EXPERIMENTAL ANALYSES OF THE ROLES OF
THE FIBROBLAST GROWTH FACTOR FAMILY
IN SKELETogenesis

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This Thesis is dedicated to the memory of Peter Thorogood who died during the course of this project. He was the inspiration for so much of this work and instilled in me his infectious enthusiasm for scientific research. I hope he would have enjoyed reading this Thesis.
Abstract

Craniosynostosis is a disease that afflicts approximately 1 in 2500 children worldwide. It is caused by the premature fusion of the cranial sutures which normally function as proliferation centres allowing the expansion of the skull during the growth of the brain and facial region. Affected children have major abnormalities including underdevelopment of the midface, limb defects, raised intracranial pressure, breathing problems as a result of airway restriction and severe learning difficulties. In 1994 a mutation in the Fibroblast Growth Factor Receptor 2 (FGFR2) gene was found in patients with Crouzon syndrome, one of the craniosynostoses and subsequently mutations in FGFRs 1-3 have accounted for many of these syndromes. Very little is known to date about the mechanisms which generate normal and abnormal phenotypes and in this Thesis, a model system has been used to elucidate the developmental pathways responsible.

A grafting technique has been used to manipulate developing embryonic chick crania and perturb morphogenesis. Implantation of beads soaked in the ligand FGF-2 did not affect normal cranial development at biological concentrations. In the limb bud however, these same beads elicited dramatic changes in morphogenesis demonstrating that these beads are biologically active. Implantation of beads soaked in a neutralising antibody to FGF-2 resulted in a graded response. When a single bead is implanted thereby reducing the active levels of endogenous FGF-2 protein, the grafts grew to a massive size as a result of increased cell division in the tissue. By using a technique to detect proliferating cell nuclei immunohistochemically it is clear that in these large grafts almost all nuclei are undergoing cell division whereas in control grafts the opposite is the case. With greater inactivation of FGF-2 protein (2-3 antiFGF-2 loaded beads implanted) further bone differentiation was blocked and the level of cell proliferation was reduced below background levels. It is proposed that a multi-stage signalling cascade operates within the skull such that at low levels of
FGF, proliferation occurs and at higher levels, these cells are further induced to differentiate into bone. Conversely, when FGF is blocked and the amount available to receptors is reduced, cranial bone morphogenesis is prevented. These results relate to the clinical situation since the majority of mutations in FGFRs in patients with craniosynostosis are thought to result in increased receptor activation equivalent to an increase in FGF signalling. Hence the effect is premature differentiation of cranial sutures into bone.
Acknowledgements

Working on this project has been a delight, I remain constantly amazed by my good fortune in obtaining this job and will be eternally grateful that Peter gave me the opportunity to work in such an exciting environment. The first thanks must go to my co-supervisors, Andy Copp and Patrizia Ferretti who have had to take over my project under tragic circumstances. Both have given up their valuable time to help with my final experiments, writing the paper and this Thesis. I hope I haven’t been too much of a burden for them and I remain grateful for their ongoing support and friendship. Special thanks are also due to Paul Hunt who whilst at ICH instilled in me the fundamentals of laboratory practice and taught me so many techniques. He has also freely donated his own time to read various manuscripts.

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## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin-biotin peroxidase complex</td>
</tr>
<tr>
<td>AER</td>
<td>apical ectodermal ridge</td>
</tr>
<tr>
<td>Al₂(SO₄)₃</td>
<td>aluminium sulphate</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indoyl-phosphate</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-Bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>chorio-allantoic membrane</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine tetrahydrochloride</td>
</tr>
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<td>diethylpyrocarbonate</td>
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<td>horseradish peroxidase</td>
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<tr>
<td>HSPG</td>
<td>heparan sulphate proteoglycan</td>
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<td>alpha minimal essential medium</td>
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<tr>
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<td>neural cell adhesion molecule</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>NP40</td>
<td>nonidet P-40</td>
</tr>
<tr>
<td>ns</td>
<td>not significant</td>
</tr>
<tr>
<td>NTMT</td>
<td>sodium chloride/Tris/magnesium chloride/Tween buffer</td>
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</tbody>
</table>
OD  optical density
OIF  osteogenic induction front
PAGE  polyacrylamide gel electrophoresis
PBMT  phosphate buffered saline with Marvel™ and Tween-20
PBS  phosphate buffered saline
PBST/PBT  phosphate buffered saline with Tween-20
PCNA  proliferating cell nuclear antigen
PFA  paraformaldehyde
pmol  picomole
PMSF  phenylmethylsulphonylfluoride
PNPP  p-Nitrophenyl phosphate
RIPA  radioimmune precipitation assay
RNA  ribonucleic acid
RNase  ribonuclease
rpm  revolutions per minute
SDS  sodium dodecyl sulphate
SE  standard error
SEM  scanning electron microscopy
Shh  sonic hedgehog
S phase  synthesis phase
SSC  sodium chloride/sodium citrate buffer
TAE  Tris acetate EDTA electrophoresis buffer
TBS  Tris-buffered saline
TBST  Tris-buffered saline with Tween-20
TE  Tris-EDTA
TEMED  N,N,N',N’tetramethylethylenediamine
TGF-β  transforming growth factor-beta
UTP  uridine tri-phosphate
Chapter One: Introduction

1.1 Skull morphogenesis

The vertebrate skull is a modular structure consisting of three component parts which emerged at different evolutionary timepoints and which appear to use different developmental mechanisms to specify form and pattern (Hanken and Thorogood, 1993). The neurocranium forms the cranial base and the olfactory, optic and otic capsules, the viscerocranium forms the jaws and much of the facial skeleton, and the dermatocranium forms the series of flat bones that comprise the cranial vault and afford protection to the brain.

Historically, skeletal structures were thought to be derived exclusively from mesoderm and this opinion persisted until relatively recently. However, careful fate mapping by a number of groups has now confirmed that the skull is composed of four tissue types derived from two cell mesenchymal lineages. Mesoderm provides one of these lineages but the contribution of mesodermal mesenchyme to skeletogenesis in the head is relatively minor (Couly et al. 1993; Thorogood, 1997). In fact, a considerably greater contribution comes from the second of these two lineages, that derived from the cephalic or cranial neural crest. This so-called 'ectomesenchyme' reflects its ectodermal origin as a transient migratory cell population arising from the margin of the neural plate.

1.1.1 The neural crest

The presence of a distinctive band of cells lying along the developing neural tube close to the ectoderm was first reported by His (1868, quoted in Hörstadius, 1950) who named this band Zwischentrang, the neural crest (Langille and Hall, 1993). He noted that these cells migrated from the neural tube ventrolaterally to form the spinal ganglia. Subsequently, other reports
discussed the possible roles of these cells and their contribution to varying tissue types.

It is now known that the neural crest forms as a thickened region of ectoderm just prior to neural tube closure. Crest cells then become trapped between the closed neural tube and the overlying ectoderm (Fig 1.1) before coming to rest on the dorsal surface of the neural tube. Over the next 24 hours, this apparently homogenous population of cells migrates away from the neural tube along distinct pathways to populate defined structures (Bellairs and Osmond, 1998).

Using vital dye staining and selective extirpation of regions of the neural crest, Hörstadius and Sellman (1946) divided urodele neural crest into regions which contributed to specific skeletal elements of the head. They found that neural crest from different axial levels had distinct chondrogenic capabilities such that trunk neural crest was unable to form cartilaginous structures whereas cranial neural crest could (Hall and Hörstadius, 1988). The route of neural crest emigration in the chick has since been mapped by grafting radioisotopically-labelled cells into host embryos. The migratory pathway has been shown to be dependent on the axial level at which the cells emerge and is highly predictable. Hence, cranial or mesencephalic neural crest migrate cranial, caudal and medial to the developing eye (Johnston, 1966). Trunk neural crest migrate in two streams, dorsolaterally into the superficial ectoderm, or ventrally between the neural tube and the somitic myotome (Hall and Hörstadius, 1988; Weston, 1963). The differentiative fates of neural crest cells are therefore greatly influenced by their migrating pathway and a huge range of cells and tissues are known to be derived from neural crest (Table 1.1).
**Figure 1.1 Formation of the neural crest**

Sections taken through the neural tube at three consecutive stages to illustrate the origin of the neural crest. The arrows show the paths taken by the migrating neural crest cells. nc, neural crest; nf, neural fold; nt, neural tube; not, notochord. From Bellairs and Osmond (1998).
<table>
<thead>
<tr>
<th>Pigment cells</th>
<th>Sensory nervous system</th>
<th>Autonomic nervous system</th>
<th>Skeletal and connective tissue</th>
<th>Endocrine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trunk Crest</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Melanocytes</td>
<td>Spinal ganglia</td>
<td>Sympathetic</td>
<td>Mesenchyme of dorsal fin in amphibia</td>
<td>Adrenal medulla</td>
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<td>Xanthophores</td>
<td>Some contributions to vagal (X) root ganglia</td>
<td>Superior cervical ganglion</td>
<td>Walls of aortic arches</td>
<td>Type I cells of carotid body</td>
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<tr>
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<td></td>
<td>Parasympathetic</td>
<td></td>
<td></td>
</tr>
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<td>Pelvic plexus</td>
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<tr>
<td></td>
<td></td>
<td>Visceral and enteric ganglia</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Some supportive cells</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Glia</td>
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<td>Schwann sheath cells</td>
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<tr>
<td></td>
<td>Some contribution to meninges</td>
<td></td>
<td></td>
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<tr>
<td>Cranial Crest</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Small, belated contribution</td>
<td>Trigeminal (V)</td>
<td>Parasympathetic ganglia</td>
<td>Most visceral cartilages</td>
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</tr>
<tr>
<td></td>
<td>Facial (VII) root</td>
<td>Ciliary</td>
<td>Trabeculae carnea</td>
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<td></td>
<td>Glossopharyngeal (IX) root</td>
<td>Ethmoid</td>
<td>Contributes cells to posterior trabeculae, basal plate, parachordal cartilages</td>
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<td></td>
<td>(superior ganglia)</td>
<td>Sphenopalatine</td>
<td>Odontoblasts</td>
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<td>Vagal (X) root (jugular ganglia)</td>
<td>Submandibular</td>
<td>Head mesenchyme (membrane bones)</td>
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<td></td>
<td>Intrinsic ganglia of viscera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supportive cells</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

**Table 1.1 Major neural crest derivatives**

Adapted from Gilbert (1991).
Although the majority of the visceral and facial skeleton was known to be neural crest-derived (Hörstadius, 1950; Le Douarin, 1982) until recently, uncertainty still surrounded the origins of the parietal and frontal bones. Couly and co-workers have exploited the differences in quail and chick nuclear staining with Feulgen and carried out a number of heterotopic grafts to study the fate of the cephalic neural folds (Couly et al. 1993; Le Douarin, 1973). At the 3-somite stage, defined regions of the neural folds of the paraxial cephalic and rostral somitic mesoderm in the chick were substituted for equivalent tissues from the quail. Analysis of quail cells in the resulting chicken embryos identified the cranial tissues which had originated from the grafted region (Summarised in Fig.1.2 and Table 1.2). Contrary to previous indications, this work proved that the early migration of cephalic neural crest cells entirely contributes to the formation of the dermis and membrane bones of the cranial vault, including the sutures. Substitution of the first six somites of the chick with their quail counterparts has clarified the other controversial theory; that the vertebrae participate to the occipital region of the skull. The first five somites contribute to the occipital bone so that this bone can be considered as a giant vertebra in which the brain rests. The neural arch is therefore represented by the exo-occipital and supra-occipital bones and the corpus of the vertebra by the basioccipital bone (Couly et al. 1993).
Legend for Figure 1.2
Figure 1.2 Schematic drawing of the cephalic skeleton of the chick
(A) Right external view; (B) right internal view. Red, skeleton of neural crest origin; blue, skeleton of cephalic mesoderm origin; green, skeleton of somitic origin. After Couly et al. (1993).
Table 1.2 The Origin of the Cephalic Skeleton
Adapted from Couly et al. (1993).

1.1.2 Mechanisms of Osteogenesis
The bones of the cranium originate by two different pathways although the resulting bone deposition is indistinguishable. The cranial base and a lesser part of the jaw, in addition to all other long bones in the body, form by endochondral ossification. This means literally 'in cartilage' and these structures are formed first as cartilage before later ossifying into bone. The
remaining calvarial and facial bones develop directly from mesenchymal condensates, a process known as intramembranous ossification. A minority of bones ossify by a combination of these two mechanisms, for example, the occipital bone has a dual origin comprising both membrane bone and cartilage.

1.1.2.1 Endochondral Ossification

This method of bone formation enables functional stresses to be sustained during skeletal growth. Condensing mesenchyme in the limbs and jaw form cartilaginous templates enclosed by a perichondrial sheath (Deng et al. 1996; Hamilton et al. 1966; Wong and Tuan, 1995). This cartilage undergoes appositional growth to form a template whose size and shape closely approximates the bone which will ultimately develop in its place. The presence of this cartilage can be confirmed by staining with Alcian Blue which detects a sulphated proteoglycan, aggrecan and, in addition, by detecting expression of Type II Collagen, a major cartilage marker (Wong and Tuan, 1995). Within the shaft of the cartilage template, the chondrocytes enlarge and become hypertrophic, providing the first visual indication of ossification (Deng et al. 1996). These hypertrophic chondrocytes resorb the surrounding cartilage and deposit calcium salts into the extracellular matrix prior to their own destruction. It is at this point that the perichondrium develops osteogenic potential and assumes the title of periosteum (Burkitt et al. 1997). The internal cellular layer of the periosteal sheath releases osteoblasts whose function is to synthesise and secrete osteoid matrix and these rapidly penetrate the calcified matrix. From the inner layer of the periosteum a vascular bud arises and penetrates the calcified matrix of the bone to the centre where a passage termed the irruption canal develops. The associated capillary network that develops from this bud provides an active blood supply to mesenchymal cells which are able to differentiate into either osteoblasts or chondroclasts. As more of the cartilaginous matrix is
disintegrated by the chondroclasts, osteoblasts migrate into the spaces left and orientate themselves along the calcified cartilage attracting further osteoblasts as they do so. The ossification centre extends laterally along the bone replacing degenerated calcified cartilage and establishing the primary or diaphyseal centre of ossification. As diaphyseal ossification reaches completion, giant osteoclasts are released from the vascular bud to constantly absorb and remodel the shape of the bone (Deng et al. 1996; Hamilton et al. 1966).

Towards the end of embryogenesis, the endochondral bones appear as ossified shafts with cartilaginous ends and the process of secondary or epiphyseal ossification takes over. This results in two ossification centres within the bone, the diaphysis and the epiphysis, separated by a layer of cartilage, the epiphyseal plate. Growth of the bone is then able to continue in two directions, in length from the diaphysis and by absorption of existing endochondral bone from the perichondrium to increase bone width and establish a bone marrow cavity. Towards adulthood, the epiphyseal plate thins and remains the focal point for bone growth; upon cessation of growth of any bone, this plate disappears and the epiphysis and diaphysis unite (Burkitt et al. 1997; Hamilton et al. 1966). The progression of endochondral ossification is summarised diagrammatically in Figure 1.3. On completion of endochondral ossification, the bone expresses type I collagen, stains with Alizarin red (a marker of calcified bone) and is histologically identical to bone formed directly from mesenchyme.
Figure 1.3 Endochondral ossification

The progression of endochondral bone formation demonstrated by the development of a long bone. From Burkitt et al. (1997).
1.2.2 Intramembranous ossification

As discussed previously (Section 1.1.2.1) the majority of the calvarial bones form through a process termed intramembranous ossification in which bone is deposited directly, without the formation of a cartilaginous model (Thorogood, 1993). Undifferentiated mesenchyme rapidly proliferates and condenses at defined points within the skull to form the ‘membranes’ in which bone will form. These condensates deposit collagenous fibres extracellularly providing a template upon which differentiating osteoblasts can orientate. Osteoblasts are thought to derive from stromal stem cells which are in turn derived from a sub-population of mesenchymal cells and have features typical of protein-secreting cells (Williams, 1995). Osteoblasts synthesise and deposit large amounts of osteoid matrix and this is rapidly mineralised by the deposition of calcium salts. As the osteoid grows, a proportion of osteoblasts become trapped within the matrix and become osteocytes whose role is subsequently to maintain the bone matrix (Burkitt et al. 1997). There is evidence to suggest that osteoblasts may also indirectly regulate bone resorption since they have receptors for parathyroid hormone, 1,25-dihydroxy vitamin D3, and other stimulants of bone resorption (Williams, 1995). The progress of intramembranous ossification is diagrammatically represented in Fig. 1.4.

As ossification proceeds, mesenchymal transformation into osteoblasts results in two-dimensional growth of the bone from central foci until the majority of bone growth is completed. The proliferative capacity of any remaining mesenchyme then decreases and a number of bone marker genes are expressed. These include alkaline phosphatase, osteocalcin and osteopontin although the expression of alkaline phosphatase is downregulated towards the completion of mineralisation (Stein et al. 1989). This initial stage of bone deposition is completed relatively quickly and is followed by a lengthy period of maturation which continues postnatally. Spongy embryonic bone is surrounded by a layer of compact periosteal bone, a condensed sheet of mesenchyme, the internal layer of which is
1.2.2.2 Intramembranous ossification

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Figure 1.4 Intramembranous ossification

This schematic displays the cellular changes associated with intramembranous ossification. (A) Mesenchymal cells differentiate into osteoprogenitor cells to form condensations within the cranium. (B) These progenitor cells differentiate into osteoblasts which deposit osteoid matrix from their internal surfaces. This matrix is mineralised to form bone. (C) The periosteum is formed on the outer surface of the bone by a compact layer of osteoblasts. Those osteoblasts which become trapped in the matrix form osteocytes and maintain intercellular contacts by means of their surface processes. Blood vessels are also incorporated into the bone at this stage. (D) Osteoclasts continuously remodel bone by resorption of bone apatite and proteolytic degradation of the matrix. bv, blood vessel; cb, calcified bone; m, mesenchyme; ob, osteoblast; oc, osteocyte; ol, osteoclast; os, osteoid. Adapted from (Williams, 1995).
covered with osteoblasts allowing growth by accretion. The original spongy layer of bone is later remodelled by osteoclastic activity (Blair, 1998). This results in spongy or cancellous bone within the central portion of the bone surrounded by more compact bone formed by the continual addition of new ossification layers. All spaces in the cancellous bone are eventually invaded by vascular tissue to become haematopoietic, the basis of red bone marrow. Membrane bones are continually shaped throughout development by the differentiation of mitotic stem cells and their subsequent release from the outer periosteal layer replacing any loss of osteoblasts from the inner periosteum (Hamilton et al. 1966; Williams, 1995). Bone growth is solely appositional, new layers are added to pre-existing surfaces, remodelling of bone therefore depends on a delicate balance between deposition and removal. This allows the growth of the cranium to keep pace with brain expansion during early fetal development; a function eventually taken over by the sutures.

1.1.3 Cranial sutures

The definition of a suture varies depending upon exactly which tissues are included in its structure. Since there is as yet no firm agreement upon this point, there is a certain degree of vagueness surrounding these descriptions. Goss in Gray’s Anatomy states that it is “an articulation in which contiguous margins of adjacent bones are united by a thin layer of fibrous tissue” (Cohen Jr, 1993; Goss, 1959). Probably a more comprehensive definition is provided by Wagemans et al. who describe a suture as “the entire complex of cellular and fibrous tissues lying between and surrounding the opposing edges of two skull bones, including the bony edges” (Wagemans et al. 1988).

The skull must expand in response to the growing brain. In humans, the brain reaches 50% of adult size by 6 months and 80% after 2 years. This enlargement is possible because of the cranial and facial sutures. While the sutures
themselves remain patent, they provide the material necessary for calvarial growth by cell differentiation at their margins (Fig. 1.5) (Cohen Jr, 1993; Hockley, 1993). Although this ability to maintain skull growth is the major function of the sutures, they also play a protective role. They prevent damage to the brain from external forces by acting as an interlocking sheet, which allows a relative degree of movement between bones, but which can absorb mechanical stresses in childhood (Cohen Jr, 1993; Wagemans et al. 1988).

1.1.3.1 The structure of cranial sutures

Sutural development and composition varies not only between different sutures but also within the same suture over time (Cohen Jr, 1993). This is mainly because sutures form an intermediate structure somewhere between bony and soft tissues. Consequently, although there have been numerous microscopic descriptions of sutural morphogenesis and anatomy, the details are often conflicting (Fürtwangler et al. 1985).

As the developing cranial bones approach each other, three distinct layers can be identified. On the dorsal surface lies the ectocranial periosteum, beneath which is a central cambial layer. This is a site of very active osteogenesis and the fibrous capsule which surrounds it must expand to keep pace with the growing bone (Pritchard et al. 1956). On the ventral surface of the cambial layer lies the dura mater which forms the remainder of the fibrous membrane. The dura mater is a dense cellular layer formed from the ectomeninx, the non-vascularised portion of the meninges (Tyler, 1983). The dura mater and the periosteal layer which form the fibrous membrane do not encapsulate the margins of the cranial bones as they do in the facial region. Instead they merge slightly ahead of the advancing bones and the single membrane crosses the presumptive sutural region parallel to the plane of the bones.
In addition to vertical stratification of the mineralised areas, there are three morphological zones evident in cross-section (Fig. 1.5). The inner zone is mineralised and formed from highly differentiated and packed bone, a midzone of inner osteoid accumulation follows and a zone of undifferentiated cells is located peripherally. This zone of cellular differentiation which precedes ossification has been termed the osteogenic induction front (OIF) (Delashaw et al. 1989; Johansen and Hall, 1982).

As the brain grows and expands, the calvarial bones continue to grow along their margins by deposition of osteoid tissue. Osteoid becomes mineralised and transformed into bone and these bone fronts eventually meet in the midline at the suture site (Delashaw et al. 1989). As the OIF proceeds through the membrane, the width of the undelaminated ectomeninx between the bones decreases until the cambial layers of the bones are almost touching. The remaining portion of ectomeninx slightly separates allowing a slightly looser structure to form. Although at this point it would appear that the cambial layers could cross the suture and fuse, this is prevented by the appearance of fibrous capsules which separate the two bones. It is proposed by Pritchard and co-workers that this capsule has developed from peripheral cambial cells which are transformed into fibroblasts. Collagen fibres are deposited around the fibroblasts and sit sagittally, at right angles to the uniting layers of the bones. The structure of the suture at this stage consists of five intervening layers - the first cambial layer and the first fibrous capsule, followed by the loose middle zone and the second fibrous capsule and cambial layer. It also has two uniting layers comprised of fibrous laminae which form as the bone fronts approach each other (Pritchard et al. 1956).

It should be pointed out here that although Pritchard and co-workers have described a five layer structure, the number of sutural layers varies from one
(Weinmann and Sicher, 1955) to three (Moss, 1954) to seven layers (Enlow, 1990; Wagemans et al. 1988) and many investigators simply describe the structure of a suture as "highly variable" (Persson, 1973). Obviously during sutural maturation the number of layers alters becoming highly laminated during development before stabilising in adult life.

The bony edges of a differentiated suture exhibit two forms macroscopically. These have been widely described as end to end (butt, flat or plane) or overlapping (bevelled or squamous) sutures. The shape of these sutures is determined by the plane of passage of the OIF through the loose connective tissue of the skull (Wagemans et al. 1988). It is generally held that end-to-end sutures develop in the midline region of the skull. For example in the human, the sagittal and midpalatal sutures are of the end-to-end type (Cohen Jr, 1993) as are the metopic and internasal in the rat (Koskinen, 1977). All other sutures are of the overlapping type and include the coronal and frontozygomatic (Kokich, 1976; Koskinen, 1977). It has been proposed that midline sutures may be of the end-to-end type because the biomechanical forces on either side of this region are quite likely to be equal in magnitude. Sutures such as the coronal which are located away from the midline are subjected to wide differences in force magnitude which results in an overlapping phenotype.

There appears to be no differentiative activity of cells or fibres to indicate the future location of sutural development (Johansen and Hall, 1982; Wagemans et al. 1988). Markens (1975) however reported the appearance of a characteristic fusiform blastema in humans and rats. He proposed that this temporary structure indicated the site of and the relative vertical position of the bony edges forming the coronal suture (Johansen and Hall, 1982; Markens, 1975; Wagemans et al. 1988). Although the presence of this blastema is not disputed, it appears to be unimportant in determining relative bone position within the suture. Johansen
Figure 1.5 Normal cranial suture development

(A) Dorsal view of a child’s skull, showing the position of the major sutures. Coronal craniosynostosis leads to a short, broad skull; conversely, sagittal synostosis results in a long narrow skull. (B) Diagrammatic cross-section through a coronal suture. The skull bones overlap slightly. OIF, osteogenic induction front. Adapted from Wilkie (1997).
and Hall have indicated that the blastema does not appear until considerably after the initial overlap of the frontal and parietal bones in mice (Johansen and Hall, 1982; Wagemans et al. 1988). This would suggest that at least in this, and likely in other species, the location of the cranial suture is defined long before the growth of the bones and before it is visually identifiable histologically.

### 1.1.3.2 Mechanisms of sutural patency

Very little is known about the mechanisms which underlie sutural development. For example, why do growing bones stop at the edges of a suture when they would be expected to grow across it, and how does a suture know where to form.

