investigated.

Prolonging the life of the AER

FGF-4 has been shown to replace the function of AER and to mimic the activity of the other FGFs during limb development (Kuhlman and Niswander, 1997). It was therefore chosen as the most likely signalling molecule able to prolong the life of the AER.

Application of FGF-4 to the distal tip of the limb at stage 26 resulted in the consistent elongation of element 5 proximodistally in the form of a digit. The result showed that prolonging the action of the apical ectodermal

ridge it could induce experimental atavisms in chick digit morphology. By these means 'element 5' was shown to be a rudimentary digit.

Soft tissue analysis of the experimental limbs were carried out. Also three dimensional surface models of the limbs were created to provide information about effects of FGF-4 on the limb as a whole. In addition experiments were carried out to investigate the molecular mechanisms involved in elongation of element 5.

Soft tissue analysis

Soft tissue analysis of the limb revealed that the ectopic FGF-4 had not caused any disturbance, formation of novel elements or elongation of already existing elements in the anterior region of the limb. Therefore, the molecular information for development involving FGF-4 was perhaps only present in the posterior region.

Since the ectopic FGF-4 had only brought changes to the posterior region the molecular mechanism operating in that region were investigated. Normal expression of FGF-4 in the AER is posteriorly biased. *shh* is expressed in the posterior region of the limb, the ZPA region. The molecular mechanisms in the posterior region that involved both the AER and the ZPA was the *shh*/FGF-4 positive feedback loop (Laufer et al., 1994). The *shh*/FGF-4 positive feedback loop is thought to allow the co-ordination of mesodermal outgrowth and patterning. This co-ordination is only possible because *shh* patterns mesodermal tissue and regulates FGF-4 expression. FGF-4 protein in turn induces mesodermal proliferation and maintains *shh* expression. Thus mesodermal tissue can

only be patterned by *shh* in conjunction with the activity provided with FGF-4, indicating that patterning always coincides with proliferation.

Signalling molecules

Originally, it was thought that the ectopic FGF-4 may have sustained or re-established the positive *shh* /FGF-4 feedback loop.
Whole mount in situs labelled for FGF-4 showed an anteroposterior ectopic expression of FGF-4 in the AER. This ectopic expression indicated that perhaps the active life of the AER had been extended. Ectopic FGF-4 was not detected in embryos older than stage 28. In order to examine the size of the AER, FGF-8 was used as a marker. FGF-8 is present along the entire length of the AER throughout its existence (Mahmood et al ., 1995; Vogel et al., 1996). In the limbs implanted with FGF-4, the AER was less regressed supporting the hypothesis that application of ectopic FGF-4 had indeed delayed the regression process of the AER.
The result showed that extended life of the AER had failed to sustain or re-establish the *shh* /FGF-4 loop. This indicates that perhaps the fate of the cells was determined irreversibly by the polarising region. Perhaps, by stage 26 most of the positioning of the limb was well established and once the positive feed back loop is diminished it can no longer be re-established. The whole mount in situs hybridization for *shh* expression failed to explain the reason behind the elongation of element 5.
Clearly prolonging the active life of the AER had brought about elongation of element 5 but without the involvement of the limb morphogen. This is of particular interest since it suggests that other mechanisms yet unknown may have been involved.

Another group of genes involved in anterposterior patterning and digit formation are certain members of Hoxd gene family cluster in particular *Hoxd-11*. However, as can be seen in chapter 6, element 5 elongation had not occurred due to an alteration to the normal expression pattern of the *Hoxd-11*, confirming that the ectopic source of FGF-4 had failed to vary the expression pattern of *Hoxd-11*. Therefore prolonging the active life of the AER does not alter the molecular pathways involved in anteroposterior patterning or digit formation in the chick limb.

