THE RELATIONSHIP OF FIBROBLAST GROWTH FACTOR 4 (FGF-4) TO EMBRYONIC FACIAL GROWTH

by 47

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ABSTRACT

Patterning of the undifferentiated mesenchyme of the chick wing bud is guided by interactions between the apical ectodermal ridge and the polarizing region. FGF-4 has been shown to maintain the polarizing activity in the chick wing bud when the apical ectodermal ridge is removed. FGF-2, another member in this family of growth factors, is known to stimulate frontal nasal mass mesenchyme. The possible role of FGF-4 in facial development was investigated by grafting facial mesenchyme from two different facial primordia from stage 24 embryos into the wing bud of stage 22 host embryos. Since FGF-4 is an epithelium-derived factor, mesenchyme with its attached epithelium was grafted as a positive control.

As the frontal nasal mass (FNM) develops, the prenasal cartilage forms and an egg tooth (a specialized epithelial structure) appears. When the FNM with its epithelium is grafted into the wing bud, 84.6% produce an outgrowth with an egg tooth. If the FNM is grafted without the epithelium, the incidence of egg tooth formation drops to 64.3%. When a bead soaked in FGF-4 is placed in the FNM graft without the epithelium, the incidence of egg tooth formation rises to 92.9%. The mean length of the cartilage rod in the outgrowth is not statistically different between the FNM with epithelium group and the FNM mesenchyme plus FGF-4-soaked bead group but

both are significantly longer than the FNM mesenchyme only group.

Mandibular graft experiments show a different response pattern: the mandibular (Md) mesenchyme with an FGF-4-soaked bead does not produce a significantly longer cartilage rod in the outgrowth compared to Md mesenchyme alone: both are significantly shorter than the Md mesenchyme with its intact epithelium. Qualitatively, the shape of the cartilage also differs. Long, straight rods result from Md mesenchyme with intact epithelium grafts whereas rectangular, triangular, and circular rods of cartilage develop in the Md mesenchyme plus FGF-4-soaked bead grafts.

These results could mean that either: 1) FGF-4 affects the FNM more than the mandible or 2) FGF-4 expresses its action at a different stage of development within these primordia. In these experiments, FGF-4 appears to play a role in patterning and differentiation of the facial primordia and has a lesser effect on proliferation and outgrowth.

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GENERAL INTRODUCTION

Facial growth and development is important to understand as a practitioner of orthodontics especially as it relates to treatment. Abnormal growth and development of the face and head presents with unique problems in planning treatment in terms of structures and tissues that are present, structures and tissues that are not present, lack of outgrowth of the facial primordia, lack of fusion of facial primordia, and premature fusion of growth areas. "The successful rehabilitation of the patient with abnormal development of the cranium, face, and mouth is contingent on accurate diagnosis and an understanding of the aetiopathogenesis of the basic defect. As the scientific basis of malformation is clarified it will be easier for the surgeon and the orthodontist to improve the quality and stability of treatment."[1] Recent discoveries have linked fibroblast growth factor receptor mutations to a number of syndromes involving the face (and limbs) such as Crouzon's Syndrome (Reardon *et al.*, 1994a, 1994b; Rutland *et al.*, 1995; Wilkie *et al.*, 1995; Jabs *et al.*, 1994), Jackson-Weiss Syndrome (Jabs *et al.*, 1994), Pfeiffer Syndrome (Rutland *et al.*, 1995; Muenke *et al.*, 1994), Apert's Syndrome (Wilkie *et al.*, 1995), and achondroplasia (Shiang *et*

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[1] Poswillo, D. (1988). The aetiology and pathogenesis of craniofacial deformity. **DEVELOPMENT** *103 Supplement*, p. 212.

al., 1994). Thus it is important to clarify the role that fibroblast growth factors play in normal development.

Embryonic Development of the Chick Face

The face of the chick embryo is first recognized at stage 14 (≈2.25 days incubation) of *in ovo* development when the first and second branchial arches and clefts are distinct. Facial primordia - mesenchyme covered by epithelium - grow outward around the stomodeum and fuse together. The final facial form is achieved through growth, morphogenesis, cell differentiation, and pattern formation (spatial ordering of cell differentiation) (Wedden *et al.*, 1988) of the facial primordia. These primordia are: 1) the frontonasal mass which forms the upper beak, prenasal cartilage, nasal septum, and premaxilla, 2) the paired lateral nasal processes that form the conchae of the nose and external nares, 3) the paired maxillae which form the corners of the beak, tissues under the eyes, and the palatal shelves, and 4) the paired mandibular processes that fuse together to form the mandible (Tamarin *et al.*, 1984). Both the frontonasal mass and mandibular primordia produce distinct cartilage rods - the prenasal cartilage and Meckel's cartilage - which make these primordia good candidates for studying outgrowth of the face. Cellular differentiation and chondrogenesis within these primordia

begin at stage 24. At stage 30 (6.5 days incubation), the egg tooth appears (Tamarin *et al.*, 1984) at the distal tip of the prenasal cartilage. It is a specialized ectodermal structure on the dorsal surface of the upper beak used by the chick to break out of the shell but then disappears shortly after hatching (Yee and Abbott, 1978).

Epithelium permits the outgrowth of the mesenchyme of the facial primordia: if the epithelium is removed, mesenchymal outgrowth is truncated (Wedden, 1987). Either limb bud epithelium or facial epithelium will permit outgrowth but the mesenchyme determines the shape (Richman and Tickle, 1989) of the prominence.

The mesenchyme within the facial primordia arises from two sources: the neural crest and the somitomeres. The migration of neural crest cells from the edges of the neural folds occurs at the time when the neural tube is closing (Noden, 1984; Lumsden *et al.*, 1991). The fate of the neural crest cells is pre-programmed prior to migration from the neural epithelium (Noden, 1983b; Wedden *et al.*, 1988). As these neural crest cells migrate, they transform into mesenchymal cells capable of forming cartilage, bone, glial cells, sensory and autonomic ganglia, or pigment cells. Mapping the migration of neural crest cells using tritiated thymidine (Johnston, 1966; Noden, 1975), grafted quail cells with distinguishable nuclei (LeDouarin, 1973; LeLievre and LeDouarin, 1975), or DiI (Lumsden, 1991) have shown that the

neural crest cells contributing to the face arise in the region from the midbrain caudally to the second rhombomere.

The first branchial arch was filled by neural crest cells migrating from the level of the midbrain to rhombomere 2. The neural crest cells fill the arch ventral-dorsally and rostral-caudally (Lumsden, 1991). The first branchial arch subsequently develops into the lower half of the maxilla and the mandible.

Not all structures within the head are derived from the neural crest cells: the voluntary muscles of the face are derived from somitomere mesoderm (Noden, 1983a, 1984) although the connective tissues around these muscles may originate from the neural crest. Other non-neural crest derivatives include motor neurons, the cranial vault, and endothelial cells of blood vessels (Noden, 1988).

In the frontonasal mass, the neural crest cells and the cells from the somitomeres intermingle but in the mandible, the neural crest cells maintain a peripheral position around a core of somitomere-derived mesoderm (Trainor and Tam, 1995).

Although the neural crest contribution of cells is unique to the head and neck regions, the outgrowth of the facial primordia is thought to follow a pattern similar to that of the limbs.

Embryonic Development of the Chick Forelimb

The paraxial mesoderm, adjacent to the notochord, divides into segments called somites and each somite has a dermatome (→feathers), sclerotome (→cartilage), and myotome (→muscles).

At stage 15 (Hamburger and Hamilton, 1951), an inconspicuous condensation of mesoderm begins at the prospective limb areas. The wing bud becomes visible at stage 16 as a thickened ridge. By stage 22, the wing buds are easily distinguished outgrowths but cell differentiation and cartilage formation have not yet started. As in the face, the epithelium signals outgrowth of the mesoderm. Carrington and Fallon (1984) showed that by removing the epithelium over the presumptive limb bud at stage 15, wing development was suppressed. This denuded mesoderm could be re-induced if ectoderm was replaced: however, this ectoderm had to be from an embryo that had not developed past stage 20. As well, they found that the older the ectoderm (stage 15 → stage 20) the less likelihood of a normal wing developing.

The developing limb bud contains a specialized epithelial structure of pseudostratified columnar epithelium at the apex known as the apical ectodermal ridge (AER) (figure 1). Removal of the AER resulted in stunted growth of the wing (Saunders, 1949; Summerbell *et al.*, 1973). The aging AER of the older embryo maintained its ability to promote wing outgrowth as shown by Rubin and Saunders (1972): AER's from older chick

embryos, up to stage 29, that were transplanted into younger embryos were able to promote normal wing outgrowth. This phenomenon further suggested that the mesenchyme was responsible for patterning. An area of rapidly dividing mesenchymal cells at the tip of the wing bud called the progress zone (Summerbell *et al.*, 1973) was the source of mesenchymal cells populating the developing wing. The longer the cell spent in the progress zone, the more likely that cell was to become a distal structure. The progress zone and AER were shown to work together and that by removing the AER, the development of distal structures was affected in a time-dependant manner: removal of the AER at stage 18 resulted in absence of the radius, ulna, and digits but by stage 24, removal of the AER resulted in normal formation of the radius and ulna, however, the digits were absent. Tickle *et al.* (1985) showed that the AER needed the underlying mesenchyme to maintain itself. Therefore, not only did signals pass from the AER to the mesenchyme but the mesenchyme sent signals to the AER.

A polarizing region located at the posterior junction of the limb bud and trunk was shown to be important in antero-posterior patterning of the wing. Transplanted polarizing regions, from either the leg or wing bud, into the wing resulted in duplicated wing structures forming from the surrounding mesenchyme. Tickle *et al.* (1975) demonstrated that the polarizing region exerted its effect via a concentration

gradient but it wasn't until seven years later (Tickle *et al.*, 1982) that the substance thought to create the gradient might be retinoic acid. Using retinoic acid-soaked beads implanted under the AER, Tickle *et al.* (1985) showed that the concentration of retinoic acid, the length of exposure, and bead position all affected the patterning of the wing. Thaller and Eichele (1987) showed that in the chick embryo, endogenous retinoic acid is present in a gradient distribution with the highest concentration at the posterior of the wing bud. However, Wanek *et al.* (1991) and Noji *et al.* (1991) conclusively demonstrated that retinoic acid converted surrounding mesenchyme into a polarizing region thus causing the duplication of wing structures.

Growth factors also play a role in the outgrowth of the wing buds and the growth of facial mesenchyme cells in culture. Thus, growth factors are also likely to play a part in normal facial growth of the embryo.

Growth Factors

Growth factors are broadly defined as proteins that promote growth. The main families of growth factors are: 1) transforming growth factors, 2) insulin-like growth factors, 3) epidermal growth factors, 4) platelet-derived growth factors, and 5) heparin-binding growth factors, also called the fibroblast growth factors (Baird and Klagsbrun, 1991).

The Family of Fibroblast Growth Factors

Although acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) were the first members of the fibroblast growth factor (FGF) family to be recognized, FGF's played a role in many tissue culture experiments prior to their characterization. Hoffman (1940) used extracts from adult rat brain to maintain growth and cell division within chicken fibroblast cell cultures. Embryonic rat extracts, however, were effective irrespective of the organ from which the extract was derived. Armelin (1973), using 3T3 mouse cell cultures, found that a bovine pituitary extract, NIH-LH-B8, would promote growth *in vitro* but highly purified luteinizing hormone would not reproduce these results. Heat or protease treatment of NIH-LH-B8 also negated this effect. Consequently, another protein factor in the NIH-LH-B8 extract was suspected although never isolated. Gospodarowicz (1974), also using 3T3 cell cultures, found bovine brain extract ten-fold more efficacious than pituitary extract in promoting cell culture growth. He was the first to use the term fibroblast growth factor and attempts to purify FGF (Gospodarowicz *et al.*, 1978) led him to believe that there were 2 different proteins - a protein of 128 amino acids which he called FGF1 and a second protein of 107 amino acids, FGF2. These proteins were found to stimulate mitosis of fibroblasts in culture, increase amino acid and nucleotide transport, increase protein synthesis,

increase MRNA and DNA synthesis, increase formation of polyribosomes, and cause a reversible de-differentiation of cells in culture.

Maciag *et al.* (1984) and Shing *et al.* (1984) discovered a simple way of purifying endothelial growth factors by utilizing heparin affinity chromatography. Using a similar protocol, the amino acid sequences of purified aFGF (Gimenez-Gallego *et al.*, 1985; Thomas *et al.*, 1985) and bFGF (Esch *et al.*, 1985) were determined. Clones of complementary deoxyribonucleic acid (cDNA) were made to be used as probes to locate the bovine FGF genes (Abraham *et al.*, 1986a) and the human bFGF gene (Abraham *et al.*, 1986b). A single gene with 99% homology to the bovine bFGF was found. Thomas (1987) and Kurokawa *et al.* (1987) surmised that there were other genes similar to bFGF as a result of their cDNA assays. Jaye *et al.* (1986) mapped the endothelial cell growth factor to human chromosome 5q31.3-33.2 but when Mergia *et al.* (1986) mapped the aFGF and bFGF genes to human chromosomes 5 and 4 respectively, there was speculation that endothelial cell growth factor might actually be a member of the family of fibroblast growth factors. Indeed, through DNA and amino acid sequencing, aFGF and bFGF were determined to be the same as a number of other growth factors (table I), (Abraham *et al.*, 1986b; Thomas, 1987).

The proteins encoded for were 155 amino acids in length but did not contain signal peptides so their mechanism of

secretion from the cell was unknown. Cell lysis or membrane transport via an unknown mechanism were the prime candidates. The amino acid sequences of human aFGF and human bFGF were found to be 55% homologous and homology across species for each growth factor was also found to be quite high (Thomas, 1987; Abraham *et al.*, 1986a).

FGF's were shown to be mitogenic and stimulated production of collagenases and plasminogen activator - proteolytic enzymes involved with tissue remodelling - (Gross *et al.*, 1982; Presta *et al.*, 1985; Moscatelli *et al.*, 1986) and could thus play a role in tumour invasiveness and neovascularization. Using purified or recombinant aFGF and bFGF, both factors were found to be chemotactic for endothelial cells *in vitro* and as mitogens for embryonal cells of neuroectodermal and mesenchymal origin *in vitro* (Burgess and Maciag, 1989; Gospodarowicz *et al.*, 1986). Basic FGF elicited neurite extension in culture and increased cell survival of neurons (Morrison *et al.*, 1986). Folkman and Klagsbrun (1987) suggested that aFGF and bFGF likely played a role in development of the vascular system based on their *in vivo* angiogenic effects and tumour neovascularization. FGF's were shown to both inhibit and enhance cellular differentiation, depending on the cell type.

As FGF's had the ability to influence so many different cell types, their role in embryologic development was studied. Risau (1986) detected FGF in the developing chick brain.

Kimelman and Kirschner (1987) and Slack *et al.* (1987) showed that by adding FGF's to explants of ectoderm from the animal pole of *Xenopus* embryos, mesoderm induction could be stimulated. This effect was blocked when heparin was added. Kimelman *et al.* (1988) demonstrated the presence of endogenous FGF in early *Xenopus* embryos. By inhibiting the binding of FGF to their receptors using a dominant negative mutant form of FGF receptor, Amaya *et al.* (1991) inhibited mesoderm induction and produced developmental defects in *Xenopus* embryos.

Currently, 9 members of the FGF family have been identified (table I). Since the FGF's have been known by a variety of different names, the new standardized terminology will be used throughout the remainder of the thesis.

The *int*-2 oncogene, originally identified by Peters *et al.* (1983) and sequenced by Moore *et al.* (1986), was not identified as a member of the FGF family until Dickson and Peters (1987) and Smith *et al.* (1988) showed that its sequence was similar to bFGF but with a large middle segment missing. The *int*-2 oncogene product is now known as FGF-3.

FGF-3 was found in cells from embryonic mice but not in normal adult tissues (Wilkinson *et al.*, 1988). The insertion of the mouse mammary tumour provirus adjacent to the murine FGF-3 gene caused the gene to be transcribed in the adult.

In the embryo, *in situ* hybridization showed high levels of FGF-3 in cells migrating through the primitive streak, non-

migrating cells in the epithelial endoderm of the pharyngeal pouches, and neural ectoderm of the hindbrain, but was not accumulated by neural crest cells. FGF-3 appeared to play a role in signalling the otocyst to begin development of the inner ear (Wilkinson *et al.*, 1988). Mansour (1994) showed that disruption of the FGF-3 gene caused developmental defects in the inner ear of mice.

I have selected FGF-4 for my experiments and will discuss it in a separate section.

FGF-5 was isolated and characterized by Zhan and Goldfarb (1986; Zhan *et al.*, 1988). FGF-5 was 40%-50% homologous to FGF-1, FGF-2, FGF-3, and FGF-4 and like FGF-3, it was a secreted protein with signal peptide sequence.

FGF-5 expression increased in the embryonic ectoderm just prior to gastrulation (Hébert *et al.*, 1991) and was found in the acoustic branch of the 8th cranial ganglion (Goldfarb *et al.*, 1991) and cochlea (Haub and Golfarb, 1991). Hughes *et al.* (1993a) demonstrated that FGF-5 supported survival of chick embryo spinal motoneurons in culture but *in vivo*, FGF-5 could not prevent the loss of lesioned facial nerves in newborn rats (Hughes *et al.*, 1993b). Lindholm *et al.* (1994) suggested that FGF-5 may act as a neurotrophic factor *in vivo* as it is present at increased levels in the newborn rat hippocampus and was shown to have neurotrophic effects on septal cholinergic and raphe serotonergic neurons in culture.

FGF-6 was discovered by hybridization of a *hst*/KFGF (FGF-

4) probe to the FGF-6 DNA coding sequence (Marics, 1989). FGF-6 had a signal peptide sequence and was secreted from cells. FGF-6 was first detected in the mouse embryo at E9.5 (Han and Martin, 1993) and peaked at 15.5 days of embryonic development (de-Lapeyriere *et al.*, 1990). It was localized in the myotomal compartment of the somite in the murine embryo and FGF-6 was found in fetal skeletal muscle which suggested a role in skeletal muscle innervation. Isaacs *et al.* (1992) similarly detected FGF-6 in myotomes of *Xenopus* embryos.

FGF-7, also called keratinocyte growth factor (KGF), was cloned from the amino acid sequence of the purified protein from embryonic lung fibroblasts (Finch *et al.*, 1989; Rubin *et al.*, 1989). FGF-7 was found to be mitogenic for epithelial cells but not fibroblasts or endothelial cells and elicited keratinocyte migration in vitro (Tsuboi *et al.*, 1993). During wound healing experiments, mRNA for FGF-7 increased 160-fold in the dermis and hypodermis (Werner *et al.*, 1992) and this FGF-7 acted as a mitogen on the epithelial cells - the cells that produce the receptors. FGF-7 increased the rate of re-epithelialization, increased the number of serrated basal cells (with increased collagen deposition), and resulted in a thicker epidermis when healed (Staiano-Coico *et al.*, 1993).

FGF-7 secretion decreased after cellular differentiation (Tiesman and Rizzino, 1989). However, FGF-7 is an oncogene product in adults and was produced by rat prostate tumour (stromal) cells *in vitro* after exposure to dihydrotestosterone

(Fukabori *et al.*, 1994).

FGF-8 was detected as an androgen-inducible growth factor in mammary carcinoma cells in culture. Northern blot analysis detected FGF-8 mRNA transcripts in embryonic tissues associated with outgrowth and patterning, including the face and limb, but none in normal adult tissues of the mouse. The presence of FGF-8 in the murine embryo face was detected after the migration of neural crest cells into the branchial arches was completed. The frontonasal mass and surface ectoderm of the lower half of the maxilla and upper half of the mandible initially expressed FGF-8 with expression localized to the nasal pits after 2 more days gestation (Heikinheimo *et al.*, 1994). Crossley and Martin (1995) also detected FGF-8 in the pharyngeal pouches and grooves. Limb outgrowth preceded the expression of FGF-8 in both the forelimb and hindlimb of the mouse embryo according to Heikinheimo *et al.* (1994) which was completely opposite to the findings of Ohuchi *et al.* (1994) and Crossley and Martin (1995) that FGF-8 was expressed prior to limb outgrowth and suggested that FGF-8 might play a role in development of the apical ectodermal ridge. In both the face and limbs, FGF-8 was located in surface epithelium only. FGF-8 was also located in the developing brain and seemed to be temporally and spatially related to the development of the pituitary gland, optic stalk, and olfactory epithelium. All FGF-8 expression in the mouse embryo ceased by day 14 (Heikinheimo *et al.*, 1994).