Initially, sutural patency is thought to be maintained by avoiding direct physical contact of OIFs by growth of the bones in different planes dorsoventrally. However, midline sutures such as the sagittal, frontal and lambdoid do not show this spatial separation and so cells of adjacent OIFs come into direct contact with each other. A secondary mechanism of separation has therefore been proposed by Joseph Furtwängler and co-workers (Furtwängler et al. 1985) who discovered evidence in the mouse to suggest that apoptosis may prevent the complete ossification of a suture. Sutural cells stained more intensely and once sectioned appeared highly eosinophilic and exhibited an irregular cellular outline. This histological picture strongly suggested that cells at the leading edges of the respective OIFs were undergoing apoptosis, preventing any further growth across the suture. Simultaneously, osteoid tissue and mineralised bone were being deposited on either the dorsal or ventral surface of one of the osteogenic plates. This allowed the pattern of growth to change direction, eventually producing the dorsoventral stratification mentioned earlier. Although this mechanism appears to occur in mice, the stages which exhibited this morphogenesis ranged from 3-7 days postnatally. Since sutural morphogenesis
occurs considerably before this time, it seems unlikely that apoptosis plays a major part in this process. Instead it appears to prevent the premature osseous obliteration of sutures, an essential process if skull growth is to continue into adulthood.

Whether this situation is isolated is unclear. Whilst Furtwängler was the first to attribute his findings to apoptosis, others may already have identified the same process. Distinct concentrations of cells had been mentioned by Moss (1954) who noted an increase in cell density of the sutural tissue as the osteogenic zones approach in the calvaria of the rat. Unfortunately, a direct comparison of these findings is impossible as the cytological properties of these cells were not investigated (Furtwängler et al. 1985). Persson and Roy (Persson and Roy, 1979) studied the formation of the maxillary and palatine bones in 22-24 day old rabbit fetuses and described cells which appeared comparable to the apoptotic cells identified by Furtwängler. In both sutures, the interosseous zone contained cells with darkly stained cytoplasm.

Two mechanisms have been proposed to explain how sutural location is controlled. Strong arguments exist for each hypothesis but recent evidence favours one only.

1. Sutures are formed at the interface of two ossifying plates.
2. Sutural position is determined by a signal originating from the underlying dura mater.

The initial theory was proposed by Moss (1954) and reiterated by Moffet (1965) who stated that calvarial sutures are formed where two bones meet. At these sites, the sutures develop by mechanisms which are presently undetermined (Opperman et al. 1993). Once the mineralised calvaria are in place, further
growth of the bones is achieved by proliferation and ossification at the sutural margins. The trigger for this growth is provided by the expanding brain beneath. As intracranial pressure increases, more pressure is transferred to the skull, in particular to the sutures. The soft tissue present there is affected by tension applied in this way and the result is bone deposition at the margins of the suture to relieve this pressure (Moss, 1954; Opperman et al. 1993; Pritchard et al. 1956; Wagemans et al. 1988).

Tension across facial sutures results in the separation of the bones and the widening of the suture producing an increased rate of bone deposition at the sutural margins. When these forces are removed, the sutures return histologically to their normal structure (Cohen Jr, 1993; Guyman et al. 1980; Jackson et al. 1979). In this way, it is perceived by many that the suture is an adaptive growth centre such that its activity is controlled mainly by surrounding structures (Wagemans et al. 1988).

If this were true however, cessation of growth of the brain would be expected to result in osseous obliteration of the sutures. Although this does occur, there is a delay of 5-10 years between completion of brain growth and fusion of the sutures. This is even more striking when related to the facial region which is fully mature by 20 years but whose sutures remain patent far into the seventh decade, see Table 1.3 (Cohen Jr, 1993). When premature closure of the calvarial sutures occurs it is the compensatory growth potential of the skull which causes any alteration in shape. Any effect on brain growth is caused secondarily by this restriction and not vice versa.
Cranial Suture Closure Begins (Years) Facial Suture Closure Begins (Years)

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<thead>
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<th>Facial Suture</th>
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<tr>
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<td>72</td>
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<td>26</td>
<td>Zygomaticotemporal</td>
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</tr>
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<td>Masto-occipital</td>
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Table 1.3 Suture Closure in Humans

*The metopic suture is usually obliterated by the third year but persists throughout life in 10% of adults. Adapted from Cohen Jr (1993).

The alternative theory stems from work by Markens and Taverne (1978) who propose that from the 21st day of rat gestation, the dura mater contains information as to the future location of the suture (Johansen and Hall, 1982). An osteogenic inhibiting mechanism found in the embryonic suture is transmitted to the dura mater allowing the ongoing maintenance and open state of the suture (Markens and Taverne, 1978). Cranial suture sites are therefore determined by dural reflections. An absence of a specific dural reflection would then culminate in the failure of that suture to form and ossification to occur (Cohen Jr, 1993; Smith and Töndury, 1978). Facial sutures, where the dura mater is absent, do not form in this manner (Cohen Jr, 1993; Pritchard et al. 1956).

Numerous pieces of evidence support this theory. Early work by Pritchard (1946) indicated that the first signs of bone regeneration after calvarial removal were observed at 2 days as clumps of osteoblasts. By 4 days the first flexiform bone was seen on an intact dura mater (Moss, 1954; Pritchard, 1946). Partial or complete removal of the cranial roof without damage to the dura mater results in
complete regeneration of the cranial bones and sutures which develop in their anatomically correct positions (Markens and Taverne, 1978; Opperman et al. 1993; Simpson et al. 1953). Although the dura can control suture position during regeneration of neonatal calvaria, its role in initial bone formation before suture induction is unknown (Opperman et al. 1993).

In mice, rotation of the posterior frontal and sagittal sutures through 180° so that the frontal suture lies over the sagittal dura mater and vice versa provided stronger evidence for the role of the dura in controlling sutural patency. Rotation of these sutures results in fusion of the sagittal suture (which normally remains patent) and continued patency of the frontal suture (typically fused by 24 days). In addition, introduction of an impermeable barrier between the dura and frontal suture delays osseous obliteration (Bradley et al. 1996; Roth et al. 1996). Organ cultures of cranial sutures indicate that signalling from the dura mater controls sutural patency. When cultured in defined media with dura mater present, all sutural grafts developed as would be expected in vivo. Removal of the dura, however, results in prolonged patency of frontal sutures which normally fuse both in vitro and in vivo (Bradley et al. 1996). The culture of rat coronal sutures in a similar system has indicated that the signal transmitted from the dura mater is heparin-soluble. Semi-permeable filters located between dura and dermis allow normal coronal sutures to develop, cranial rudiments cultured without dura show no such sutural development (Bradley et al. 1996; Opperman et al. 1993).

However, the form in which the signal is transmitted between the suture and dura has yet to be clarified. Van Limborgh suggested that sutural growth was controlled mainly by local epigenetic factors found in the skull cartilages and nearby structures and that intrinsic genetic factors played little or no part (Van Limborgh, 1970; Van Limborgh, 1972). Local environmental factors acting as tensile and compressive forces may also affect sutural growth. Oudhof (1982)
used transplantation experiments to affirm that the location and structure of sutures are determined hereditarily (Wagemans et al. 1988), however he puts greater importance on the role of environmental factors in the final determination of sutural morphogenesis. It appears therefore that there may be a multitude of influences on the normal development of a suture with the balance between various factors altering with circumstances. The role of signalling molecules such as growth factors in the development and maintenance of sutural patency is now believed to be quite significant and will be discussed in more detail later on in this Chapter.

1.2 Craniosynostosis

Craniosynostosis was the term introduced by Rudolf Virchow in 1851 to describe the premature fusion of the calvarial bones (Virchow, 1851). In 1791, Sömmering had previously observed that skull growth occurred along calvarial sutures and that failure of growth at a particular suture resulted in cranial deformity, although without naming the condition (Delashaw et al. 1989; Sömmering, 1839). Technically speaking, craniosynostosis is the process of premature sutural fusion and craniostenosis the effect, although the former term is now used to describe both processes.

To date, more than 100 causally related syndromic forms of craniosynostosis have been identified and still more which are non-syndromic combining in an incidence of approximately 1 in 2500 births; in 10-20% of these cases there is evidence of Mendelian inheritance (Cohen Jr, 1993; Hockley, 1993; Reardon and Winter, 1995). The causes of craniosynostosis are numerous, ranging from monogenic disorders to metabolic diseases and hence the pathology of the disease is highly heterogeneous (Table 1.4). Although it is caused mainly by primary congenital disorders it can also be secondary to inadequate brain growth.
as in microcephaly or appear after shunt operations for hydrocephalus (Cohen Jr, 1993; Hockley, 1993). Teratogenic exposure during pregnancy is known to cause craniosynostosis in humans with substances such as retinoic acid, aminopterin and phenytoin identified as key teratogens (Cohen Jr, 1993; Hockley, 1993). Animal models of craniosynostosis have been produced by an excess dose of vitamins A and D, and ethanol administration maternally (Hockley, 1993).

<table>
<thead>
<tr>
<th>CAUSES</th>
<th>EXAMPLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monogenic Conditions</td>
<td>Syndromic – Crouzon, Pfeiffer, Isolated Craniosynostoses</td>
</tr>
<tr>
<td>Chromosomal Disorders</td>
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</tr>
<tr>
<td>Metabolic Disorders</td>
<td>Hyperthyroidism, Rickets</td>
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<tr>
<td>Mucopolysaccharidoses</td>
<td>Mucolipidosis III</td>
</tr>
<tr>
<td>Haematological Disorders</td>
<td>Thalassaemias, Sickle Cell Anaemia, Congenital Haemolytic Icterus, Polycythemia Vera.</td>
</tr>
<tr>
<td>Teratogens</td>
<td>Aminopterin, Diphenylhydantoin, Retinoic Acid, Valproic Acid</td>
</tr>
<tr>
<td>Malformations</td>
<td>Microcephaly, Encephalocele, Holoprosencephaly</td>
</tr>
</tbody>
</table>

Table 1.4 Known Causes of Craniosynostosis
Adapted from Cohen Jr (1993)

It was originally thought that calvarial sutures did not play a major role in the determination of skull shape (Cohen Jr, 1993; Delashaw et al. 1989; Moss, 1959). Instead it was suggested by Moss that the deformation of the cranial base is the primary cause of craniofacial abnormalities resulting in secondary fusion of the cranial sutures. The evidence for this line of thought was based on several observations. First, cranial vault abnormalities can occur in the absence of a fused suture and second, skull growth in the rat is unaffected by removal of a
cranial vault suture. In addition, cranial base abnormalities have been associated with craniosynostoses particularly in patients with sagittal or bicoronal synostosis (Cohen Jr, 1993; Delashaw et al. 1989; Moss, 1959; Persing et al. 1991). This theory held great weight for several years until a number of experimental studies disproved it. Albright and Byrd concluded in a histological survey of craniosynostotic sutures that in sagittal craniosynostosis, skull base changes are secondary to the fusion along the sagittal suture (Albright and Byrd, 1981). In addition, there are many documented cases where familial, isolated, non-syndromic craniosynostoses have been observed (Cohen Jr, 1993; Cohen, 1977). In some families the degree of fusion in individuals is variable and it is difficult to explain why dramatically different abnormalities of the cranial base are seen in the same family as a dominant trait. Obviously a different mechanism must be operating in such cases.

As the pathogenesis of the craniosynostosis is so variable, even within the same syndrome, the extent to which the skull is deformed and associated problems arise must be individually assessed. Generally speaking, however, the severity of the skull deformity rises with an increase in the number of sutures prematurely synostosed. Early synostosis invariably results in raised intracranial pressure as the growing brain puts pressure on the fixed skull and there is some evidence that chronic raised intracranial pressure may result in mental retardation. Hydrocephalus is common in cases where more than one suture has closed and usually presents within 5 years of birth. Blindness may result if papilloedema caused by optic atrophy is not detected quickly. Many of the syndromes which have been identified exhibit additional deformities particularly involving the digits and facial region with the latter often resulting in airway obstruction. These patients present significant problems to clinicians despite important advances in surgical techniques over the past 20 years (Wilkie, 1997).
The most common craniosynostosis syndromes are Apert (incidence 1 in 10,000), Crouzon (1 in 60,000); Pfeiffer and Saethre-Chotzen (incidence unknown) and Jackson-Weiss (1 in 26,000). Achondroplasia (1 in 26,000) and Thanatophoric Dysplasia (1 in 20,000) are skeletal dysplasias but are included because of their close relationship with craniosynostoses, a point which will be discussed later.

Apert, Crouzon, Pfeiffer, Saethre Chotzen and Jackson-Weiss syndromes are primarily inherited in an autosomal dominant pattern with sporadic cases appearing from novel mutations. A number of features are shared between these syndromes as summarised in Table 1.5; a tower shaped skull, widely spaced protruding eyes, a hooked nose and underdeveloped midface are characteristic of these craniosynostoses (Muenke and Schell, 1995; Reardon and Winter, 1995). However, although these dominant conditions are fully penetrant, the degree to which the features are expressed remains highly variable (Lewanda et al. 1994) and consequently it is often the case that clinical diagnoses are made on the basis of hand and foot malformations.

Apert syndrome sufferers present with severe syndactyly (cutaneous and bony fusion of the digits) whilst the facial features show some similarity in Crouzon syndrome but the digits remain unaffected (Hockley, 1993; Wilkie et al. 1995). Pfeiffer syndrome is distinguished from other craniosynostoses by broad thumbs and great toes whereas the lack of hand abnormalities is diagnostic of Jackson-Weiss patients (Muenke and Schell, 1995; Reardon and Winter, 1995). Indeed, the true birth prevalence of the craniosynostoses is probably much higher as patients who are mildly affected may never come to medical attention (Lewanda et al. 1994).
<table>
<thead>
<tr>
<th>Phenotype</th>
<th>HCH</th>
<th>ACH</th>
<th>TD</th>
<th>PS</th>
<th>AS</th>
<th>JWS</th>
<th>CS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short limb dwarfism</td>
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<tr>
<td>Cloverleaf skull</td>
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<td>Underdeveloped midface</td>
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<td>+</td>
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<td>Craniosynostosis</td>
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<tr>
<td>Foot anomalies</td>
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<td>+</td>
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<tr>
<td>Hand anomalies</td>
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</table>

**Table 1.5 Phenotypes in skeletal disorders**

HCH, hypochondroplasia; ACH, achondroplasia; TD, thanatophoric dysplasia; PS, Pfeiffer syndrome; AS, Apert syndrome; JWS, Jackson-Weiss syndrome; CS, Crouzon syndrome. Adapted from Muenke and Schell (1995).

In 1994, the first report emerged linking mutations in Fibroblast Growth Factor Receptor 2 (FGFR2) with Crouzon syndrome (Reardon et al. 1994).

Subsequently, six of the major craniosynostosis syndromes (Apert, Beare Stevenson, Crouzon, Jackson-Weiss, Muenke and Pfeiffer) have now been associated with mutations in FGFR 1, 2 and 3 (reviewed Burke et al. 1998; Muenke and Schell, 1995; Wilkie, 1997).
1.3 The Fibroblast Growth Factor family

Fibroblast Growth Factors (FGFs) are low molecular weight polypeptides which function as mitogens and differentiation factors. They were originally isolated in the 1970s from bovine brain extracts for their ability to promote angiogenesis (Szebenyi and Fallon, 1999). To date, at least 19 structurally related FGFs have been identified (see Table 1.6), all with distinctive functions. The prototype members, originally named acidic (FGF-1) and basic FGF (FGF-2) differ somewhat from the rest of the family in that they are widely expressed during embryonic development and lack a signal peptide sequence (Baird, 1994; Givol and Yayon, 1992). Although this would appear to prevent the secretion of these FGFs, they have been detected extracellularly and novel export pathways have now been proposed (Florkiewicz et al. 1995). The diversity of this family is increased further through the use of alternative initiation codons for translation and alternative splicing of the gene sequence (Mason, 1994). Post-translational modifications are also thought to play a role in FGF activity with several family members known to be glycosylated. The role of FGFs in limb and skeletal development will be discussed in more detail later in this Chapter (Section 1.4). However, the action of FGFs are not just restricted to these tissues and it is prudent to provide some evidence of such diversity.

A fundamental function of FGF-2 was described by Slack and co-workers (Slack et al. 1987) who investigated the role of this factor in the induction of mesoderm in *Xenopus*. Ectoderm was removed from the animal pole of *Xenopus* blastulae and exposed to FGF-2. Normally, these cells would differentiate into epidermis or remain undifferentiated. However, at low concentrations of FGF-2, ventral mesoderm was clearly induced. Endogenous FGF-2 has since been isolated from *Xenopus* embryos and its mesoderm-inducing activity can be neutralised by antiFGF-2 antibodies (Slack et al. 1989).
<table>
<thead>
<tr>
<th>FGF</th>
<th>Gene or protein pseudonyms</th>
<th>Chromosomal map location</th>
<th>Associated functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF-1</td>
<td>acidic fibroblast growth factor; endothelial cell growth factor; heparin-binding growth factor 1; ECGF; ECGFA; ECGFB; HBGF1; aFGF</td>
<td>5q31</td>
<td>Endothelial cell migration and proliferation; Angiogenesis; Astrocytomas Delayed-early gene activation;</td>
</tr>
<tr>
<td>FGF-2</td>
<td>basic fibroblast growth factor; bFGF; HBFG2</td>
<td>4q25</td>
<td>Angiogenesis; Astrocytomas; Limb development; Neurogenesis</td>
</tr>
<tr>
<td>FGF-3</td>
<td>Oncogene int2</td>
<td>11q13</td>
<td>Mouse mammary carcinoma; Formation of the inner ear; spatial patterning processes</td>
</tr>
<tr>
<td>FGF-4</td>
<td>Oncogene hst; FGF-related oncogene hstfl; K-FGF</td>
<td>11q13</td>
<td>Human stomach cancer; Melanoma; Teratoma; Germ cell tumours; Kaposi's sarcomas; spatial patterning processes; Limb development</td>
</tr>
<tr>
<td>FGF-5</td>
<td>Oncogene fibroblast growth factor 5</td>
<td>4q21</td>
<td>Inhibitor of hair elongation</td>
</tr>
<tr>
<td>FGF-6</td>
<td>Oncogene hst2</td>
<td>12p13</td>
<td>Muscle development</td>
</tr>
<tr>
<td>FGF-7</td>
<td>Keratinocyte growth factor (KGF)</td>
<td>15q-q21</td>
<td>Epidermal growth and wound healing; Branching morphogenesis of lung, salivary and prostate gland</td>
</tr>
<tr>
<td>FGF-8</td>
<td>Androgen-induced growth factor (AIGF)</td>
<td>10q25-q26</td>
<td>Androgen-dependent tumour cell proliferation; Midbrain development; Gastrulation</td>
</tr>
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<td>FGF-9</td>
<td>Gliia-activating factor (GAF)</td>
<td>13q12</td>
<td>Gliial cell proliferation</td>
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<td>FGF-10</td>
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<td>5p12-p13</td>
<td>Limb and lung development; wound healing</td>
</tr>
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<td>FGF-11</td>
<td>Fibroblast growth factor homologous factor-3 (FHF-3)</td>
<td>17p12</td>
<td>Development of the nervous system</td>
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<td>FGF-12</td>
<td>Fibroblast growth factor homologous factor-1 (FHF-1)</td>
<td>3q28</td>
<td>Limb, heart and neural tube development</td>
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<tr>
<td>FGF-13</td>
<td>Fibroblast growth factor homologous factor-2 (FHF-2)</td>
<td>Xq26</td>
<td>Limb, heart and neural tube development</td>
</tr>
<tr>
<td>FGF-14</td>
<td>Fibroblast growth factor homologous factor-4 (FHF-4)</td>
<td>13</td>
<td>Limb, heart and neural tube development</td>
</tr>
</tbody>
</table>
Table 1.6 Nomenclature and functions of the Fibroblast Growth Factor family

This table summarises the FGFs identified to date, their human chromosomal map location (where known), and associated functions.


<table>
<thead>
<tr>
<th>FGF</th>
<th>Gene or protein pseudonyms</th>
<th>Chromosomal map location</th>
<th>Associated functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGF-15</td>
<td></td>
<td>nd</td>
<td>Brain development</td>
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<td>FGF-16</td>
<td></td>
<td>nd</td>
<td>Development of heart and brown adipose tissue; oligodendrocyte and hepatocellular proliferation</td>
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<td>FGF-17</td>
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<td>nd</td>
<td>Gastrulation</td>
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<td>FGF-18</td>
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<td>nd</td>
<td>Mesoderm patterning</td>
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<td>FGF-19</td>
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<td>nd</td>
<td>Brain development</td>
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</tbody>
</table>
Roles have also been proposed for FGFs in the regionalisation of the midbrain during chick development. FGF-2 is expressed by the notochord and its presence is required for the induction of En-1, the earliest marker of midbrain tissue (Shamim et al. 1999). Subsequently, FGF-8 is induced by En-1 and is expressed by the isthmus, providing a polarising signal for the developing midbrain. The introduction of ectopic FGF-8 into the posterior diencephalon is sufficient to respecify this tissue into midbrain. The normal function of FGF-8 is thought to be in maintaining patterns of gene expression in the developing midbrain (Shamim et al. 1999).

FGF-7 is implicated in the development of several organs which form by branching morphogenesis. Transcripts for FGF-7 have been detected in lung and salivary gland mesenchyme during epithelial development (Mason et al. 1994) and it is also thought to be involved in the formation of the prostate gland (Sugimura et al. 1996). Normally, development of the prostate epithelium is regulated by testosterone which is able to induce FGF-7 expression in the underlying mesenchyme. Addition of a neutralising antibody to FGF-7 inhibits the branching morphogenesis of the prostate epithelium whereas addition of ectopic FGF-7 stimulates epithelial development in the absence of hormone (Sugimura et al. 1996). FGF-10 is also thought to play a role in lung morphogenesis; mesodermal expression of this factor regulates endodermal proliferation and lung bud outgrowth (Bellusci et al. 1997).

This brief overview of FGF bioactivity provides only a small range of their functions during development. Several FGFs are known to be expressed at the same time in developing tissues and there must be a level of redundancy between family members. This will be discussed later in the Thesis (Chapter 10).
1.3.1 FGFRs

The transduction of FGF signals to the cytoplasm is mediated by a group of high affinity tyrosine kinase receptors – the FGFRs (Muenke and Schell, 1995). These receptors are characterised by multiple extracellular immunoglobulin (Ig)-like loops maintained by disulphide bridges, an acid box between Ig-loops I and II, a single transmembrane domain and a split intracellular tyrosine kinase domain (Fig. 1.6). Although only 4 FGFRs have been identified, alternative splicing increases the number of receptors available and each splice variant has variable ligand binding specificities (Table 1.7). This ligand-receptor promiscuity makes the relationships between FGFs and FGFRs difficult to analyse.

FGF-FGFR binding and activation requires the presence of a co-factor, heparan sulphate proteoglycan (HSPG). HSPGs consist of sulphated glycosaminoglycans (GAGs) covalently bound to a core protein (Szebenyi and Fallon, 1999). This requirement for HSPG is clearly illustrated by mutant Chinese hamster ovary (CHO) cells in which FGF-2 cannot bind to its receptor due to defective GAG metabolism (Aviezer et al. 1994; Yayon et al. 1991). There is also evidence to suggest that HSPG binds a specific sequence in the extracellular domain of the ligand and once bound, protects the growth factor from degradation, thereby enhancing its activity. The developmental significance of the sequestration of FGF within the extracellular matrix is that it creates a reservoir of FGF and provides an immediate source of growth factor for cells to use (Ruoslhti, 1989). Aviezer and co-workers (1994) have suggested that in the case of FGF-2 at least, perlecan appears to be the HSPG involved in sequestering the growth factor to permit receptor binding. The HSPG-ligand complex is able to bind several receptor molecules simultaneously resulting in FGFR dimerisation (Spivak-Kroizman et al. 1994).
Figure 1.6 Generalised structure of an FGFR

A schematic diagram of the general structure of the FGFRs. CAM, neural cell adhesion molecule binding site; CHD, CAM homology domain. Adapted from Green et al. (1996).
<table>
<thead>
<tr>
<th>FGFR Variant</th>
<th>FGF-1</th>
<th>FGF-2</th>
<th>FGF-3</th>
<th>FGF-4</th>
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**Table 1.7 Promiscuity of FGF/FGFR binding**

The following table summarises the published data from direct binding experiments and biological assays (as measured by the mitogenic responses of BaF3 cells expressing various FGFRs to various FGFs). Biological assays: *** >75%; ** 40-75%; * 10-40%; - <10%. Direct binding: +++ >75%; ++ 20-75%; + 10%, ± <10%; 0 no response. Data taken from Chellaiah et al. 1994; MacArthur et al. 1995; Mathieu et al. 1995; Ornitz and Leder, 1992; Vainikka et al. 1992; Werner et al. 1992.
Receptor dimerisation leads to an increase in kinase activity ultimately causing autophosphorylation and biological signalling. Mutations in cysteines in the extracellular domain of FGFRs resulting in intermolecular disulphide bonds (i.e. dimerisation) produce constitutively activated receptors. This illustrates a possible mechanism by which dimerisation can initiate signal transduction and will be discussed in relation to craniosynostosis later in the Chapter (Neilson and Friesel, 1995).