Apoptosis

It has been suggested that the morphogenesis of digits in the avian limb is controlled by FGFs, TGFβ, and *noggin* via *BMP* signalling (Merino et al., 1998). *BMP*s are thought to be involved in programmed cell death during limb development. Throughout the AER apoptotic and proliferating cells are present. The AER cells are continuously undergoing cells death and cell proliferation suggesting that, PCD may play an important role in maintaining AER activity (Ferrari et al., 1998). *BMP*s and FGFs are expressed in the AER and FGF-4 and *BMP-2* are known to encode secreted signalling molecules (Niswander and Martin, 1993). *BMP-4* expression is detected in the PNZ, ANZ and INZ suggesting that it may be involved in programmed cell death in different regions of the embryo and at different times during development (Niswander and Martin, 1993). Interactions between these signals control the formation of digital rays. This is achieved by regulating the spatial distribution of *BMP*R-1b gene expression in the digit forming mesenchyme (Merino et al., 1998). Thus an ectopic source of FGF-4 might have eliminated *BMP-4* expression in the mesenchyme and as a result inhibited programmed cell death in the PNZ in particular. The FGF-4 implanted limbs stained for programmed cell

death patterns showed complete elimination of posterior necrotic zone but the cell death pattern observed in the anterior necrotic zone was larger and more distally located. Perhaps application of ectopic FGF-4 had delayed the onset of *BMP-4* activation. This would explain the more distally situated anterior necrotic zone. If the onset of programmed cell death in the posterior necrotic zone is eliminated or delayed less limb material is removed. Soft tissue analysis of the limb showed that the ulnametacarpalis dorsalis (UMD) muscle had increased substantially and the number of blood vessels to the region of element 5 had also increased. Angiogenesis is a property of FGF: however angiogenesis observed in the FGF-4 implanted limbs had occurred in the posterior region of the limb and not just locally at the site of implant. It is of particular interest that no additional elements had formed in the anterior regions of the implanted limbs.

These results show conclusively that known molecular mechanisms and pathways that involve FGF-4 had not been involved in bringing about the elongation of element 5.

Investigating the ectopic expression pattern of *BMPs* might provide an alternative molecular pathway through which FGF-4 operated in the limb. If *BMPs* are involved then PCD has played a greater role in providing the molecular environmental conditions necessary for the elongation of element 5. Conceivably, the molecular information for digital development may only exists in the posterior region suggesting that digit reduction in the avian wing has occurred posteriorly.

The results in chapter 7 showed that effects of ectopic FGF-4 on cartilage formation is observed at stage 30, 48 hours after implantation. Prolonging the active life of the AER could have supported a prolonged graded influence of FGF-4 proximodistally in the limb.

The molecular changes caused by prolonging the active life of the AER and the subsequent proximodistal elongation of element 5 need to be investigated further. Studying the molecular pathways involving *BMPs* and *noggin* might provide invaluable information as to what occurs in the limb on a molecular level if the active life of the AER is prolonged.

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Appendices

Recipes for solutions used in chapter 6

Solutions:

PBT

10ml, of 10X PBS (made with DEPC treated water)

400ml, DEPC water

400ml, Tween-20 to a final concentration of 0.1%

6% hydrogen peroxide

1000µl, 6% hydrogen peroxide (30% stock)

5ml, PBT

Proteinase K

15µl, Proteinase K

30ml, PBT

Pre-hybridisation/ Hybridisation solution

25ml, 50% formamide

12.5ml, 20X SSC pH 4.5

50µl, 50mg/ml yeast RNA

5ml, 1% SDS

50µl, 50mg /ml heparin

Solution 1

25ml, 100% formamide

12.5ml, 20X SSC (pH 4.5)

5ml, 10% SDS

7.5ml, Millipore water

Solution 3

25ml, 100% formamide

5ml, 20X SSC (pH 4.5)

20ml, Millipore water

10X TBST

16g, NaCl

0.4g, KCl

50ml, 1M Tris-HCl (pH 7.5)

20µl, Tween-20

NTMT

pH 9.5 always prepare fresh.

1ml, 5M NaCl

5ml, 1M Tris-HCl (pH 9.5)

2.5ml, 1M $MgCl_2$

50ml, Tween-20

0.024g, 2mM Levamisole

20X SSC pH 4.5

175.3g, NaCl

88.2g Na_3Citrate

800ml, ddH_2O

Adjust Volume to 1 Litre and DEPC treat and autoclave

10X TBE

in 1.61L of ddH_2O add in order, boric acid will not dissolve in ddH_2O unless TriZma base is dissolved first.

215.6g, TriZma Base (sigma T1503)

90g, Boric Acid (sigma B7660)

15g, EDTA (disodiumsalt, sigma E5134)

Make volume to 2 L.