FGF-9 (glial activating factor) was isolated from the supernatant of a human glioma cell line (Miyamoto *et al.*, 1993). This protein lacked a signal sequence and was 30% homologous to FGF-1 and FGF-2. In situ studies detected rat FGF-9 in the adult brain and kidney.

FGF-4

FGF-4 was isolated independently in two labs and as such, was also called K-FGF and *hst-1*. Bovi *et al.* (1987) transfected DNA from human Kaposi's sarcoma into NIH 3T3 cells and found a protein (K-FGF) homologous to FGF-1 and FGF-2 that was capable of stimulating fibroblast growth in culture. Yoshida *et al.* (1987) located the human *hst-1* by transfecting NIH 3T3 cells with DNA from human stomach cancer. The *hst*-FGF gene sequence was 38% homologous with human FGF-1, 43% homologous with human FGF-2, and 40% homologous with murine FGF-3.

FGF-4 was shown to have a long hydrophobic leader and was secreted via a standard signal peptide-dependent mechanism (Thomas, 1987).

In situ hybridization studies have shown FGF-4 maternal transcripts to be present in the ovum (Rappolee *et al.*, 1994) and may function to maintain the pluripotency of the cells (Drucker and Goldfarb, 1993). FGF-4 was not mitogenic in the

blastocyst (Rappolee *et al.*, 1994) but was critical for development of the inner cell mass (Feldman *et al.*, 1995). Null mutants for FGF-4 in mice could develop to the blastocyst stage but then degenerated when unable to form an inner cell mass. This lethal mutation was overcome by adding recombinant human FGF-4 protein: it allowed inner cell mass proliferation and cell differentiation. FGF-4 transcripts became restricted to the primitive streak (Niswander and Martin, 1992), progressing caudally with time, and were transiently expressed in the anterior ectoderm of branchial arches one and two, the pharyngeal endoderm, and in the posterior half of the early AER. Since the role of FGF-4 in the limb is known and since FGF-4 is found in the embryonic face, this study will investigate the role of FGF-4 in the face.

As an oncogene in adults, FGF-4 was shown to play a role in the progression of breast tumours in mice to a metastatic, oestrogen independent, tamoxifen-resistant phenotype (McLeskey *et al.*, 1993).

The FGF Receptors

FGF's exert their effects on the cells by binding to high affinity receptors (FGFR) on the cell surface. FGF's also bind to low affinity receptors (heparin sulphate proteoglycan molecules) on the cell surface and in the extracellular matrix

(Moscatelli, 1987, 1988; Bashkin *et al.*, 1989). Heparinase or incubating cells with heparin was shown to selectively block these low affinity receptors (Moscatelli, 1987). Rapraeger *et al.* (1991), Yayon *et al.* (1991), and Partanen *et al.* (1993) demonstrated that this low affinity binding potentiates the binding of FGF's to the high affinity binding sites.

The suspicion that all FGF's share a common high affinity receptor was rejected when Rubin *et al.* (1989) found that KGF did not bind to the same receptors as FGF-1 and FGF-2. Lee *et al.* (1989) isolated the first FGF receptor cDNA and since that time, three additional FGF receptors have been identified. These high affinity FGF receptors all have the same basic structure (figure 2) consisting of an amino acid signal peptide, three extracellular immunoglobulin-like (Ig-like) domains with an "acid box" between the first and second Ig-like domains, a membrane spanning region, and two tyrosine kinase sequences intracellularly (Johnson and Williams, 1993). The FGFR's have varying degrees of homology with the most highly conserved regions being the intracellular kinase domains while the extracellular signal peptide was the least conserved.

FGFR1 was purified (Lee *et al.*, 1989) from chick embryos using heparin to isolate the high affinity receptors while excluding the low affinity receptors. Previous partial cDNA clones - human *flg* cDNA (Ruta *et al.*, 1989) and mouse bacterially expressed kinase (*bek*) cDNA (Kornbluth *et al.*,

1988) - showed a similar amino acid sequence to the FGFR1 cDNA. The whole human *flg* cDNA was found to be a splice variant of FGFR1 (Table II). FGFR1 cDNA clones have been found for chick, humans (Dionne *et al.*, 1990; Johnson *et al.*, 1990; Eisemann *et al.*, 1991; Hou *et al.*, 1991), mice (Mansukhani *et al.*, 1990; Reid *et al.*, 1990), and *Xenopus* (Musci *et al.*, 1990) and all of these proteins were highly conserved across the species - mouse:human 981%, chicken:human 91%, *Xenopus*:human 78%.

In cross-linking experiments, receptors of 125 kDa and 145 kDa were found on SDS-polyacrylamide gels. Alternative splicing of the mRNA produced two variants of FGFR1 - the 125 kDA receptor had only 2 Ig-like domains whereas the 145 kDa receptor had three but the binding to either of these variants was similar (Johnson *et al.*, 1990). A third secreted FGFR1 variant was isolated by Eisemann *et al.* (1991). Additional variants of FGFR1 have been found but the most interesting differences were in the second half of the third Ig-like domain. The three different exons - IIIa, IIIb, IIIc - were shown to bind the different FGF's with varying affinities. In other words, specificity was conferred via these different isoforms of the receptor.

The mouse *bek* partial sequence isolated by Kornbluth *et al.* (1988) exhibited some differences in the DNA and transcribed amino acid sequence compared to the FGFR1. Dionne *et al.* (1990) isolated the full *bek* cDNA and showed it to be

from a different gene than the gene coding for FGFR1. This receptor is now called FGFR2. FGFR2 also had splice variations that bestowed specificity by incorporating either the IIIb or IIIc exon into the second half of the third Ig-like domain. The KGF receptor was determined to be a unique splice variant of FGFR2 in which the first Ig-like domain and the acid box sequence were removed (Johnson and Williams, 1993).

Keegan *et al.* (1991), probing with a v-*sea* oncogene in a chronic myelogenous leukaemia cell line, isolated the third FGFR gene. The FGFR3 contained the IIIc exon in the third Ig-like domain.

The FGFR4 gene was also located by probing an HEL leukaemia cell cDNA library (Partanen *et al.*, 1991). Its unique feature was the smaller number of acid residues found in the acid box sequence of the protein.

Tissue and cell specificity in the expression of the FGFR genes and specific splicing mRNA within these cells and tissues, in addition to temporal expression of slice variants, have been demonstrated *in vivo* and may be the mechanisms for selective FGF binding (Fantl *et al.*, 1993). For example, during mouse embryogenesis, the 3 Ig domain forms of FGFR1 are expressed but 2 Ig domain forms do not appear until after birth (Reid *et al.*, 1990). Rat prostate tumours became metastatic when the FGFR2(IIIb) on the epithelial cells switched to the FGFR2(IIIc) splice variant (Yan *et al.*, 1993).

The regulation of FGF receptors may be via an autocrine

mechanism; as the cell produces the FGF ligand, the production of FGF receptors decreases. Once the cell differentiated and decreased production of FGF's, the number of FGF receptors at the cell surface increased (Moscatelli, 1994). Conversely, Olwin and Hauschka (1988) found that terminal differentiation of skeletal muscle myoblasts in culture correlated time-wise with an irreversible loss of FGF receptors at the cell surface.

FGFR1 transcripts were localized to the brain and mesenchymal tissues in the embryo (Heuer *et al.*, 1990; Wanaka *et al.*, 1990, 1991; Peters *et al.*, 1992c). FGFR2 was found in the perichordal sclerotome (Han and Martin, 1993), brain, and epithelial tissue (Peters *et al.*, 1992c). FGFR3 was localized to brain, spinal cord, and cartilage precursors of developing bone (Peters *et al.*, 1993) while FGFR4 transcripts were found in endoderm, developing skeletal muscle, and lung tissues (Stark *et al.*, 1991; Partanen *et al.*, 1991; Han and Martin, 1993).

The presence of FGF receptors in the face has been shown by *in situ* hybridization studies (Richman, 1995).

Ligand Binding Specificity of FGF-4

FGF-4 exhibited a decreased affinity for the 3 Ig/(IIIc-isoform) of FGFR1 and the 2 Ig/IIIc/FGFR1 but high affinity

for 3 Ig/IIIc/FGFR2. This altered affinity was most likely due to structural differences between FGFR1 and FGFR2 and not a result of alternative splicing. Both splice variants of FGFR2 (*bek* and KGFR) bind FGF-4 equally well (Orr-Urtreger *et al.*, 1991, 1993). Downregulation of the 3 Ig/IIIc/FGFR2 receptor by FGF-4 decreased the binding of FGF-2. FGF-4 would bind to FGFR4 but its binding affinity was variable (Han and Martin, 1993; Vainikka *et al.*, 1992).

Receptor Activation

When an FGF would bind to an FGF receptor, the receptor would undergo dimerization and phosphorylation (Bellot *et al.*, 1991) and the activated the tyrosine kinase would begin tyrosine phosphorylation of intracellular proteins. Binding and cross-linking caused down-regulation of FGFR1. Klagsbrun and Baird (1991) suggested the FGF binding to a high affinity receptor may be mediated by first binding to a cell surface glycosaminoglycan (figure 3).

PLC-γ was identified by Burgess *et al.* (1990) as a substrate of an FGF receptor and was phosphorylated following FGF activation of the receptor. A mutation of the receptor did not permit phosphorylation of PLC-γ although other intracellular proteins were phosphorylated (Peters *et al.*, 1992a; Mohammadi *et al.*, 1992) and mitosis was stimulated.

Raf-1 was another protein phophorylated by FGF's. A mutant form of *Raf-1* serine/threonine kinase was shown to be responsible for failed mesoderm induction (Fantl *et al.*, 1993).

Other effects of FGF binding were increased intracellular pH, activation of the Na^+/K^+ pump, (Halperin and Lobb, 1987), increased gene transcription (Müller *et al.*, 1984), and increased hydrolysis of polyphosphoinositides (Brown *et al.*, 1989).

The exact mechanisms and proteins involved after receptor activation have yet to be fully elucidated. Future studies could involve knocking out the FGF receptors by cleavage of the proteins with cyanogen bromide or trypsin (Friesel *et al.*, 1986), blocking the receptors with suramin (Huang *et al.*, 1986; Coffey *et al.*, 1987), protamine sulfate (Huang *et al.*, 1986), wheat-germ agglutinin (FGFR2 specifically) (Feige and Baird, 1988), targeted over-expression (Wilkie *et al.*, 1995), or separating the intracellular mechanisms using the Y/F766 (=Y766F) mutant receptor (Peters *et al.*, 1992b; Mohammadi *et al.*, 1992) or dominant negative mutants (Amaya *et al.*, 1993).

Growth Factors in Vertebrate Limb Development

In *Xenopus* embryos, growth factors are critical for normal limb development. *Xenopus* embryos with mutant FGF

receptors had normal head development but little or no tail formation with deficiencies in mesodermal tissue and lack of somite development (Amaya *et al.*, 1991); hence, no limb development.

In the chick wing bud, a retinoic acid gradient is present and is involved in converting the posterior-proximal cells into the zone of polarizing activity (ZPA). The apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA) send signals which maintain the progress zone. The ZPA and the progress zone are both needed to maintain a functional AER and the AER is required to maintain the ZPA. Therefore, communication between these three areas is essential for normal limb development (Johnson *et al.*, 1994).

Suzuki *et al.* (1992) demonstrated that FGF-4 (*hst-1*) was produced in the posterior half of the AER and speculated that FGF-4 may be responsible for mesenchyme outgrowth. This result was confirmed by Niswander *et al.* (1993) and Niswander and Martin (1993a, 1993b) who cultured intact trunks of mice with attached limb buds minus their AER in defined media. FGF-4 added to the media resulted in mesenchyme outgrowth. Bone morphogenic protein 2 (BMP-2), however, was found to inhibit mesenchyme outgrowth. By combining FGF-4 and BMP-2 in various proportions, the extent of the outgrowth could be controlled and may play a role in defining limb shape. FGF-1, FGF-2 or FGF-5 could also stimulate mesenchyme outgrowth so outgrowth is not specific to FGF-4. FGF-2 is also found in the AER

(Riley *et al.*, 1993) and may play a role in both proximodistal and anteroposterior patterning, especially in the anterior regions where FGF-4 is not expressed. The level of FGF-2 in the wing is highest during cell proliferation (mitogenic effect) and directs development of an extensive capillary network (angiogenic effect) and the migration of muscle precursor cells into the wing (cell motility effect) (Munaim *et al.*, 1988). Extracellular matrix hyaluronate, which may play a role in limb morphogenesis, is abundant when the level of FGF-2 is high.

FGF-4, however, is required to maintain the ZPA (Vogel and Tickle, 1993; Niswander *et al.*, 1994). *Sonic hedgehog* (Shh) is a protein secreted by the ZPA that maintains the AER in a positive feedback loop. Establishment of this positive feedback loop may be dependent on retinoic acid (Niswander *et al.*, 1994) and functions to maintain the progress zone. Shh may act as a diffusible morphogen or may induce other signalling molecules locally. Shh and FGF-4 together activate the Hox genes and BMP-2 (Laufer *et al.*, 1994).

A signal from the dorsal ectoderm may also be needed in addition to FGF-4 to maintain Shh expression. Yang and Niswander (1995) showed that by removing the dorsal ectoderm, Shh expression decreased and posterior skeletal elements were not formed. Ectopic expression of the *Wnt7a* gene can replace the dorsal ectoderm and restore Shh expression.

Hox gene expression is linked spatially and temporally to

limb patterning (Johnson *et al.*, 1994) and could influence the number and arrangement of the precartilagenous blastemas that will eventually form the bones. The BMP's are the likely targets of the Hox genes as the BMP's promote cartilage and bone development (Francis *et al.*, 1994). Disruption of *Hoxd-13* delays limb development, delays ossification of distal skeletal elements, and affects morphology in terms of fused and missing bones. Defects in *Hoxd-11* result in shortened and misshapen metacarpals and inappropriate fusion of wrist bones (Davis and Capecchi, 1994). Tickle (1991) demonstrated expansion of the domain of homeobox gene *XlHbox 1* when exposed to retinoic acid. An article by Morgan and Tabin (1994) reviews the role of Hox genes in limb morphogenesis.

What causes initiation of limb bud formation? Cohn *et al.* (1995) showed that FGF-1, FGF-2, or FGF-4 were all capable of inducing ectopic limb buds in stage 13-17 chick embryos as is FGF-8 (Ohuchi *et al.*, 1994). Alternatively, activation of Hoxb-8 (a homeobox gene) expression in the flank mesoderm by retinoic acid defines the ZPA-competent cells which produce SHH and in combination with dividing mesenchyme cells, direct the ectoderm to form an AER (Tabin, 1995). The FGF-4 produced by the AER maintains the mesenchymal outgrowth and together with Shh from the ZPA, activates patterning gene expression (Homeobox genes). See figure 4.

Growth Factors in Chick Facial Development

The growth factors involved in facial development have not been as well studied as those in the limb bud but it is suspected that their roles may be similar. The pattern of cellular differentiation along the proximodistal axes of the beaks of chick embryos also involves epithelial-mesenchymal interactions although a distinct structure similar to the AER is not present on any facial primordium. The presence of the eyes is known to contribute to the medio-lateral axis patterning: removal of the eyes results in a bifid upper beak (Silver, 1962).

In recombination experiments of epithelia and mesenchyme grafted to host wing buds, Richman and Tickle (1992) showed that the stage of development was important in epithelial-mesenchymal signalling. The presence of an egg tooth indicates distal differentiation of the frontonasal mass (FNM): in combinations of stage 24 AER with stage 24 FNM mesenchyme, an egg tooth formed at slightly over half the rate that egg tooth formation occurred in grafts of stage 20 AER with stage 24 FNM. Also, in graft combinations of limb mesenchyme with facial epithelia, the mandibular epithelia combination resulted in cartilage rods that were similar in length to the FNM epithelia combination but the pattern was not as "limb-like" in morphology. This finding may suggest a temporal effect in pattern signalling because at stage 24, the mandible

resembles its final morphology much more than the FNM does. Thus the mandibular epithelia may have been past the point at which signals for pattern formation are secreted but not signals for outgrowth.

Basic fibroblast growth factor (FGF-2) may be involved in differential growth of the facial primordia. FGF-2 stimulates mitosis and increases cell numbers of cultured frontonasal mass mesenchymal cells (Richman and Crosby, 1990) but not cultured mandibular mesenchyme cells nor cultured mesenchymal cells from the lower half of the maxilla (Walin, 1993). It is unclear whether this is due to FGF-2 distribution within the facial primordia or receptor type and/or distribution (both of which could be a reflection of the neural crest origins of these primordia), or efficacy of signal transduction (the pattern and/or relative contributions of neural crest and somitomere mesoderm).

FGF-3, FGF-4, and FGF-8 are also found in the embryonic face.

Exogenous retinoic acid is known to affect pattern formation and outgrowth of the upper beak in a dose-dependent fashion (Richman, 1992). Retinoic acid affects the mesenchyme, not the epithelium as shown in recombination experiments (Wedden, 1987). Three retinoic acid receptors (RAR's) (Osumi *et al.*, 1990) were localized by *in situ* hybridization in murine embryos: RARα showed weak, uniform distribution throughout the craniofacial region, RARβ was expressed in

cells committing programmed cell death and in cells developing into parts of sensory organs (retina, optic nerve, olfactory epithelium), and RARγ was found in differentiating skin, cartilage, and bone. The neural crest origin of the cells correlated with the presence of RARβ and RARγ. Therefore, retinoic acid or, more likely, a signalled molecule probably contributes to facial patterning in a manner similar to the wing bud. However, excess retinoic acid in the face is known to cause facial clefting (Leon-Delgado, 1992).

Other molecules that are expressed in the face and have a known role in limb outgrowth and pattern formation are the bone morphogenic proteins. Francis-West *et al.* (1994) mapped BMP-2 and BMP-4 in chick facial primordia. BMP-4 transcripts were found only in the epithelium in younger embryos but later appeared in the mesenchyme at the distal tips of the primordia. BMP-4 may regulate *Msx-1* expression and in mice, has a role in tooth development. BMP-2 was expressed in the epithelium and mesenchyme of young embryos and later in the FNM and lateral nasal processes with epithelial expression occurring before mesenchymal expression. Both BMP-2 and BMP-4 are suspected of playing a role in cartilage modelling.

Brown *et al.* (1993) demonstrated *Msx-1* expression in the same regions as the BMP-4 distribution. *Msx-1* plays a role in epithelial-mesenchymal interactions and appears to be regulated by signals from the epithelium - possibly BMP-4?. Indeed, Brown *et al.* (1993) showed that for the mesenchyme to

maintain expression of *Msx-1*, the cells must be in just below the AER in the limb or the equivalent to the AER in the facial primordia. It is interesting to note in their study that limb mesenchyme grafted into the maxilla did not maintain *Msx-1* expression. This result may be due to temporal regulation - at the same stage of development, the limb is farther down the patterning/morphogenesis path than the upper facial primordia.

Theoretically, the limb and facial primordia likely follow similar morphogenic signalling pathways but the limbs and each of the facial primordia may have their own temporal activation.

SPECIFIC AIMS

In this study, the ability of fibroblast growth factor 4 to replace the epithelium in the growth and development of two facial primordia (the frontonasal mass and the mandible) grafted into the wing bud of the chick embryo was investigated.

The rational for the specific aim is as follows. FGF-4 was chosen for study on the assumption that it would be operative in facial development since it is found in the face (Niswander and Martin, 1992). There is information available in the literature on the role of FGF-4 in limb development (as discussed in the introduction), however, nothing is known about this member of an important growth factor family (the fibroblast growth factor family) in the face. We assume that FGF-4 will replace the role of the epithelium in facial development in a manner similar to that found in the limb.

The chick embryo was chosen as it is easy to manipulate and its development is well studied. Hamburger and Hamilton (1951) outlined the development of the chick based on external morphological characteristics which allowed precise timing of the experiments.

FIGURE 1:

Schematic diagram illustrating the stage 22 wing bud and locations of the apical ectodermal ridge (AER), the progress zone (PZ), and the zone of polarizing activity (ZPA). Pr = proximal, D = distal, A = anterior, P = posterior.