It should be mentioned at this point that FGFs are not the only ligands able to bind and activate FGFRs. Cell adhesion molecules (CAMs), in particular N-CAM and L1 have been shown to promote axonal growth by activating the FGFR in neurons (Doherty and Walsh, 1996; Green et al. 1996). Activation of N-CAM and L1 leads to phosphorylation of the FGFR and neurite outgrowth, responses which are lost when a kinase-deleted, dominant negative form of FGFR1 is expressed in PC12 cells (Saffell et al. 1997).

1.3.2 FGFR mutations and craniosynostosis

Although the functional consequences of the various \(FGFR\) mutations associated with craniosynostosis remain to be elucidated, an increasing body of evidence suggests that these mutations result in constitutive activation of the receptor. Primarily, \(FGFR\) mutations cannot be loss-of-function because chromosomal deletions [del(8p), del(10q), del(4p)] which result in multiple anomalies do not include craniosynostosis (Cohen, 1997). Loss of FGFR3 in mice causes prolonged and rapid bone growth, a phenotype which sharply contrasts with the dwarfism typically seen in skeletal dysplasias (Deng et al. 1996). The production of chimeric FGFR3 containing a mutation found in achondroplasia confirms this theory. This point mutation causes ligand-independent activation of the tyrosine kinase activity of FGFR3 and results in increased levels of phosphotyrosine on the receptor itself (Webster and Donoghue, 1996). Further experiments have
indicated that the transcription factor Stat1, is constitutively activated in cells transfected with an engineered mutation in FGFR3 found in patients with thanatophoric dysplasia (Su et al. 1997). In addition, an increased number of apoptotic chondrocytes have been detected in fetuses with thanatophoric dysplasia, suggesting that their differentiative fate is altered by FGFR3 mutations (Legeai-Mallet et al. 1998). The severity of the phenotype seen in patients with skeletal dysplasias has also been linked to the level of FGFR3 activation. Thanatophoric dysplasia is invariably lethal and such mutations are more strongly activating than those which cause achondroplasia (Naski et al. 1996).

It seems likely that FGFR mutations in syndromic craniosynostosis also result in constitutive activation of the receptor, since the introduction of FGFR2 containing a Crouzon-type mutation into Xenopus embryonic animal pole explants leads to FGF-independent induction of mesoderm (Neilson and Friesel, 1995). Moreover, FGFR/Neu chimeric receptors incorporating various Crouzon-type mutations have an increased tyrosine kinase activity (Galvin et al. 1996). There is also experimental evidence to suggest that such mutations may increase the affinity of FGFRs for ligand, enabling the activation of signalling where the availability of ligand is limiting. Engineered Apert mutations, compared with wild-type, exhibit a selective decrease in the disassociation kinetics of FGF-2, but not of other ligands. Another mutation in FGFR2, previously identified in asymptomatic individuals showed wild-type kinetics (Anderson et al. 1998). Biochemical analysis of calvarial cells taken from Apert fetuses indicated that the expression of both alkaline phosphatase and type I collagen were 2-10 fold greater than normal. This suggests that Apert FGFR2 mutations result in an increase in the number of precursor cells which enter the osteogenic pathway, ultimately leading to an increase in bone formation and premature cranial ossification during fetal development (Lomri et al. 1998).
Targeted disruption of \textit{FGFR1} and 2 in mice both result in homozygous mutant embryos which die just prior to gastrulation (Arman et al. 1998; Deng et al. 1994; Yamaguchi et al. 1994). One group has overcome the problem of embryonic lethality and functional redundancy by producing mice which express a secreted dominant-negative FGFR. This mutant receptor can bind to a wide subset of FGFs and hence disrupt the signalling of virtually all FGFR isoforms \textit{in vivo} by reducing the availability of ligand. These mice have craniofacial and limb abnormalities reminiscent of human skeletal disorders which are associated with FGFR mutations (Celli et al. 1998).

FGF-2 knockout mice are morphologically normal (Zhou et al. 1998), however, over-expression of FGF ligands in mice is providing information about their functions \textit{in vivo}. A retrovirally-mediated insertional mutation in the intragenic region between the \textit{Fgf3} and \textit{Fgf4} genes (high affinity ligands for \textit{FGFR2}) has led to the production of the \textit{Bulgy-eye (Bey)} mouse. This mutation results in the up-regulation of both \textit{Fgf3} and \textit{Fgf4} in the cranial sutures and phenotypically the \textit{Bey} mouse shows features comparable to those found in Crouzon syndrome (Carlton et al. 1998). Overexpression of FGF-2 in mice also results in skeletal malformations. These included shortening and flattening of the long bones, malformed ribs and vertebrae and most interestingly, enlarged calvaria (macrocephaly) (Coffin et al. 1995). These data provide indirect evidence for the theory that \textit{FGFR} mutations in humans are constitutively activating.

\textbf{1.3.3 Other genes involved in craniosynostosis}

Although FGFR mutations are known to be responsible for the majority of syndromic craniosynostoses, two other genes have been identified in craniosynostotic patients. The first is the homeobox gene \textit{MSX2} (muscle segment homeobox 2) which is mutated in Boston-type craniosynostosis. This syndrome is restricted to a large three-generation family and has a variable and non-specific phenotype (Jabs et al. 1993; Wilkie, 1997). The mutation in \textit{MSX2}
is also thought to result in a gain of function of the gene. Transgenic mice engineered with the same mutation in Msx2 exhibit precocious fusion of the cranial bones (Liu et al. 1995) and evidence suggests that this may partly be due to enhanced DNA binding activity (Ma et al. 1996). A recent study has found that ectopic Msx2 overexpression inhibits calvarial osteoblast differentiation in vitro. In contrast, expression of antisense Msx2 RNA decreased proliferation and accelerated differentiation (Dodig et al. 1999). One role of Msx2 in vivo may be to inhibit differentiation and stimulate the proliferation of osteoblastic precursors in the osteogenic front at cranial sutures.

Mutations in FGFR2 have already been described in patients with Saethre-Chotzen syndrome. However, recent work has linked another gene, TWIST, to a subset of patients with this disease (El Ghouzzi et al. 1997; Howard et al. 1997). The Twist gene product is a basic-helix-loop-helix (b-HLH) transcription factor required by head mesenchyme for cranial neural tube morphogenesis in mice. Homozygous null embryos fail to close their neural tubes (Chen and Behringer, 1995), however, heterozygous embryos exhibit a moderate phenotype including minor skull and limb abnormalities (Bourgeois et al. 1998). These transgenic mice provide evidence that mutations of TWIST in humans cause a loss of function of this gene (haploinsufficiency) (Wilkie, 1997). This is in sharp contrast with FGFR2 mutations in Saethre-Chotzen which are likely to be gain of function and illustrates the genetic heterogeneity of this disease (Paznekas et al. 1998). The genes known to be mutated in different forms of craniosynostosis and their proposed mechanisms of action are illustrated in Table 1.8.

The linkage between FGFRs, Msx2 and Twist in terms of normal skeletogenesis is not yet known. It is thought that expression of one the Drosophila FGFRs, DFR1, may be dependent on Twist (Casal and Leptin, 1996; Shishido et al. 1993) although there remains disagreement over whether Twist down-regulates or up-regulates FGFRs in humans (Paznekas et al. 1998).
Mechanism | Example | Associated Syndrome
--- | --- | ---
Haploinsufficiency | TWIST | Saethre-Chotzen
Reduced disassociation of ligand | MSX2 (DNA binding) FGFR2 (FGF-2 binding) | Boston-type Apert
Covalent cross-linking of cysteines | FGFR2 | Crouzon, Pfeiffer
Transmembrane hydrogen bonding | FGFR2 FGFR3 | Unclassified Crouzon/acanthosis nigra

Table 1.8 Proposed mechanisms of dominance in craniosynostosis mutations
Adapted from Wilkie (1997).

1.4 Expression of FGF and FGFRs during skeletal development

Despite the accumulating evidence regarding the molecular pathology of craniosynostosis, the functional role of FGF-mediated signalling in the skeletogenic differentiation of cranial neural crest cells has been largely neglected. Historically, attention had been focused principally on other growth factors – particularly Transforming Growth Factor-βs (TGF-β) (Hall and Ekanayake, 1991). Although these are now thought to play some role in sutural patency, they have yet to be linked with craniosynostosis syndromes (Cohen, 1997).

1.4.1 FGFs and limb development

The most comprehensively studied tissue in relation to the expression and function of FGFs has been the developing chick limb. From expression studies and classical experimental manipulations, the role of FGFs in limb specification, outgrowth and differentiation is now known to be fundamental and may explain why limb malformations are commonly associated with FGFR mutations in humans. Transgenic embryos lacking the entire immunoglobulin-like domain of
Fgfr2 do not form limb buds (Xu et al. 1998). These homozygous mutant embryos die between E10 and 11, contrasting with a previously published report indicating that Fgfr2 knockout mice died much earlier (Arman et al. 1998). It is therefore suggested that this targeted deletion may not result in a completely null phenotype.

To date, FGFs 2, 4, 8, and 10 and FGFRs 1-3 have all been detected at critical stages of limb morphogenesis (Xu et al. 1998). FGFs 2, 4 and 8 are expressed by the apical ectodermal ridge (AER) at the tip of the developing limb bud, a region thought to control limb outgrowth (Tickle, 1996). In both humans and mice, Fgfr2 mRNA is expressed in limb ectoderm whereas Fgfr1 is diffusely localised throughout the underlying mesenchyme (Delezoide et al. 1998; Peters et al. 1992). At later stages of development when mesenchymal cells differentiate into chondrocytes, expression of FGFR1 and 2 is lost and restricted to the perichondrium whereas FGFR3 transcripts remain abundant (Delezoide et al. 1998). This pattern of expression differs from that reported in mice since FGFR3 can not be detected in chondrocytes at this stage (Deng et al. 1996; Peters et al. 1992).

Classical experiments involving manipulation of developing limb buds and implantation of beads soaked in FGFs have produced some remarkable results. Beads soaked in either FGF-1, FGF-2 or FGF-4 placed into the flank of chick embryos are able to induce the formation of ectopic limbs. Depending upon the axial level at which the beads are implanted, either complete wings or legs develop (Cohn et al. 1995). This suggested at the time that one of these FGFs was the signal for limb initiation. However, none appear to be expressed early enough for this to be the case and this role is now thought be played by FGF-10 (Xu et al. 1998). FGF-2 and 4 are thought to play prominent roles in limb outgrowth since removal of the AER followed by the application of an FGF-coated bead enables limb outgrowth to continue unchecked (Fallon et al. 1994; Niswander et al. 1993). Retroviral expression of FGF-2 in the anterior portion of
stage 20-22 chick limb buds causes duplications of anterior skeletal elements. The frequency of duplication declined dramatically when FGF-2 was ectopically expressed in more posterior sites in the limb. *In vitro* experiments have also implicated FGF-2 in limb differentiation. Addition of FGF-2 protein to organ cultures of rat metatarsals inhibits the longitudinal growth of these limb elements. It appears that exogenous FGF-2 prevents chondrocytic proliferation and matrix production (Mancilla et al. 1998). These data implicate FGF-2 in limb outgrowth, anterior-posterior patterning and differentiation.

### 1.4.2 FGFs and craniofacial morphogenesis

Although several groups have attempted to map the expression of FGF/FGFRs during craniofacial development, most have centred on earlier stages of development, prior to skeletogenesis. In addition, many studies do not distinguish between different FGFR splice variants, or sometimes even which FGFR is being detected (Wanaka et al. 1991). This is clearly important as FGFR splice variants are differentially expressed and have distinct roles in skeletogenesis. Early reports suggested that FGFs/FGFRs are widely expressed during embryogenesis, and may play a role in the development of the brain, limb, kidney, lung, heart, vascular system and musculature (Patstone et al. 1993; Peters et al. 1992; Wanaka et al. 1991). Work by Wanaka and co-workers implicated a non-specific FGFR in skeletal patterning in the rat. FGFR expression was detected in the perichondria surrounding the vertebral column but not in the cartilage itself. This expression pattern was also seen in the developing limb with weak labelling in the ossification centres surrounded by strong mesenchymal staining (Wanaka et al. 1991). Differential expression of FGFR1 and 2 was subsequently observed in numerous tissues during mouse development (Peters et al. 1992). However, expression of these genes during intramembranous ossification was not assessed. Analysis of FGFR distribution in the early chick embryo has revealed that FGFRs 1, 2 and 3 are expressed in unique patterns during the outgrowth of the frontonasal mass. Although the
stages analysed were earlier than the period of skeletogenesis, particularly strong expression of FGFR2 was observed in the centre of the frontonasal mesenchyme suggesting a role for this receptor in the outgrowth of the prenasal cartilage (Wilke et al. 1997). At later stages of chick development, high levels of both FGFR1 and 2 mRNA can be detected in the periosteum surrounding long bones and around differentiating membrane bones contrasting with relatively lower levels in the bones themselves (Patstone et al. 1993).

The first study to look at the distribution of FGFR splice variants clearly identified subtle, but distinct differences in mRNA expression during skeletogenesis. During mouse development, the k-exon form of FGFR2 selectively localises to the skin and epithelial linings of inner spaces. This form of FGFR2 was originally called keratinocyte growth factor receptor (KGFR) and is the only receptor known to specifically bind FGF-7 (also known as KGF). Although KGFR is also detected in long and cranial bones, the level of expression of the b-exon form of FGFR2 (known as Bek) in these tissues is much higher (Orr-Urtreger et al. 1993). These splice variants have also been shown to occupy characteristic expression domains in many other organs and tissues. At later stages (E16.5), Bek mRNA is highly expressed by the growing osteogenic fronts as they approach at sutures (Kim et al. 1998). Whole mount in situ hybridisation of whole mouse heads at this stage clearly shows the developing cranial bones outlined by Fgfr2 expression. In addition, the expression domain of osteopontin, an early osteogenic marker is contained within this Fgfr2 outline whilst remaining mutually exclusive from it (Iseki et al. 1997).

Work from our laboratory further investigated the spatial distribution of FGFR1 and -2 expression in the human embryo from 6 weeks of development. Both genes are expressed in sheets of condensed mesenchyme before overt chondrogenic differentiation and distinct patterns of expression are established by 8 weeks. FGFR2 is expressed evenly throughout developing cartilage and
bone, whereas FGFR1 transcripts predominate in perichondria and periostea. Complementary patterns of FGFR1 and FGFR2 expression are also observed in the enamel epithelium and papilla mesenchyme of the tooth germ, at a stage when morphogenetic tissue interactions are occurring (Chan and Thorogood, 1999).

The majority of experimental studies have centred around FGF-2, partly because it is readily available and also because earlier work in the limb had implicated it in skeletal patterning. In one such study, chick frontonasal mass mesenchyme was grafted onto a host limb bud and a bead soaked in either FGF-2 or FGF-4 placed on top. Both growth factors stimulated an increase in the length of cartilage rods. Mandibular mesenchyme grafted without ectoderm did not show a corresponding increase in cartilage length. FGF-2 is expressed by facial ectoderm and is thought to be a likely candidate for promoting outgrowth of the facial prominences (Richman et al. 1997). The facial ectoderm may therefore have similar properties to the AER in the limb. Interestingly, FGF-8 which was previously thought to be a candidate for facial outgrowth is not expressed by the facial ectoderm (Richman et al. 1997). Subsequent experiments using the same grafting system have indicated that the expression of FGFR2 and Type II Collagen (a marker of chondrogenesis) decrease following epithelial removal. This down-regulation of expression suggests that frontonasal mass mesenchyme requires continuous signalling from the facial epithelium to maintain expression (Matovinovic and Richman, 1997).

Clearly, these experiments focus on regions of the head where endochondral ossification is the primary osteogenic pathway. Two experiments in mice have attempted to manipulate calvarial bone development by the addition of FGF-beads. FGF-2 soaked beads were implanted onto the coronal suture of E15 mice by ex utero surgery. After 24 hours, the embryos were removed and stained for Fgfr2 or osteopontin by whole mount in situ hybridisation. Bead implantation induces ectopic expression of osteopontin and down-regulates Fgfr2 expression.
The expression of these two genes is mutually exclusive and it is suggested that an increase in FGF-2 shifts the cell proliferation/differentiation balance towards differentiation by down-regulating Fgfr2. Fgfr2 expression normally coincides with BrdU, a marker of cell proliferation (Iseki et al. 1997).

Similar experiments have been carried out with FGF-4 (Kim et al. 1998). Beads soaked in FGF-4 accelerated sutural closure when placed on the osteogenic fronts of calvarial explants in vitro. The same beads had no such effect when placed on the mid-sutural mesenchyme. One explanation for this finding is that FGF-4 may stimulate the proliferation of undifferentiated mesenchymal cells in the mid-sutural mesenchyme whereas in the osteogenic fronts it may increase the number of osteoblasts and bone matrix. In addition, when the underlying dura mater was removed prior to culture, the osteogenic fronts of the parietal bones approached each other more rapidly than those cultured with intact dura mater. After 72 hours in culture, the explants without dura mater had fused parietal bones whereas in control explants the sagittal suture remained open. The ability of the dura mater to maintain sutural patency only seems to occur during embryogenesis. Postnatal calvaria cultured with and without dura mater do not show significant differences in parietal bone growth. Several genes are known to be expressed by the dura mater, including FGF9, BMP4, Msx1 and 2. The presence of Msx2 is particularly interesting since there is evidence that it regulates type I collagen and osteocalcin, genes expressed by terminally differentiated osteoblasts (Newberry et al. 1996). MSX2 is also known to be mutated in a single family afflicted with Boston-type craniosynostosis.

The final piece of evidence implicating FGFs in cranial skeletogenesis comes from in vitro experiments carried out in our lab. Cranial neural crest cells from quail embryos can be dissected out from the mesencephalic neural folds before cell migration commences and grown in culture. Addition of FGF-2 to the culture medium has a concentration-dependent effect. At low levels (1 ng/ml), the neural crest cells rapidly proliferate and such cultures are significantly larger.
than controls. At higher levels of FGF-2 (10 ng/ml), cell proliferation is inhibited. Instead the cells form condensates which over a 10-day period differentiate into cartilage nodules. These have been characterised by expression of Type I Collagen, Alcian blue staining and SEM. If co-factors such as ascorbate, dexamethasone and β-glycerophosphate are added to the culture medium, over a period of weeks, both endochondral and intramembranous bone formation occurs. A 48-hour exposure to FGF-2 is sufficient to induce cartilage formation, however, this must occur in the first 48 hours of culturing or the ability of the neural crest cells to undergo chondrogenesis is lost. Addition of FGF-4 however, did not have a significant effect on proliferation and was not able to induce cartilage formation (Sarkar and Thorogood, 1999). These data provide strong evidence for the role of FGF-2 in skeletogenesis since addition of this factor alone to premigratory neural crest cells in vitro is sufficient to cause the differentiation of the three major skeletogenic lineages.
1.5 Experimental Strategy

The aim of this Thesis was to investigate the roles of Fibroblast Growth Factors in normal and abnormal skeletogenesis. Throughout this Thesis, I have used the chick as a model system, mostly because of its availability, but also because of its relatively fast developmental rate. This enables the manipulation of embryos \textit{in ovo} whereas such procedures are more technically difficult in mice. The mechanisms by which bone and cartilage form during embryogenesis have been assessed in many species, particularly in mice, rats and rabbits. However, the timing of critical events in both cranial and long bone had not been discussed in relation to chick development. Initially therefore, it was important to map cranial development. This was achieved using a combination of histological techniques and these results form the start of this Thesis.

At the inception of this project, the first mutation in \textit{FGFR2} had been linked to Crouzon syndrome, one of the craniosynostoses. Although \textit{MSX2} had previously been linked with another class of craniosynostoses, mutations in this gene could not be found in other syndromes, suggesting that this was not a major factor in cranial development. Expression studies had indicated that FGFs and their receptors were expressed in the developing head and face at early stages of development, when facial patterning processes were occurring. Little information was available on their expression during skeletogenesis, and this was a logical next step in the Thesis. Both FGF mRNA and protein localisation are analysed, the former at earlier stages of development, the latter at critical points in cranial differentiation. These data pinpoint FGF-2 specifically in cranial patterning.

To analyse the role of specific members of the FGFs in calvarial bone development, an experimental system was then set up. This technique had previously been used for the \textit{in ovo} culture of small pieces of tissue. By the use of carrier beads, ectopic FGF-2 is added to developing crania and its effect on
skeletal patterning assessed by histological staining. These beads are then implanted into developing limb buds to confirm that they are biologically active.

In subsequent experiments a blocking antibody to FGF-2 is applied to this system via a novel bead release system. The effect of varying levels of this growth factor on proliferation and differentiation are then analysed using an immunohistochemical marker. Finally, the kinetics of the release of antiFGF-2 in cranial tissue from beads is analysed using qualitative and quantitative techniques. This work provides strong evidence of a role for FGF-2 in skeletal patterning and may explain how the balance between proliferation and differentiation is controlled *in vivo* and how it is disrupted in abnormal skeletogenesis.
1.6 Summary

The vertebrate skull provides an essential protective case for the brain and sense organs. During normal development, fibrous junctions between the bones known as cranial sutures enable the skull to expand with the growing brain. In later life, these sutures naturally fuse, however, premature fusion results in the clinical condition craniosynostosis. This group of diseases typically cause severe facial deformation, mental retardation and breathing difficulties and major surgery is usually required. Within the last 5 years, the genes responsible for the major syndromic craniosynostoses and skeletal dysplasias have been identified as Fibroblast Growth Factor Receptors (FGFRs). Experimental evidence suggests that these mutations result in the constitutive activation of the receptor in question leading to uncontrolled signalling and premature ossification. The ligands thought to bind to FGFRs and to induce signal transduction are known as Fibroblast Growth Factors (FGFs). To date, 19 family members have been identified, and there is a significant amount of ligand-receptor promiscuity with many FGFs able to bind to several receptors. FGF-2 has emerged as the most likely family member to be involved in cranial skeletogenesis. Experimental evidence has already linked this growth factor with limb induction, patterning and differentiation. FGF-2 is present at high levels in the facial region of early chick embryos and addition of the protein in vivo induces the activation of a bone marker, osteopontin. In vitro experiments have indicated that addition of high concentrations of FGF-2 is sufficient to promote the skeletogenic differentiation of neural crest cells into both cartilage and bone. Lower concentrations promote cell proliferation only. The cranial vault and sutures are now known to be derived almost entirely from neural crest so it seems likely that a similar situation would exist in vivo. In this Thesis, I have investigated the role of FGF-2 in both normal and abnormal skeletogenesis and discovered that a similar concentration-dependent response can be elicited in chick crania. These data provide indirect evidence that mutations in FGFR genes causing craniosynostosis are constitutively activating.
Chapter Two: Materials

2.1 General Reagents

All reagents apart from those listed below were Analar grade supplied by BDH Ltd (Poole, Dorset, UK). Sigma-Aldrich (Poole, UK) supplied the following: Aprotinin, bovine serum albumin (BSA), bromophenol blue, carbonate-bicarbonate coating buffer, diethylpyrocarbonate (DEPC), dithiothreitol (DTT), ethidium bromide, formamide, glutaraldehyde, heparin, levamisole, β-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED), Orange G, paraformaldehyde (PFA), phenylmethylsulphonyl fluoride (PMSF), proteinase K, Ponceau S concentrate, sodium dodecyl sulphate (SDS), trypsin tablets, Tween-20, Trizma base, 30% hydrogen peroxide, yeast t-RNA.

Other reagents were supplied by: enhanced chemiluminescence reagent (ECL, Amersham International, Little Chalfont, Bucks, UK); non-fat milk (Marvel™, Premier Beverage, Stafford, UK).

2.2 Bacterial Plasmids

Chicken FGF-2 and FGF-4 plasmids were kindly provided by Ivor Mason at UMDS Guy’s Hospital. Human FGFR2 (B and K exon) plasmids were kindly provided by Joe Chan, Developmental Biology Unit, ICH. The plasmid maps are displayed below.

2.3 Enzymes

All restriction enzymes, T3 and T7 polymerases, RNasin ribonuclease inhibitor, RNase A, ribonuclease DNase I and DH5α competent cells were purchased from Promega (Promega Corporation, Southampton, Hants, UK).
**Fig. 2.1 Vector map of plasmid pBluescript SK+** (Courtesy of Stratagene, La Jolla, USA)
Fig. 2.2 Restriction map of chicken FGF-2 plasmid
This plasmid consists of the full length FGF-2 alt clone isolated from stage 7/8 chick embryos.

Fig. 2.3 Restriction map of chicken FGF-4 plasmid
This plasmid consists of the full length coding sequence of FGF-4 isolated from chick embryos.
Fig. 2.4 Restriction map of human FGFR2 (B exon) plasmid
A PCR fragment was amplified from human genomic DNA using primers to intronic sequences flanking FGFR2 exon B. The blunt end was then cloned into the SmaI site of pBluescript SK+.

Fig. 2.5 Restriction map of human FGFR2 (K exon) plasmid
A PCR fragment was amplified from human genomic DNA using primers to intronic sequences flanking FGFR2 exon K. The blunt end was then cloned into the EcoRV site of pBluescript SK+.
2.4 Molecular size markers

Rainbow™ markers (RPN 756; Amersham International, Little Chalfont, Bucks, UK) were used to size proteins. 1Kb and 123 base pair ladders were used for sizing DNA and RNA bands (Gibco BRL).