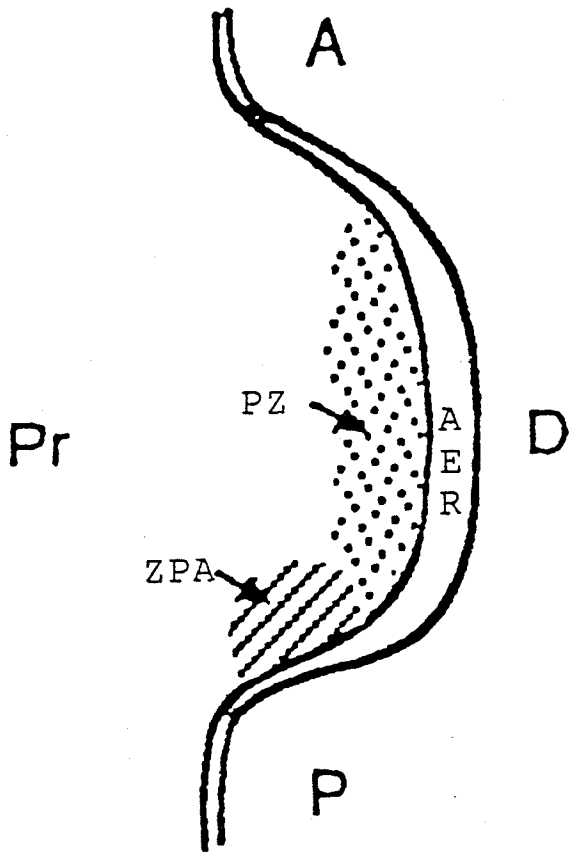

A
Pr
PZ
AER
D
ZPA
P

TABLE I: lists the names previously used, in various species, for the fibroblast growth factors and whether or not a signal peptide sequence is present.

TABLE I: Former Names of the Fibroblast Growth Factors

New Name	Former Names	Signal Peptide Present
FGF-1	acidic fibroblast growth factor, endothelial cell growth factor, α-heparin binding growth factor, eye-derived growth factor II, α-retina-derived growth factor, anionic hypothalamus-derived growth factor, astroglial growth factor 1, prostatropin	no
FGF-2	basic fibroblast growth factor, ß-heparin binding growth factor, tumour angiogenesis factor, eye-derived growth factor, ß-retina-derived growth factor, cartilage-derived growth factor, cationic hypothalamus-derived growth factor, a component of macrophage-derived growth factor, astroglial growth factor 2, chondrosarcoma-derived growth factor	no
FGF-3	*int*-2 (oncogene)	yes
FGF-4	K-FGF, *hst*-1, Kaposi's sarcoma FGF	yes
FGF-5	proto-oncogene	yes
FGF-6	*hst*-2 (oncogene)	yes
FGF-7	keratinocyte growth factor (KGF)	yes
FGF-8	androgen-inducible growth factor	yes
FGF-9	glial activating factor	no

TABLE II: lists the names previously used, in various species, for the fibroblast growth factor receptor genes.

TABLE II: Former Names of the Fibroblast Growth Factor Receptor Genes

FGFR1	**FGFR2**	**FGFR3**	**FGFR4**
flg	*bek*	*Cek2*	FGFR4
bFGFR	*Cek3*	FGFR3	
Cek1	K-sam		
N-bFGFR	K-sam'		
h2	TK14		
h3	TK25		
h4	KGFR		
h5	FGFR2		
FGFR1			

FGFR1=fibroblast growth factor receptor 1
FGFR2=fibroblast growth factor receptor 2
FGFR3=fibroblast growth factor receptor 3
FGFR4=fibroblast growth factor receptor 4

FIGURE 2:

Schematic diagram of a fibroblast growth factor receptor protein consisting of the following structures: the signal peptide (solid box), the three extracellular Ig-like domains (labelled I, II, and III), the variable region of the third Ig-like domain (thick line), the acid box domain (striped box), the transmembrane region (open box), the intracellular kinase domains (stippled boxes, labelled K1 and K2), and the C-tail peptide (labelled C).

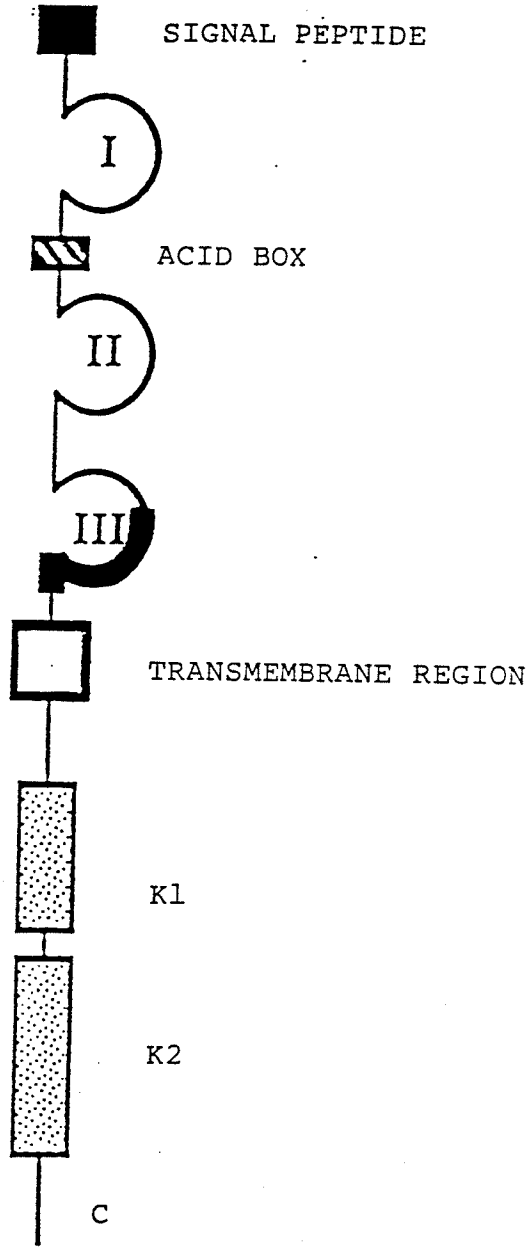
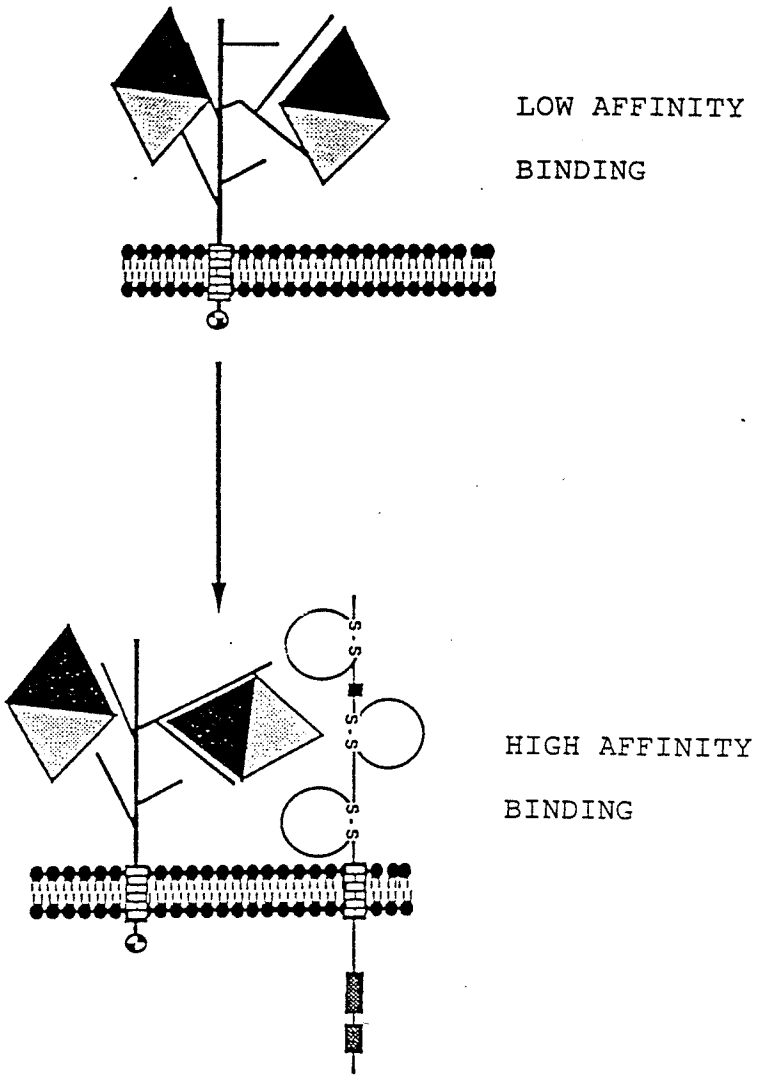

SIGNAL PEPTIDE
I
ACID BOX
II
III
TRANSMEMBRANE REGION
K1
K2
C

FIGURE 3:

Schematic diagram of the binding of FGF's to FGF receptors as proposed by Klagsbrun and Baird (1991). The FGF first binds to the low affinity heparin sulphate proteoglycan on the cell surface which then presents the FGF to the high affinity FGF receptor extracellular domains.


LOW AFFINITY
BINDING
S-S
S-S
S-S
HIGH AFFINITY
BINDING

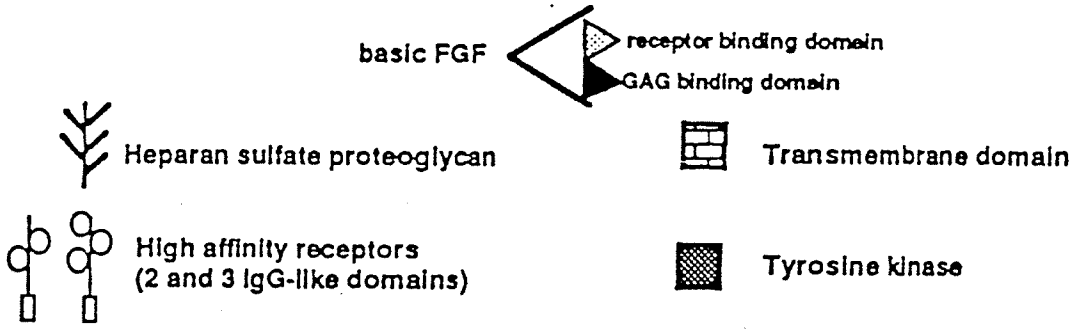

basic FGF
receptor binding domain
GAG binding domain
Heparan sulfate proteoglycan
Transmembrane domain
High affinity receptors
(2 and 3 IgG-like domains)
Tyrosine kinase

FIGURE 4:

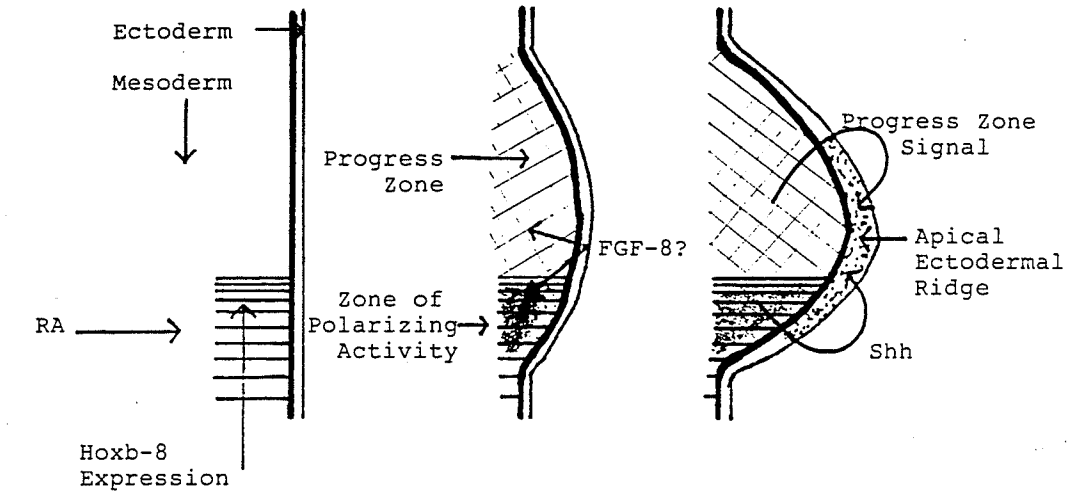
Schematic model for wing bud induction and growth adapted from Tabin (1995).

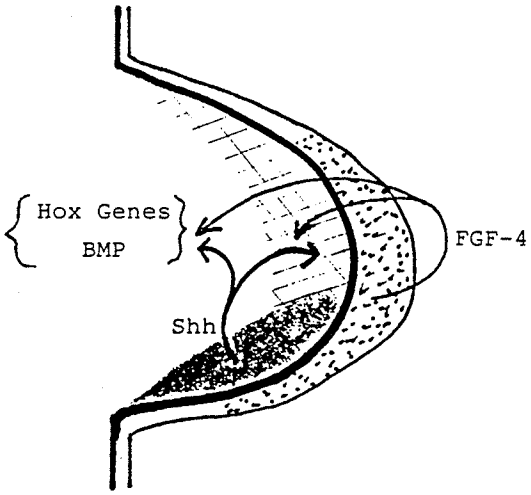
(A) Flank area of a stage 15 embryo with retinoic acid (RA) activating *Hoxb-8* expression (horizontal stripes).

(B) Local FGF-8 (debatable) directs formation of the zone of polarizing activity (ZPA) in areas of *Hoxb-8* expression and induces formation of the progress zone (PZ) resulting in mesenchyme proliferation. *Wnt7a* gene expression may influence the ZPA also.

(C) *Sonic hedgehog* (Shh) from the ZPA and signals from the PZ together induce the formation of the apical ectodermal ridge (AER) and Shh stimulates FGF production by the AER.

(D) Shh from the ZPA and FGF from the AER form a positive feedback loop to maintain limb outgrowth and pattern formation by activating the *Hox* genes and bone morphogenic proteins (BMP).


Ectoderm
Mesoderm
RA
Hoxb-8
Expression
Progress
Zone
Zone of
Polarizing
Activity
FGF-8?
Progress Zone
Signal
Apical
Ectodermal
Ridge
Shh
A
B
C


Hox Genes
BMP
Shh
FGF-4
D

MATERIALS AND METHODS

I. Incubation and Staging of Embryos

Fertilized White Leghorn chicken eggs were obtained from the Department of Poultry Science, University of Manitoba and incubated at 99.5°F and 100% humidity. The embryos were staged (Hamburger and Hamilton, 1951) after 3.5 days of incubation to synchronize their development so that the embryos would reach either stage 22 or 24 the following day. By leaving the more developmentally advanced embryos out of the incubator, their rate of growth was slowed.

The eggs were rotated to release the embryo from the inner shell membrane and then candled with a fibre-optic light to locate the embryo. A hole was made into the air cell, located at the large end of the egg and a second hole was carefully made over the embryo which allowed the air space to move over top of the embryo. A piece of tape was placed over this second hole to allow a window to be cut into the egg shell. The embryo was viewed under a dissecting microscope to determine its stage of development.

Stage 22 embryos (figure 5) were used as the host embryos because the limb mesenchyme is still undifferentiated. Stage 24 embryos served as the tissue donors as this is the earliest stage at which cell differentiation and cartilage formation begins in the face. At stage 22, the wing bud can be seen as

a semi-circular outgrowth from the trunk. At stage 24, the wing bud is longer than it is wide and the allantois is considerably larger, approaching the level of the wing bud.

II. Dissection of Embryos

The donor tissue was obtained by removing the stage 24 embryo from the egg and dissecting in sterile cold Hanks' Buffered Saline Solution (Gibco) with 10% fetal calf serum (Gibco) on a cold slab. The head was separated from the membranes and body of the embryo. The middle third of the frontal nasal mass and the medial half of both halves of the mandible were retrieved (figure 6). These tissues, approximately 500 μm x 500 μm, were lightly stained with Neutral Red and then placed into fresh Hanks' with serum, at room temperature, if the epithelium was to remain intact. If the epithelium was to be removed, the donor tissue was placed in 2% trypsin in Hanks' Buffered Saline Solution at 4°C for 20-90 minutes, depending on the batch of trypsin, until the epithelium could be peeled away easily from the mesenchyme. Pre-staining with Neutral Red was done to distinguish the epithelium (orange) from the mesenchyme (red) and thus facilitate complete removal of the epithelium. Then the mesenchyme was immediately placed in fresh Hanks' with serum at 4°C.

III. Grafting

The graft sites were prepared in the right wing buds of the stage 22 host embryos after carefully dissecting through the membranes. An approximately square site (500 μm x 500 μm) was delineated with a fine dissecting needle and undermined to remove the square of tissue from the wing bud. The donor tissue was placed immediately into this prepared site to minimize the bleeding (figure 5).

In the specimens that were to receive beads, the grafts were allowed to anneal to the host wing bud at room temperature for a minimum of 30 minutes before proceeding with bead placement.

IV. Bead Preparation and Placement

Heparin acrylic beads (Sigma), 200 μm - 250 μm, were selected. To maintain the humidity and prevent the beads from drying out, one covered Petri dish was used to soak all the beads required each day. 3 droplets per dish of FGF-4 (0.85 $\mu g/\mu l$)[2], 6 beads per 4.5 μl drop, were surrounded by 7 droplets of Dulbecco's saline solution, 2-3 beads per 8 μl

[2] FGF-4 supplied by Dr. Elizabeth Wang, The Genetics Institute, Cambridge, MA. This FGF-4 protein is genetically engineered from the mouse DNA sequence.

drop, for a minimum of one hour at room temperature.

A cut was made in the graft tissue with the dissecting needle and the bead was placed into the middle of the grafted tissue (figure 7).

The window was taped over and the host embryos were returned to the incubator to continue growing. The embryos were checked at 24 hours to confirm that the graft tissue and/or bead was still in the right wing bud (figure 8). The embryos were allowed to grow for another 6 days. In normal development, the final facial form would have been achieved by 10 days incubation so by sacrificing the embryos at 10.5 days, the grafted tissue should have reached a similar stage.

V. Preparation of Tissue for Staining

The embryos were removed from the eggs and placed in a dish of cold Hanks' solution on a frozen slab. The right and left (control) wings were detached and viewed under the microscope to locate any outgrowths (=discrete tissue projections not found in the untreated wing), determine the presence of the bead if one had been placed, and determine the presence of any egg teeth. The bead and the egg tooth are quite opaque and easiest to see at this time: once stained and cleared, they are more difficult to detect.

The pairs of wings were fixed together in either 5% TCA

or half strength Karnovsky's fixative for 24 hours. The wings were placed in acid alcohol (70% ethanol, 1% HCl) for one day and then stained for cartilage with 0.5% Alcian Blue for 3 days. The wings were destained in acid alcohol and several changes of 70% ethanol, then dehydrated through a series of increasing ethanol concentrations (90%,100%), and finally cleared in methyl salicylate. The cartilage rods were stained blue while the rest of the tissue became transparent.

VI. Measurement and Photography of the Grafts

The right wings were placed under a glass slide in a petri dish filled with methyl salicylate and compressed slightly, parallel to the long axis of the outgrowth. Utilizing a Camera Lucida mounted on the microscope and a digitizing pad, the length of the cartilage rod was measured using the SigmaScan Scientific Measurement System. This program also calculated the statistical data. The following statistical tests were performed in order to compare means between the various types of grafts: independent student's t-test and Sign test.