2.5 Bacterial growth media and plasmid preparation

Lennox L Broth (LB Broth), Lennox L Agar (LB Agar) and ampicillin were from Gibco (Gibco BRL, Paisley, UK). Plasmids were isolated using a Qiagen tip-20 kit (Qiagen Inc, Chatsworth, CA, USA).

2.6 Gel electrophoresis and equipment

Agarose was supplied by Gibco BRL, Protogel™ (37.5:1 polyacrylamide to bisacrylamide stabilised solution) from National Diagnostics (Atlanta, GA, USA). Agarose gels were run in the Horizon horizontal gel electrophoresis system (Gibco BRL).

2.7 Photography, autoradiography and blotting

X-ray film used was Kodak X-OMAT AR from Genetic Research Instrumentation (GRI, Braintree, Essex, UK). Autoradiographic cassettes and intensifying screens were supplied by GRI, Hybond-C Extra nitrocellulose membrane from Amersham and 3MM chromatography paper by Whatman International Ltd (Maidstone, Kent, UK). Kodak Ektachrome 64T-MAX photographic film was used for colour slides (Leeds Photovisual, London, UK).

2.8 RNA labelling

RNA was labelled using digoxygenin RNA labelling mix (Boehringer Mannheim).
2.9 Histological Reagents and Equipment
Glass staining troughs and racks, storage containers and all histological stains were obtained from BDH. Sections were cut on a Microm HM330 rotary microtome using disposable blades (both from Raymond Lamb, UK). Glass microscope slides, coverslips and paraffin wax, melting point 57-58°C (Lambwax,) were obtained from Raymond Lamb. HistoClear was from National Diagnostics (Atlanta, GA, USA). Slides were mounted in dextropropoxyphene (DPX; BDH).

2.10 Growth Factors
Bovine brain-derived FGF-2 (FGF basic) was purchased from R&D Systems (133-FB-025; R&D Systems, Abingdon, Oxon, UK).

2.11 Primary Antibodies
Primary antibodies raised against the following proteins were used in this study. The dilution of antibodies used in immunohistochemistry or Western blotting is given in square brackets.

2.11.1 FGF-2 (bFGF)
AntiFGF-2 neutralising polyclonal antibody raised in rabbit using bovine FGF-2 as an immunogen (AB-33-NA; R&D Systems) [50 µg/ml for immunohistochemistry; 2 µg/ml for Western Blotting]. This antibody has been shown not to cross-react against a wide range of growth factors tested (Eichele et al. 1984).

2.11.2 FGFR1 (Flg)
Flg (C-15) is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping to the COOH terminus of the precursor form of the human
FGFR1 protein (sc-121; Santa Cruz Biotechnology, Santa Cruz, CA, USA) [1:100 for immunohistochemistry; 1 \( \mu g/ml \) for Western blotting].

### 2.11.3 FGFR2 (Bek)

Bek (C-17) is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping to the COOH terminus of the human FGFR2 protein (sc-122; Santa Cruz Biotechnology) [1:100 for immunohistochemistry; 1 \( \mu g/ml \) for Western blotting].

### 2.11.4 PCNA

PCNA (PC10) is a mouse monoclonal IgG2a antibody derived by fusion of spleen cells from a BALB/c mouse with recombinant PCNA with Sp2/0-Ag14 myeloma cells (Santa Cruz Biotechnology). It reacts against the PCNA p36 protein expressed at high levels in proliferating cells of human, murine, insect and yeast origin [1:100 for immunohistochemistry].

### 2.12 Secondary antibodies

Alkaline phosphatase (AP)-conjugated anti-digoxigenin antibody was obtained from Boehringer Mannheim (Boehringer Mannheim UK Ltd, Lewes, East Sussex, UK). Horseradish peroxidase (HRP)-conjugated anti-rabbit antibody was purchased from Sigma and used at a 1:16,000 dilution for Western blotting. DAKO StreptABComplex/HRP duet kit was used for the labelling and signal amplification of primary antibodies used in immunohistochemistry (ABC Kit; DAKO Ltd, High Wycombe, Bucks, UK). A HRP-conjugated goat anti-rabbit antibody was used specifically for whole mount immunohistochemistry at a concentration of 1.6 \( \mu g/ml \) (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA). Alkaline phosphatase–conjugated anti-rabbit antibody was obtained from Sigma and used at a 1:10,000 dilution for ELISA.
2.13 Sera

Mouse IgG$_2a$ serum (X0943) was obtained from DAKO Ltd; rabbit serum (R-9133) was purchased from Sigma. Normal goat serum was from Jackson Labs (Jackson ImmunoResearch Laboratories Inc, West Grove, PA, USA). Fetal calf serum (FCS) was supplied by Sigma.

2.14 Detection Reagents

4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP/ X-Phosphate) were from Boehringer Mannheim. Diaminobenzidine tetrahydrochlorodihydrate (DAB) and p-Nitrophenyl phosphate (p-NPP), both in tablet form were obtained from Sigma.

2.15 Beads

Heparin-coated acrylic beads (H-5263) were from Sigma. AffiGel blue agarose beads were from Bio-Rad (Mesh 100-200, diameter 75-100µm; Bio-Rad, Hercules, CA, USA).

2.16 Tissue culture media and equipment

Alpha modification of Eagle's minimal essential medium with ribosides and deoxyribonucleosides (α-MEM) and 100x antibiotic/antimycotic were provided by Gibco BRL. Tissue culture grade petri dishes and other plastics were supplied by Philip Harris (Stone, Staffs, UK) and Nunc Inc (Naperville, IL, USA). Millipore filters were obtained from Millipore Corp (Bedford, MA, USA).
2.17 Western blotting equipment

Mini-Protean II apparatus was used to run polyacrylamide gels. Blotting of protein from the gel to membrane was carried out using a Trans-blot SD semi-dry transfer cell (both from Bio-Rad).

2.18 ELISA

Dynatech MRX Elisa plate reader was from Becton Dickinson (Oxford, UK)

2.19 Microscopy

Microdissections were carried out under a Zeiss SV6 stereomicroscope (Carl Zeiss, 7082 Oberkochen, Germany). Photography of large specimens was performed on a Zeiss SV11 stereomicroscope. Brightfield microscopy was performed on a Olympus BH2 microscope, using objective lenses of 4x, 10x and 20x magnification (Olympus, Chiyoda-Ku, Tokyo 101, Japan). Digital images were captured electronically using a Zeiss Kontron ProgRes 3012 digital camera (Imaging Associates Ltd, Thame, Oxon, UK), version 2.0 of the associated software and stored and labelled in Adobe Photoshop v3.0 (Adobe Systems Europe, Edinburgh, UK).

2.20 Source of chicken tissues

Fertilised white leghorn chicken eggs were provided by J.K. Needle and Co, Polyndon Farm, Herts, UK. Eggs were incubated in a humidified forced draft incubator at 37.5°C (Curfew Ltd., Essex, UK).

2.21 Centrifuges

Samples were centrifuged in a Heraeus Biofuge 13R (Heraeus instruments, Brentwood, Essex, UK) or a Sigma 113 microfuge (Sigma, Osterode, Germany).
Chapter Three: Methods

3.1 Whole mount skeletal preparations

The method used was adapted from Klymkowsky and Hanken (1991). Chick embryos were incubated from 7 to 16 days at 37.5°C, recovered under sterile conditions in phosphate buffered saline (PBS) and the amniotic membranes removed. If the embryo was older than 8 days, the head was removed by severing the spinal cord at the level of the neck. The tissue was immediately fixed in Alcian blue 8GX (0.15 mg/ml Alcian blue, 20% acetic acid, 96% ethanol) for 2-3 days, rocking at 4°C to ensure all tissues are fixed evenly. The time of fixation depended on the size of the tissue fixed. Alcian blue simultaneously fixes and stains cartilage.

The tissue was rehydrated through an ethanol series as follows: 100% ethanol (twice) over 2 days, followed by 95%, 70%, 40%, 15% ethanol, MilliQ for around 4 hours each or until the tissue has dropped to the bottom of the tube. Bone was then stained by immersing in Alizarin red S solution, 0.1 mg/ml in 0.5% potassium hydroxide (KOH), until the skeletal tissues were visible. This was normally around 1-2 hours. Care was taken not to leave the tissue in this stain for an extended period as the tissue starts to disintegrate as a result of potassium hydroxide in the solution. The tissue was then cleared further in 20% glycerol in 1% aqueous KOH and the solution changed regularly until all non-skeletal tissues became transparent. Again it was important to avoid a lengthy immersion in this solution.

The tissue was transferred to progressively higher concentrations of glycerol in 1% KOH: 50%, 80% and 100%, changing solutions when the tissue had sunk to the bottom of the tube. The stained embryos were stored in 100% glycerol at 4°C.
3.2 Fixation, wax embedding and sectioning

Tissue was dissected out in ice-cold PBS and transferred to freshly made 4% paraformaldehyde (PFA) in PBS. It was then fixed from between 3 hours to 3 days depending on size: typically fixation took place overnight with rocking at 4°C. Fixed samples were then washed twice in PBS at 4°C, followed by 50% ethanol in PBS at 4°C with rocking, and both for at least 30 minutes each. The samples were then dehydrated through 70% (twice), 85%, 95% and 100% (twice) ethanol. Solutions were changed at 30 minute intervals and kept at 4°C until the 85% ethanol stage. Again, the times used depended on the size of the tissue, generally once the tissue had sunk to the bottom of the tube it was thought to be fully equilibrated to the solution. After the second 70% ethanol wash, the specimens can be stored indefinitely at 4°C.

Next, the samples were immersed in HistoClear in glass containers, twice for 30 minutes each. This was replaced with a 1:1 HistoClear:Paraffin wax mixture at 60°C for 20 minutes. The wax was then changed 3 times, each for 20 minutes at 60°C. The samples were then transferred to a mould where they were orientated with warmed needles and the wax allowed to set. The timing of the incubations in wax are particularly critical. Short incubations may result in incomplete penetration of the tissue with wax, lengthy incubations will cause the tissue to disintegrate upon sectioning. Wax blocks were stored at 4°C until required.

Excess wax was trimmed from the block and the sides of the block angled like a kite. This promotes “ribboning” allowing sections to adhere to each other in a strip. Sections were cut on a microtome at 6 μm and placed on distilled water on Poly-L-lysine coated slides. Poly-L-lysine interacts with the anionic sites of tissue sections resulting in strong adhesive properties. The sections were floated out at 40°C until all creases had disappeared. Excess water was removed and the slides dried vertically in a rack at 37°C overnight. Slides were stored at 4°C until use.
3.3 Histochemical staining

3.3.1 Haematoxylin and eosin

The sections were placed in a polythene rack and rehydrated and dewaxed by sequentially passing through HistoClear twice, for 10 minutes each and a graded alcohol series of 100% twice, 95%, 85%, 70%, 50% and 30% each for 5 minutes. The slides were then washed in MilliRO water for 5 minutes to equilibrate the tissue. Nuclei were stained by immersing the slides in Erhlich’s Haematoxylin for 5 minutes followed by ‘blueing’ in running tap water for 5 minutes. The sections were then dehydrated back through to 95% ethanol, 5 minutes in each grade. The slides were stained in 1% eosin in 95% ethanol for 5 minutes followed by a wash in 95% ethanol and dehydration in 100% ethanol. Finally, the slides were immersed in HistoClear, twice, for 10 minutes each before mounting in DPX and drying overnight in a fume hood.

3.3.2 Alcian blue and Durazol red

The sections were dewaxed and rehydrated to 70% ethanol (see Section 3.3.1. for details). Bone was decalcified by a 30 minute incubation in acid alcohol (1% HCl in 70% ethanol) before rehydrating to water. Nuclei were stained by immersing the slides in Mayer’s Haematoxylin for 15 minutes followed by a 15 minute rinse under running tap water. Cartilage was stained by immersing in Alcian Blue (1 g/ml in 1% acetic acid in MilliRO) for 10 minutes before a quick rinse in MilliRO water. The stain was then differentiated by incubating in freshly made 1% (w/v) phosphomolybdic acid for 10 minutes and rinsed in MilliRO. Bone was stained with 0.5% (w/v) Durazol Red, again for 10 minutes, rinsed with MilliRO and then dehydrated back through to 100% ethanol before immersing in HistoClear, twice, for 10 minutes each. Slides were mounted in DPX and dried overnight in a fume hood.
3.3.3 Nuclear fast red

The protocol was essentially that of Mansour et al. (1993). Sections were
dewaxed and rehydrated as described in Section 3.3.1. Slides were then washed
in MilliRO water for 5 minutes to equilibrate before counterstaining in nuclear
fast red (0.02% nuclear fast red, 5% Al\textsubscript{2}(SO\textsubscript{4})\textsubscript{3}.(14-18H\textsubscript{2}O)) for 10 minutes.
Excess stain was removed by washing, 3 times in MilliRO, before a 10 minute
incubation in HistoClear. Slides were mounted in DPX and allowed to dry
overnight in a fume hood. This stain was used to counterstain sections of whole
mount \textit{in situ} hybridised embryos in which positively stained cells appeared blue
(see Section 3.7).

3.4 Transformation of foreign DNA into bacterial cells

The transformation tubes were chilled and competent cells simultaneously
thawed on ice. For each transformation, 50 \(\mu l\) of DH5\textalpha\ competent cells and 50-
100 ng of plasmid DNA were added to the pre-cooled tubes. The contents were
gently mixed by tapping the tube, to avoid shearing of the competent cells and
then stored on ice for 45 minutes. At this stage, the L Agar plates prepared
earlier were warmed to 37°C. The tubes were then heat-shocked for 45 seconds
at 37°C in a waterbath before immediately chilling on ice for 2 minutes. 0.5 ml
of L Broth was then added to the tubes which were then incubated at 37°C for at
least 1 hour. This period allows the cells to recover following heat shock. The
cells were then spun down at 2000 rpm at 4°C for 5 minutes, the supernatant was
removed and the pellet resuspended in residual supernatant. The transformed
bacteria were then plated out onto L Agar containing 50 mg/ml ampicillin with a
flamed glass spreader. The presence of ampicillin in the agar enables only
transformed bacteria to grow so that all colonies formed will contain the
plasmid. Once the residue had soaked into the agar, the plates were inverted and
incubated at 37°C overnight.
3.5 Small-scale isolation of plasmid DNA

Single bacterial colonies were removed from agar plates using a clean pipette tip. Colonies appear white and an isolated colony was picked if possible. The tip was placed in a 15 ml tube containing 3 ml of L Broth and 50 mg/ml ampicillin. The lid was loosely replaced and the tubes placed in a 37°C shaker incubator overnight. The L Broth should have turned cloudy overnight indicating bacterial growth. Cells were harvested by centrifugation at 2500 rpm for 10 minutes at 4°C. The DNA was then ready to mini-prep using a Qiagen plasmid purification kit.

This method is based on the optimised alkaline lysis method of Birnboim and Doly (1979). The pellet was resuspended in 0.3 ml of buffer P1 (containing 100 μg/ml RNase A, 50 mM Tris/HCL and 10 mM EDTA pH 8.0). The cells were then lysed with 0.3 ml of buffer P2 (containing 200 mM NaOH and 1% SDS) and incubated at room temperature for 5 minutes. The SDS denatures the cellular proteins, while the alkaline conditions result in the denaturation of the chromosomal and plasmid DNA. The timing here is critical. Overexposure of the plasmid to alkaline conditions may result in the production of more denatured supercoiled DNA, which does not renature properly, and runs faster on an agarose gel.

The lysate is neutralised by the addition of 0.3 ml of chilled buffer P3 (containing 3M Potassium acetate, pH 5.5) for 10 minutes on ice. The solution must be gently mixed several times during the incubation to enhance the precipitation. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate while the shorter plasmid DNA renatures correctly and stays in solution. The RNase A added at the beginning of the procedure, digests any RNA ensuring that these fragments do not bind to the column. The sample was centrifuged for 15 minutes at 10,000 rpm to pellet the precipitated debris leaving a clear lysate. The supernatant was
removed promptly. The separation column was then equilibrated by applying 1ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% ethanol, and 15% Triton X-100). The column consists of a patented anion-exchange resin which allows the isolation of plasmid DNA by gravity flow. The supernatant was then applied to the equilibrated column followed by 4 washes each of 1 ml buffer QC (containing 1 M NaCl, 50 mM MOPS pH 7.0 and 15% ethanol). The DNA remains bound to the resin while the washes remove any remaining contaminants from the supernatant. The DNA was then eluted by the addition of 0.8 ml of buffer QF (containing 1.25 M NaCl, 50 mM Tris/HCl pH 8.5 and 15% ethanol). The DNA in the eluant was then precipitated by the addition of 0.7 volumes of isopropanol at room temperature to minimise coprecipitation of salt and centrifuged immediately at 13,000 rpm for 30 minutes. The supernatant was then discarded and the DNA pellet washed briefly in 70% ethanol before centrifuging again. The ethanol replaces any precipitated salt and isopropanol making the DNA easier to redissolve. After removal of the ethanol supernatant, the pellet was air-dried briefly (for approximately 10 minutes) and then redissolved in approximately 40 µl of Tris-EDTA pH 8.0 (TE, 10 mM Tris /HCl, 1 mM EDTA pH 8.0). The purified DNA was then ready for use.

### 3.6 Agarose Gel Preparation

1% agarose gels were used for these procedures. 0.5 g agarose was dissolved in 50 ml Tris-acetate EDTA (TAE) buffer by microwaving briefly. The gel was cooled slightly, 1 µl of 1 mg/ml ethidium bromide was added and then poured into a casting tray. Combs were inserted and the gel left to set for an hour. Once set, the combs were removed and the gel placed into an electrophoresis tank and covered with TAE buffer. The samples were mixed with a loading buffer such as bromophenol blue or orange G, loaded into the wells and electrophoresed at 75 mV until the dye front approached the end of the gel. A molecular weight marker was loaded in the end lanes and used to determine the size of the fragments seen. The gel was visualised on an ultra-violet transilluminator and
photographed. The ethidium bromide in the gel intercalates between the base pairs of the DNA and emits ultra-violet induced fluorescence on a transilluminator.

3.7 Whole mount in situ hybridisation

Labelled RNA probes are synthesised by in vitro transcription of DNA, which has been cloned downstream of (in this case) a T7 promoter, by T7 RNA polymerase using digoxygenin-labelled uridine-triphosphate (DIG-UTP) as a substrate. The DIG-labelled RNA probe is hybridised to target nucleic acids in the tissue and detected by enzyme-linked immunoassay using an antibody-conjugate. An enzyme-catalysed colour reaction with 5-bromo-4-chloro-3-indoyl phosphate (BCIP/X-phosphate) and nitroblue tetrazolium salt (NBT) produces an insoluble blue precipitate which visualises the labelled molecules. The details of this procedure are shown schematically in Fig. 3.1.

3.7.1 Preparation of equipment and solutions for RNA work

All solutions for RNA work were made, where possible, from solids which were kept separate from the general chemicals in the laboratory. The solutions were then treated with 0.1 % diethylpyrocarbonate (DEPC) at room temperature overnight and autoclaved. DEPC is a potent inhibitor of ribonuclease (RNase) and is broken down to ethanol and carbon dioxide by autoclaving (Fedorcsak and Ehrenberg, 1966). The only aqueous solutions which were not DEPC-treated were those containing Tris or amines and those which could not be autoclaved. These solutions were made up in bottles which had been DEPC treated, autoclaved and baked. All glassware was baked overnight at 200°C before use. Sterile plasticware was assumed to be RNase-free.

Many of the solutions used in whole mount in situ hybridisation have numerous constituents and are described below to maintain the clarity of presentation.
Figure 3.1 Non-radioactive DIG-RNA labelling by *in vitro* transcription
Cloned DNA is transcribed *in vitro* with T7 or T3 RNA polymerases in the presence of DIG-UTP. This is used as a substrate and is incorporated in the transcript - every 20-25th nucleotide of the newly synthesised RNA is a DIG-UTP. Since the nucleotide concentration does not become limiting in the standard transcription assay, a large amount of labelled RNA can be generated (Courtesy of Boehringer Mannheim).
### 3.7.1.1 Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TE (Tris/EDTA)</strong></td>
<td>10mM (2M) Tris pH 8.0</td>
<td>0.05 ml</td>
</tr>
<tr>
<td></td>
<td>5mM (0.5M) EDTA</td>
<td>0.1 ml</td>
</tr>
<tr>
<td></td>
<td>DEPC-H₂O</td>
<td>9.85 ml</td>
</tr>
<tr>
<td><strong>Prehybridisation Mix</strong></td>
<td>50% formamide</td>
<td>25 ml</td>
</tr>
<tr>
<td></td>
<td>5x SSC (20x) pH 4.5</td>
<td>12.5 ml</td>
</tr>
<tr>
<td></td>
<td>50 mg/ml heparin</td>
<td>50 μl</td>
</tr>
<tr>
<td></td>
<td>10 mg/ml yeast t-RNA</td>
<td>250 μl</td>
</tr>
<tr>
<td></td>
<td>1% SDS</td>
<td>0.5 ml</td>
</tr>
<tr>
<td></td>
<td>DEPC-H₂O</td>
<td>11.7 ml</td>
</tr>
<tr>
<td><strong>Solution 1</strong></td>
<td>50% formamide</td>
<td>25 ml</td>
</tr>
<tr>
<td></td>
<td>5x SSC (20x) pH 4.5</td>
<td>12.5 ml</td>
</tr>
<tr>
<td></td>
<td>1% SDS (10%)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td></td>
<td>DEPC-H₂O</td>
<td>12 ml</td>
</tr>
<tr>
<td><strong>Solution 3</strong></td>
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<tr>
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<td>2x SSC (20x) pH 4.5</td>
<td>5 ml</td>
</tr>
<tr>
<td></td>
<td>DEPC-H₂O</td>
<td>20 ml</td>
</tr>
<tr>
<td><strong>TBST (10x stock)</strong></td>
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<tr>
<td></td>
<td>KCl</td>
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</tr>
<tr>
<td></td>
<td>1M Tris HCl pH 7.5</td>
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<td></td>
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<tr>
<td><strong>TBST working solution</strong></td>
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<tr>
<td></td>
<td>1% Tween-20</td>
<td>0.5 ml</td>
</tr>
<tr>
<td></td>
<td>Levamisole</td>
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</tr>
<tr>
<td></td>
<td>DEPC-H₂O</td>
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<td><strong>NTMT working solution</strong></td>
<td>100 mM (5M) NaCl</td>
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<td></td>
<td>100 mM (2M) Tris pH 9.5</td>
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<tr>
<td></td>
<td>50 mM (2M) MgCl₂</td>
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<td></td>
<td>1% Tween-20</td>
<td>0.5 ml</td>
</tr>
<tr>
<td></td>
<td>Levamisole</td>
<td>0.0024 g</td>
</tr>
<tr>
<td></td>
<td>DEPC-H₂O</td>
<td>44.75 ml</td>
</tr>
</tbody>
</table>

1 Stock concentrations.
3.7.2 Plasmid linearisation

It is important to ensure that the plasmid preparation actually contains an insert of the correct size before proceeding with any labelling steps. Using the plasmid map, the gene insert was cut out of the plasmid with the appropriate restriction enzymes in the following solution:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid preparation</td>
<td>2 µl</td>
</tr>
<tr>
<td>10x enzyme buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>DEPC-H₂O</td>
<td>14 µl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

To linearise the plasmid before labelling (so that the RNA polymerase is able to access the DNA), the following components were mixed:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid</td>
<td>5 µl</td>
</tr>
<tr>
<td>10x enzyme buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>DEPC-H₂O</td>
<td>12 µl</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

The solutions were vortexed and incubated at 37°C for at least 1 hour. 1 µl samples were taken and run on an agarose gel (see Section 3.6) to check that the correct fragment size was present and complete linearisation had occurred.

3.7.3 Synthesis of digoxygenin-labelled riboprobe

The transcription was carried out in a 20 µl volume essentially as described (Holtke and Kessler, 1990). The following reagents were mixed at room temperature:
DEPC-H₂O 13 μl
10x transcription buffer 1 μl
0.2M DTT 1 μl
Nucleotide mix (pH 8.0) 2 μl
1 μg/μl linearised plasmid 1 μl
100 U/μl placental ribonuclease inhibitor 0.5 μl
RNA polymerase 1 μl

Typically, T7 polymerase was used for the transcription of antisense and T3 polymerase for transcription of sense probes (see plasmid maps in Chapter 2). The mixture was then incubated at 37°C for 2 hours. A 1 μl aliquot was then removed and run on a 1% agarose gel to estimate the amount of probe synthesised. An RNA band approximately 10x denser than the plasmid band indicated that approximately 10 μg probe had been synthesised and confirmed that transcription was successful. The probe was then incubated with 2 μl ribonuclease-free DNase I at 37°C for 15 minutes. Next, 100 μl TE, 10 μl 4M LiCl and 300 μl ethanol were added to precipitate the RNA and the mixture incubated at -20°C for 30 minutes. The pellet was then collected by centrifuging at 13,000 rpm for 10 minutes, washed in 100 μl 70% ethanol and then air-dried briefly. Finally, the pellet was resuspended in TE to a final DIG-RNA concentration of 0.1 μg/μl and stored at -20°C until use. 10 μl of probe was added per ml of hybridisation mix.