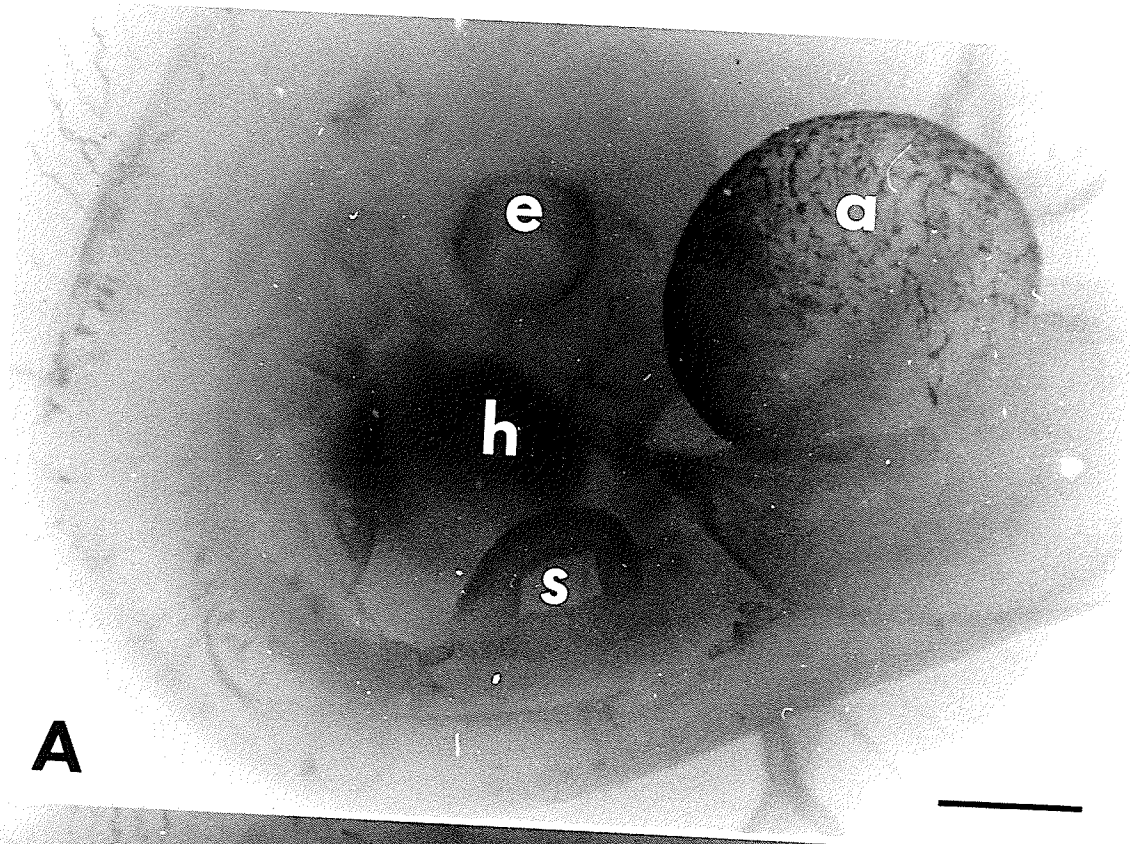
Photographs were taken with Ilford FP4 125 black and white film using a Leica camera mounted on the microscope with substage illumination.

FIGURE 5:

Photographs of the stage 22 host chick embryo.

(A) Preparation of the graft site (s) in the right wing bud. Scale bar = 1 mm, e = eye, h = heart, a = allantois.

(B) Graft tissue stained with neutral red placed into the prepared graft site. g = graft tissue.


e
a
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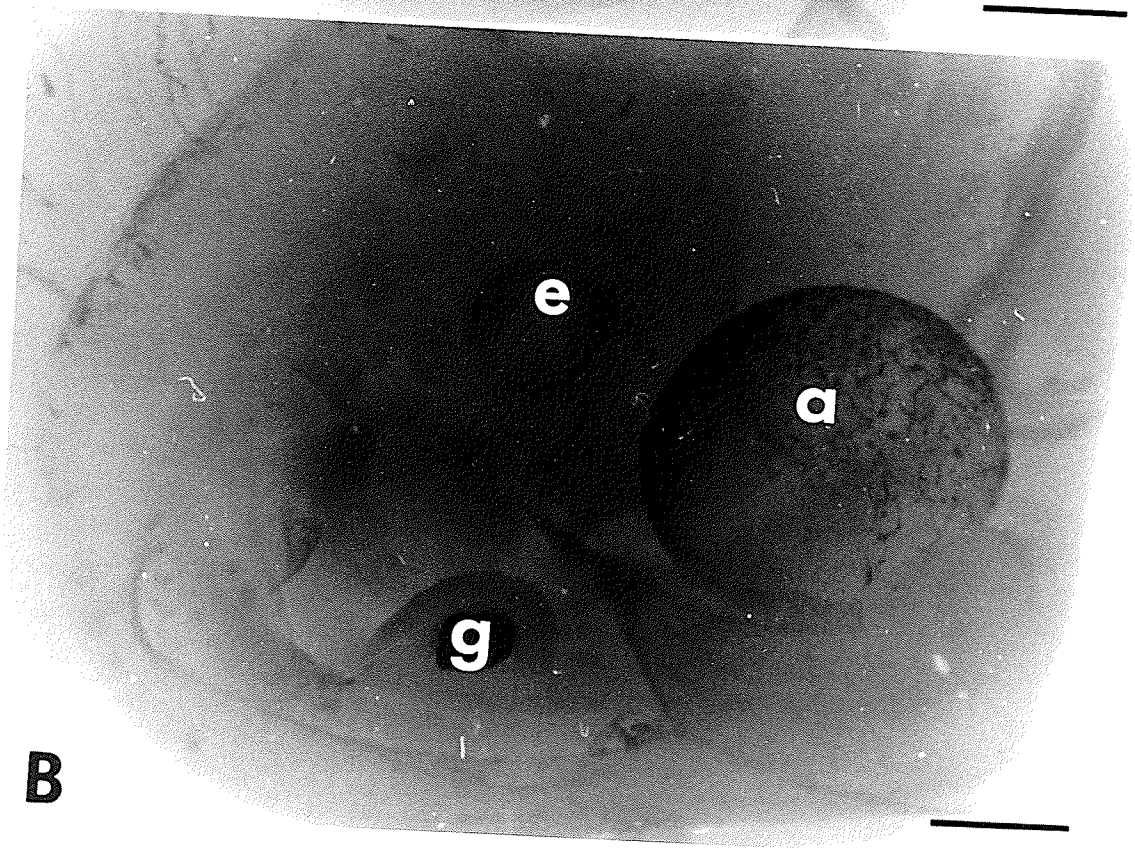

e
a
g
B

FIGURE 6:

Schematic frontal view of the stage 24 donor embryo with the middle third of the frontonasal mass and the medial halves of both sides of the mandible harvested for grafting then stained with neutral red in Hanks' with fetal calf serum. Dashed lines = dissection cuts. FNM = frontonasal mass, MD = mandible, NP = nasal pit, E = eye.

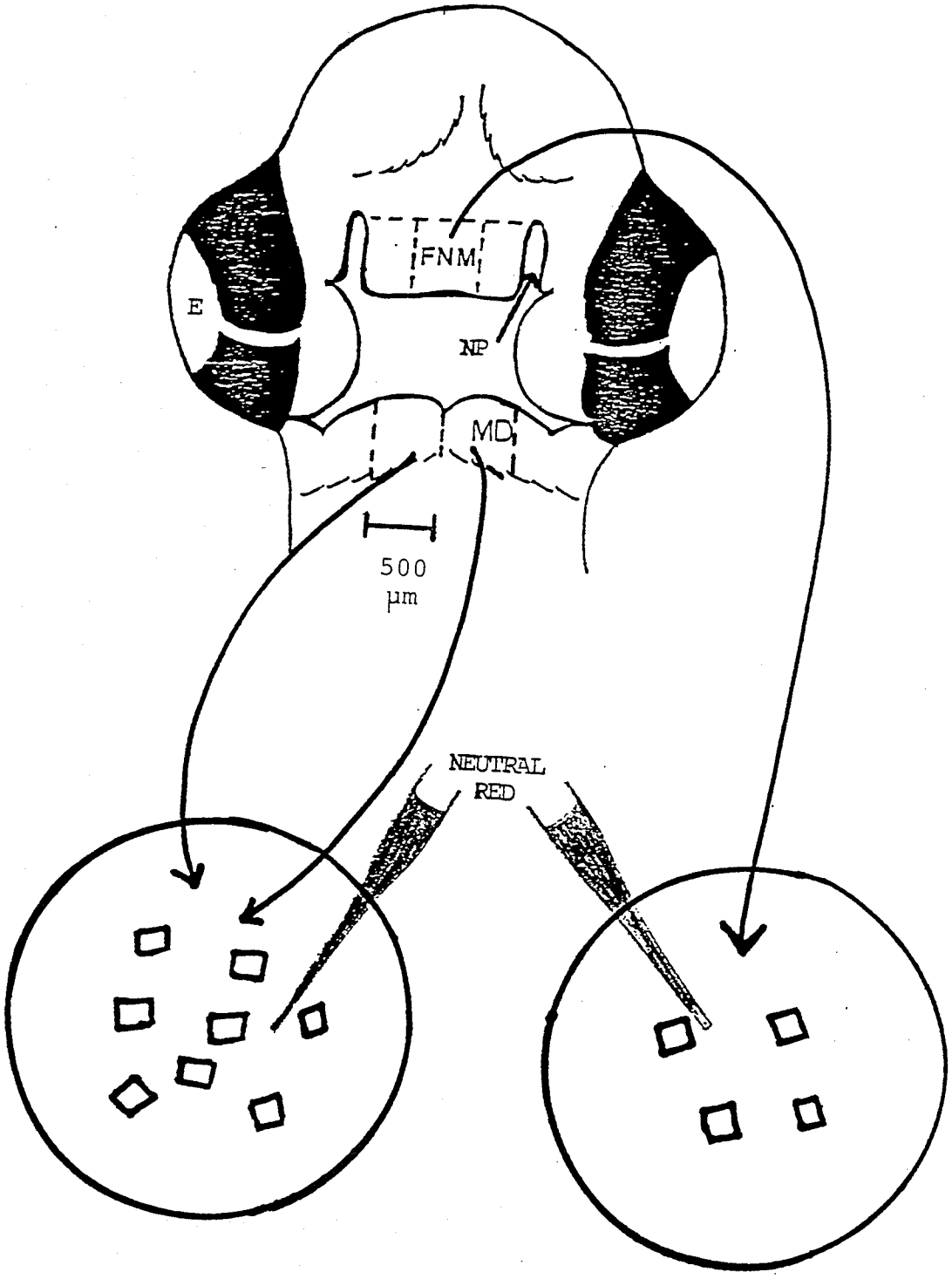

FNM
E
NP
MD
500
µm
NEUTRAL
RED

FIGURE 7:

Flow chart of the experimental method. **Not drawn to scale.**

(A) Donor tissue stained lightly with neutral red in Hanks' balanced salt solution with 5% fetal calf serum.

(B) The stage 22 host wing bud graft site is prepared.

(C) Donor tissue that is to have the epithelium removed follows this pathway:
1) Donor tissue is soaked in 2% trypsin in Hanks' buffered saline solution at 4°C until the epithelium separated from the mesenchyme.
2) To stop the action of the trypsin, the donor tissue is transferred into fresh Hanks' with serum at 4°C.
3) The epithelium is stripped off.
4) The mesenchyme is now ready to graft into the host wing bud.

(D) Donor tissue that is to have the epithelium remain intact is placed directly into the graft site.

(E) 200 μm - 250 μm heparin acrylic beads are selected,
1) rinsed in Dulbecco's saline, then
2) soaked in either FGF-4 (5-6 beads per 4.5 μl of .85 μg/μl FGF-4) or in 8 μl of Dulbecco's saline for a minimum of 1 hour.

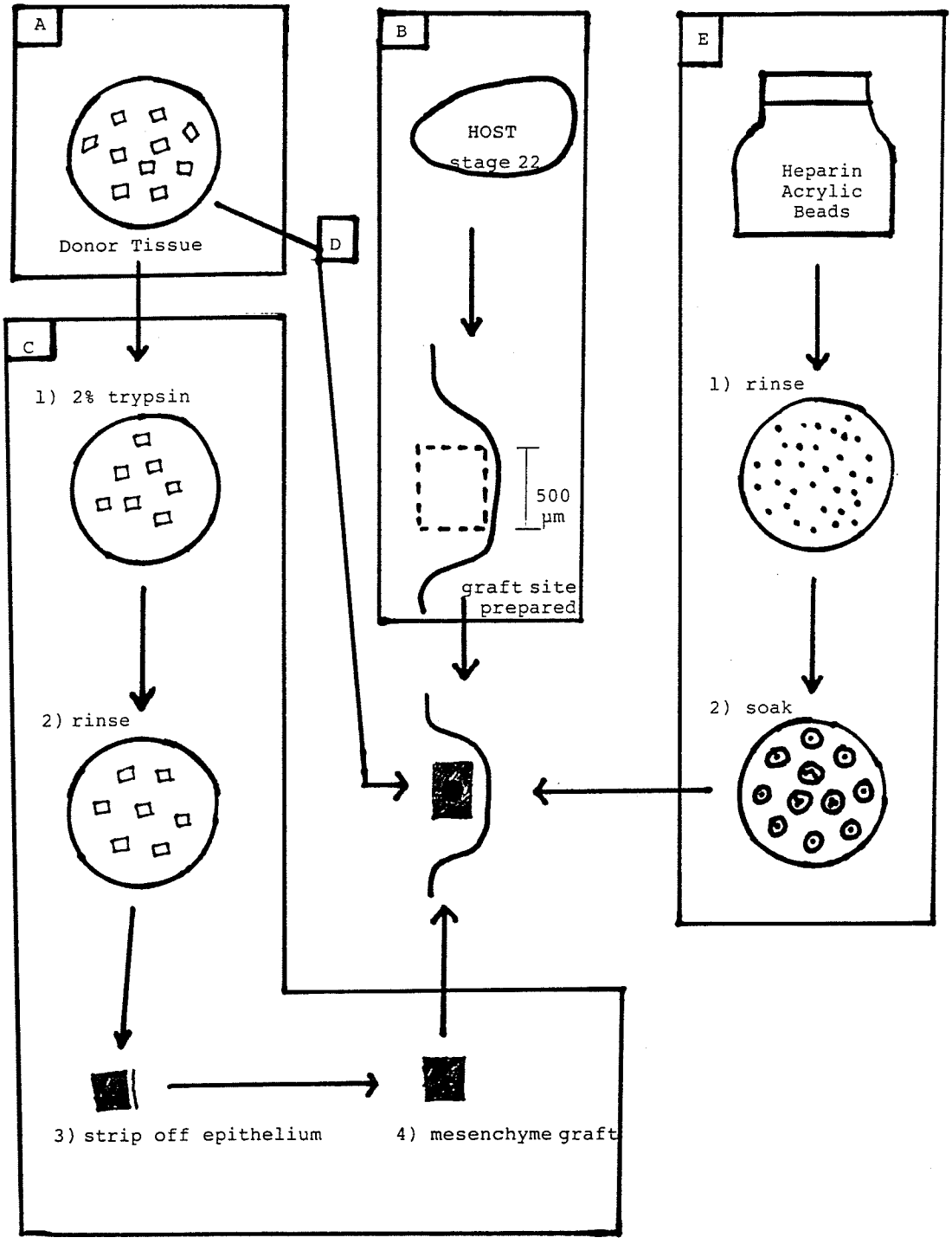

A
Donor Tissue
D
B
HOST
stage 22
500
μm
graft site
prepared
E
Heparin
Acrylic
Beads
1) rinse
2) soak
C
1) 2% trypsin
2) rinse
3) strip off epithelium
4) mesenchyme graft

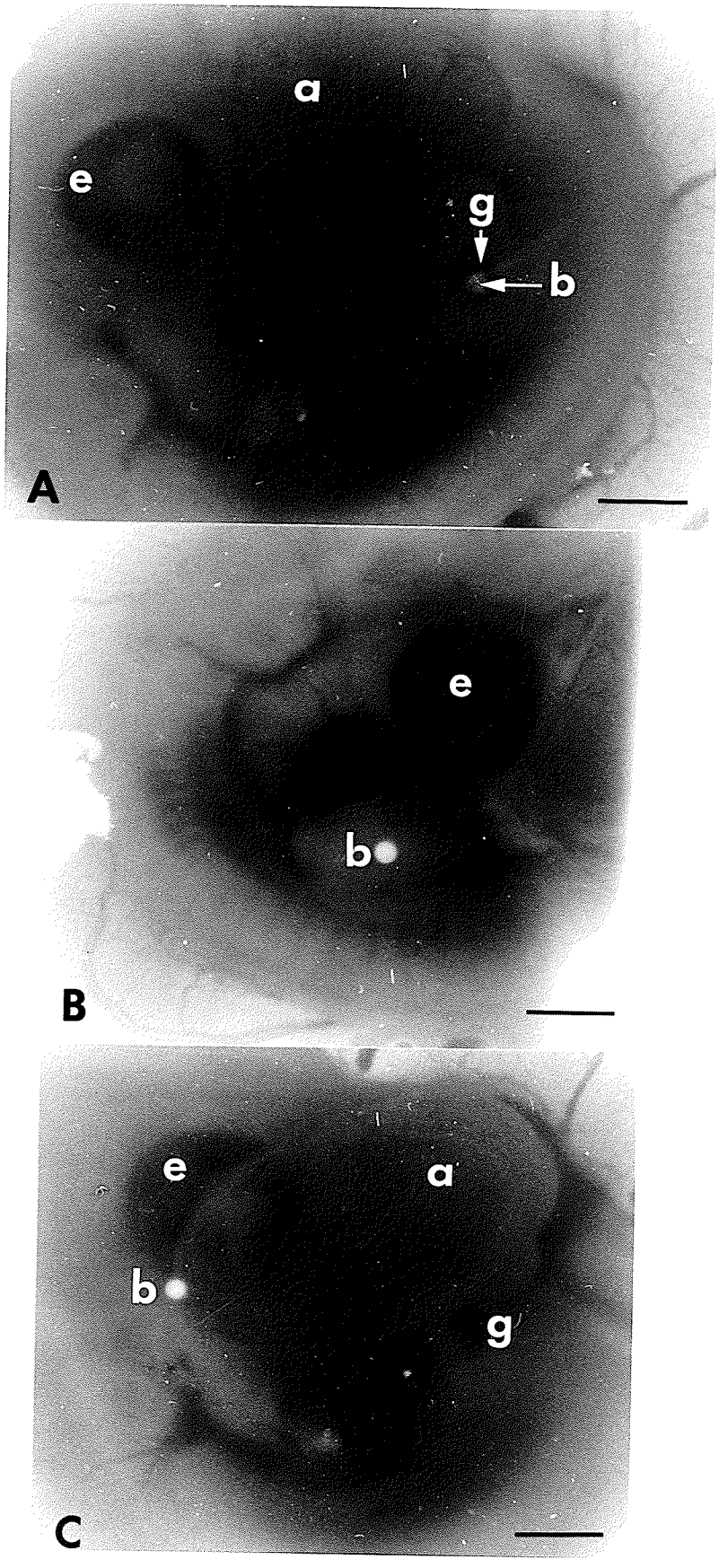
FIGURE 8:

Chick embryo 24 hours post-graft:

(A) With graft tissue (g) and the bead (b) in the right wing bud. a = allantois, e = eye.

(B) With the bead only in the right wing bud.

(C) With graft tissue remaining in the right wing bud but the bead has fallen out and is sitting on the allantois.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A
e
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a
b
g
C

RESULTS

Growth of Frontonasal Mass Grafts on the Limb Bud

The frontal nasal mass gives rise to the pre-nasal cartilage and an egg tooth in normal embryonic development of the chicken. To investigate the contribution of the epithelium and mesenchyme to these structures, the middle third of the frontonasal mass of stage 24 chick embryos was grafted into the right wing bud of stage 22 chick embryos either with the epithelium removed or with the epithelium left intact. The frontonasal mass mesenchyme without epithelium plus a heparin acrylic bead soaked in FGF-4 was grafted into host wing buds to assess whether or not FGF-4 was capable of replacing the epithelium. A sham control group received a frontonasal mass mesenchyme without epithelium graft plus a bead soaked in Dulbecco's saline solution. The results are summarized in Table III.

The untreated control left wings of all the embryos in all groups exhibited normal growth (not illustrated).

The frequency of recognizable, soft tissue outgrowths on the host limb ranged from 70% in the mesenchyme only group to 100% in the mesenchyme plus FGF-4-soaked bead group. The outgrowth due to the grafted tissue is easily recognized at day 6 post-graft because the feather germs have not yet started to grow. The presence of cartilage in the outgrowth

ranged from 69.2% in the grafts with epithelium to 96.4% in the grafts with FGF-4-soaked beads.

Egg teeth were present more often in the grafts with epithelium (84.6%) and grafts with FGF-4-soaked beads (92.9%) than in the grafts of mesenchyme only (64.3%) and grafts with a saline-soaked bead (28.6%).

Also, the presence of an egg tooth was positively correlated with the presence of a cartilage rod >.75 mm in length in the grafts of mesenchyme only and grafts with a saline-soaked bead groups (Sign Test, $p<0.05$). However, in the grafts with epithelium and grafts with FGF-4-soaked bead groups, the presence of an egg tooth showed no correlation with the length of the cartilage rod (Sign Test, $p>0.05$).

The length of the cartilage rods (Table IV) was not significantly different at the 0.05 level between the grafts of mesenchyme only (0.67 ± 0.32 mm) and the grafts with a saline-soaked bead (0.70 ± 0.31 mm) at 6 days post-graft. Because the mean length of cartilage rods in the grafts of mesenchyme only group and grafts with a saline-soaked bead group were indistinguishable ($p>0.05$), the data from both groups will be combined ($n=9$ at 6 days post-graft). This combination makes sense as the bead soaked in the Dulbecco's only should have no effect on the growth of the grafted tissue except for surgical trauma (ie. loss of some cell-to-cell contact) as the bead was placed (figure 9). This combined group will be called frontonasal mass mesenchyme grafts.

The mean length of the cartilage rods formed in the frontonasal mass mesenchyme grafts (0.69 ± 0.30 mm) was significantly smaller than both the grafts with epithelium (1.35 ± 0.68 mm) and the grafts with FGF-4-soaked beads (1.06 ± 0.50 mm) at the $p<0.05$ level (figures 9,10,11). Thus, both the epithelium and the FGF-4-soaked bead were able to stimulate outgrowth of the cartilage rod compared to what would form in isolated frontonasal mass mesenchyme.

The effectiveness of the FGF-4 bead in replacing the epithelium can be determined by comparing the mean length of cartilage rods in grafts with epithelium and grafts treated with FGF-4. The means between the grafts with epithelium and grafts with FGF-4-soaked beads appear different (grafts with epithelium longer than grafts with FGF-4-soaked beads) but the difference is not statistically significant at the 0.05 level. The wider distribution of the lengths of the cartilage rods in the grafts with epithelium group (figure 13) might be the reason for the lack of significance.

The position of the bead was usually at or near the elbow at the time of sacrifice.

The shape of the cartilage rod (straight or curved) and the position of the egg tooth at the end of the cartilage rod showed relatively the same distribution amongst all the frontonasal mass groups.

It would appear that the placement of the FGF-4-soaked bead did substitute for the contribution made by the

epithelium to the growth of the grafted mesenchyme both in terms of length of the cartilage rod and the presence of an egg tooth.

Growth of Mandibular Grafts on the Limb Bud

The mandible gives rise to Meckel's cartilage in the normal embryonic development of the chicken. To investigate the contribution of the epithelium and mesenchyme to Meckel's cartilage, the medial 500 μm of the mandibular prominence, with or without the epithelium of a stage 24 embryo was grafted into the right wing bud of a stage 22 chick embryo. To elucidate the possible role of FGF-4 contributed by the epithelium, mandibular grafts without epithelium were placed with either a Dulbecco's saline-soaked bead or an FGF-4-soaked bead. The results are summarized in Table V.

The untreated control left wings of all the embryos in all groups exhibited normal growth (not illustrated).

The frequency of outgrowths was consistently high in all groups, ranging from 86.7% in the mesenchyme with saline-soaked beads group to 100% in the mesenchyme with epithelium group. The presence of cartilage in the outgrowth was also consistently high. Both the mesenchyme with saline-soaked beads group and mesenchyme with FGF-4-soaked beads group had one specimen each that had an extra cartilage rod growing but

not as an outgrowth. In other words, these wings looked normal at the time of dissection but when stained, each had an extra rod of cartilage present within the normal confines of the wing.

Egg teeth do not normally form in the mandible of the chicken and no egg teeth were found in the outgrowths of the mandibular grafts in this experiment.

The length of the cartilage rods (Table VI) was not significantly different at the .05 level between the mesenchyme only group (n=2; mean=1.56 ± 0.28 mm) and the mesenchyme with saline-soaked bead group (n=13; mean=0.86 ± 0.48 mm). As these 2 groups are not statistically different and the plain bead should have no effect on the outgrowth, these two groups will be combined and called the mandibular mesenchyme grafts (figure 14).

The mean length of the cartilage rods formed in the mandibular mesenchyme grafts (0.95 ± 0.51 mm) was significantly shorter at the $p<.05$ level than the mesenchyme with epithelium grafts (3.73 ± 1.27 mm) but was not significantly different at the .05 level from the mesenchyme with FGF-4-soaked bead grafts (1.30 ± 0.43 mm). Thus, FGF-4 was not able to stimulate outgrowth of the cartilage rod compared to what would form in isolated mandibular mesenchyme (figure 15).

The effectiveness of the FGF-4 bead in replacing the epithelium can be determined by comparing the mean length of

the cartilage rods in the mesenchyme with epithelium grafts (3.73 ± 1.27 mm) to the mesenchyme with FGF-4-soaked bead grafts (1.30 ± 0.43 mm). The cartilage rods of the mesenchyme with epithelium grafts (figure 16) are significantly longer than the mesenchyme with FGF-4-soaked bead grafts, $p<0.05$.

Although the difference was not statistically significant between the mandibular mesenchyme grafts and mesenchyme with FGF-4-soaked bead grafts, their distributions (figure 17) do appear different - ie. mandibular mesenchyme grafts are shorter than mesenchyme treated with FGF-4-soaked beads.

The placement of the FGF-4 bead in the mandibular mesenchyme grafts did not replace the role of the epithelium in terms of the length of the cartilage rod. However, the shape of the cartilage rod did appear to be affected by the presence of FGF-4. The cartilage rods in the mandibular mesenchyme grafts and mesenchyme with epithelium grafts were all straight or curvilinear. In the mesenchyme with FGF-4-soaked bead group, there was a range of shapes from square to triangular to donut-shaped as well as curvilinear rods (figure 18).

Effect of FGF-4 Beads on the Host Limb Bud

To investigate whether or not the bead itself had any effect, graft sites were prepared in stage 22 host right wing

buds and a heparin acrylic bead soaked in FGF-4 or Dulbecco's saline was placed.

The FGF-4-soaked bead did not result in the development of any egg teeth. Extra cartilage rods, duplicated digits, duplicated radii and ulnae, duplicated humeri, fusion of the elbow joint (complete and partial), and misshapen radii and ulnae were all detected in this group (figure 19). In fact, only one specimen within this group appeared normal.

There was fusion of the elbow joint (ie. no joint space between the humerus and radius and ulna) in approximately 24% of the specimens that had an FGF-4-soaked bead (with or without a graft) (Table VII and figure 20). As well, the only specimens that had 4 or more extra bits of cartilage were ones that had an FGF-4-soaked bead (Table VIII).

No host limbs receiving a saline-soaked bead (n=15) showed a true outgrowth (one wing had a small bump) and none developed egg teeth. No extra cartilage rods were seen (figure 21, C and D).

Also note in the above figure (A and B) that the surgical preparation of the graft site did not alter normal growth of the wing.

TABLE III: summarizes the results of the frontonasal mass graft experiments showing the number of specimens per group (n), the absolute number and proportion that had an outgrowth visible, the absolute number and proportion that had cartilage within the outgrowth if an outgrowth was present, the absolute number and proportion that had an egg tooth on the outgrowth, and the absolute number and proportion that had both an egg tooth and a cartilage rod if an outgrowth was present.

TABLE III: Frontonasal Mass Grafts

Group	(n)	Outgrowth	Cartilage in Outgrowth	Egg Tooth	Outgrowth with cartilage that had egg tooth
F	(20)	14/20 (70%)	12/14 (85.7%)	9/14 (64.3%)	8/12 (66.7%)
FB	(15)	14/15 (93.3%)	11/14 (78.6%)	4/14 (28.6%)	4/11 (36.4%)
FE	(15)	13/15 (86.7%)	9/13 (69.2%)	11/13 (84.6%)	9/9 (100%)
4FB	(28)	28/28 (100%)	27/28 (96.4%)	26/28 (92.9%)	25/27 (92.6%)

F=frontonasal mass mesenchyme only
FB=frontonasal mass mesenchyme plus plain bead
FE=frontonasal mass mesenchyme with epithelium intact
4FB=frontonasal mass mesenchyme plus FGF-4-soaked bead

TABLE IV: shows the mean length of the cartilage rods in each group and number of specimens per group.
NB. Does not include specimens that didn't have a cartilage rod.

TABLE IV: Length of Cartilage Rods in Frontonasal Mass Graft Outgrowths

Group	**(n)**	**mean (mm)**	**standard deviation (± mm)**
F	(5)	0.68	0.32
FB	(4)	0.70	0.31
FE	(9)	1.35	0.68
4FB	(27)	1.06	0.50
F+FB	(9)	0.69	0.30

F=frontonasal mass mesenchyme
FB=frontonasal mass mesenchyme plus plain bead
FE=frontonasal mass mesenchyme with epithelium intact
4FB=frontonasal mass mesenchyme plus FGF-4 soaked bead
F+FB=frontonasal mass mesenchyme and frontonasal mass mesenchyme plus plain bead
=frontonasal mass mesenchyme grafts

FIGURE 9:

Wholemounts of wings bearing grafts of frontonasal mass mesenchyme without epithelium, stained with Alcian Blue. Scale bar = 0.5 mm, h = humerus, r = radius, u = ulna, g = graft, e = egg tooth, st = soft tissue outgrowth.

(A) Graft plus a plain bead. A soft tissue outgrowth has formed.

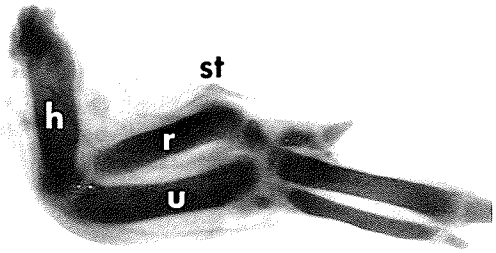
(B) Graft plus a plain bead. A short cartilage rod has formed. Surgical trauma (haematoma) likely disrupted the normal development of the humerus resulting in the shortened, bulbous morphology.

(C) Graft plus a plain bead. A slightly longer cartilage rod has formed with an egg tooth at the distal end.

(D) Graft only. A short cartilage rod has formed. Note the misshapen radius and ulna.

(E) Graft only. A short cartilage rod with an egg tooth at the distal end has formed. Note the bowed radius and short ulna.

(F) Graft only. A long cartilage rod has formed with an egg tooth at the distal end. The ulna is short and the digits articulate at a more acute angle than normal.



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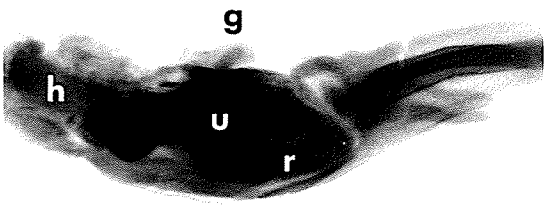

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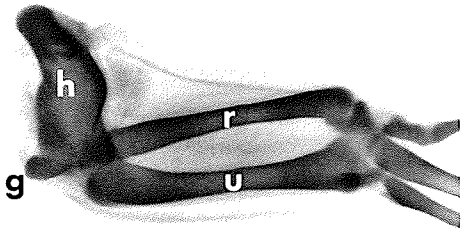

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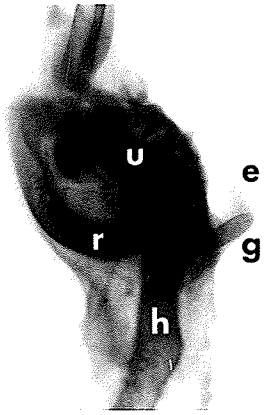

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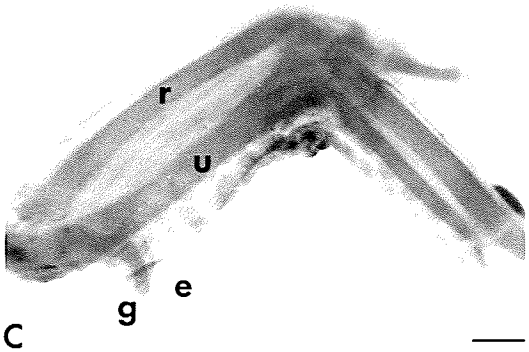

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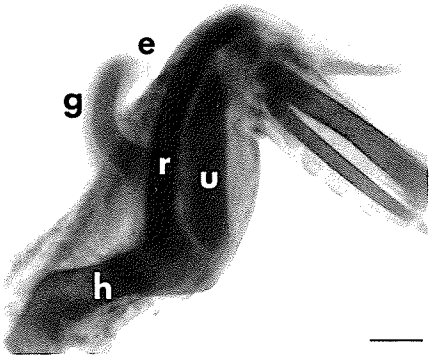


F

FIGURE 10:

Wholemounts of wings bearing grafts of frontonasal mass mesenchyme with intact epithelium, stained with Alcian Blue, illustrating the range of outgrowths. Scale bar = 0.5 mm, h = humerus, r = radius, u = ulna, g = graft, e = egg tooth, st = soft tissue outgrowth.

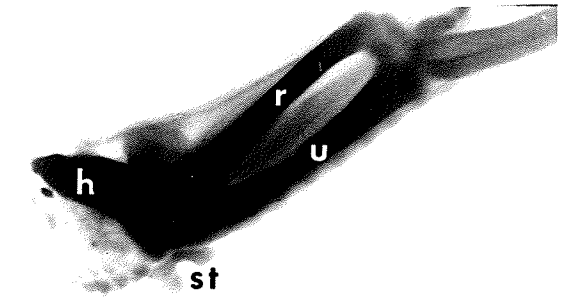
(A) A small outgrowth of soft tissue only.

(B) A medium, soft tissue outgrowth with cartilage at the base only and an egg tooth at the distal end.

(C) A long, straight cartilage rod has formed in the outgrowth with an egg tooth at the distal tip.

(D) A long, straight cartilage rod has formed with a large egg tooth at the distal end.

(E) A long, straight cartilage rod with a bulbous distal end and an egg tooth at the tip. The radius and ulna are short.



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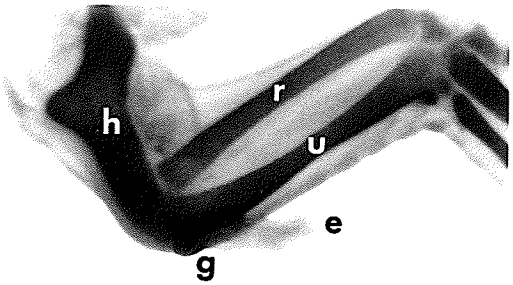

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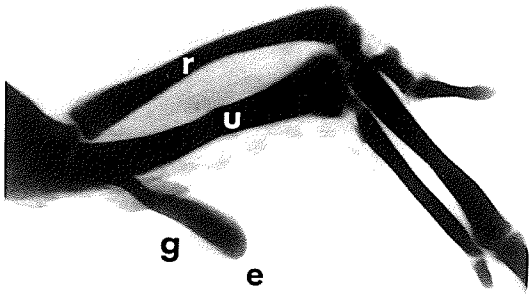

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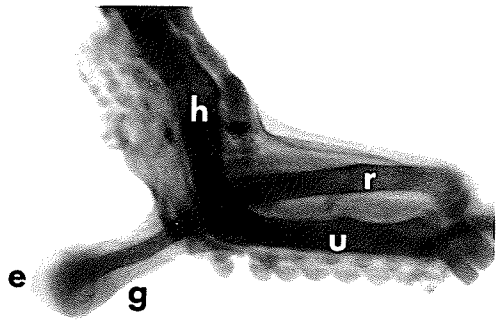

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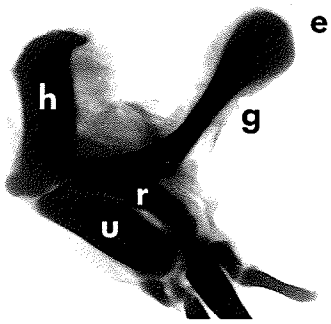

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FIGURE 11:

Wholemounts of wings bearing frontonasal mass mesenchyme grafts without epithelium and an FGF-4-soaked bead. Scale bar = 0.5 mm, h = humerus, r = radius, u = ulna, g = graft, e = egg tooth.

(A) An egg tooth formed but no outgrowth. Note the bowed radius and short ulna.

(B) Multiple cartilages formed within the limb and an outgrowth at 90° to the ulna had an egg tooth at the distal tip.

(C) Medium length, curved outgrowth with an egg tooth. Again notice the bowed radius and ulna.

(D) Long curved outgrowth with an egg tooth.

(E) Long outgrowth with an egg tooth and a second nodule of cartilage. Note the bent shape of the radius and ulna.

(F) Long outgrowth with an egg tooth. Note the abnormal elbow formation.

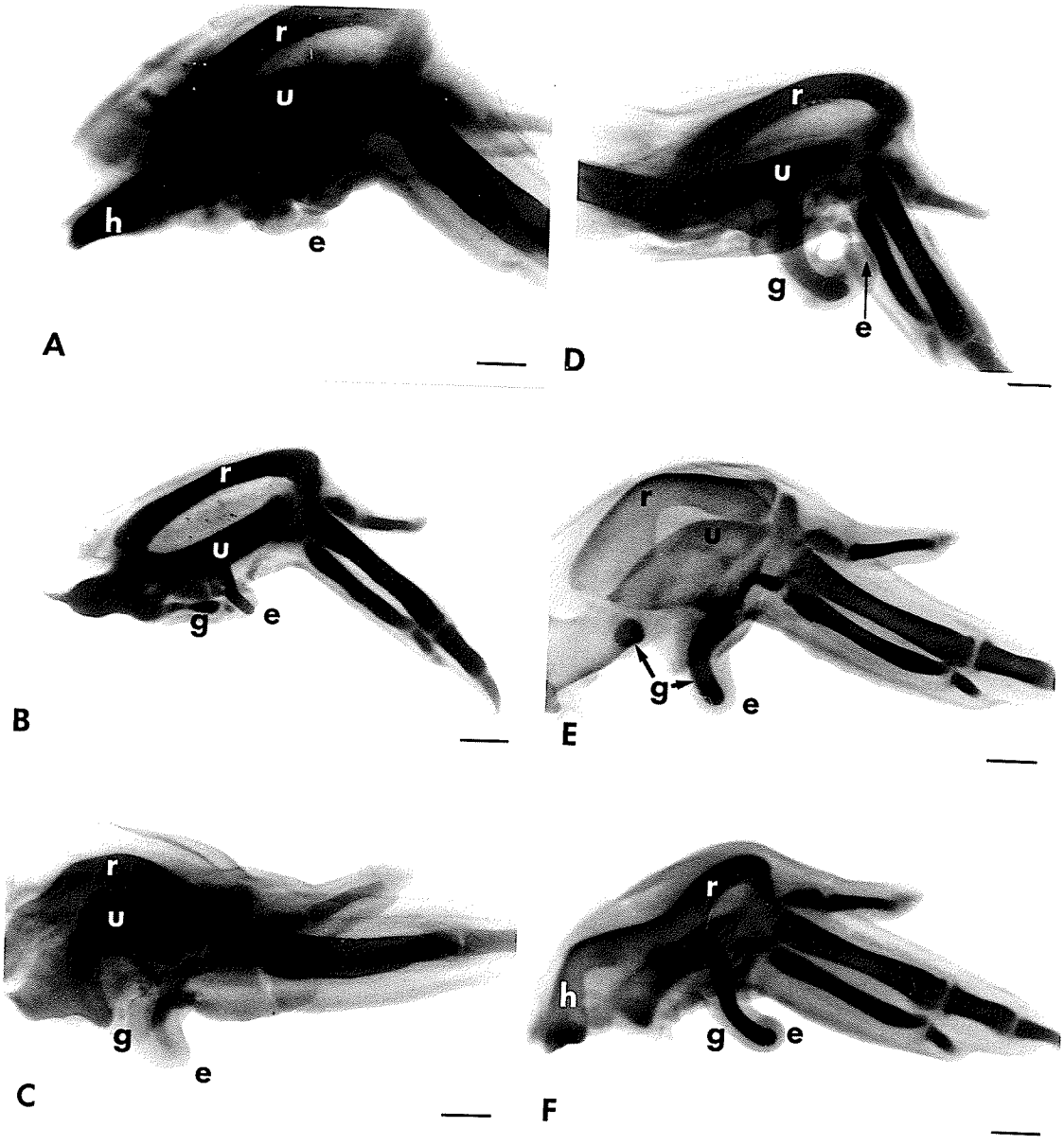
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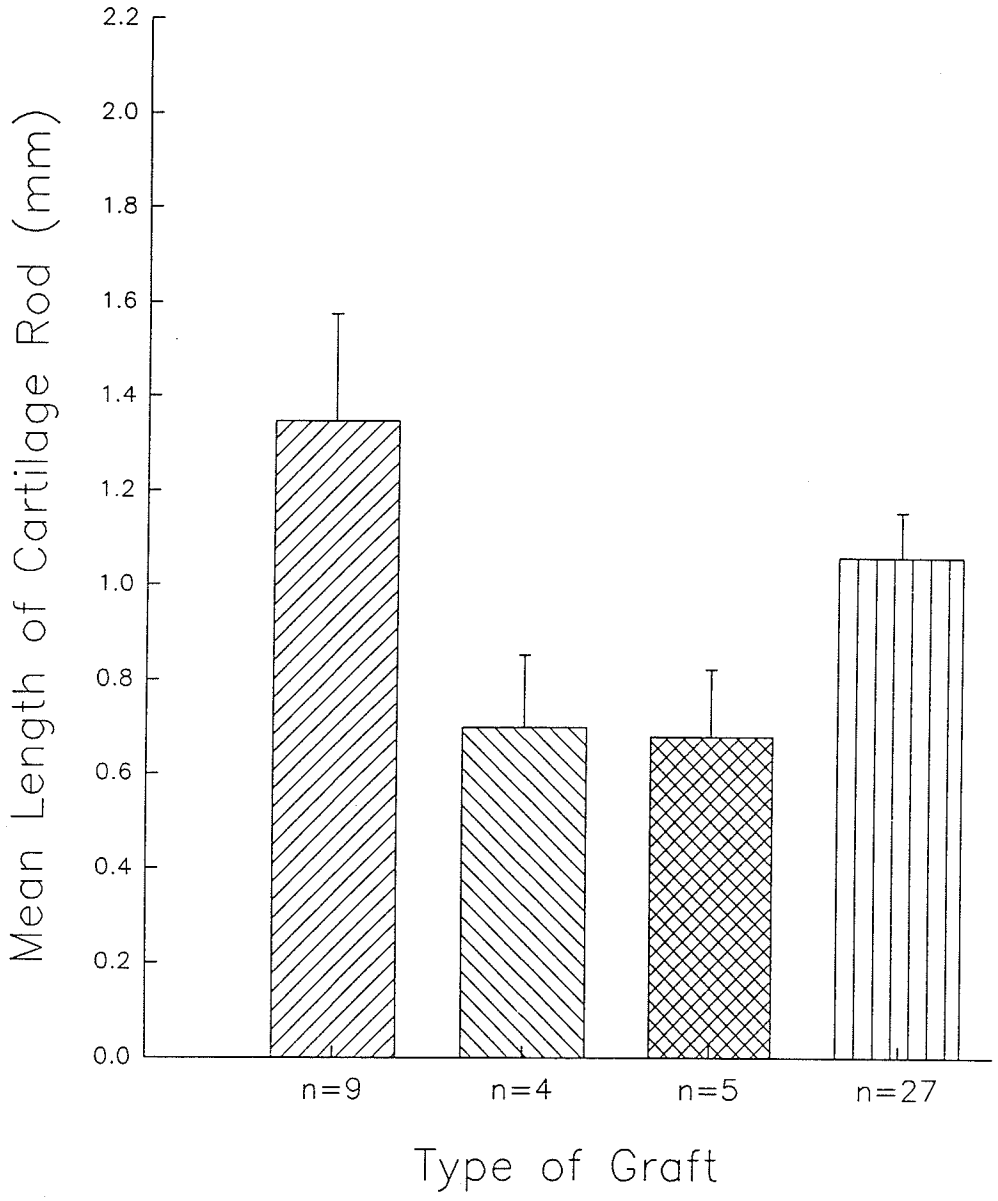
FIGURE 12: Mean Length of Cartilage Rods in Frontonasal Mass Grafts