3.7.4 Embryo Pre-treatment

All pretreatments were carried out in 5 or 10 ml polypropylene round bottomed tubes. Embryos from stages 17-25 (Hamburger and Hamilton, 1951) were dissected out in DEPC-PBS and all extraembryonic membranes removed before staging accurately. The roof plate of the hind brain was punctured to reduce trapping of probe or antibody. Embryos were fixed in ice-cold 4% PFA in PBT (0.1% Tween-20 in PBS), rocking at 4°C for at least 3 hours, preferably
overnight. The PFA was rinsed off twice in PBT, for 5 minutes each, before dehydrating through a methanol series (25%, 50%, 75% in PBT) and twice in 100% methanol. The embryos were then stored in methanol at -20°C overnight before rehydrating through the reverse series of methanol in PBT followed by 2 washes in PBT. Following this, the embryos were bleached in 6% hydrogen peroxide in PBT for 1 hour with rocking and then washed 3 times in PBT. Embryos were treated with Proteinase K, the duration being dependent on the stage of the embryos used. Typically, embryos around stage 18 were incubated for 15 minutes in 10 μg/ml Proteinase K in PBT. The purpose of this treatment is to enable more efficient probe penetration, particularly in larger embryos. Embryos were then rinsed in freshly prepared 2 mg/ml glycine in PBT followed by 2 washes in PBT. Since the embryos are fragile after Proteinase K treatment, they are then refixed in fresh 0.2% glutaraldehyde in 4% PFA in PBS for 20 minutes. The fixative was removed by 2 washes in PBT before transfer of the embryos to 2 ml microtubes. Next, the embryos were rinsed in 1 ml of prehybridisation mix before replacing with a fresh 1 ml and incubating for 1 hour at 70°C. At this stage, embryos can be stored at -20°C before hybridisation. 10 μl/ml of hybridisation mix including 1 μg/ml digoxigenin-labelled RNA probe was then added and embryos were incubated overnight at 70°C.

3.7.5 Post-hybridisation washes

Embryos were washed in Solution 1, twice for 30 minutes at 60°C, followed by the same regime with Solution 3. The embryos were then washed three times with TBST for 5 minutes each. Following this, the embryos were preblocked with 10% goat serum in TBST for 60-90 minutes, rocking at room temperature. During this time, 3 mg of embryo powder per microtube was weighed out, to which 0.5ml TBST was added. This solution was heated at 70°C for 30 minutes before cooling on ice. Then 5 μl goat serum and 1 μl anti-digoxigenin antibody (conjugated to alkaline phosphatase) were added and the mixture rocked gently at 4°C for 1 hour to preabsorb the antibody. This antibody solution was then
spun at 13,000 rpm for 10 minutes and the supernatant collected. The supernatant was diluted to 2 ml with 1% goat serum in TBST. After removal of 10% goat serum/TBST, this antibody solution was added and the embryos were rocked at 4°C overnight (or longer) to allow the antibody to penetrate the tissues.

### 3.7.6 Post-antibody washes and histochemistry

Three washes for 5 minutes with TBST were followed by 5 washes during the day to rinse out any unbound antibody. Washing with TBST was continued overnight. The following morning, the embryos were washed in NTMT, 3 times for 10 minutes each, before addition of a final solution of NTMT containing 4.5 μl NBT and 3.5 μl BCIP per ml. The embryos were rocked in the dark for approximately 20 minutes (depending on how fast the colour reaction proceeded) in a glass embryo dish. This allowed the monitoring of the colour reaction under a microscope. If the development was very slow (for low copy transcripts particularly) the reaction was allowed to proceed overnight at 4°C. Once the colour had developed to the desired extent, and before the background had started to colour, the reaction was stopped in PBT, washing several times. Embryos were then stored in the dark in PBT containing a small amount of sodium azide to prevent fungal growth. Representative embryos were wax embedded and sectioned to determine the exact cellular localisation of RNA transcripts (see Section 3.3.3 for methodology).

### 3.8 CAM-Grafting

#### 3.8.1 Bead preparation

Experimental dishes were prepared as followed: 2 μl of either FGF-2, AntiFGF-2, pre-immune serum or PBS were pipetted into the centre of a sterile tissue culture grade petri dish. Around this, 10 μl drops of PBS were pipetted to maintain the humidity of the dish and prevent the beads drying out. Dishes
containing 10 μl of either heparin-coated acrylic or Affi-Gel blue agarose beads were set up in the same manner. The largest beads were removed with sterilised fine forceps and placed into a wash drop of PBS to rinse off debris. The beads were then evenly distributed amongst the pre-prepared experimental dishes, taking care not to carry over any contaminating solution which would alter the effective concentration. Approximately 10-15 beads can be accommodated in a 2 μl drop. The dishes were then covered with parafilm and incubated at room temperature for at least 1 hour.

### 3.8.2 Host preparation

Fertilised brown chicken eggs were incubated for 8 days at 37.5°C in a humidified forced draft incubator. A hole was made in the blunt end of the egg, into the airspace to release air from the egg and make more space when the egg was opened. A small (1 cm²) square of shell was carefully sawn using a sterilised hacksaw blade and removed with fine forceps. It was crucial that only the shell was cut and not the underlying chorio-allantoic membrane (CAM). Usually, once the shell had been removed the change in pressure in the egg resulted in the drop of the CAM without damage. However, if the CAM remained stuck to the shell membrane, the latter was carefully peeled away without damaging the CAM. Damage to the CAM resulted in a loss of the host, and typically, more hosts were set up than donors to compensate for this loss. After removing excess shell membrane from around the window, clean Sellotape™ was used to seal the egg and the egg was returned to the incubator until needed.

### 3.8.3 Donor preparation and grafting

Fertilised eggs were incubated for between 7-11 days at 37.5°C as described previously. The donor embryos were removed from the egg under sterile conditions and placed in a dish of sterile PBS. Any remaining amniotic
membranes were removed, donors were sacrificed by decapitation and the torsos discarded. After washing in a fresh dish of PBS to remove excess yolk, the embryos were transferred to cold α-Minimal Essential Medium (α-MEM) ready for dissection. Using flamed iridectomy scissors, incisions were made behind the eye on each side, caudally to (and including) the occipital cartilages and rostrally to the nasal cartilage. This tissue included dermis, dura mater, mesenchyme, bone, cartilage and brain. The tissue was removed, turned ventrally and cuts made to the meninges surrounding the brain to allow the brain to be removed intact. A small incision was then made dorsally with a sharpened tungsten needle within the frontal region, either in the presumptive suture, undifferentiated mesenchyme or into an area where bone differentiation was occurring. 1-3 coated beads were implanted and the dermis pushed back in place. The implanted grafts were then placed onto the CAM of host embryos and a few drops of Tyrodes solution added. Tyrodes (500 ml stock) contains: 4 g NaCl, 0.1 g KCl, 0.25 g NaH$_2$PO$_4$2H$_2$O. On day of use 1 g/L glucose, 1 g/L NaHCO$_3$ and 1% antibiotic/antimycotic were added and the solution filter sterilised. The hosts were then resealed with Sellotape™, ensuring that the window was completely sealed and returned to the incubator for 6-9 days. This grafting period was dependent on the initial age of the donor tissue but generally, the grafts were recovered when 16 days old. Maintenance of the humidity of the incubator over this period was essential to recover a significant number of viable grafts.

3.8.4 Graft recovery

At the end of the grafting period, hosts were opened and viable grafts (those which had healthy hosts and complete graft incorporation) were cut out from the CAM. The grafts were rinsed in PBS before trimming away excess host-derived membranes. The grafts were then fixed in Alcian Blue and stained for bone and cartilage as described in Section 3.1. Once staining was completed, all grafts were measured under a stereomicroscope at the same magnification and their
dimensions were recorded. A small proportion of grafts were fixed in 4% PFA, wax embedded, and sectioned for general histology and immunohistochemistry.

3.9 Limb Implantation

Fertilised brown chicken eggs were incubated for the appropriate time periods for stages 18-25 (Hamburger and Hamilton, 1951). The eggs were windowed as described in Section 3.8.2, and if necessary, to make more room in the egg, 1 ml of albumin was removed with a sterile syringe through a hole made into the airspace. The vitelline membrane was opened with iridectomy scissors over the upper limb bud and an incision made in roughly the centre of the limb bud with a sharpened tungsten needle. 1-2 previously soaked (see Section 3.8.1) heparin or agarose beads were implanted into the upper left limb bud and when satisfied that they would remain in place, a few drops of Tyrodes were added and the egg sealed with Sellotape™. The operated embryos were then returned to the incubator and allowed to develop to 10 days when skeletal patterning is completed. The embryos were then recovered and sacrificed, washed in PBS to remove excess albumin and fixed in Alcian Blue. Whole mount skeletal staining was then carried out as described in Section 3.1. Individual limb elements (humerus, radius, and ulna) were accurately measured and recorded for statistical analysis.

3.10 Immunoperoxidase

Immunohistochemistry is a well established technique which allows the detection of protein epitopes in a tissue section using an antibody which is specific for the protein of interest (Coons et al. 1955).

For conventional immunohistochemistry using tissue sections, the tissues are first fixed to preserve morphology, then dehydrated and embedded in a wax block. Thin sections of the block (6 μm) are cut on a microtome and transferred
to a microscope slide. Next, the sections are rehydrated, pre-treated to increase penetration of the antibodies (optional depending on the antibody used), blocked to avoid non-specific binding and the antibody is applied. The antibody is then detected using secondary antibodies or other amplifying secondary detection techniques (for example, ABC kit, see Fig. 3.2). The distribution of the protein can be determined using fluorescent or light microscopy.

3.10.1 Fixation, wax embedding and sectioning
Carried out as described previously in Section 3.2. The time of incubation in each solution depended on the size of the tissue to be fixed.

3.10.2 Rehydration and pretreatment
Rehydration was carried out as described in Section 3.3.1.

The sections were then treated to improve antibody penetration. In the majority of cases this was achieved by treatment with trypsin and in a few cases by microwaving the sections which causes antigen unmasking (Shi et al. 1991). The technique used depended on the individual antibody, trypsin treatment was used for all antibodies except PCNA. Trypsinisation was carried out by incubating with 0.1% trypsin for 15-30 minutes at 37°C. Incubation time depended on the age of the tissue and was increased for older specimens. The trypsin was washed off in MilliRO water, twice for 5 minutes followed by PBS.

To unmask by microwaving, slides were transferred into citric acid buffer (2.1 g citric acid in 1 litre MilliRO water, adjusted to pH 6.0). They were then microwaved at full power in a 700 W oven in a covered plastic container for several minutes. Once again the time was optimised for the number and type of specimens, ranging between 6 minutes for small friable embryonic tissues to
10 minutes for larger numbers of samples. Slides were left to stand and cool for 10 minutes in a tray of cold running tap water, before briefly washing, twice in MilliRO water and once in PBS for 5 minutes.

3.10.3 Blocking steps

The following stages eliminate non-specific signals by blocking endogenous peroxidase and non-specific binding by blocking charged proteins (Fig. 3.3-3.4). Slides were placed flat in a humidified container and 1 ml of 3% hydrogen peroxide in PBS placed over each slide. After a 15 minute incubation at room temperature, the hydrogen peroxide was washed off with PBS, before two further washes for 5 minutes. Charged proteins were then blocked by incubating with a non-specific protein rich solution for 30 minutes at room temperature. Typically this consisted of 1% bovine serum albumin (BSA) and 10% fetal calf serum (FCS) in PBS.

3.10.4 Primary antibody

After briefly rinsing in PBS, the primary antibody was applied. Since only 100 µl of antibody solution was added per slide, it was necessary to carefully remove excess liquid using a clean tissue to avoid inadvertently altering the antibody concentration. All antibodies were diluted in the blocking solution used above (Section 3.10.3). Each slide was covered with a plastic coverslip and incubated in a humid chamber overnight at 4°C. This incubation protocol gives relatively slow molecular motion and maximum opportunity for the antibody and antigen to find their complementary spatial structures and bind. A list of antibodies used and their dilutions can be found in Chapter 2.11. Negative controls were run with all experiments and preimmune serum, either mouse or rabbit was used in place of the primary antibody at the same concentration.
**Figure 3.2 Avidin-Biotin method of signal amplification**

This method enables the signal to be amplified by the production of an avidin-biotin conjugate.

**Figure 3.3 Blocking endogenous peroxidase**

Endogenous peroxidase in the tissue will show staining if not inhibited by the addition of hydrogen peroxide prior to the application of primary antibody.

**Figure 3.4 Blocking charged proteins**

Non-specific binding of primary antibody to charged collagens and connective tissue can be avoided if the specimen is pre-treated with a blocking solution rich in animal sera. Diagram courtesy of Dako Corporation.
3.10.5 Washes and secondary antibody

Post-antibody washes consisted of PBS containing 1% Triton X-100, a detergent which reduces background staining. Initially, the slides were washed 3 times for 5 minutes in this solution with rocking, before rinsing in PBS to remove excess detergent. This step was important and enabled the next solution to adhere to the sections. The detection protocol involved the use of an ABC kit. This system utilises an avidin-biotin-peroxidase system to amplify the signal. Avidin is a large glycoprotein found in egg white which has four high affinity binding sites for biotin. A secondary biotinylated antibody (reagent C) is applied, followed by an avidin-peroxidase conjugate (reagents A and B) which binds to the biotin attached to the secondary antibody. Peroxidase activity is then detected by colorimetric changes in diaminobenzidine (Fig. 3.2).

Reagent C was applied to slides at a 1 in 100 dilution in PBS and the sections covered with plastic cover slips. Simultaneously, a mixture containing reagents A and B, each diluted 1 in 100 in PBS, was prepared. After incubating for 30 minutes at room temperature in a humid chamber, the slides were washed twice in 1% Triton/PBS, rinsed in PBS and then the AB mix applied for 30 minutes, again applying coverslips. The slides were then washed in Triton/PBS and rinsed in PBS before beginning the colour reaction.

3.10.6 Detection, counterstaining and mounting

Approximately 500 µl of filtered DAB solution was added to each slide. An insoluble brown precipitate is produced by a reaction between the peroxidase linked to the AB complex, DAB and hydrogen peroxide and can be monitored by eye or microscopically. The slides were allowed to develop for between 1 and 10 minutes, depending on the rate of colour development, and washed in MilliRO water, twice, for 5 minutes to stop the reaction and wash off any excess
DAB. If background staining appeared to be high, 1 μl of hydrogen peroxide per ml of DAB was subsequently added to prevent overexposure of the background.

The slides were then counterstained in Methyl Green, a blue nuclear stain which contrasts against the brown DAB stain. After 10 minutes, the slides were dipped 10 times each in three troughs of MilliRO water followed by at least 10 dips in butan-1-ol (in a fume hood). The butanol causes the stain to differentiate. Once the desired level of staining was reached, the slides were equilibrated in HistoClear for 10 minutes and mounted in DPX before drying in a fume hood overnight.

3.11 Western Blotting

Western blotting uses specific antibodies to identify proteins within tissue extracts. The proteins are denatured or kept in their native form and separated on SDS-polyacrylamide gels. The proteins are then transferred from the gel onto a solid support (nitrocellulose filter) and the filter is probed with a monoclonal or polyclonal antibody which adheres to the protein of interest. A secondary antibody conjugated to horseradish peroxidase or alkaline phosphatase is then used to detect the protein which is shown as a band on the filter. The size of the detected protein is determined by comparison with molecular weight markers.

3.11.1 Protein Extraction

The first stage in protein extraction is to homogenise the tissue in lysis buffer which breaks up the cells and causes solubilisation of the proteins. This buffer contains proteinase inhibitors which prevent protein degradation. After homogenisation, the sample is centrifuged to pellet the solid debris and the supernatant contains the protein suspension.
Samples were collected and placed in radioimmune precipitation assay buffer (RIPA) consisting of 0.1% SDS, 0.5% DOC, 1% NP40, 150 mM NaCl, 50 mM Tris pH 8 to which was added fresh proteinase inhibitors: 30 μl/ml aprotonin and 10 mg/ml phenylmethylsulphonylfluoride (PMSF). Tissues were thoroughly homogenised by repeated syringing through needles of decreasing calibre and left to stand on ice for 30 minutes to allow the proteins to dissociate. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatant removed. Protein extracts were then stored at -20°C until required.

3.11.2 Polyacrylamide gel preparation

Glass plates with 1 mm spacers were assembled using the Mini-Protean II apparatus. The resolving gel was mixed first and poured between the plates until 2/3 full. The percentage of resolving gel required is dependent on the size of the protein to be analysed: 8 % gels were used for FGFR1 and 2 (80-100 kD) and 15 % gels for FGF-2 (15-35 kD) protein separation. The gel consisted of MilliRO water, Protogel™, 1.5 M Tris pH 8.8, 10 % SDS, 10 % fresh ammonium persulfate and N,N,N’,N’ tetramethylethylenediamine (TEMED). TEMED accelerates the polymerisation of acrylamide and bisacrylamide by catalysing the formation of free radicals from ammonium persulfate. The quantity of Protogel varied depending on the percentage of gel required. After the resolving gel was poured it was covered with water saturated butan-1-ol which flattens the top of the gel and prevents the formation of air bubbles which would distort protein migration. The butanol must be water saturated to avoid dehydrating the gel. The gels were left at room temperature for 30 minutes to set, the overlay was poured off and the top of the gel was washed several times with MilliRO water to remove any unpolymerized acrylamide and butanol. The area above the gel was carefully dried with pieces of 3MM filter paper.

The stacking gel was next poured above the resolving gel. This aligns the proteins into a very thin layer on the surface of the resolving gel, increasing the
resolution of the proteins before they are separated. The percentage of gel used is always 5% with a 5 ml mix consisting of 3.4 ml MilliRO water, 0.83 ml Protogel, 0.63 ml 1 M Tris pH 6.8, 0.05 ml 10% SDS, 0.05 ml 10% ammonium persulfate and 0.005 ml TEMED. A 10-well comb was inserted, taking care not to trap bubbles in the wells, and the gel left to set for 30 minutes at room temperature. If larger wells were required, the level of stacking gel was topped up every few minutes to compensate for shrinkage of the gel as it set. Once the gels were ready for running, they were transferred to the running tanks and immersed in 350 ml running buffer (diluted 5x stock; 25 mM Tris, 250 mM glycine pH 8.3, 0.1% SDS). The Laemmli discontinuous buffer system was used (Laemmli, 1970) where part of the buffer is located between the gels and the rest is in the running tank.

3.11.3 Sample preparation and loading

The protein homogenates were thawed and mixed with an equal volume of loading buffer (1 ml glycerol, 0.5 ml β-mercaptoethanol, 3 ml 10% SDS, 1.25 ml 1 M Tris pH 6.7 and 1.2 mg bromophenol blue). The samples were then heated to 95°C for 5 minutes on a hot block and immediately quenched on ice to reduce the proteins. Each sample was carefully loaded into a well, avoiding cross-contamination and coloured molecular weight markers loaded in the 2 end lanes. Gels were run at 45 V until the samples reached the resolving gel and the markers began to separate. The voltage was then increased to 70 V until the loading buffer had run off the bottom of the gel.

3.11.4 Blotting

The glass plates were carefully removed and the stacking gel discarded. The resolving gel was placed in transfer buffer (5.8 g Tris, 2.9 g glycine, 10% SDS, 200 ml methanol, 1 L MilliQ water) for 10 minutes. One piece of nitrocellulose
membrane and 6 pieces of 3MM filter paper were cut to the exact size of the gel and soaked in the transfer buffer simultaneously.

The proteins were transferred from the gels to the nitrocellulose membrane using a semi-dry electroblotter. This apparatus was set up in the following way; 3 pieces of 3MM filter paper were placed on the anode, the nitrocellulose membrane was placed on top of the filters and any air bubbles were rolled out with a 1 ml pipette. Next, the gel was carefully picked up and set on top of the nitrocellulose membrane followed by the remaining 3 pieces of filter paper. The stack was carefully rolled again to avoid damaging the gel. Any excess transfer buffer on the anode was dried off with a paper towel. The lid containing the cathode was then fitted and the proteins blotted at 12 volts for 30 minutes (20 kD) or 45 minutes (200 kD). Larger proteins take longer to blot.

Once blotting was completed, the protein transfer was initially assessed by the colour transfer of the marker proteins. If these had not transferred completely then further blotting was performed. The membrane was then removed and soaked in Ponceau Red S solution for 5 minutes before washing in MilliRO to clear the membrane. Blotted proteins can be visualised as pink bands on the membrane. This stain does not interfere with the immunological detection methods which follow.

3.11.5 Immunological detection

The remaining steps are similar to immunohistochemistry since they involve the blocking of non-specific binding, application of primary antibody and a conjugated secondary antibody/ enzymatic detection system.

The corner of each membrane was cut off to help orientate the proteins and the lanes labelled in pencil. At this stage, the lanes to be used as negative controls were separated from the rest of the membrane. The membranes were incubated
in blocking solution (5% non-fat milk, 0.01% Tween-20, 1% thimerosal in PBS), with rocking, at 4°C overnight. The primary antibody was then applied at the correct dilution in block (to reduce non-specific background staining) and rocked for at least 4 hours at room temperature.

The primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies diluted 1:16,000 in blocking solution. Membranes were incubated in this solution for 30 minutes before three 5 minute washes in 0.05 % Tween-20 in TBS, and a final wash in TBS for 5 minutes. The membranes were incubated in Enhanced Chemi-Luminescence reagent for precisely 1 minute. Excess reagent was drained away and the membrane wrapped in cling film and exposed to X-ray film from between 30 seconds to 1 hour depending on the intensity of the signal. Proteins were sized with Rainbow markers.

3.12 Cell counting

To quantitatively assess the proportion of dividing cells in each graft, positive and negative cell nuclei stained with PCNA were counted in the following areas: 5 fields of mesenchyme from one section, 1 section per slide and 5 slides from each graft making a total of 25 counts per graft. Five grafts, for each of four treatments, were assessed in this way. Equivalent fields of bone, cartilage and epidermis were counted in each section and used as positive controls. For the purpose of measuring proliferation, osteogenic cells were defined as those contained within the periosteum. All counts were carried out blind. Data were analysed using the G-test for independence. CAM graft surface area was measured by taking the mean of the greatest length and the greatest width and then converting to surface area using the formula \( \pi r^2 \). The data were analysed using Student’s t-test. Limb size was analysed using Student’s t-test for matched pairs.
3.13 Whole mount immunohistochemistry

The protocol used was essentially that of Wheatley et al. (1993) with some modifications. Cranial tissue was dissected and bead implantation was carried out described in Section 3.8.2. Dissected tissues were then either incubated in media at 37°C for 15 minutes, 30 minutes, 1 hour, 2 hours or 6 hours or, alternatively, placed on the CAM as described in Section 3.8.3 and incubated from 12 hours to 24 hours before fixing in 4% PFA in PBS. Following fixation at 4°C overnight, grafts were washed at least 3 times in PBS for an hour each. The tissues were then dehydrated through a methanol series; 25%, 50%, 75% in PBS for an hour each, followed by 100% methanol for 30 minutes and overnight at -20°C. Tissue could be stored at this stage indefinitely. The tissue was then bleached in 6% hydrogen peroxide in methanol for an hour at room temperature before rehydrating back through the reverse dilutions of methanol to PBS. Non-specific antibody binding was then blocked by incubation in PBMT, (PBS containing 2% non-fat milk and 0.1% Triton-X100) twice for 1 hour each.

Secondary antibody (HRP-conjugated anti-rabbit IgG) was diluted to 1.6 μg/ml (1:500) in PBMT containing 10% FCS and tissues were incubated for 2 days at 4°C with rocking. Excess secondary antibody was removed by washing 6 times, for a hour each, with PBMT and then overnight with rocking. Tissue was then washed twice for an hour each with PBT (PBS containing 0.1% Triton-X100) prior to colour detection. Tissue was incubated with inactive DAB (PBT containing 0.08% Nickel Chloride (metal enhancer) and 250 μg/ml DAB) in the dark for 30 minutes at room temperature. Hydrogen peroxide was then added to a final concentration of 0.03% and the colour reaction allowed to proceed for 10 minutes in the dark. The reaction was stopped by washing the tissues in PBT twice, for 10 minutes, or longer if the background was high. The tissue was post-fixed in 4% PFA in PBS overnight at 4°C to prevent loss of stain when clearing. The tissue was washed twice in PBS, then cleared by passing through increasing concentrations of glycerol in PBS (over several days) before storing in glycerol at 4°C.
3.14 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a relatively simple technique by which unknown concentrations of antibody can be quantitatively measured. In the indirect method used in this Thesis, an excess of antigen (FGF-2) is first bound to the microtitre plate. The analyte (antiFGF-2) is then added to the sensitised wells and binds to immobilised antigen. Alkaline phosphatase-conjugated secondary antibody reacts with the captured antibody and a colour reaction is then developed using an enzymatic substrate. The amount of colour generated is proportional to the amount of antibody bound. The analysis can be quantified by including standards of known concentration.

3.14.1 Preparation of antigen-coated plate

Bovine FGF-2 was diluted in carbonate-bicarbonate coating buffer to a concentration of 250 ng/ml and 50 µl added to each well of a 96-well polystyrene microtitre plate. The plate was incubated overnight at 4°C in a humid atmosphere before washing twice with PBST (0.1% Tween-20 in PBS) and briefly drying. Binding sites were blocked by adding 200 µl 3% BSA in PBS per well for 2 hours at room temperature. Excess block was removed by washing with PBST before allowing the plate to dry.

3.14.2 Preparation of test solutions

To prepare the test solutions, washed agarose beads were transferred to 2 µl drops of either PBS, or antiFGF-2 at concentrations of 10 mg/ml, 1 mg/ml or 100 µg/ml. After 1 hour, either 1 or 3 beads were transferred to 100 µl of PBST in a microtube and the contents of the beads allowed to diffuse out without shaking for defined time points. After the allotted time, 75 µl of the supernatant was removed and stored at 4°C prior to assay.
3.14.3 Binding and detection

Known concentrations of antiFGF-2 were serially diluted in PBST and assayed with each plate. 50 µl of the solution to be tested was added to each well and allowed to bind for 2 hours at room temperature. Unbound antibody was then removed by several washes in PBST before addition of a secondary antibody conjugated to alkaline phosphatase (1:10,000 in PBST) for 2 hours at room temperature. After further washes, the reaction was detected by the addition of 200 µl 1 g/l p-nitrophenylphosphate substrate (pNPP) for approximately 30 minutes in the dark. Optical densities of the wells were read immediately at 410 nm on an Elisa Plate Reader. A standard curve was plotted using the readings of known antibody concentrations. The unknown readings were then converted using this graph into antibody concentrations.
4.1 Introduction

Many accounts detail the progression of intramembranous ossification in human fetal development (e.g. Bryan, 1996; Slavkin, 1979) but these often provide little information regarding the timing of critical events. Those reports which do discuss this, often provide conflicting information. In the developing chick, limb differentiation has been widely studied, however, less is known about the temporal and spatial formation of the cranial bones. The premise of this project was to analyse skull development and perturb it in some way, and so it was crucial to map the development of cranial bones and sutures before starting experimental analyses.

To fulfil this, a series of embryonic chick embryos from 8 to 16 days of development were stained for bone and cartilage using a whole mount skeletal technique (Klymkowsky and Hanken, 1991). This technique makes use of differential stains for bone (red) and cartilage (blue) whilst simultaneously clearing all non-skeletal tissues. This allows the visualisation of each element, a necessary requirement given that some elements (particularly those within the jaw) are quite delicate and would be impossible to identify otherwise. The tissue was first fixed in Alcian Blue, which binds to sulphated glycosaminoglycans (GAGs), the presence of which is characteristic of cartilage matrix. Calcified bone was subsequently stained with Alizarin red and the preparation cleared. Each stage was then analysed to assess the degree of ossification in both the appendicular and cranial skeletons.

To assess cranial osteogenesis at the microscopical level, whole chick heads were embedded and sectioned before staining with either haematoxylin and
eosin (for general histology), or Alcian blue and Durazol red (for cartilage and bone respectively). The latter stains earlier components of bone than those detected by Alizarin red (which is a marker of mineralised matrix) and therefore reveals the presence of bone at an earlier stage.

4.2 Results

4.2.1 Whole mount skeletal preparations

At 8 days of development, the first stage analysed, there is already a considerable amount of cartilage (stained blue) formed within the appendicular skeleton (Fig 4.1, A). This clearly illustrates the first stage of endochondral ossification, that of the formation of a cartilage template. Although bone is not detectable in the limbs at this stage, the individual limb elements can already be identified. Within the axial skeleton, the vertebrae also ossify endochondrally and these are clearly defined as cartilaginous rings at 8 days. Those bones in the skull which develop from cartilage, the occipital bone at the base of the skull, and the premaxilla, are also distinctive at this stage.

Midway through gestation at 10 days, (Fig.4.1, B) the occipital cartilages at the base of the skull have fused together. Although there has been a considerable increase in the size of the head, the morphology of the jaw has changed little, and ossification has yet to commence. When the cranial region is removed and viewed from a dorsal perspective (Fig.4.1, C), it is clear that the majority of the skull at this stage is undifferentiated tissue. By 11 days of development, the vertebrae have expanded in size and are more complex in shape (Fig. 4.1, D) but it is not until 12 days that the first signs of ossification can be detected (Fig 4.1, E). At this stage, the supra-angular begins to stain pink (arrow) and this marks the initiation of intramembranous ossification since clearly this bone is
Figure 4.1 Whole mount skeletal preparations (1)

Chick embryos from 8 to 13 days were stained for bone (pink) and cartilage (blue) to assess skeletal development. At 8 days (A), cartilage has formed the template of the limbs and vertebral column. By 10 days (B) the occipital cartilages are distinctive at the base of the skull. This is clearly shown in a dorsal view of the skull at this stage (C). There is still no bone apparent at 11 days (D) but by the next day (E), the first condensation of mineralised bone marks the differentiation of the supra-angular bone (arrow). Intramembranous bones in the jaw rapidly develop, and by 13 days (F), several more bones can be identified. Bar=2mm.
Figure 4.2 Whole mount skeletal preparations (2)

From 14 to 15 days, the cranial bones begin differentiating intramembranously. These first appear at 14 days (A-C) around the eye and anterior to the occipital cartilage (A). A dorsal view illustrates the frontal bones (B), which at higher magnification (C) appear only partially mineralised at this stage. At 15 days (D-F), most of the viscerocranium is strongly mineralised, only a few cartilaginous structures remain. The cranial bones are rapidly growing toward the midline (D). At high magnification, isolated osteoblasts delineate the edges of the future bones but are absent from the presumptive sutures (E, suture marked by arrows). Osteoid matrix (os) deposited at the leading edges of parietal bones illustrates the progression of intramembranous ossification (F). f: frontal bone; os: osteoid matrix; p: parietal bone. Bar=5mm except C=2.15mm and E,F=1.6mm.
Figure 4.3 Whole mount skeletal preparations (3)

By 16 days of development, the viscerocranium is almost completely ossified (A). Although the paired structure of the frontal and parietal bones is now apparent, there is still a large region of the skull which is undifferentiated mesenchyme (B,D). The osteoblasts observed a day previously are not evident and appear to be a transient landmark. The supraoccipital bone, which differentiates endochondrally from the upper portion of the occipital cartilage, can be identified at higher magnification (C). The arrangement of the major bones and sutures at 16 days is illustrated in (D). f: frontal bone; m: mesenchyme; p: parietal bone; so: supraoccipital bone. Bar=5mm, except C=1.25mm.
differentiating from membrane, without the assistance of a cartilaginous template.

Bone deposition then proceeds rapidly, and the following day (Fig. 4.1, F), the majority of the bones within the jaw are showing signs of mineralisation. These data are illustrated in Table 4.1 and show, perhaps surprisingly, that the cranial bones have yet to ossify although by this stage, large areas of osteoid matrix are visible around the eyes.

At 14 days of development (Fig. 4.2, A-C), the first mineralised cranial bones become visible. Initially, small regions of condensing mesenchyme appear around the squamosal region and in the frontal region immediately adjacent to the eye (Fig. 4.2, A). Frontal bone formation then proceeds posteriorally (Fig. 4.2, B) toward the occipital region and laterally toward the midline of the skull. The following day, ossification of the jaw is almost complete, whilst differentiation of the cranial bones is rapidly progressing (Fig. 4.2, D). The parietal bones, which with the frontal bones will ultimately constitute the major portion of the skull, are rapidly expanding towards the midline of the skull.

Between 14 and 15 days, isolated cells, stained pink with Alizarin red first appear (Fig. 4.2, C,E,F). These cells are likely to be osteoblasts, perhaps not a surprising discovery given their location. However, they are distributed throughout the cranial mesenchyme, displaying spatial patterning (although the tissue appears structureless at this stage) whilst remaining absent from the presumptive cranial sutures. This is most apparent at 15 days (Fig 4.2, E,F), where these cells clearly define the lateral edges of bones which have yet to form. At higher magnification, osteoid matrix (os) can be observed at the leading edge of the growing parietal bones (Fig 4.2, F), behind which mineralised bone stains strongly with Alizarin red.
Table 4.1 Progression of ossification in the chick

Chick embryos from 8 to 16 days were stained for bone and cartilage. The level of staining in each skeletal element was assessed and is displayed above. Bones labelled in blue form endochondrally via a cartilaginous template. Bones labelled in red form intramembranously, directly in mesenchyme. The start of endochondral ossification is marked by an area, usually in the centre of the skeletal element which does not stain for either bone or cartilage. This is referred to as the zone of degradation and is produced by the breakdown of the cartilaginous matrix prior to invasion by osteoblasts.
<table>
<thead>
<tr>
<th>Embryonic Age (Days)</th>
<th>Bone</th>
<th>Level of Mineralisation (Staining Intensity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Centre of tibia.</td>
<td>Isolated osteoblasts seen at high magnification.</td>
</tr>
<tr>
<td>10</td>
<td>Femur, tibia, hip, tarsal, humerus, ulna, radius.</td>
<td>No staining, zone of degradation present in the centre of all cartilages</td>
</tr>
<tr>
<td>11</td>
<td>Femur, tibia, tarsals. Hip, fibula. Humerus, ulna, radius, carpals.</td>
<td>High Zone of degradation increased in size.</td>
</tr>
<tr>
<td>12</td>
<td>Femur, tibia, tarsals, hip, fibula, humerus, ulna, radius, carpals. Quadratojugal, jugal, dentary, angular Supra-angular</td>
<td>High Osteoid Low</td>
</tr>
<tr>
<td>13</td>
<td>Quadrate, epibranchial (hyoid) Squamosal, prefrontal, nasal, dentary, quadratojugal, jugal Frontal - around eyes Premaxilla</td>
<td>Low Medium</td>
</tr>
<tr>
<td>14</td>
<td>Quadrate Epibranchial (hyoid) Parietal Palatine, vomer, pterygoid, squamosal, nasal, frontal - around eyes angular, quadratojugal, jugal, prefrontal, maxilla Dentary, premaxilla</td>
<td>Low Osteoid Low Medium High</td>
</tr>
<tr>
<td>15</td>
<td>Quadrate, epibranchial (hyoid), pterygoid Parietal, frontal - over mesencephalon Frontal - around eyes Squamosal, prefrontal, nasal, palatine, vomer Supra-angular, dentary, splenial, angular maxilla, premaxilla</td>
<td>Medium Low Medium Medium High</td>
</tr>
<tr>
<td>16</td>
<td>Vertebral arches, supraoccipital, exoccipital, epibranchial, pterygoid Scleral ossicles Palatine, vomer, prefrontal, nasal, articular, quadratojugal, jugal, supra-angular, splenial, frontal - over brain</td>
<td>High Low High</td>
</tr>
</tbody>
</table>
By 16 days, the last stage analysed, the distinctive paired structure of the frontal and parietal bones is emerging (Fig 4.3A, B and diagrammatically in D). In the centre of the skull, there is still a large area of undifferentiated mesenchyme which will remain so until hatching (Fig 4.3B). At this stage (16 days), the term ‘suture’ may be a misnomer since it refers to the broad region of undifferentiated tissue between osteogenic fronts. There appears no defined structure or cell type which will delineate where a suture will eventually form. The distribution of osteoblasts which previously seemed to define future sutural locations has now disappeared, and appears to be only a transient landmark. A small amount of cartilage remains in the skull, but by this stage, most of the chondrocranium has ossified to form the bones of the skull base. The morphology of bone at this level is that of a flat sheet, often described as trabeculated due to the large amount of space observed within the bony matrix (Fig. 4.3C). This structure is more apparent at the microscopical level and will be discussed later in this Chapter.

4.2.2 Microscopical progression of skeletogenesis

At 8 days of development (Fig. 4.4A), the tissue destined to differentiate into the cranial bones is homogenous in appearance. Blood vessels (bv) are the only structures within the mesenchyme. By staining for bone and cartilage, it is clear that the layer of tissue immediately subjacent to the epidermis is rich in sulphated GAGs (blue stain). It is within this mesenchymal layer that osteogenesis will take place. By 9 days (Fig. 4.4B) this proteoglycan-rich region has widened caudally. At the base of the skull, the developing occipital cartilage can be seen (Fig. 4.4C, c) and the first mesenchymal condensations are forming (as delineated by arrows). This marks the initiation of intramembranous ossification and this condensate will eventually differentiate into the squamosal bone. At 10 days (Fig. 4.4D), at a more medial location in the skull, there is still no sign of ossification.
Two days later (Fig. 4.5A,B), at the most anterior edge of the frontal bones, osteoid matrix has been deposited by osteoblasts and osteocytes into these mesenchymal condensates and stains strongly with Durazol red. This binds to premineralised bone and hence is an earlier marker of osteogenic differentiation than Alizarin red. As the frontal bones grow, they crumple at the margins, a process which enables them to expand quickly as the brain grows beneath them. At the ossification fronts (Fig. 4.5B), new matrix is being deposited and will continue to do so until the bones almost meet at the midline. The frontal or metopic suture (s) at this stage appears structureless and consists of mesenchymal cells. At the coronal suture a day later, these osteogenic fronts overlap (Fig. 4.5C) and the patency of the suture is maintained by proliferating mesenchymal cells. These two panels depict the typical arrangement of bones seen at cranial sutures. The midline sutures (metopic and sagittal) tend to be 'butt-ended' (Fig. 4.5B) whereas lateral sutures (coronal and lambdoid) are characterised by overlapping bones (Fig 4.5C). At the more lateral edges, the earliest formed bone displays the woven or trabeculated phenotype typical of membrane bone. The occipital cartilage has now extended dorsally (Fig 4.5D, compare with 4.4C) and the early signs of endochondral ossification can be identified by the presence of hypertrophic chondrocytes in more basal regions of the cartilage. At 15 days (Fig. 4.5E,F), the frontal suture is very small and to ensure that this suture does not overlap or fuse, excess bone is pushed ventrally. This is another mechanism by which the cranial bones are able to rapidly expand as the brain grows. Finally, at the articulation between the occipital bone and cartilage (Fig. 4.5F), the base of the occipital bone is ossifying endochondrally. The layer of hypertrophic chondrocytes is degrading and at its leading edge is being invaded by osteoblasts depositing osteoid matrix.
Figure 4.4 Histology of the developing skull (1)

Transverse sections of chick skulls were stained for bone (in red) and cartilage (in blue). At 8 days (A), blood vessels (bv) are the only structures found in the otherwise featureless mesenchyme. Prior to osteogenesis, at 9 days, this layer appears to be rich in proteoglycans as evidenced by the strong Alcian blue staining (B). At the base of the skull, the occipital cartilage is forming, and condensing mesenchyme (arrows) illustrates the initiation of intramembranous ossification (C). In more medial sections at 10 days, this condensation has yet to commence (D). br: brain; bv: blood vessel; c: cartilage; cp: choroid plexus; ep: epidermis; m: mesenchyme. Bar=12.5μm.
Figure 4.5 Histology of the developing skull (2)

At 12 days (A,B), in the most anterior region of the cranium, the frontal bones are clearly differentiating. These bones have folded toward the midline, enabling them to rapidly expand as the brain grows beneath them. The frontal suture at this stage is comprised of mesenchymal cells (B) and shows a morphology typical of a midline suture. In contrast, more lateral sutures (such as the coronal suture, C, shown at 13 days) are characterised by overlapping bones. At the base of the skull at this stage (D), the occipital cartilage shows early signs of endochondral ossification with the appearance of hypertrophic chondrocytes. By 15 days (E), the suture is kept open by proliferating mesenchymal cells, preventing the frontal bones from fusing. The occipital bone is almost ossified at 15 days (F), however, there is still a region of hypertrophic cartilage which is being invaded by osteoblasts. bo:bone; c:cartilage; fg:feather germ; hc:hypertrophic cartilage; pc:prehypertrophic cartilage; s:suture. Bar=50μm, except A,E=12.5μm.
4.3 Discussion

Using both macroscopical and microscopical staining techniques, the critical stages in skeletal differentiation within the chick skull have been analysed. These two techniques provide complementary information. Whole mount skeletal staining facilitates the study of the gross morphology of bone and cartilage and also the timing of the ossification of specific structures. This reveals that mineralised matrix is first apparent at 12 days within the jaw, whereupon ossification progresses rapidly, and from 14 days in the cranial region. The transient presence of osteoblasts distributed widely throughout the cranial mesenchyme is an interesting discovery since these cells seem to be specifically absent from presumptive sutures.

Sections of developing crania stained for bone and cartilage illustrate the different stages in both intramembranous and endochondral ossification. This staining technique which uses Durazol red and Alcian blue detects different components of bone from the method used on whole mount specimens (Alizarin red and Alcian blue). Hence, bone differentiation is apparent at earlier stages. At 11 days, the first mesenchymal condensations form, and by 12 days, these have been replaced by osteoid matrix (compare with 13 days as seen in whole mount preparations). The bones grow toward each other as a result of bone deposition at osteogenic fronts, until they almost meet at a suture. The patency of these sutures is thought to be maintained by mesenchymal cell proliferation (see Chapter 1). The analysis of the temporal progression of cranial patterning was crucial in deciding which stage of development would be suitable for use in experimental work. Tissue from 7 to 11 days of development, at stages before overt osteogenic differentiation commences, was therefore used in CAM-grafting experiments (see Chapter 6).
Chapter Five: Expression of FGFs in the developing chick embryo

5.1 Introduction

The importance of FGFs and FGF receptors in the development of a variety of tissues and organs has been well documented. They play a significant role in limb outgrowth and patterning (Cohn et al. 1995; Olwin et al. 1994), heart development (Sugi et al. 1995), tooth initiation and patterning (Vaahtokari et al. 1996) and brain development (Shamim et al. 1999) to name a few (discussed further in Chapter 1). Although experimental data implicates FGFs in skeletogenesis (reviewed by Szebenyi and Fallon, 1999), the focus of this research has been directed towards endochondral, not intramembranous ossification and there is little information regarding their role in cranial development.

The following criteria must therefore be established for a growth factor or other signalling molecule to be demonstrated to be of importance in normal skeletogenesis: 1) the ligand and its receptor must be expressed by the developing skeletal tissues in an appropriate spatial and temporal manner and 2) functional experiments should demonstrate that the signalling system is important in vitro and / or in vivo.

The aim of the work described in this chapter was to investigate whether the growth factors FGF-2 and FGF-4 fulfilled the first of these criteria, namely that they and their receptors were expressed in the chick embryo at critical stages of skeletal differentiation.

Both RNA and protein detection techniques have been used. Whole mount in situ hybridisation was used to examine mRNA expression at early stages of
development, before skeletal differentiation had commenced. Immunohistochemistry was then employed to assess protein expression at the cellular level, at the initiation of cranial bone differentiation. Finally, Western blotting was carried out to confirm the specificity of the antibodies used in immunohistochemical studies.

5.2 Results

5.2.1 mRNA expression of FGF-2, FGF-4 and FGFR2

5.2.1.1 Preparation of digoxygenin-labelled RNA probes

There are 2 stages in the preparation of digoxygenin (DIG)-labelled probes. The DNA sequence must be linearised to allow the RNA polymerase access for transcription. The linearised plasmid is then simultaneously labelled with DIG-UTP (which incorporates at the 3’-end of the nucleotide sequence) and transcribed with the appropriate enzyme.

Since all the plasmids used in this study were provided by different sources, it was initially important to check the presence of the insert by cutting it out from the plasmid with the appropriate restriction enzyme/s and assessing its size on an agarose gel.

5.2.1.1.1 Chicken FGF-2

The chicken FGF-2 construct contains an insert which is approximately 445 base pairs (bp) in length. The restriction map (Methods, Fig. 2.2), indicates that the enzyme Pst I cuts into the middle of this insert and produces a fragment of around 280 bp. When the insert is cut out from the plasmid, two bands are detected on the gel (Fig. 5.1). The band of larger molecular weight corresponds to the size of the vector used (in this case, around 3Kb, see Methods, Fig. 2.1 for details) and a band of lower size corresponds to the insert (lane 1). Once linearised, the plasmid migrates as a single band equal in size to the vector and
insert combined (lane 2). Transcription of linearised plasmid using T7 RNA polymerase results in a band approximately 10 times the intensity of the plasmid band, signifying that approximately 10 μg of RNA has been transcribed (Fig. 5.3). The size of the transcribed probe (475 bp) corresponds to the full length sequence of FGF-2 (lanes 1 and 2).

5.2.1.1.2 Chicken FGF-4
This construct contains a 585 bp fragment of FGF-4 which was excised with EcoR I and Hind III (Fig. 5.2, lane 1). The plasmid was then linearised in both sense (Fig. 5.2, lane 2) and antisense (Fig. 5.2, lane 3) orientations before transcribing with T7 or T3 polymerase (Fig. 5.3). After transcription, a faint band is visible at 3 Kb (corresponding to the vector) and a intense band is seen at 585 bp (corresponding to the transcribed sequence).

5.2.1.1.3 Human FGFR2 B exon
This plasmid contains an insert of approximately 250 bp specific to the IIIC exon of FGFR2 (see Introduction, Fig. 1.5). This splice variant is also known as the Bek form of FGFR2. The insert was cut out with Sac II and Pst I (Fig. 5.1, lane 3). The probe was linearised with Hind III (sense) or Xba I (antisense, Fig. 5.1, lane 4) before transcribing with T3 polymerase (sense, Fig. 5.4, lane 5) or T7 polymerase (antisense, Fig. 5.4, lane 6).

5.2.1.1.4 Human FGFR2 K exon
This plasmid contains a sequence which is specific for the IIIB exon of FGFR2 (see Introduction, Fig. 1.5) and is also known as KGFR. This insert was cut out from the plasmid with EcoR I and Hind III and migrated on the gel to 210 bp, (Fig. 5.1, lane 5) equivalent to the size shown on the restriction map (Methods, Fig. 2.5). The plasmid was then linearised using Hind III (sense) or Xba I (antisense, Fig. 5.1, lane 6) and transcribed with either T3 (sense, Fig. 5.4, lane 2) or T7 (antisense, lane 3) polymerase.
Figure 5.1 Linearisation of FGF-2 and FGFR2 plasmids

The insert in each plasmid was cut out with restriction enzymes and its size assessed on a gel before linearisation of the plasmid in either sense or antisense orientations. The insert in FGF-2 (lane 1) was cut out with Pst I which cuts into the insert and produces a band of predicted size (270 bp). The plasmid was linearised with Not I (antisense, lane 2). The insert in FGFR2 exon B was checked with Sac II and Pst I (250 bp, lane 3), for FGFR2 exon K, EcoR I and Hind III were used (210 bp, lane 5). Both FGFR2 plasmids were linearised with Xba I (antisense, lanes 4 and 6). 123 bp ladder was used as a marker (M).

Figure 5.2 Linearisation of FGF-4 plasmid

The insert size was checked with EcoR I and Hind III (arrow at 585 bp, lane 1). The plasmid was then linearised with either Hind III (lane 2) or BamH I (lane 3) for sense and antisense probes respectively. 1 Kb ladder was used as a marker (M).
Figure 5.3 Transcription of FGF-2 and FGF-4

Linearised plasmids in antisense (lanes 1 and 3) and sense (lanes 2 and 4) orientations were transcribed using T7 (antisense) or T3 RNA polymerase (sense) and DIG-UTP as a substrate. This produced a band corresponding to the full length of the insert, 475 bp for FGF-2 (lanes 1 and 2) and 585 bp for FGF-4 (lanes 3 and 4) which was 10x stronger than the band corresponding to the plasmid (3 Kb, arrow). It should be noted that the extra bands seen in lanes 1 and 2 are likely to have resulted from incomplete linearisation of the plasmid and the subsequent transcription of these fragments. Although one of these bands at 585 bp is the same size as that seen in lanes 3 and 4, it is highly unlikely to be due to contamination with FGF-4 plasmid. The expression patterns of these two probes are different and correspond to previously published data (see section 5.2.2.1 for details)
Figure 5.4 Transcription of FGFR2 probes

The insert of the K exon of FGFR2 was excised using EcoRI and HindIII and produced a band of 210 bp (lane 1). Linearised plasmid was then transcribed using T3 (lane 2) or T7 RNA polymerase (lane 3) for sense and antisense probes respectively. The B exon insert was cut out with BamHI and XhoI and produced a fragment of 250 bp (lane 4). The plasmid was then transcribed with either T3 (sense, lane 5) or T7 (antisense, lane 6) polymerase. The arrow indicates bands at around 250 bp corresponding to the transcribed probes. The less intense bands at around 400 bp seen in lanes 2,3,5 and 6 are probably due to transcription of incompletely linearised plasmid. 1 Kb ladder was used as a marker (M).
5.2.2.1 Whole mount in situ hybridisation

5.2.2.1.1 FGF-2

Expression of FGF-2 mRNA is first detected at late stage 17/early stage 18 in the cranial mesenchyme of the developing chick embryo. This expression intensifies by stage 19 (Fig. 5.5, A) and low levels of FGF-2 can also be detected in the heart. A stage later (Fig. 5.5, B), distinct expression is seen in the branchial arches, particularly proximally, where the arches bud out from the neck. It should be noted that the expression seen in the otocyst at this stage is a result of probe trapping and does not represent a true location of FGF-2 mRNA. This non-specific staining was observed with most probes used. FGF-2 transcripts also seem to be diffusely localised around the developing limb buds in the lateral plate mesoderm. This expression pattern is perhaps clearer in (Fig. 5.5, C), where a clear demarcation of FGF-2 mRNA is seen between the limb buds. At around this time, defined points of expression can be seen in the different regions of the brain. These condensations of FGF-2 mRNA do not appear to correspond to a specific structure or cell type and it is not clear what their function may be. Strong expression of FGF-2 is seen at the most distal and proximal tips of the branchial arches (Fig. 5.5, C), and weakly in more medial areas. By stage 21 (Fig. 5.5, D), FGF-2 is first detected in the neural tube and developing somites. Low levels of expression can now be seen in the limb buds.