Histogram showing the mean length of the cartilage rods in the frontonasal mass graft experiments.

error bar = 1 standard deviation



= frontonasal mass mesenchyme with epithelium

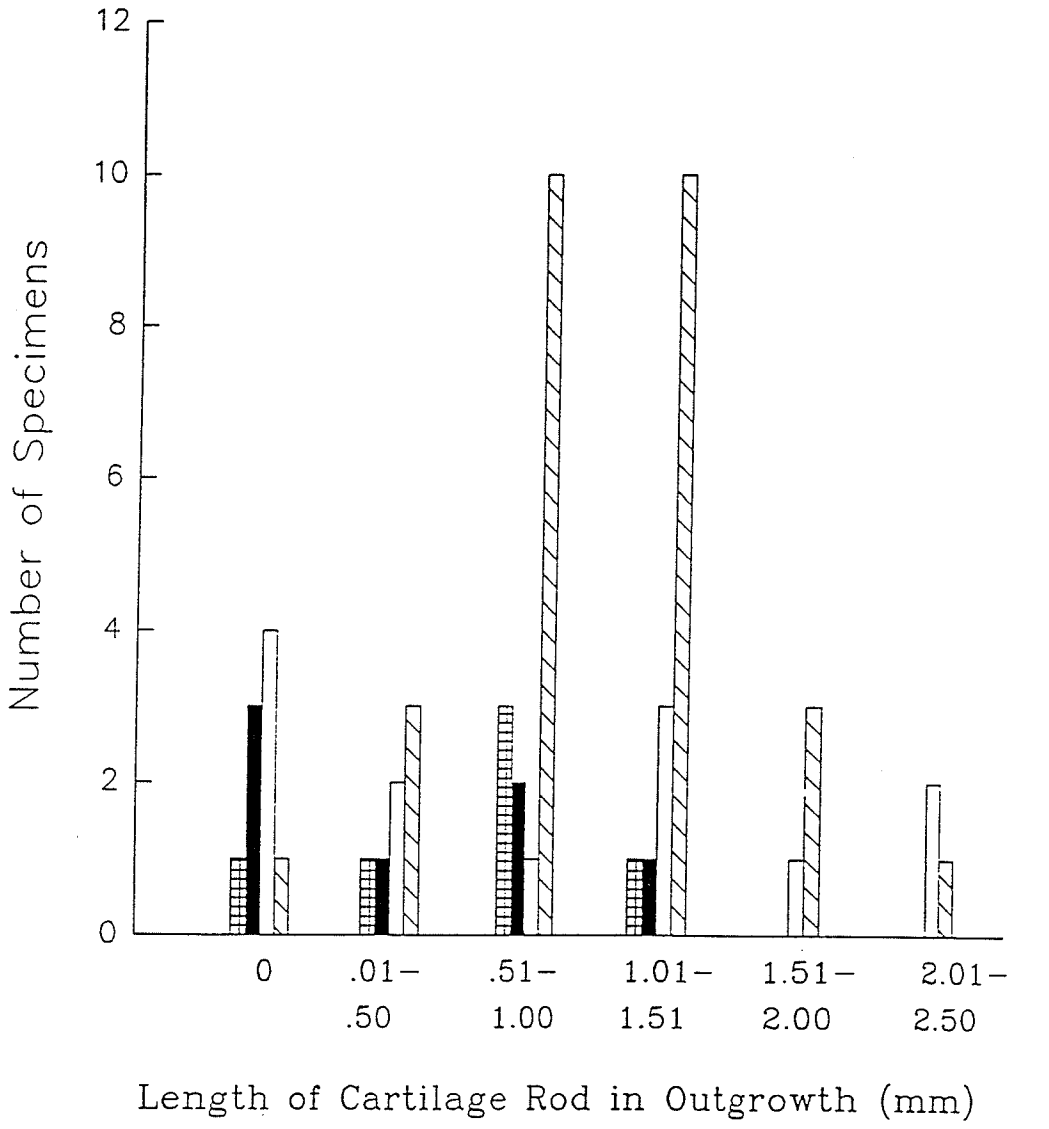
= frontonasal mass mesenchyme with plain bead

= frontonasal mass mesenchyme

= frontonasal mass mesenchyme with FGF-4 bead

FIGURE 13: Frontonasal Mass Grafts - Distribution of the Cartilage Rods by Length.

Histogram showing the distribution of the lengths of the cartilage rods formed in each treatment group.


12
10
8
6
4
2
0
Number of Specimens
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.50
.51–
1.00
1.01–
1.51
1.51–
2.00
2.01–
2.50
Length of Cartilage Rod in Outgrowth (mm)
= frontonasal mass graft
= frontonasal mass graft plus plain bead
= frontonasal mass graft with epithelium
= frontonasal mass graft plus FGF–4 bead

TABLE V: summarizes the results of the mandibular graft experiments showing the number of specimens per group (n), the proportion that had an outgrowth visible, and the absolute number and proportion that had cartilage within the outgrowth if an outgrowth was present.

TABLE V: Mandibular Grafts

Group	(n)	Outgrowth Present	Cartilage in Outgrowth
M	(3)	3/3 (100%)	2/3 (66.7%)
MB *	(15)	13/15 (86.7%)	12/13 (92.3%)
ME	(11)	11/11 (100%)	11/11 (100%)
4MB *	(14)	13/14 (92.9%)	13/13 (100%)

M=mandibular mesenchyme only
MB=mandibular mesenchyme plus plain bead
ME=mandibular mesenchyme with intact epithelium
4MB=mandibular mesenchyme plus FGF-4 soaked bead

*=group contained one wing with no apparent outgrowth but an extra cartilage rod was found when stained.
Not included in Cartilage in Outgrowth column.

TABLE VI: shows the mean length of the cartilage rods in each group and number of specimens per group.
NB. Does not include specimens that didn't have a cartilage rod.

TABLE VI: Length of Cartilage Rods in Mandibular Graft Outgrowths

Group	**(n)**	**mean (mm)**	**standard deviation (± mm)**
M	(2)	1.56	0.28
MB	(13)	0.86	0.48
M+MB	(15)	0.95	0.51
ME	(7)	3.73	1.27
4MB	(14)	1.30	0.43

M=mandibular mesenchyme only
MB=mandibular mesenchyme plus plain bead
M+MB=mandibular mesenchyme only and mandibular mesenchyme plus plain bead
=mandibular mesenchyme grafts
ME=mandibular mesenchyme with intact epithelium
4MB=mandibular mesenchyme plus FGF-4-soaked bead

FIGURE 14:

Wholemounts of wings bearing grafts of mandibular mesenchyme without epithelium, stained with Alcian Blue. Scale bar = 0.5 mm, h = humerus, r = radius, u = ulna, g = graft, b = bead.

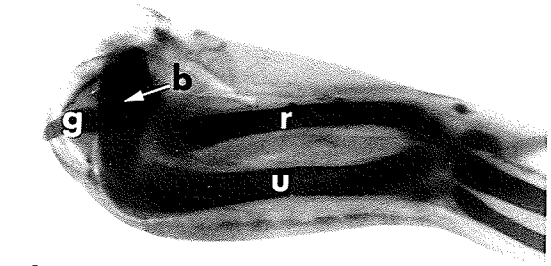
(A) Graft plus a plain bead (indicated) that has formed a short outgrowth.

(B) Graft plus a plain bead (indicated) that has formed two outgrowths off of the ulna and a small island of cartilage next to the bead. Note the bowed radius and acute articulation of the digits at the wrist.

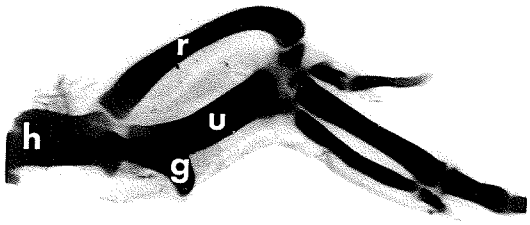
(C) Graft plus a plain bead (indicated) that has formed a very short cartilage outgrowth.

(D) Graft that has formed a long rod of cartilage but the outgrowth appears small.

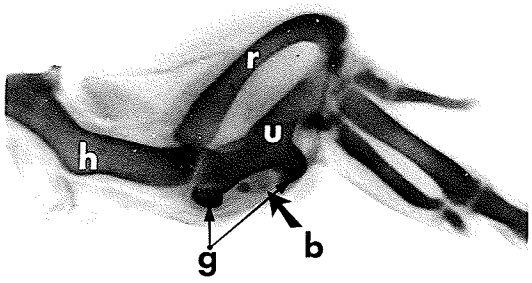
(E) Graft that has formed a long rod of cartilage within the confines of the wing.



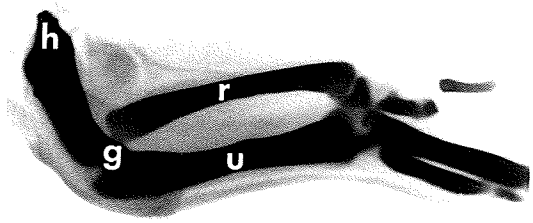
A



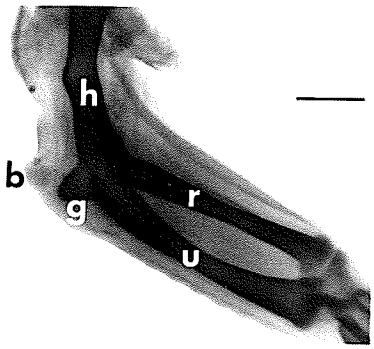
D



B



E

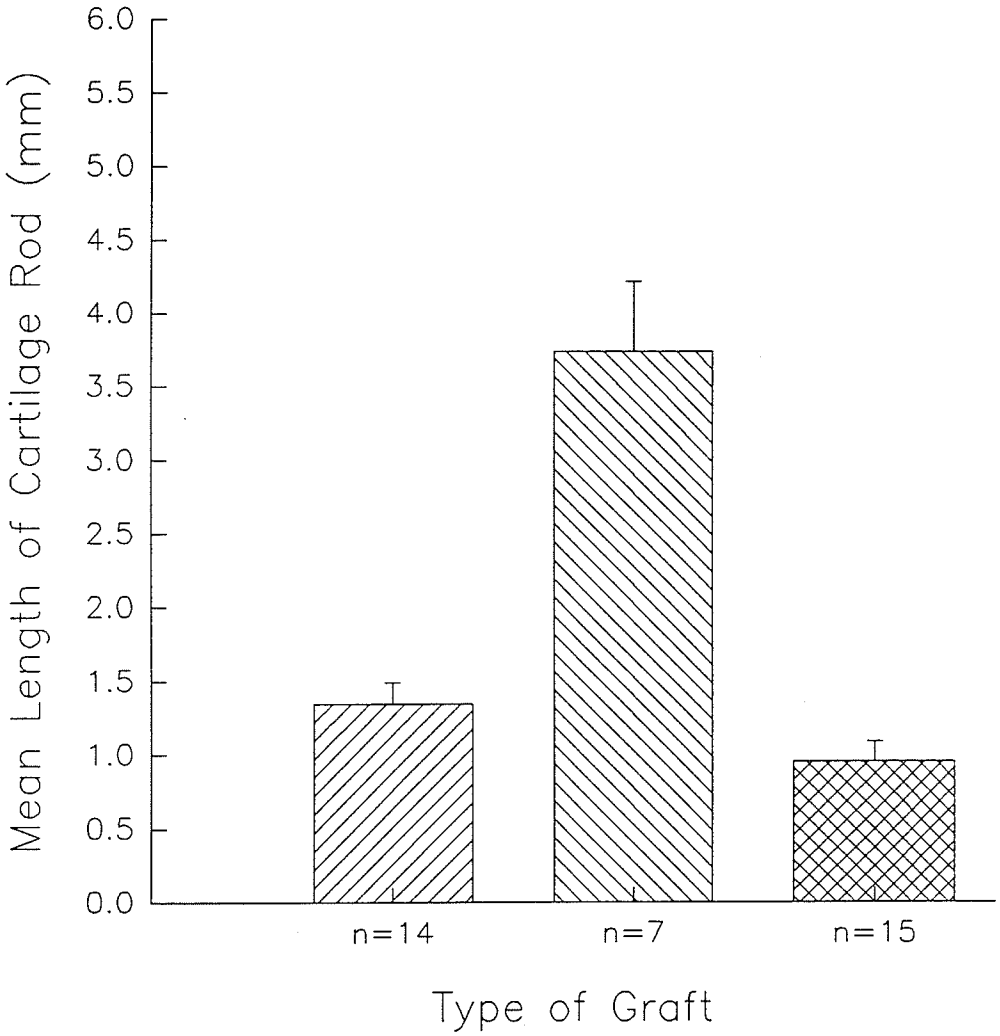


C

FIGURE 15: Mean Length of Cartilage Rods in Mandibular Grafts

Histogram showing the mean lengths of the cartilage rods formed in the mandibular grafting experiments.

error bar = 1 standard deviation



= mandibular mesenchyme plus FGF−4 bead

= mandibular mesenchyme with epithelium

= mandibular mesenchyme only and mandibular mesenchyme plus plain bead

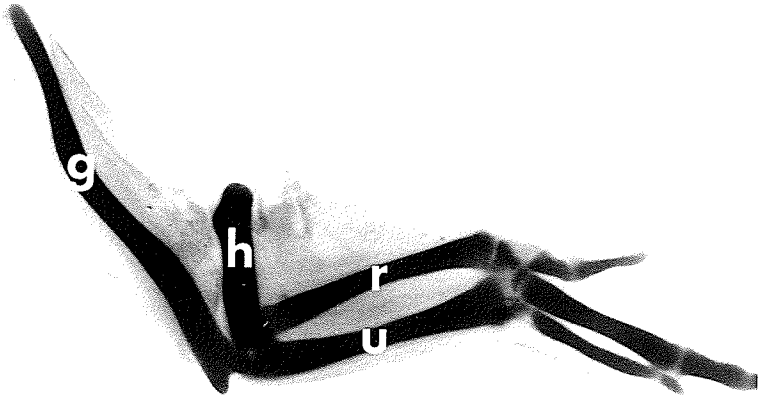
FIGURE 16:

Wholemounts of wings bearing grafts of mandibular mesenchyme with intact epithelium, stained with Alcian Blue. Scale bar = 1.0 mm, h = humerus, r = radius, u = ulna, g = graft.

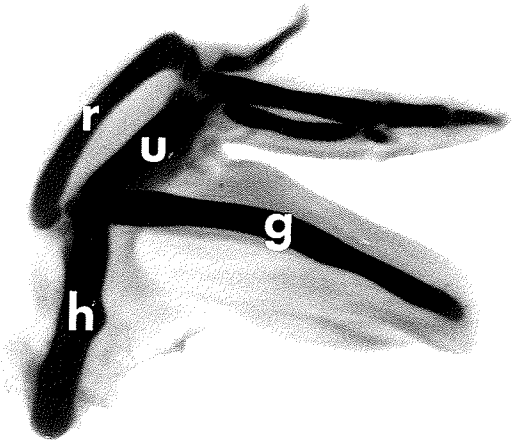
(A) Long, straight cartilage rod has formed with a structure resembling a ramus with a condyle and a coronoid process at the elbow.

(B) Long, straight cartilage rod has formed. Note the short ulna and the articulation of the elbow.

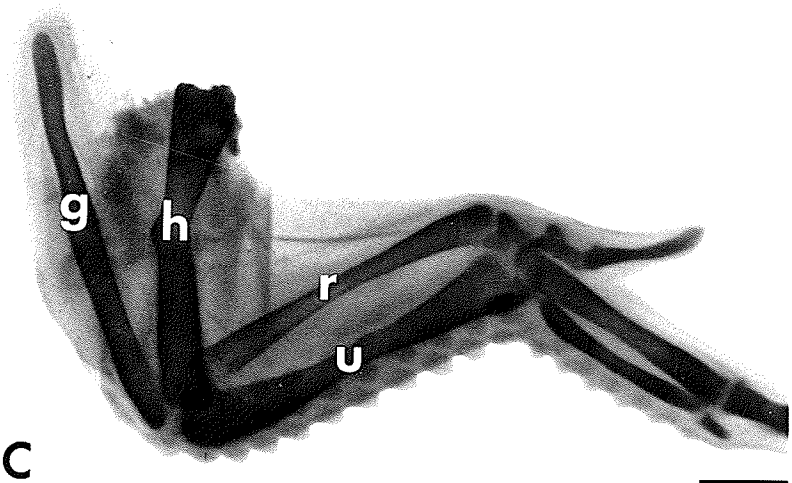
(C) Another long, straight cartilage rod has formed. This embryo was 7 days post-graft.