A few embryos were embedded, sectioned and counterstained with nuclear fast red to assess FGF-2 expression at the cellular level. Sagittal sections of stage 19 embryos (Fig. 5.6, A) clearly show mRNA expression within the head mesenchyme but the brain is largely negative. The epithelia lining the ventricles of the brain, however, show strong FGF-2 expression. At the proximal edges and in the aortae of the branchial arches (Fig. 5.6, B), staining is also visible. In the dorsal aorta, clumps of blood cells are distinctly expressing FGF-2 mRNA. Mesenchyme at other locations in the embryo (i.e. below the branchial arches, Fig. 5.6, B) does not express FGF-2, confirming that the expression seen in the cranial region is specific to this location.
It became increasingly difficult to carry out in situ hybridisation on later embryonic stages, mostly due to the size of the tissue and the large amount of matrix present which promote high levels of background staining. The pattern of FGF-2 mRNA expression at stage 25 in the head was therefore impossible to map using whole mount in situ hybridisation. FGF protein expression patterns are described later in this chapter (Section 5.3). Lower levels of expression were seen in the torso at this stage, providing some information, particularly about limb development (Fig. 5.7). FGF-2 mRNA is detected in the neural tube, the developing vertebrae and in the heart (Fig. 5.7, A,B). However, perhaps the most interesting pattern of expression is seen in the limb (Fig. 5.7, C). At this stage, condensing mesenchyme is forming the template of skeletal elements, soon to be replaced by cartilage. Expression of FGF-2 is seen in undifferentiated mesenchyme but is switched off in condensing mesenchyme enabling the visualisation of the developing proximal limb elements.

5.2.2.2 FGF-4
Expression of FGF-4 mRNA is first seen at stage 17, initially in cranial mesenchyme. By stage 18, this mesenchymal staining intensifies (Fig. 5.8, A,B) and strong expression is also seen throughout the dorsal aorta. At the tip of the developing limb buds, a discrete band of FGF-4 mRNA can be seen and this marks the location of the apical ectodermal ridge (AER). FGF-4 is known to be an essential signalling molecule in limb outgrowth and addition of this growth factor to developing limbs can substitute for the AER if it is removed (Niswander et al. 1993). Sections through the limb bud at this stage (Fig. 5.9, A) clearly illustrate that the ectodermal thickening at the distal tip of the limb bud stains positively for FGF-4 mRNA. At stage 19, FGF-4 mRNA is detectable in the developing heart (Fig. 5.8, C and Fig. 5.9, B) in addition to the strong expression maintained in the cranial mesenchyme. By stage 21, the expression in
Figure 5.5 *FGF-2* mRNA expression from 3 to 4 days
Expression of *FGF-2* (denoted by pink or purple staining) is seen at stage 19 (A) in the cranial mesenchyme. By stage 20 (B,C) this staining has intensified and expression can also be seen in the lateral plate mesoderm adjacent to the developing limbs (arrowhead), in the branchial arches and in the heart. Discrete patches of expression over the brain (marked by arrows) appear at this stage and are maintained at stage 21 (D). The neural tube and developing somites also show strong expression of *FGF-2* at this stage. Bar=0.5mm in A, 0.55mm in B-D.
Figure 5.6 Cellular localisation of FGF-2 mRNA

Embryos were probed for FGF-2 mRNA before sectioning sagittally and counterstaining with nuclear fast red (stained pink). At stage 19, expression of FGF-2 mRNA (shown as blue/purple staining) is clearly restricted to cranial mesenchyme and is not seen in mesenchyme in other regions of the embryo (A) The neuroepithelia lining the ventricles of the brain show strong expression of FGF-2. At higher magnification, the proximal edges (lining the buccal cavity) and the aortae of the branchial arches show distinct expression of FGF-2 mRNA (B). Clumps of blood cells in the dorsal aorta also strongly express FGF-2 (arrows). ba, branchial arch; br, brain; cm, cranial mesenchyme; da, dorsal aorta; ne, neuroepithelium; v, ventricle. Bar=125μm in A, 50μm in B.
Figure 5.7 Expression of FGF-2 mRNA at 5 days of development

Expression is seen in the neural tube, heart and weakly in the vertebrae at stage 25 (A,B). FGF-2 mRNA is expressed in the developing limb but is absent from areas of condensing mesenchyme (C). Bar=1mm in A and B, 0.66mm in C.
Figure 5.8 Expression of *FGF-4* mRNA from 3 to 4 days

Strong expression of *FGF-4* is seen at stage 18 (A,B) in the cranial mesenchyme, lateral plate mesoderm and dorsal aorta. A distinct strip of expression can be seen in the AER (arrowheads) at this stage. By stage 19, *FGF-4* is switched on in the heart but is no longer detectable in the AER (C). By the end of 4 days (stage 21, D), the dermomyotomes can be identified by the position of stripes of *FGF-4* transverse to the embryonic axis (arrows). Bar=0.66mm in A, 0.7mm in B-D.
Figure 5.9 Cellular localisation of FGF-4 mRNA

The thickened ectoderm which marks the position of the AER at the distal edge of the limb (arrow) is strongly positive for FGF-4 mRNA at stage 18 (A). This expression disappears by stage 20. At stage 19, expression of FGF-4 mRNA is visible in the heart, particularly in the endocardium (B) and stripes of FGF-4 can be seen in the developing dermomyotomes (arrows, C). AER: apical ectodermal ridge; ec: endocardium; mc: myocardium. Bar=50μm.
Figure 5.10 Expression of FGF-4 mRNA at 5 days of development

The band of expression of FGF-4 in the developing vertebrae has now extended mediolaterally to outline the ribs (A,B). The heart does not express FGF-4 at this stage. Both the upper (C) and lower (D) limbs express FGF-4 mRNA in undifferentiated mesenchyme. Expression is not seen in mesenchymal condensates at early stages of chondrogenesis. Bar=0.86mm in A and B, 0.5mm in C and D.
the AER is lost, however, defined bands of FGF-4 mRNA mark the developing vertebrae (D). Sagittal sections of embryos illustrate bands of FGF-4 expression corresponding to early prechondrogenic mesenchymal condensates (Fig. 5.9, C).

By stage 25, the expression of FGF-4 in the vertebrae has widened ventrolaterally and the developing ribs can be identified (Fig. 5.10, A,B). FGF-4 is not detectable within the heart at this stage. The pattern of expression in the developing limbs has switched from a discrete band in the AER at stage 18 to a diffuse staining throughout the majority of the limb (Fig. 5.10, C,D). However, FGF-4 mRNA, like FGF-2, is not present in condensing mesenchyme and hence the outline of the differentiating cartilage templates can be clearly identified.

5.2.2.2.3 FGFR2
Expression of the B exon of FGFR2 is first detected at late stage 17 in the cranial mesenchyme. It is not as strongly expressed at stage 18 and 19 in the head as FGF-4 is at these stages (Fig. 5.11 A,B compare with Fig. 5.8 A,B). FGFR2 B exon mRNA is switched on in the heart and lateral plate mesoderm around stage 20 (Fig. 5.11, C).

FGFR2 exon K is expressed in largely the same locations as its splice variant. Faint expression can first be identified in the head at stage 17 (Fig. 5.12, A) and this staining intensifies a stage later (Fig. 5.12, B). Expression is also detected in the branchial arches and in the lateral plate mesoderm adjacent to the developing limb buds at this stage (Fig. 5.12, B). By stage 20 (Fig. 5.12, C), this expression is stronger although the limb buds themselves are not positively stained. The K exon, unlike the B exon, is not localised to the heart at this stage of development. At stage 23 (Fig. 5.12, D), both the upper and lower limb buds stain diffusely for the K exon of FGFR2, and mRNA can also be detected in the heart. It should be noted that at the stages analysed, FGFR2 B exon was not seen in the branchial arches, unlike its splice variant.
Figure 5.11 Expression of *FGFR2* (B exon) from 3 to 4 days

As expected, the expression of *FGFR2* closely maps that of its ligands. The B exon splice variant is switched on at stage 17 and is faintly expressed in the cranial mesenchyme by stage 18 (A). This expression intensifies by stage 19 (B), although it still remains the only site of *FGFR2* expression at this time. By stage 20 (C), this splice variant is also detected in the heart and in the lateral plate mesoderm adjacent to the limb buds. Bar=0.5mm in A, 0.62mm in B, 0.57mm in C.
Figure 5.12 Expression of FGFR2 (K exon) from 3 to 4.5 days

*FGFR2* exon K mRNA is first detected at stage 17 in the head mesenchyme (A). A stage later (B), faint expression is visible in the branchial arches and in the lateral plate mesoderm. At stage 20 (C), the major site of *FGFR2* K exon expression is still in the cranial mesenchyme. It is not until stage 23 (D) that expression can be detected in the heart, and throughout the limb buds. Bar=0.66mm in A and B, 1mm in C and D.
At stages later than 25, the size of the embryo and the large amount of matrix prevented efficient probe penetration and resulted in high background staining. Therefore, to assess the expression of FGFs in developing cranial tissues at the time of osteogenesis, (which occurs at least 5 days later than stage 25), sections of chick skulls were stained for FGF proteins using immunohistochemistry.

5.2.3 Protein expression of FGF family members

5.2.3.1 Immunoperoxidase

5.2.3.1.1 10 Days

FGF-2 protein is widely expressed in the developing head (Fig 5.13). It is strongly expressed in the brain (Fig 5.13, A), in developing bones and cartilages (Fig 5.13, B) and in the presumptive cranial sutures (Fig 5.13, C). The choroid plexus, the structure in the brain produces cerebrospinal fluid (CSF) shows high expression of FGF-2 (Fig 5.13, A). This is an interesting observation given that patients with craniosynostosis show an increased production of CSF. Both the periosteum (the layer of cells surrounding bone) and the perichondrium (the layer around developing cartilage) stain strongly for FGF-2. However, expression is weaker in cartilage itself (Fig 5.13, B) since only a proportion of chondrocytes appear positively stained. A high level of FGF-2 protein is maintained throughout the structure of the bone (Fig 5.13, C). The increase in staining observed in the cranial suture (compared with the surrounding mesenchyme) (Fig 5.13, C) is particularly interesting, since there is no defined structure or overtly specialised cell type present at this time. It cannot be attributed solely to an increase in cell density in this region. Negative control sections, in which pre-immune serum was used in place of the primary antibody, showed no staining (Fig. 5.13, D).

As perhaps expected, expression of the FGFRs closely matches that of the ligand. FGFR1 is also strongly expressed in the brain, choroid plexus,
Figure 5.13 FGF-2 protein expression at 10 days
Immunoperoxidase detection of FGF-2 in transverse sections through 10 day chick skulls. Expression of FGF-2 is high in the developing brain and choroid plexus (A). Strong staining is observed in all regions of developing bone, but appears to be higher in the periosteum (arrow, B). There is a lower level of FGF-2 protein in cartilage. At the site of the presumptive frontal suture, FGF-2 expression is upregulated (C). Sections treated with preimmune serum as negative controls show no staining (D).
b:bone; br:brain; c:cartilage; cp:choroid plexus; s:suture. Bar=50μm.
Figure 5.14 Detection of FGFR1 protein at 10 days of development

FGFR1 protein is strongly expressed by the choroid plexus and neuroepithelium but only at lower levels in the rest of the brain (A). FGFR1 protein is expressed at low levels by chondrocytes within developing cartilage. The periosteum (arrows) shows distinctive protein expression which is absent from the bone itself (B,C). Although there is an increase in the level of FGFR1 in the suture, it is not as marked as that seen with the ligand (C). Negative control sections treated with preimmune serum did not show staining (D). b: bone; br: brain, cp: choroid plexus; s: suture. Bar = 125 μm in A and D, 50 μm in B and C.
Figure 5.15 Detection of FGFR2 protein at 10 days of development

Expression of FGFR2 is higher in the perichondrium (arrows) and chondrocytes than FGFR1 at this stage (A). In bone, however, FGFR2 has the same pattern of expression and is seen at high levels in the periosteum (B). Sections treated with preimmune serum as negative controls show that this staining is specific (C). b: bone; c: cartilage.

Bar = 50μm.
developing bones and cartilages (Fig. 5.14). However, there are subtle differences in their expression patterns. Generally, FGFR1 protein is localised to specific cell types, rather than displaying a diffuse distribution. In the brain, expression is strongest in the neuroepithelium (Fig. 5.14, A) and at a lower level in the rest of the brain. The choroid plexus still shows strong expression (Fig. 5.14, A). FGFR1 protein is highly expressed in the periosteum, but not in the perichondrium, and only at a very low level in cartilage itself (Fig. 5.14, B). It is also absent from bone and, although there seems a definite upregulation of FGFR1 in the suture (Fig. 5.14, C), it is not as distinctive as that seen with FGF-2 (compare with Fig. 5.13, C). Negative controls (Fig. 5.14, D) show no staining when the primary antibody is substituted for pre-immune serum.

FGFR2 expression is almost identical to that of FGFR1 (Fig. 5.15). It appears to be more highly expressed in the perichondrial layer and in chondrocytes (Fig. 5.15, A) (compare with Fig. 5.14, B), although overall, the expression is still lower in cartilaginous tissues than in bone (Fig. 5.15, B). Sections treated with pre-inunune serum (negative controls) showed no staining (Fig. 5.15, C).

5.2.3.1.2 13 Days

At this stage of development, the bones overlap each other at the sutures (Fig. 5.16). Expression of FGF-2 is strong in the periosteum but seems to have decreased in bone itself (Fig. 5.16, A). The earliest formed bone (i.e. that furthest away from the suture) does not express the ligand at this time. Both FGF-2 and FGFR mesenchymal expression is much stronger and more specific to the cranial sutures than 3 days previously (Fig. 5.16, A,B). However, staining of FGFR1 protein at the suture remains less intense than that of its ligand (Fig. 5.16, B). There appears a slight upregulation in the level of FGFR1 protein in the perichondrium (Fig. 5.16, C) at this stage.

FGFR2 protein is also specifically upregulated at sutures (Fig. 5.17, A). In older, trabeculated bone (Fig. 5.17, B), high levels of protein are seen in the periosteum
and by osteocytes at the margins of the bone. Unlike FGFR1, FGFR2 expression remains high in cartilage. In prehypertrophic cartilage (Fig. 5.17, C), FGFR2 protein is strongly expressed. As this cartilage becomes hypertrophic (Fig. 5.17, D), a stage typified by large degenerating chondrocytes, expression of FGFR2 protein is lost. Negative control sections which were incubated with pre-immune serum in place of the primary antibody show no staining, indicating that the signal seen with antiFGF-2 and antiFGFR antibodies is specific (Fig. 5.18).

5.2.3.1.3 16 Days

FGF-2 expression within bone is now more localised to sites of active bone deposition and resorption (Fig. 5.19). In the centre of trabeculated bone, there is very little staining, whereas at the margins and within the periosteum, expression remains high (Fig. 5.19, A). Chondrocytes express FGF-2 protein at a lower level (Fig. 5.19, B) and staining is also seen within cartilage matrix at the boundary between prehypertrophic and hypertrophic chondrocytes (Fig. 5.19, C), suggesting a role for this ligand in the switch towards endochondral ossification.

Expression of FGFR1 and 2 (Fig. 5.20) is fairly similar. The choroid plexus still stains strongly for FGFR1 (Fig. 5.20, A), though only the cells around the edges rather than whole vesicles are now positive (compare with Fig. 5.14 A). In the brain, both FGFR1 and FGFR2 proteins are specifically expressed by the neuroepithelium at the junction between the cerebral hemispheres (Fig. 5.20, D) and individual neurons (arrows). Both FGFR1 and 2 proteins are found at sites of active osteogenesis (Fig. 5.20, B,E) but not in resting bone. Although FGFR1 expression in cartilage seems higher at this stage than at 13 days (Fig. 5.20, C), overall, lower levels of receptor proteins are seen in developing cartilage than in
Figure 5.16 FGF-2 (A) and FGFR1 (B, C) protein expression at 13 days
Sutural expression of FGF-2 is more specific at 13 days than at earlier stages (A). Staining remains strong in the periosteum (arrow) but is now absent from bone. FGFR1 protein is similarly located, although it is not as strongly expressed in the suture (B). There has been an upregulation of FGFR1 protein in both the perichondrium and in chondrocytes (C). b: bone; pc: prehypertrophic cartilage; s: suture. Bar=50μm.
Figure 5.17 Expression of FGFR2 protein at 13 days

Strong expression of FGFR2 protein is seen at the cranial suture and in the periosteum whereas protein is only present at low levels in bone (A,B). In earlier formed trabeculated bone, FGFR2 is found at sites of active bone deposition and resorption (B). Expression is higher in chondrocytes and the perichondrium at 13 days when compared with earlier stages (C). However, as these chondrocytes become hypertrophic, FGFR2 protein is lost (D). b: bone; hc: hypertrophic cartilage; pc: prehypertrophic cartilage; s: suture. Bar = 50 μm.
Figure 5.18 Pre-immune serum treated sections at 13 days
Sections were treated with pre-immune serum of the same species as that in which the primary antibody was raised. This was used in place of the primary antibody to assess whether staining was due to non-specific binding. Adjacent sections were used where possible. Sections were counterstained with methyl green. All tissues tested showed no staining. There is an increase in cell density in the suture, however, this is not sufficient to explain the high level of staining seen with FGF-2 and its receptors (A). Trabeculated bone and cartilage are stained only with the counterstain (B,C). The progression of apoptosis can be seen in hypertrophic cartilage as the cell nuclei degenerate, explaining why expression of FGFRs are low at this stage (D). b: bone; hc: hypertrophic cartilage; pc: prehypertrophic cartilage; s: suture. Bar=50µm.
Figure 5.19 Expression of FGF-2 protein at 16 days

FGF-2 expression is now strongest at sites of bone deposition and resorption (arrows) at this stage and is lower in inactive regions of bone (arrowheads, A). Prehypertrophic chondrocytes continue to show high levels of protein (B), however, at the boundary between prehypertrophic and hypertrophic cartilage, the matrix itself starts to express FGF-2 protein (C). b: bone; c: cartilage; hc: hypertrophic cartilage. Bar=50μm in A, 125μm in B and 25μm in C.
Figure 5.20 Expression of FGFR proteins at 16 days
A-C, FGFR1; D-F, FGFR2. FGFR1 protein is now localised to areas of cell proliferation and tissue modelling. It is expressed at vesicular edges in the choroid plexus and weakly by the neuroepithelium (A). It is absent from bone itself but is strongly expressed by the periosteum (B). There appears however to be an upregulation of FGFR1 protein in chondrocytes (C) although this expression is still relatively low. FGFR2 is seen in the layer of cells marking the point of contact between the cerebral hemispheres in the brain and in neurons (D, arrows). It is also expressed in the periosteum where osteogenic patterning is occurring (E) and at a lower level in cartilage (F). Overall, a higher level of FGFR2 protein is expressed by bone compared with cartilage (F). b:bone; br:brain; c:cartilage; cp:choroid plexus. Bar=125µm in A and F, 50µm in B, D and E, and 25 µm in C.
bone. This is clearest when bone and cartilage are in close proximity (Fig. 5.20, F) illustrating that FGFR2 expression is much higher in differentiating bone.

5.2.4 PCNA Protein expression

For reasons which will become apparent later, a marker of cell proliferation was used to assess sites of active cell division within the developing head. Given that the expression pattern of the marker used closely matches that displayed by FGF-2 and FGFRs 1 and 2, it is a logical point at which to discuss this in the Thesis. Proliferating cell nuclear antigen (PCNA) is a cyclin-associated protein and is synthesised in early G1 and S phases of the cell cycle. It is specifically located to the nuclei of cells in which cell division is occurring (Hall et al. 1990).

At 10 days of development, PCNA protein is found in cell types also positive for FGF proteins (Fig. 5.21). The developing choroid plexus and ependymal layer are sites of rapid proliferation (Fig. 5.21, A), as are the developing bones and cartilage (Fig. 5.21, B). This expression pattern is similar at 13 days (Fig. 5.21, C-E), PCNA protein marks the junction of the left and right cerebral hemispheres (Fig. 5.21, C). Cell proliferation is rapid at the periosteum and staining is strongest at the growing edges of bone (Fig. 5.21, D). The perichondrium, however, is not an active site of cell proliferation (Fig. 5.21, E) although chondrocytic nuclei are proliferating. By 16 days (Fig. 5.22), distinct regions of the developing brain are undergoing cell proliferation and are delineated by PCNA staining (Fig. 5.22, A). At the leading edge of differentiating bone (Fig. 5.22, B), the level of proliferation is maximal, as new bone is deposited. More laterally, cell division declines and in trabeculated bone (Fig. 5.22, C), there is a lower level of proliferation in the periosteum. Much of the cell division within the bone itself can be attributed to vasculogenesis. Chondrocytes are now becoming hypertrophic (Fig. 5.22, D) and as such, the level of cell proliferation is naturally low.
Rapid cell proliferation is seen in the developing choroid plexus and in the ependymal layer of the brain at 10 days (A). The leading edge of the developing parietal bone is outlined by PCNA-positive cells (B). By 13 days, cell proliferation marks out different regions of the brain and the pia which separates the two ventricles can be identified (C). In newly formed bone, the level of proliferation is reduced and PCNA protein is only seen around the periosteum (D, arrowhead). By contrast, the perichondrium (arrows) is not proliferating at this stage, although the majority of chondrocytes are (E). A,B 10 days; C-E, 13 days. b:bone; br:brain; c:cartilage; cp:choroid plexus; el:ependymal layer; p:pia; v:ventricle. Bar=25 μm in A and C, 50μm in B, D and E.
Figure 5.22 Expression of PCNA protein at 16 days

Cell proliferation is still high at boundaries between distinct regions of the brain, particularly in the ependyma lining the ventricles and the pia at the ventricular junction (A). As at 13 days, PCNA expression is maximal at the leading edge of developing bone (the osteogenic induction front) and decreases more laterally (B). In trabeculated bone, expression in the periosteum is now very low (C). Cartilage is becoming hypertrophic at this stage and chondrocytes are undergoing apoptosis, the level of cell proliferation is therefore low (D). b: bone; br: brain; c: cartilage; el: ependymal layer; OIF: osteogenic induction front; p: pia; v: ventricle. Bar= 125 µm in A, 50 µm in B-D.
5.2.5 Western Blotting

To assess the specificity of the antibody used in subsequent blocking experiments, and to assess biochemically the presence of FGF and FGFR proteins in the developing skull, Western blotting was carried out. Proteins were extracted from 10 day embryonic chick heads and separated by SDS-PAGE. Brain, presumptive cranial region, or both fractions combined were analysed for the presence of FGF-2, FGFR1 and FGFR2.

![Western blot images](image)

**Figure 5.23 Western blotting of FGF-2, FGFR1 and FGFR2 at 10 days**

M: molecular weight positions, as indicated by molecular weight markers run in the experiments but not shown in the Figure; W: whole (brain and skull fractions combined); B: brain; S: skull. The distinct band recognised by antiFGF-2, at 18kD, corresponds to that of the positive control (+ve; bovine FGF-2). In addition the antibody appears to detect several other isoforms of FGF-2. These high molecular weight forms show distinct expression patterns in different regions of the head with the band with highest molecular weight seen in the brain, whereas two others with lower molecular weights are detected mainly in the skull. All three are detected in the whole head. These may be dimers or post-translationally modified forms of FGF-2. The bands detected by antiFGFR1 (at 110kD) and antiFGFR2 (at 94kD) correspond to protein species already described for these antibodies.
FGF-2 was present in all fractions tested (Fig. 5.23), however, different molecular weight forms were seen between the tissue types. All three fractions contain a common 18 kD form of FGF-2 which migrates at the same rate on the gel as bovine derived FGF-2 (+ve lane in Fig. 5.23). In the skull, two larger molecular weight forms are also prominent at approximately 36 kD and 60 kD. The former is likely to be a dimer of the 18 kD form since FGFs dimerise when binding to receptors. The larger isoform may be a result of a post-translational modification, possibly glycosylation since FGFs are known to have putative glycosylation sites.

FGFR1 and 2 antibodies detect two major bands by Western blotting. The lower weight band in each case corresponds to the immature protein at 110 kD (FGFR1) and 94 kD (FGFR2) as reported by Dionne et al. (1990). It is likely that the larger bands are glycosylated forms at 130 kD (FGFR1) and 150 kD (FGFR2) (Callard and Gearing, 1994).

5.3 Discussion

The expression of mRNAs for FGF-2 and 4 and the B and K splice variants of FGFR2 have been mapped at early stages of skeletogenesis. It is clear that prior to overt osteogenesis in the skull, strong expression of FGF-2, FGF-4 and FGFR2 can be detected in the neural crest-derived cranial mesenchyme from which the cranial bones and cartilages will ultimately differentiate. Outside the skull, FGFs are implicated in heart morphogenesis and are present in the branchial arches, which will eventually form the majority of the facial region.