A



B

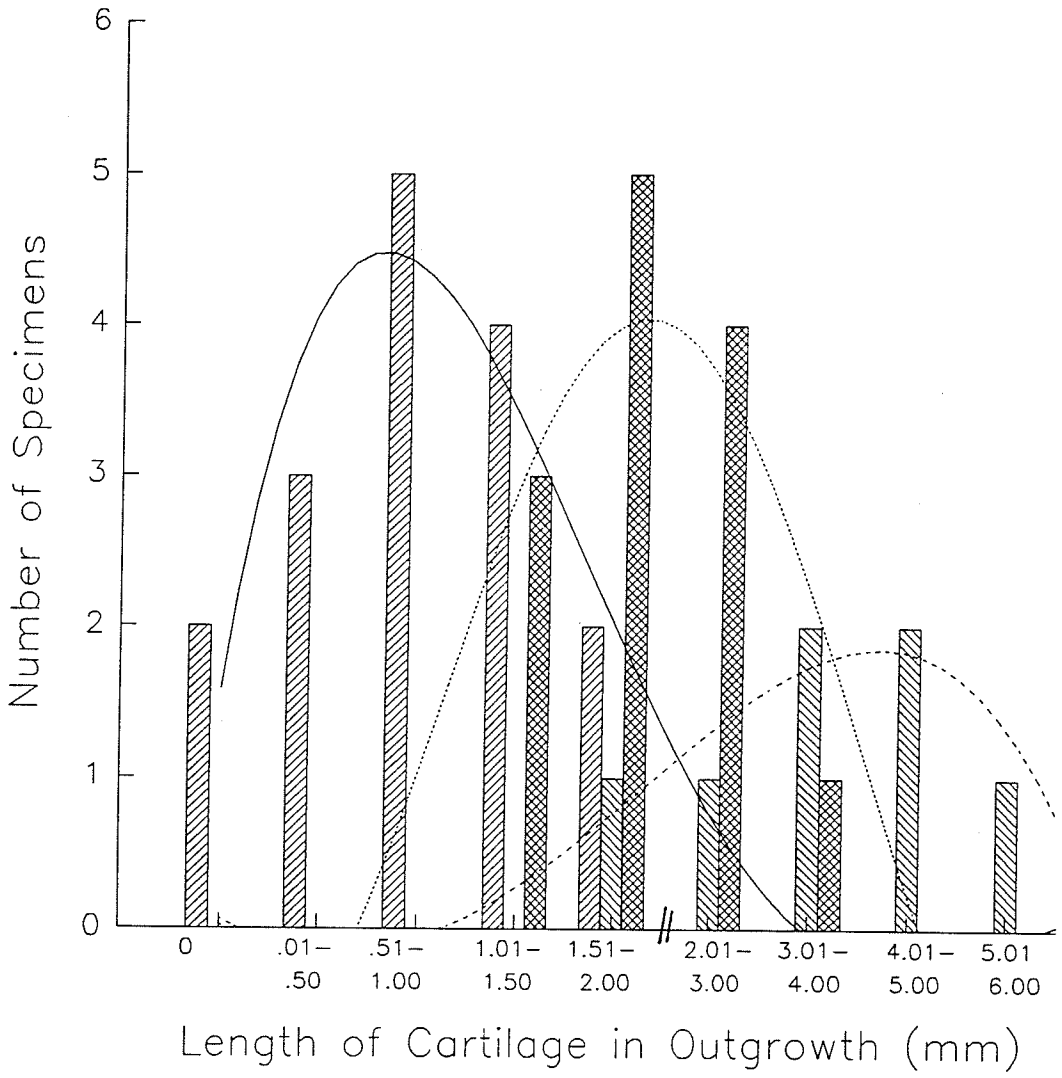


C

FIGURE 17: Mandibular Grafts - Distribution of the Cartilage Rods by Length.

Distribution of the length of cartilage rods formed in each treatment group. Note the separation of the normal curves which might indicate some effect on the outgrowth by the FGF-4-soaked bead.

NB. Change in scale on the x-axis after 2.00 mm.



= mandibular graft and mandibular graft with plain bead

= mandibular graft with epithelium

= mandibular graft with FGF–4 bead

FIGURE 18:

Wholemounts of wings bearing grafts of mandibular mesenchyme plus an FGF-4-soaked bead, stained with Alcian Blue. Scale bar = 0.5 mm, h = humerus, r = radius, u = ulna, g = graft, b = bead.

(A) A triangular-shaped cartilage formed.

(B) A donut-shaped cartilage formed.

(C) A semi-circular cartilage formed around the bead (indicated).

(D) A thick rod of cartilage formed.

(E) An Ω-shaped loop of cartilage formed between the radius and ulna.

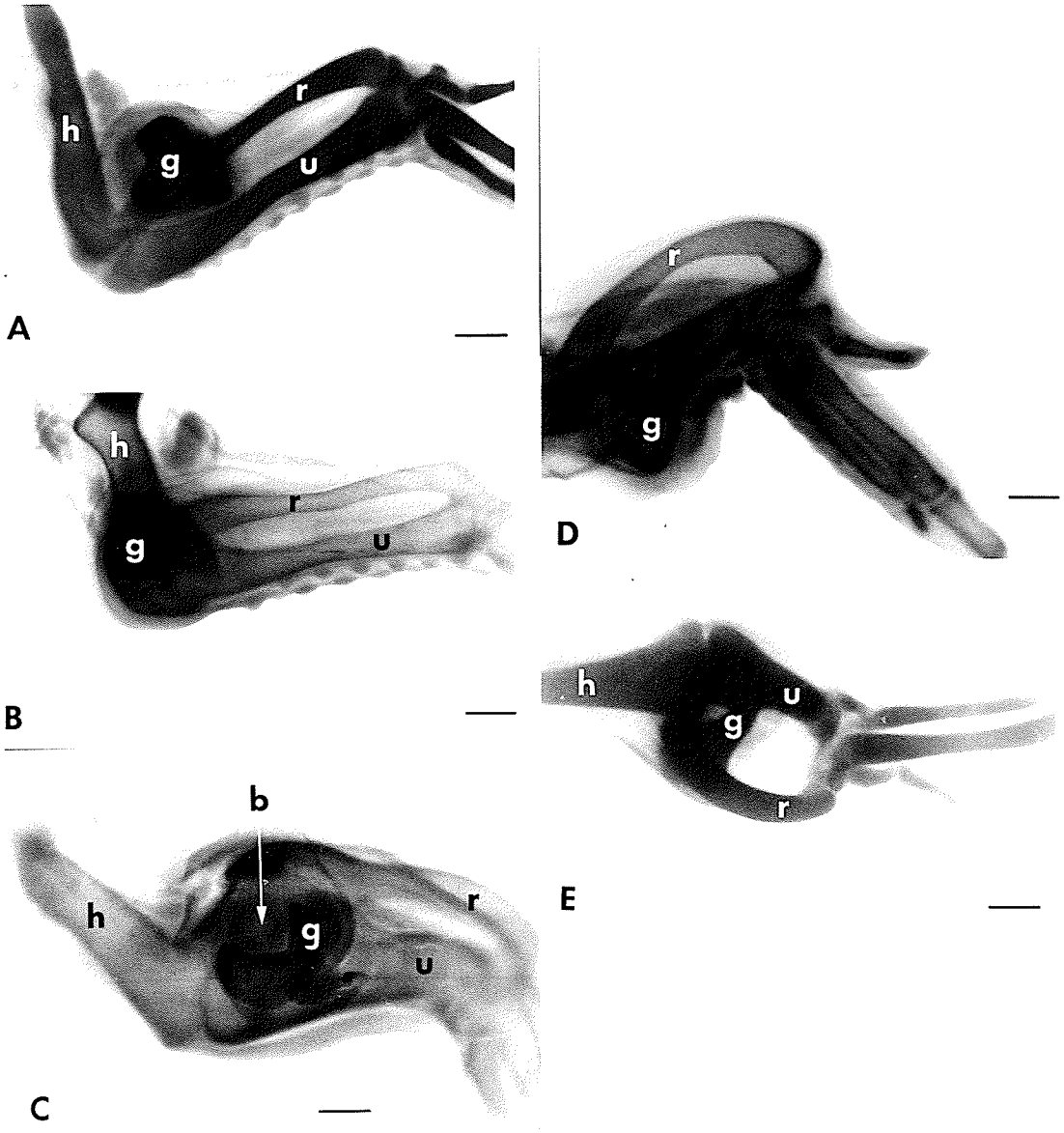

h
r
g
u
A
h
r
g
u
B
b
h
g
r
u
C
r
g
D
h
u
g
r
E

FIGURE 19:

Wholemounts of wings with an FGF-4-soaked bead only, stained with Alcian Blue. Scale bar = 0.5 mm, h = humerus, r = radius, u = ulna, xc = extra cartilage.

(A) Two extra rods of cartilage formed - a straight rod and an Ω-shaped one.

(B) Two outgrowths formed with 2 rods of cartilage in each (denoted by the black and white arrowheads) which resemble extra digits.

(C) Multiple extra rods of cartilage which may be duplicated digits. Note the short radius and ulna.

(D) Two extra rods of cartilage that look like duplications of digits 2 and 3.

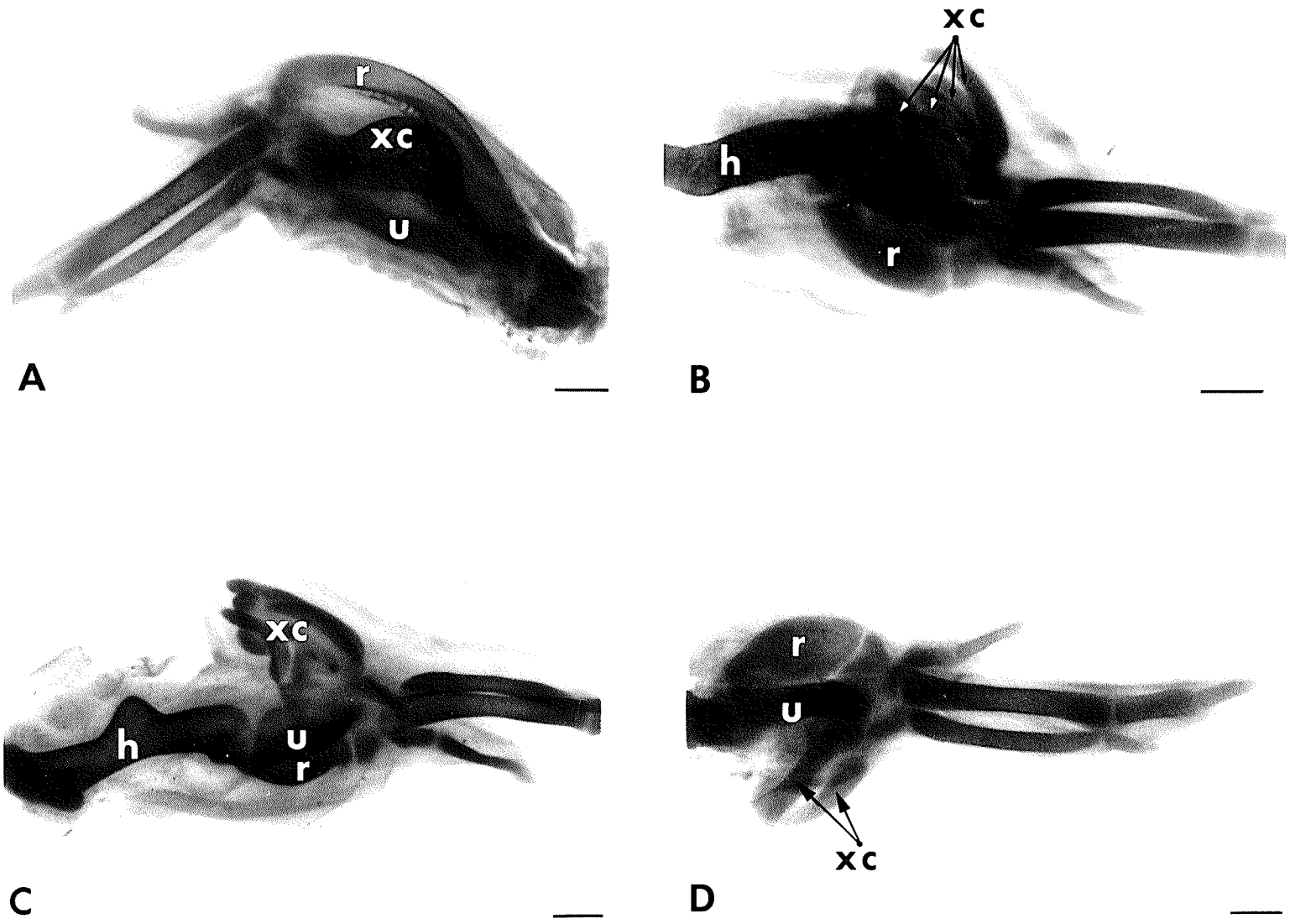

r
xc
u
A
xc
h
r
B
xc
h
u
r
C
r
u
xc
D

TABLE VII: summarizes the incidence of fused elbow joints, partial or complete. Note that in most instances of a complete or partial joint fusion, an FGF-4-soaked bead was present. ? = uncertain as to whether joint space was visible.

Table VII: Incidence of Fused Elbow Joint

Group	(n)	No	?	Partial	Yes
B	(15)	15 (100%)	0	0	0
4B	(15)	9 (60%)	1 (6.7%)	1 (R&H) (6.7%)	4 (26.7%)
4MB	(14)	9 (64.3%)	1 (7.1%)	1 (U&H) (7.1%)	3 (21.4%)
ME	(11)	9 (81.8%)	1 (no R&U) (9.1%)	1 (U&H) (9.1%)	0
MB	(15)	14 (93.3%)	0	0	1 (6.7%)
M	(4)	4 (100%)	0	0	0
4FB	(29)	21 (72.4%)	1 (3.4%)	0	7 (24.1%)
F	(13)	13 (100%)	0	0	0
FB	(12)	10 (83.3%)	0	1 (R&H) (8.3%)	1 (8.3%)
FE	(13)	13 (100%)	0	0	0

B=plain bead only
4B=FGF-4 soaked bead only
F=frontonasal mass mesenchyme only
FB=frontonasal mass mesenchyme plus plain bead
FE=frontonasal mass mesenchyme with epithelium intact
4FB=frontonasal mass mesenchyme plus FGF-4 soaked bead
M=mandibular mesenchyme only
MB=mandibular mesenchyme plus plain bead
ME=mandibular mesenchyme with intact epithelium
4MB=mandibular mesenchyme plus FGF-4 soaked bead

R&H=radius and humerus fused
U&H=ulna and humerus fused
R&U=radius and ulna fused

FIGURE 20:

Wholemounts of wings with an FGF-4-soaked bead stained with Alcian Blue. h = humerus, r = radius, u = ulna, xc = extra cartilage, b = bead, e = egg tooth, g = graft. Scale bar = 0.5 mm.
NB. Refer to figure 14 for pictures of normal joint morphology.

(A) Extra cartilage has formed between the radius and ulna. No joint space is present.

(B) In addition to the bead, this specimen had a frontonasal mass mesenchyme graft. A cartilage-containing outgrowth with an egg tooth has formed. No elbow joint space is present.

(C) This specimen received a quail frontonasal mass mesenchyme graft in addition to the bead. Note the absence of a joint space between the radius and humerus.

(D) Another graft of quail frontonasal mass mesenchyme. Note the complete fusion of the radius and ulna to the humerus.

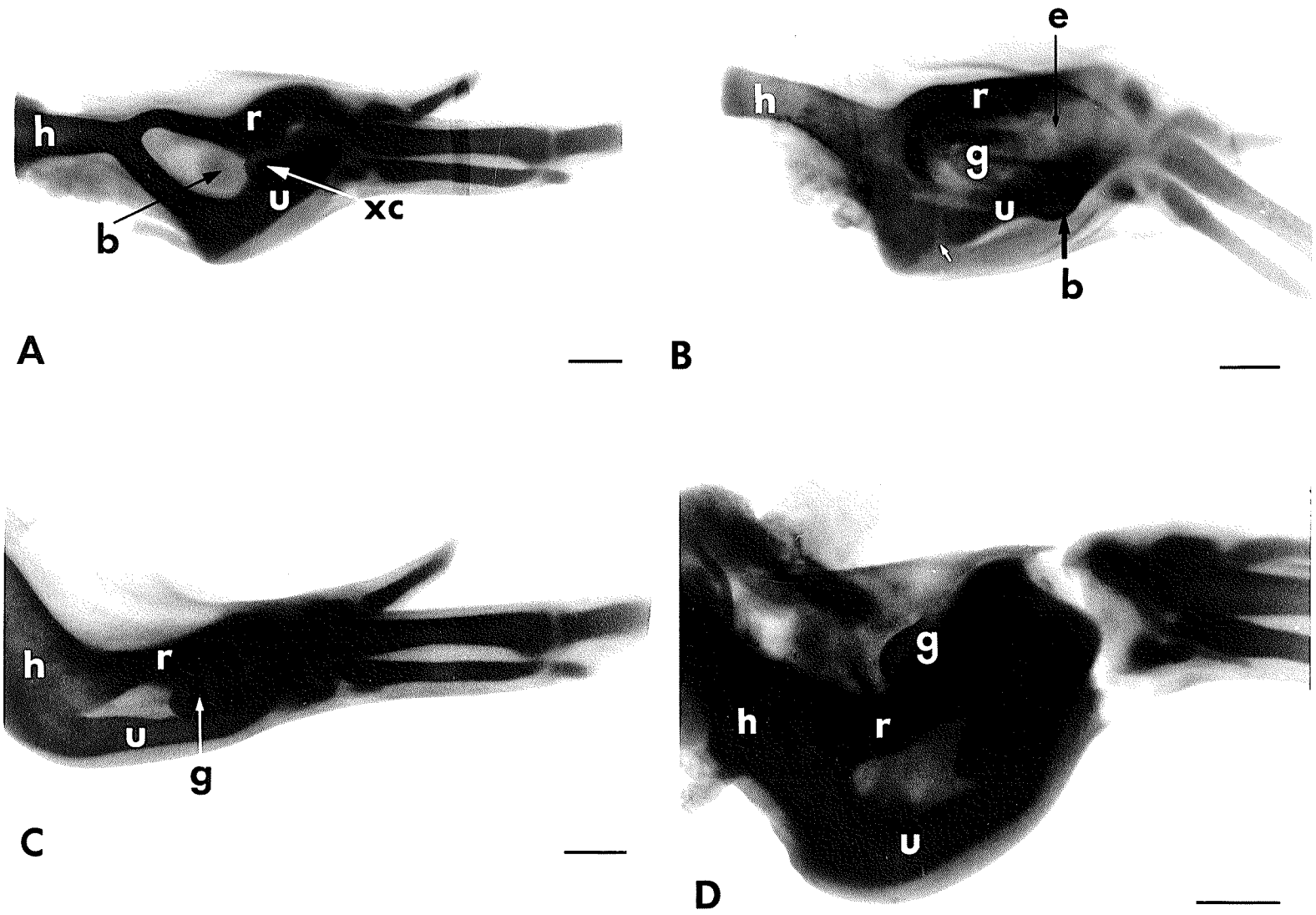

h
r
u
xc
b
A
e
h
r
g
u
b
B
h
r
u
g
C
g
h
r
u
D

TABLE VIII: shows the number of extra cartilages formed in each group. Note that in all instances where four or more extra cartilage bits formed, an FGF-4-soaked bead was present.

TABLE VIII: Number of Extra Cartilage Rods

Group	(n)	0	1	2-3	4+
B	(15)	15	0	0	0
4B	(15)	3	1	4	7
4MB	(14)	0	4	8	2
ME	(11)	0	9	2	0
MB	(15)	2	10	3	0
M	(4)	2	2	0	0
4FB	(29)	0	9	14	6
F	(13)	1	12	0	0
FB	(13)	3	8	2	0
FE	(13)	4	9	0	0

B=plain bead only
4B=FGF-4 soaked bead only
F=frontonasal mass mesenchyme only
FB=frontonasal mass mesenchyme plus plain bead
FE=frontonasal mass mesenchyme with epithelium intact
4FB=frontonasal mass mesenchyme plus FGF-4 soaked bead
M=mandibular mesenchyme only
MB=mandibular mesenchyme plus plain bead
ME=mandibular mesenchyme with intact epithelium
4MB=mandibular mesenchyme plus FGF-4 soaked bead

FIGURE 21:

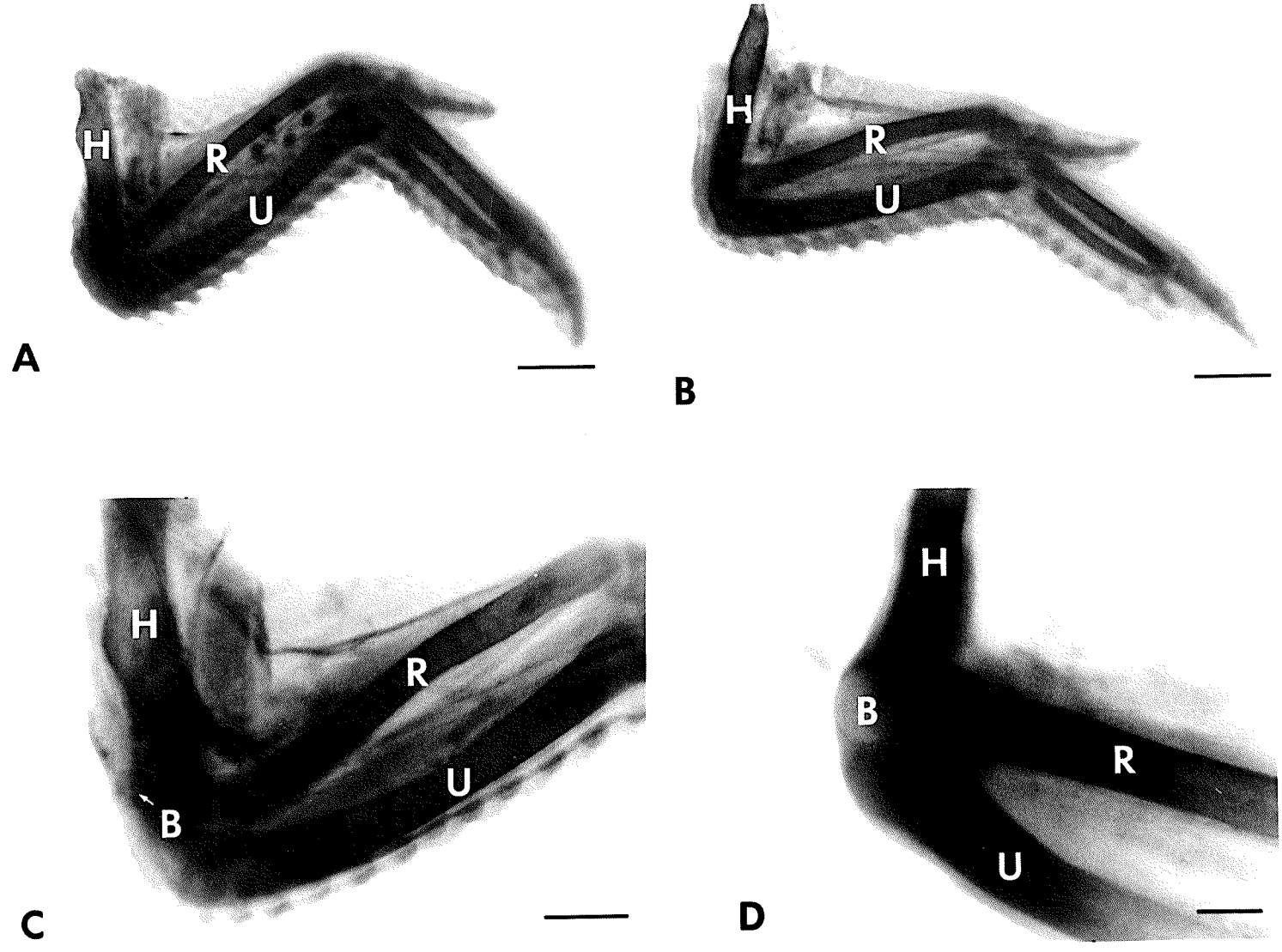
Wholemounts of wings stained with Alcian Blue. H = humerus, R = radius, U = ulna, B = bead.

(A) A graft site was prepared in this wing but nothing else was done. The wing grew normally. Scale bar = 1 mm.

(B) A second example illustrating that preparation of the graft site did not have an effect on the normal development of the wing. Scale bar = 1 mm.

(C) A graft site was prepared and a plain bead was placed. The wing grew normally. Scale bar = 0.5 mm.

(D) In this example of a plain bead placed into a prepared graft site, the bead is clearly seen at the elbow joint and no extra cartilage is present. Scale bar = 0.2 mm.


H
R
U
A
H
R
U
B
H
R
B
U
C
H
B
R
U
D

DISCUSSION

Effects of Surgical Manipulation of the Embryo

Preparation of the graft site generally did not affect normal development of the wing nor did preparation of the graft site plus placement of a saline-soaked bead. Occasionally, the marginal vein was cut during the graft site preparation and a large haematoma formed. These limbs did not show normal growth and pattern formation and were omitted from the study. As the membranes were healing (ie. incision closing), a wing bud would periodically become strangulated by the membranes: these specimens were also eliminated from the study. Thus, the outgrowths and extra cartilages found in the grafted embryos and the embryos receiving an FGF-4-soaked bead were not a result of surgical trauma to the embryo.

The fusion of the elbow joint (complete or partial) was not a result of surgical trauma since it was observed in specimens that received an FGF-4-soaked bead and not as a general finding (which one would expect if fusions resulted from surgical trauma). This phenomenon may be a result of the induction of terminal differentiation by FGF-4 or the FGF-4 disruption of the genes responsible for patterning the elbow. This effect of FGF-4 may help explain the syndactyly found in most of the craniosynostosis syndromes and the fused elbow joints occasionally found in Pfeiffer's Syndrome.

Differences in the Response of Facial Primordia to Exogenous FGF-4

In the frontonasal mass experiments, placement of the FGF-4-soaked bead with the frontonasal mass mesenchyme resulted in development of a cartilage rod that was not statistically different in length from the frontonasal mass mesenchyme with its intact epithelium. In contrast, the mandibular experiments did not show the same response. The FGF-4-soaked bead did not produce a long, straight outgrowth of cartilage like the mandibular mesenchyme with epithelium produced. The length of the cartilage outgrowth was statistically no different from the mesenchyme only grafts. Therefore, the frontonasal mass and the mandible were shown to respond differently to FGF-4. One possible explanation for the difference in response to FGF-4 between primordia may be due to differences in relative stage of development.

At stage 24 when the graft tissues were harvested, the mandible morphologically resembles its final form much more than the frontonasal mass does, suggesting the facial primordia have their own unique time-frames for development. The mandibular tissues used in these experiments may be past the point at which they are sensitive to FGF-4. This difference in sensitivity could be due to receptor types (FGFR1 versus FGFR2), receptor distribution within the tissue,

downregulation of receptors, or efficacy of signal transduction. Similar results were noted in epithelial/mesenchymal recombination experiments (Richman and Tickle, 1992) and tissue cultures with FGF-2 (Richman and Crosby, 1990; Walin, 1993).

The specific sensitivity of different facial prominences may reflect the neural crest origins of the mesenchyme. The above noted tissue culture experiments may also support the hypothesis that tissues of different neural crest origin may respond differently to FGF-4. The frontonasal mass mesenchyme is derived from the area of the prosencephalic-mesencephalic junction whereas the mandibular mesenchyme is derived from the mesencephalic neural crest. Therefore, extremely precise mapping of neural crest migration is needed to verify this hypothesis.

Cartilage Pattern Formation in the Mandible

The pattern of cartilage formation was quite different in the presence of FGF-4. In both the mandibular mesenchyme only and the mandibular mesenchyme with intact epithelium groups, the cartilage rod was straight or slightly curved. In the mandibular mesenchyme plus FGF-4-soaked bead group, the cartilage rods were triangular, round, or substantially curved. The failure of FGF-4 to restore outgrowth development

may indicate that another factor such as FGF-2 is responsible for proliferation within the mandibular mesenchyme. In the *Xenopus* blastocyst, FGF-4 was needed for cell mass differentiation (Feldman *et al.*, 1995) but was not mitogenic (Rappolee *et al.*, 1994). Similarly, in this series of experiments with grafts of mandibular tissues, FGF-4 appears to have a greater effect on cartilaginous pattern development than on outgrowth of the grafts.

Epithelial Effects of FGF-4 - Egg Tooth Formation

In the frontonasal mass grafting experiments, addition of an FGF-4-soaked bead to a frontonasal mass mesenchyme graft produced an outgrowth similar in both length and shape to the outgrowth produced by a frontonasal mass mesenchyme graft with intact epithelium. Differentiation within the frontonasal mass is most easily assessed by the presence of an egg tooth - an ectodermal specialization. The incidence of egg teeth (Table III) was high for both the frontonasal mass grafts with intact epithelium (84.6%) and the frontonasal mass grafts with an FGF-4-soaked bead (92.9%). In both these groups, the presence of an egg tooth appeared to be independent of the extent of cartilage rod outgrowth. In the frontonasal mass mesenchyme grafts and frontonasal mass mesenchyme implanted with saline-soaked beads, an egg tooth was rarely present (64.3% and 28.6%

respectively) if the length of the cartilage rod outgrowth was less than .75 mm in length which indicates that normally, growth precedes differentiation. This finding could suggest that FGF-4 plays a major role in the terminal differentiation of the cells within the ectoderm of the frontonasal mass as well as a minor role in cell proliferation in the mesenchyme.

Release Characteristics of the Beads and Their Relationship to Cartilage Outgrowths

In these experiments, a cut was made into the grafted tissue with the bead placed into the incision. Consequently, the FGF-4 concentration produced in the graft would be uniformly high around the bead, decreasing in all directions moving away from the bead. This location of the bead is in contrast to previous recombination experiments where the epithelium was placed at the surface of the graft which would produce a gradient of epithelial factors that was high at the surface and decreased through the thickness of the graft. This lack of a polarized gradient may explain why the outgrowths in this experiment were shorter than in previous experiments. In addition, the strain of chicken used by Richman and Tickle (1989) was different. In order to achieve similar growth in control grafts in Manitoba, it is necessary to incubate the host embryos for 7 days, not 6. Due to these differences in

the lengths of the controls, the data is not directly comparable to previously published results (Richman and Tickle, 1989; 1992).

An alternate explanation may be that FGF-4 does not have as great an effect on the proliferation of cells of the face as do other epithelial factors (ie. FGF-2, FGF-8). In other words, FGF-2 and FGF-4 may have different roles in the growth and development of the facial primordia, as they do in the wing - FGF-2 stimulates cell proliferation (Richman and Crosby, 1990; Walin, 1993; Munain *et al.*, 1988) while FGF-4 directs patterning and terminal differentiation (Laufer *et al.*, 1994; Niswander *et al.*, 1994). In the intact embryo, the presence of these growth factors is likely to be temporally regulated within each facial primordium by other factors such as the expression of transcription factors.

Receptors For FGF's May Mediate the Effects of Exogenous FGF-4 on Facial Mesenchyme

FGF-2 and FGF-4 have their highest binding affinities to different receptors: FGF-2 binds to FGFR1 (isoform IIIc) and FGF-4 binds to FGFR2 (isoform IIIc). Therefore, the differences observed could be due to the differential transcription of the FGFR1 and FGFR2 genes.

FGF-4 Induces Ectopic Cartilage in the Host Limb Bud

Direct placement of an FGF-4-soaked bead into the prepared graft site resulted in extra bits of cartilage growth in the wing. This effect could be expected as FGF-2 has been shown to induce extra digits when implanted into the anterior margin of the wing bud away from the normal polarizing region (Riley *et al.*, 1993). In my experiments, the position of the bead within the relatively large graft site was variable which accounts for the differences in pattern of cartilage formed. Since extra cartilage was induced in the absence of placing a graft of facial mesenchyme, it is possible that it was difficult to distinguish the donor from host tissue. In the case of the frontonasal mass grafts, the presence of an egg tooth associated with a cartilage rod confirms that it was derived from graft tissue. In the mandible, we are lacking such a marker so I relied instead on the position of the extra cartilage in relation to the host limb. All grafts ultimately are positioned at the elbow of the host limb, therefore, ectopic cartilage found in this location is most likely derived from the grafted tissue.

Future experiments could be designed using donor tissue from quail embryos grafts placed in chick wing buds. Quail cells have distinct nuclei and can be differentiated from chick cells in section using the Feulgen staining method (LeDouarin, 1973).

FGF's and The Cause of Craniosynostosis

Recent studies using DNA sequencing have shown a relationship between mutations in the fibroblast growth factor receptor genes and various craniosynostosis syndromes (figure 22).

Mutations in the third Ig domain of the FGFR2 gene have been identified in Crouzon's Syndrome (Reardon *et al.*, 1994a, 1994b; Rutland *et al.*, 1995; Wilkie *et al.*, 1995; Jabs *et al.*, 1994; Preston *et al.*, 1994), Jackson-Weiss Syndrome (Jabs *et al.*, 1994; Li *et al.*, 1994), and Pfeiffer Syndrome (Rutland *et al.*, 1995). A second genetic variant of Pfeiffer Syndrome is due to a mutation in the FGFR1 gene (Muenke *et al.*, 1994) coding for the link region between Ig II and Ig III. The syndactyly found in most of the craniosynostosis syndromes and the fused elbow joints found occasionally in Pfeiffer's Syndrome may be linked to the fused elbow joints produced by FGF-4 in the present study.

Crouzon's Syndrome, to date, shows the most genetic variability with 9 different mutations producing the Crouzon phenotype (abnormal skull shape due to premature fusion of cranial sutures, prominent eyes, but no digit abnormalities) on 2 different chromosomes - 10q25-q26 and 5q. The 5q mutation (the Boston type of craniosynostosis) is unique in that it affects the *Msx-2* gene, not the FGFR2 gene (Jabs *et al.*,

1993). The FGFR2 mutations are related to the cysteine amino acid at position 342; this is a highly conserved region and the Cys-Cys bonds are needed to maintain the structural integrity of FGFR2. The various mutations may cause a deletion of the cysteine residue, add a new cysteine amino acid which could form a Cys-Cys bond, or cause other amino acid substitutions very close to the critical Cys-Cys bonding region, altering the stereotaxis of the protein.

All of the previously mentioned craniosynostosis syndromes are autosomal dominant. Therefore, the patient is likely to have one normal copy of the gene. As such, to produce the mutant phenotype, it seems most likely that these mutations result in abnormal activation of the receptor causing it to be super-responsive to endogenous FGF's or for it to remain permanently in an activated state. Phenotypic variation may be due to ligand specificity, the genetic background in which the mutation exists (genetic variations at other loci may produce gene products that interact differently with the mutant receptor), or the degree of receptor gene activation during different temporal stages of development.

In mice with syndactyly syndromes, the AER is thickened (Grüneberg, 1960). Could this be due to the AER over-producing FGF's as a result of a disrupted feed-back mechanism?

Fusion of sutures and closure of the growth plates in the long bones normally correspond with the cessation of growth and terminal differentiation of cartilage into bone. These

phenomena would fit with the theory that FGF-4 is involved in terminal differentiation and caused the fusion of the elbow joints in the specimens receiving an FGF-4-soaked bead. Permanent activation of the FGFR2 would produce a similar effect and could explain the premature fusion of the cranial sutures common to the craniosynostosis syndromes.

Proposed Model For the Role of FGF-4 in Patterning Facial Mesenchyme

The round and "doubled back" patterns of cartilage found in the mandibular mesenchyme grafts implanted with FGF-4 beads may represent a duplication of the patterning of the mandibular cartilage and if it is indeed the case, and pattern development of the facial primordia is similar to that in the limb, then the polarizing region of the mandible is most likely located at the proximal ends of the developing mandible, away from the source of the grafted tissue. To test this hypothesis, transplant this putative zone of polarizing activity to the anterior margin of the wing bud and observe its effects. If it is indeed a polarizing region, it should induce duplications within the wing.

The progress zone would likely be located at the distal (= midline) structures of the mandible with the apical ectodermal ridge-like region overlying this. Removal of the

epithelium (mandibular mesenchyme only grafts) resulted in truncated outgrowth of the cartilage compared to the mandibular mesenchyme with epithelium grafts: this alteration is analogous to removal of the AER producing truncated limb outgrowth.

If the zone of polarizing activity within the frontonasal mass is similar to the distribution pattern of endogenous retinoic acid as proposed by Leon-Delgado (1992) (**NB.** Thaller and Eichele (1987) demonstrated a similar retinoic acid gradient in the limb), then placement of the FGF-4 bead in these experiments was into the already existing polarizing region and should not result in alterations of normal pattern development. An interesting future experiment would be to implant a retinoic acid-soaked bead into the nasal pit of a stage 22 embryo for 24 hours (it is thought that retinoic acid prevents the precursor cells from becoming committed to a differentiated phenotype), remove that bead, and then implant an FGF-4-soaked bead into the same nasal pit to see what final facial morphology develops. Using more than one FGF-4 bead initially or adding a fresh FGF-4 bead each day would elucidate the concentration and temporal effects of FGF-4.

FIGURE 22:

Diagram of the genes involved and locations within the FGF receptors of the mutations found in the craniosynostosis syndromes - from Bellus *et al.*, 1995.[3]

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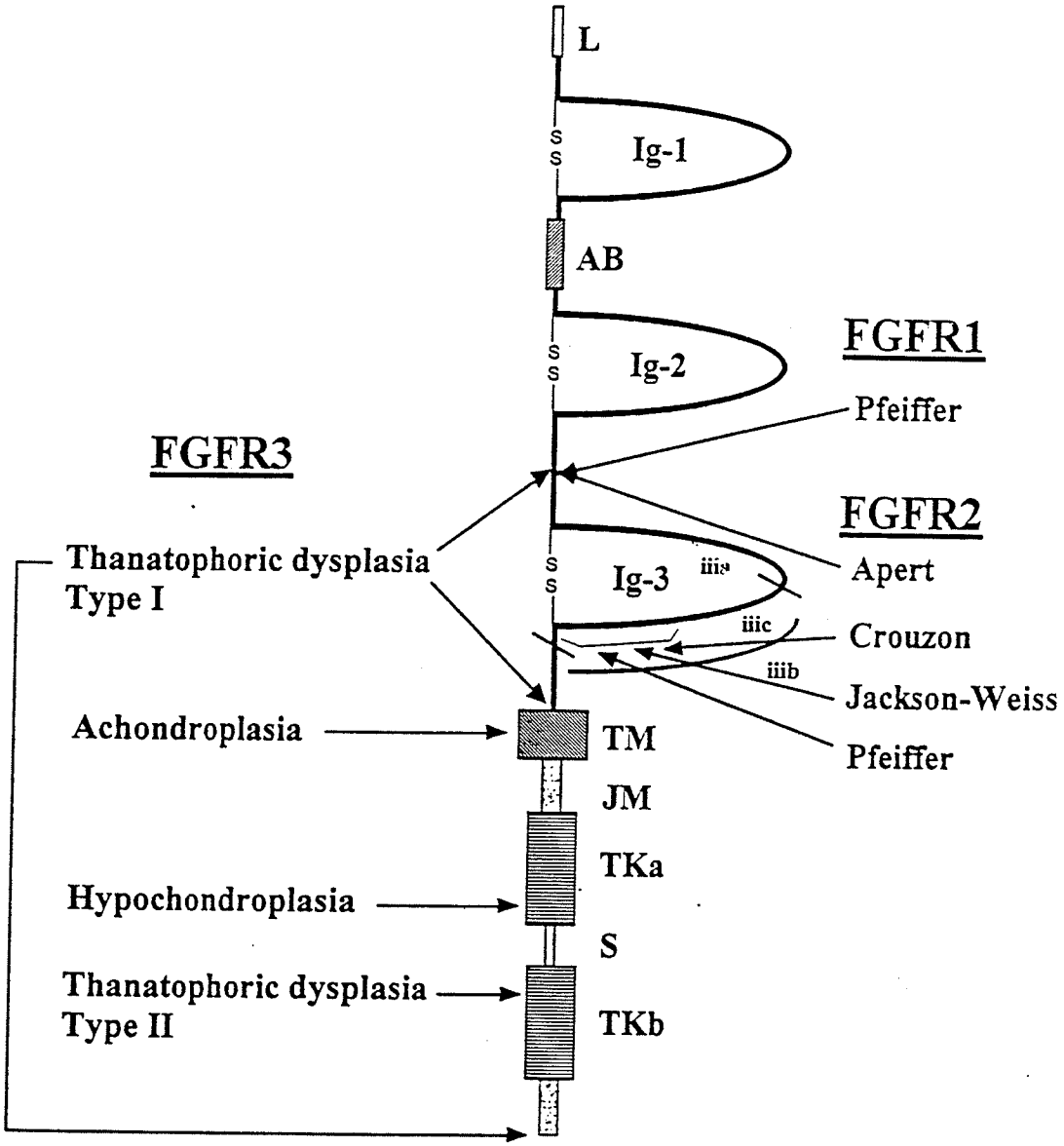
[3] Bellus, G. A., McIntosh, I., Smith, E. A., Aylsworth, A. S., Kaitila, I., Horton, W. A., Greenhaw, G. A., Hecht, J. T., Francomano, C. A. (1995). A recurrent mutation in the tyrosine kinase domain of fibroblast growth factor receptor 3 causes hypochondroplasia. **NATURE GENETICS** *10*, p 358.


L
S
S
Ig-1
AB
S
S
Ig-2
FGFR1
Pfeiffer
FGFR3
FGFR2
Thanatophoric dysplasia
Type I
S
S
Ig-3
IIIa
Apert
IIIc
Crouzon
IIIb
Jackson-Weiss
Pfeiffer
Achondroplasia
TM
JM
TKa
Hypochondroplasia
S
Thanatophoric dysplasia
Type II
TKb

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