FGFs can be detected at critical points in the development of the limb, and appear to be down-regulated at the initiation of chondrogenesis. However, the expression pattern is inconsistent with published data since FGF-2 could not be detected in the limb bud until stage 24. Other work has suggested that FGF-2 protein is present in the limb from initiation of budding through differentiation.
The dorsal AER and subjacent mesoderm often termed the ‘progress zone’ show
the strongest immunostaining, with lower levels in the centre of the bud (Dono
and Zeller, 1994; Savage et al. 1993). FGF-2 mRNA expression has yet to be
analysed, and one explanation for this discrepancy may be that there are
different isoforms of FGF-2 exhibiting distinct expression domains in the limb.
This theory is partly corroborated by Western blotting data, at least in the skull,
where several prominent bands corresponding to FGF-2 protein species have
been detected. Recent work on FGF-13, a newer member of the family, has
found that two isoforms of this gene show spatially distinct expression patterns
in the limb, neural tube and sensory ganglia (Munoz-Sanjuan et al. 1998). It is
important to note that at present, commercial antibodies are not specific for
particular splice variants or isoforms. It will be interesting to determine the
precise localisation of the different isoforms when specific antibodies become
available.

The size and cell density of embryos at later stages precluded further expression
studies using whole mount in situ hybridisation at the time of skeletogenesis.
Therefore, protein expression at these stages was analysed. Blocking
experiments discussed later (Chapter 8) involved the neutralisation of FGF-2
protein, so mapping the normal expression of FGF proteins enables comparisons
to be made between normal and abnormal development. Both FGF-2 and FGFR1
and 2 have been found to be expressed in similar locations, and are detected at
critical time points in skeletal development. Expression of FGF-2 is high
throughout bone, and is also concentrated at cranial sutures. FGFRs are also
found at higher levels in sutures (although at a later time) and this novel finding
may have significance for the maintenance of open sutures since, at this stage,
there is no obvious structure or cell type which might play a role in sutural
patency. It may be that the presence of FGFs in cranial sutures drives
mesenchymal cells to divide rather than differentiate, thereby preventing sutural
closure. Elsewhere, FGFR proteins are found in roughly the same locations as
previously reported in gene expression studies (Chan and Thorogood, 1999;
Delezoide et al. 1998; Iseki et al. 1997). Receptors are expressed by the periosteum, rather than in bone itself, suggesting a role in osteogenesis rather than bone maintenance.

Western blotting confirms the immunohistochemical data and verifies the specificity of the antibodies used in this study. This was essential in the case of FGF-2 since it must be proved that this antibody (used in later experiments, see Chapter 8) specifically recognises and blocks FGF-2 and not other closely related proteins.

The expression pattern of PCNA protein closely matches that of FGFR1 and 2 and to a lesser extent, FGF-2. The presence of PCNA staining is indicative of cell proliferation and in tissues which are undergoing growth at this time, for example the choroid plexus, staining is intense. The periosteal, but not the perichondrial layer is marked by PCNA-positive cells and at the OIF, prior to overt bone formation, the level of proliferation is elevated. This finding is in agreement with Iseki and co-workers who noted that Fgfr2 expression in the mouse strongly correlates with BrdU expression. In addition, both Fgfr2 transcription and the most active site of cell proliferation were localised to the skeletogenic membrane (Iseki et al. 1997). In cartilage, by 16 days, endochondral ossification is progressing and the level of PCNA staining is barely detectable. This downregulation of expression marks the destruction of the chondrocytes prior to the deposition of osteoid and osteoblast migration.

Clearly, all FGFs analysed are present throughout skeletogenesis in the appropriate spatial locations and can therefore be confirmed as important factors in normal skeletal differentiation. In the next Chapter, CAM-grafting is used as an experimental method to enable the manipulation of endogenous FGF-2 levels during skeletogenesis.
Chapter Six: CAM-grafting (1) – Differentiation of cranial tissues and the effect of FGF-2

6.1 Introduction

The previous Chapter demonstrated that members of the FGF family are expressed at suitable locations and at the appropriate times during development substantiating evidence for their predicted roles in skeletogenesis. Having established this, it was then necessary to use functional experiments to confirm that the signalling system was important in vivo.

At the stages at which critical skeletogenic events are occurring (9-13 days), the embryo is itself highly developed. In particular, it possesses a rich and complex vascular system which makes it technically difficult to operate on the embryo and maintain a high rate of survival, a necessary requirement for functional studies. Instead, the developing vasculature was harnessed without invasive surgery to the embryo itself.

This technique is termed Chorio-Allantoic Membrane Grafting (CAM-grafting) and makes use of the highly vascularised chorio-allantoic membrane which forms in the chick at 6 days of development. It essentially involves the establishment of vascular connections between the chorioallantoic circulation and the graft tissue or organ (Coulombre, 1967). In this instance it enables cranial tissue to be manipulated in a way that is not practical in vivo, followed by growth in a sterile vascular environment.

CAM-grafting was originally modified from a method used to inject cells from mammalian and avian sarcomas into chick embryos (Rous and Murphy, 1911). It was noted at the time that a well vascularised sarcoma developed at the point where the needle had punctured the CAM and that this was serially
Figure 6.1 Chorio-allantoic grafting
Structure of a chick egg at 10 days of development. The air space at the blunt end of the egg has been punctured to allow the chorio-allantoic membrane to drop down. The transplant (bead-implanted skull piece) is then introduced through the window in the top of the shell. Adapted from Hamburger (1973).

transplantable from host to host (Murphy, 1912). The ability of the CAM to vascularise and support exogenous tissues was then adapted to embryological investigations in 1924 by Willier and co-workers (Willier, 1924). Since then, CAM-grafting has been successfully used to assess the morphogenesis of a number of tissues, possibly the most common involving transplantation of the developing limb (Hunt, 1932). However, it has also been applied to the development of skin and eye primordia, particularly the optic vesicle and cup (Hamburger, 1973). The transplanted tissues are isolated from the structures of the host embryo so that inductive effects cannot obscure the results. However, the grafts are incorporated into the vasculature of the host and are exposed to hormones and other substances, the isolation is therefore incomplete.
In these experiments, endogenous levels of FGF-2 were manipulated in an attempt to perturb normal skeletal morphogenesis. Initially, experiments were carried out to decide upon which stage to CAM-graft by assessing normal skeletal patterning in tissue grafted at different ages. Subsequently, exogenous FGF-2 protein was added to developing skeletal tissues in different locations and the effects on morphogenesis assayed by staining the grafts for bone and cartilage (a technique previously used in Chapter 4). These experiments form the basis of this Chapter.

6.2 Results

6.2.1 Establishment of grafting conditions

Cranial tissues were dissected out at stages from 7 to 11 days of development. These were grafted onto the CAM until they had reached 16 days of development before recovering and staining for bone and cartilage. In addition to assessing skeletal patterning, the percentage of successful grafts recovered was also noted since this would determine the best stage at which to graft tissue. It is clear that grafting tissue younger than 9 days does not result in normal skeletal morphogenesis (Fig. 6.2, Table 6.1). Tissue grafted at 7 days tends to remain undifferentiated and no discernible structures can be identified (Fig. 6.2, A). In addition, these grafts do not substantially increase in size. 8 day grafted tissue occasionally formed small nodules of bone (Fig. 6.2, B, 35% of grafts), however, these could not be characterised in terms of the type of bone or its orientation. Tissue grafted at 9 days exhibits two distinct phenotypes (Fig. 6.2, C,D). A proportion of these grafts appear to proceed through osteogenesis and produce large amounts of bone (Fig. 6.2, C). However, the patterning of this bone was aberrant and the paired structure of the frontal and parietal bones was not identifiable. The remaining grafts appear normal in terms of skeletal patterning but the frontal bones do not fully differentiate. For instance, it is clear from Fig. 6.2D that the occipital cartilages have formed normally in the graft but
that only rudimentary frontal bones are present. Tissues grafted at 10 days show skeletal patterning and differentiation proceeding as normal (Fig. 6.2, E). The last stage to be grafted, 11 days, also differentiates correctly (Fig. 6.2, F). This stage was not chosen for subsequent experiments, as the size of the tissue to be grafted limits the rate of recovery (only 30%, Table 6.1) at the end of the experiment.

<table>
<thead>
<tr>
<th>Age at Grafting</th>
<th>Number of Grafts</th>
<th>% Bone</th>
<th>% Patterned</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 Days</td>
<td>18</td>
<td>0</td>
<td>0</td>
<td>82</td>
</tr>
<tr>
<td>8 Days</td>
<td>20</td>
<td>35</td>
<td>0</td>
<td>63</td>
</tr>
<tr>
<td>8.5 Days</td>
<td>6</td>
<td>33.33</td>
<td>0</td>
<td>76</td>
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<td>0</td>
<td>73</td>
</tr>
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<td>10 Days</td>
<td>10</td>
<td>100</td>
<td>90</td>
<td>69</td>
</tr>
<tr>
<td>11 Days</td>
<td>6</td>
<td>100</td>
<td>83.33</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 6.1 Summary of preliminary grafting experiments
Cranial tissue was dissected out from 7 to 11 days and grown on the CAM of host embryos until it had reached 16 days. The incidence of bone formation, whether the bone was patterned correctly and the rate of graft recovery was recorded in each category.

CAM-grafting was originally designed for the growth of small pieces of tissue which readily incorporate into the vascularised membrane. With 11 day grafts, the initial size and weight of the tissue to be grafted puts pressure on the CAM with the result that the graft either incorporates and causes the host to die, or does not incorporate at all. Tissue grafted at earlier stages exhibit relatively consistent rates of recovery (Table 6.1), although skeletal morphogenesis is aberrant. With this in mind, tissue at 10 days was chosen as the starting point for CAM-grafting since at this stage, approximately 70% of grafts are recovered and skeletal patterning is near normal. In addition, occipital cartilages are well
Figure 6.2 Establishment of normal stages to CAM-graft

A, 7 days; B, 8 days; C and D, 9 days; E, 10 days; F, 11 days. Cranial tissue was dissected out and grafted until 16 days of development. Recovered grafts were then stained for bone and cartilage. In tissues grafted at 7 (A) or 8 (B) days, growth and morphogenesis were aberrant and little, if any bone formed. In 9 day grafts (C) osteogenesis can be detected although the lack of patterning of the skeletal tissues makes orientation impossible. Alternatively, discernible bones and cartilages form in the correct locations but do not fully differentiate (D). In tissues grafted at 10 days, both differentiation and morphogenesis are normal (E). Although correctly patterned grafts are recovered when grafted at 11 days (F), the initial size of the tissue precludes an efficient recovery rate. Bar=1mm in A and C-E, 1.7mm in B and 0.7mm in F.
formed and by ensuring that the caudal region is included in the graft, the orientation of the tissue following grafting is much easier.

6.2.2 Exogenous FGF-2 has no effect on cranial skeletogenesis

Heparin-coated acrylic beads have previously been used by several groups to investigate the roles of growth factors in the development of a specific tissue/organ (particularly the limb e.g. Cohn et al. 1995). These beads are able to bind a particular class of proteins via heparin linkages on their surface before releasing the bound proteins over an extended period of time. Many growth factors, including FGFs, require heparin as a co-factor for receptor binding and/or dimerisation and as such are good candidates for exogenous application using the heparin bead-release system.

Bead soaked in FGF-2 at 1 mg/ml (a concentration used in other systems with significant results) were implanted into dissected 10 day cranial tissue in various locations. These grafts were then grown for 6 days on the CAM (until they reached 16 days), recovered and stained for bone and cartilage to assess skeletal morphogenesis. Beads were implanted into either:

1. Undifferentiated mesenchyme not destined to commence differentiation during the grafting period.
2. Early osteoid matrix which was just forming at this stage.
3. Presumptive frontal bone, yet to show overt signs of differentiation.
4. Presumptive frontal suture.

By implanting beads into these different sites, if ectopic FGF-2 protein were to induce or inhibit osteogenesis it should be relatively easy to detect.

It became clear, however, that despite implanting beads into diverse sites, skeletogenesis is unaffected by exogenous FGF-2 in terms of both spatial and
Figure 6.3 Exogenous FGF-2 has no effect on skeletogenesis in CAM-grafts

Heparin-coated acrylic beads were soaked in either 1 mg/ml (A, B) or 10 mg/ml (C) FGF-2, or PBS (D) and implanted into grafted cranial tissue at 10 days. Skeletal patterning was then assessed by staining for bone and cartilage. FGF-2 beads do not affect normal skeletal patterning when implanted into undifferentiated mesenchyme (A), or developing frontal bone (B). Higher concentrations (10 mg/ml) of FGF-2 also have no effect on differentiation (C). Control grafts were implanted with beads soaked in PBS (D). Arrows denote site of bead implantation. Bar=2mm except B=1.2mm.
temporal patterning (Fig. 6.3). If the addition of ectopic FGF-2 induced osteogenesis in an area where bone is not expected to form at this time, a region of bone around the bead should be visible. Conversely, if additional FGF-2 inhibits bone differentiation, a zone of osteogenic inhibition should be seen around the bead. Neither of these situations occur however, as seen in Fig. 6.3A and B, respectively and it appears that FGF-2 has no effect on cranial skeletogenesis. Different concentrations of FGF-2 were subsequently used. Addition of beads soaked in 5 mg/ml or 10 mg/ml FGF-2 (Fig. 6.3, C) also do not affect skeletal patterning. Grafts implanted with a PBS-soaked bead were used as controls for comparison (Fig. 6.3, D) and illustrate the patterning of skeletal elements seen at 16 days following grafting. It should be noted that some minor distortion of the bones is generally seen since grafting removes the cranial vault from the tensile forces to which it is normally exposed *in situ*. The gross morphology of the cranial bones however, is equivalent to that seen *in vivo* (compare Fig. 6.3, D with Fig. 4.3). A summary of the data generated from these experiments is displayed in Table 6.2 below.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Grafts</th>
<th>% Bone</th>
<th>% Patterned</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>14</td>
<td>100</td>
<td>92.86</td>
</tr>
<tr>
<td>1 mg/ml FGF-2</td>
<td>21</td>
<td>100</td>
<td>95.24</td>
</tr>
<tr>
<td>5 mg/ml FGF-2</td>
<td>6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>10 mg/ml FGF-2</td>
<td>7</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

*Table 6.2 Summary data of grafts treated with exogenous FGF-2*

Cranial tissue was dissected out at 10 days and implanted with either an FGF-2 or a PBS soaked heparin bead. All grafts showed some level of osteogenesis, and in almost all cases, skeletal patterning was normal.
6.3 Discussion

The experimental data in this Chapter reveal two important findings. Firstly, the most efficient stage at which to graft cranial tissue has been determined to be 10 days of incubation. This stage was chosen for its ability to pattern correctly and graft efficiently. Perhaps most significantly, it is a point in development at which the cranial bones are not yet formed and perhaps not fully determined. There are probably numerous reasons why grafts of younger stages did not pattern normally but most likely is that some level of cell commitment is required before grafting. It may also be that some as yet unidentified inductive signal from the brain is required for such a commitment. The brain is removed prior to grafting and this may be a major factor in explaining why immature cranial tissue is inadequate for these grafting experiments.

The second finding, that cranial tissues do not respond to FGF-2 was surprising since other experiments have used the same bead implantation system in the limb to good effect (Cohn et al. 1995; Riley et al. 1993). In addition, other work from the lab had implicated FGF-2 in the skeletogenic differentiation of neural crest cells in vitro (Sarkar and Thorogood, 1999). The majority of the cranial vault is derived from neural crest, and so this negative result was extremely perplexing. Therefore, to verify that these beads were releasing biologically active FGF-2 protein, they were implanted into developing limb buds. The findings of these experiments are the subject of the next Chapter.
Chapter Seven: FGF-2 and limb development

7.1 Introduction

FGFs have long been implicated in limb specification, outgrowth and differentiation with most studies focusing on the roles of FGF-2, FGF-4 and FGF-8 (see Szebenyi and Fallon, 1999 for review). There are two significant publications in terms of the data presented in this Chapter. Chick limbs implanted between stages 20-22 with anterior limb cells infected with FGF-2 expressing viruses resulted in duplication of limb elements (Riley et al. 1993). In addition, implantation of an FGF-2 soaked bead into the anterior region of the limb bud resulted in a mild duplication of limb elements. These data are highly suggestive of a role for FGF-2 in skeletal patterning.

The second piece of evidence is the dramatic discovery by Cohn et al. (1995) that the application of FGF-1, -2 or -4 beads into the presumptive flank of chick embryos can induce the formation of additional limbs. Again, the ectopic limbs themselves showed evidence of duplicated elements. These experiments used the same source of FGF-2, at the same concentration, and the same type of beads which had been used in the previous Chapter. Therefore, a crude measure by which to confirm that these beads were releasing biologically active FGF-2 was to replicate these experiments. In fact, a modification of these two techniques was used, and FGF-2 beads were implanted into the mesoderm of the developing limb bud to assess the effect on skeletal patterning.

7.2 Results

FGF-2, antiFGF-2 or PBS soaked beads were implanted into limb buds in vivo at approximately stage 21. The operated embryos were then allowed to develop until 10 days (at which time skeletal patterning is completed) before fixing and
staining for bone and cartilage. Both heparin-coated and agarose beads were used in these experiments since it was later discovered that agarose beads appeared to release higher levels of growth factor than an equivalent sized heparin bead. Individual limb elements were then measured and statistically analysed to assess whether each treatment had a significant effect on morphogenesis.

7.2.1 Heparin-coated beads

FGF-2 soaked heparin-coated acrylic beads implanted into developing limb buds have mild effects on morphogenesis. Examples of typical phenotypes are illustrated in Fig. 7.1. When beads are implanted proximally, into the site of the developing humerus (arrow), this limb element is primarily affected (Fig. 7.1, A). In this instance, the humerus is truncated and thickened. Implantation of beads into more distal sites (Fig. 7.1, B), results in aberrant morphogenesis in this region with the radius and ulna thickened and shortened whilst the humerus is unaffected. The contralateral limb (on the left side in each panel), which was used as an internal control differentiated normally. Similarly, limbs implanted with PBS-soaked beads (used as procedural controls) differentiated normally (Fig. 7.1, C).

In subsequent studies (see Chapter 8), agarose beads were used for the release of antiFGF-2 into the developing skull. These appear to soak up a solution rather like a sponge whereas heparin-coated acrylic beads bind growth factors via heparin linkages over their surface. As such, agarose beads have the potential to release larger quantities of a given solution from a similarly sized bead. In addition, these beads are much easier to handle and the remaining experiments in this Thesis were therefore completed using agarose beads.
Figure 7.1 Implantation of heparin beads soaked in FGF-2 into the limb

FGF-2 (A,B) or PBS-soaked (C) heparin acrylic beads were implanted into upper limb buds *in vivo* at stage 21, allowed to develop until 10 days before fixing and staining for cartilage. The implanted limb is on the right in all panels. At the site of bead implantation (marked by arrows), ectopic FGF-2 causes aberrant morphogenesis in the form of truncated and thickened limb elements. Proximal sites of implantation result in mispatterning of the humerus (A, right limb) whereas more medial sites affect the ulna and radius (B, right limb). PBS beads do not affect normal skeletal patterning (C). The contralateral side was used in all cases as an internal control and is shown on the left side of each panel for comparison. h:humerus; r:radius; u:ulna. Bar=1mm.
7.2.2 Agarose beads

As predicted, the effect of implanting an FGF-2 soaked agarose bead, incubated in the same concentration of FGF-2, has a more severe consequence on limb patterning than an equivalent heparin bead (Fig. 7.2). Perhaps the most noticeable difference was the ability of FGF-2 agarose beads to exert a profound influence on differentiation at distances away from the site of implantation. Therefore, implantation of a bead near to the developing radius (A, arrow) shortens and truncates not only the ulna and radius but also the humerus. Implantation of two beads typically results in a more severe phenotype (B, arrows). Again, limbs implanted with PBS beads could not be distinguished from the unoperated contralateral limb (C). In later studies, delivery of antiFGF-2 is shown to have a severe effect on cranial skeletogenesis (see Chapter 8), it was therefore interesting to analyse limb patterning following the implantation of antiFGF-2 soaked beads. Surprisingly, these beads have no detectable effect on limb morphogenesis (D, arrow) a finding which will be discussed later.

To assess the quantitative differences between FGF-2, antiFGF-2 and PBS implanted limbs, the width and length of each limb element (humerus, radius and ulna) was measured and plotted on a scatter plot. In all cases, limbs treated with FGF-2 cluster away from those treated with antiFGF-2 or PBS on a scatter plot, indicating that these elements tend to be shorter and thicker than those on the contralateral side (Figs 7.3-7.5). In addition, this effect is strongest in the humerus (Fig. 7.3), and becomes less marked in the radius (Fig. 7.4) and ulna (Fig. 7.5).

The differences between the dimensions of limb elements in each treatment were then analysed statistically using Student’s t-test for matched pairs (Table 7.1). In each case, the matched pair corresponds to the treated and contralateral limb element. This clearly shows that there are significant differences between FGF-2
Figure 7.2 Implantation of agarose beads soaked in FGF-2 into the limb

FGF-2 (A,B), PBS (B) or antiFGF-2 (C) soaked agarose beads were implanted into limb buds at stage 21. FGF-2 agarose beads have a more potent effect on limb development than do the equivalent heparin beads. Humerus, radius and ulna are all truncated and thickened (A). Implantation of two FGF-2 beads results in a more severe phenotype (B, arrows). PBS (C) or antiFGF-2 (D) beads do not affect normal skeletal patterning (right side on each panel). The unoperated contralateral side is shown for comparison (left side of each panel). Arrows denote the site of bead implantation. h:humerus; r:radius; u:ulna. Bar=1mm.
Figure 7.3 Effect of FGF-2 and antiFGF-2 coated agarose beads on humerus development

Each point on the scatter plot represents the measurement of an individual limb element (humerus). Implantation of FGF-2 beads into the limb bud (blue circles) results in truncated and thickened limb elements whereas antiFGF-2 or PBS implanted limbs do not show any deviation from the size of normal (contralateral) limbs.

Figure 7.4 Effect of FGF-2 and antiFGF-2 coated agarose beads on radius development.

See Fig. 7.3 for explanation of the graph. The effect of FGF-2 on the development of the radius is less marked than on the humerus since generally, bead implantation occurred nearer to the time of humerus development.
Figure 7.5 Effect of FGF-2 and antiFGF-2 coated agarose beads on ulna development

See Fig. 7.3 for explanation of the graph. FGF-2 has a slightly less severe effect on the development of the ulna than on the humerus and the points are therefore more skewed towards the group of control limb elements.

treated limbs and their contralateral sides, but not between antiFGF-2 or PBS treated limbs. As perhaps predicted from the scatter plots, the level of significance is lower in the radius and ulna than in the humerus. At the time of bead implantation (stage 21), specification of proximal limb elements (humerus) is occurring. Therefore, it is probable that FGF-2 will have a stronger effect on this element than on later forming structures such as the ulna and radius. The site of bead implantation also affects the degree to which individual limb elements are disrupted. In these experiments, the positioning of the bead was quite crude as the original aim was to assess whether the FGF-2 bead was capable of perturbing normal development. It is therefore likely that small adjustments to the site of bead implantation will cause large changes in morphogenesis of individual limb elements.

In these experiments, the digits are unaffected and this result is representative of the state of differentiation at the time of implantation. Digital patterning does not
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<td>0.84±0.08</td>
<td>4.45±0.16</td>
<td>0.4±0.03</td>
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<td></td>
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<tr>
<td>Treated</td>
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<td>0.39±0.03</td>
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<td>0.26±0.01</td>
<td>3.80±0.10</td>
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<td>0.26±0.01</td>
<td>3.84±0.09</td>
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<td>Significance</td>
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<tr>
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<td>4.28±0.07</td>
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<tr>
<td>Significance</td>
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<td>p&lt;0.01</td>
<td>ns</td>
<td>ns</td>
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</tr>
</tbody>
</table>

**Table 7.1. Statistical analysis of the effect of ectopic FGF-2 expression on the growth of limb elements**

Treated and contralateral limb elements were measured and compared statistically by Student’s t-test for matched pairs. The mean dimensions of each limb element (in mm ± standard error) are shown above and illustrate that there are significant differences between treated and contralateral limbs treated with FGF-2 but not between antiFGF-2 treated or PBS-treated limbs. ns: not significant
commence until 5 days by which point it is likely that the FGF-2 administered will have been sequestered or degraded and so will no be longer active. A small number of experiments were carried out where FGF-2 beads were implanted at stage 25 and this resulted in disruption to digital patterning.

7.3 Discussion

Although the effect of FGF-2 on limb development appears different from that observed by Riley et al. who discovered that FGF-2 caused limb duplications (Riley et al. 1993), it is possible that the phenotype observed in these experiments actually represents a partial duplication of limb elements. Indeed, rudimentary elements are occasionally seen, an example of which is illustrated in Fig. 7.2A. The level of activity and persistence of FGF-2 released from a bead in vivo has yet to be measured and so manipulating the level of FGF-2 delivered to tissue may effect such a change in phenotype. In any case, the experiments detailed in this Chapter confirm that implanted beads release biologically active FGF-2. Thus, the lack of an effect of FGF-2 soaked beads on cranial skeletogenesis in CAM-grafts appears to represent a significant resistance to the effects of exogenous FGF-2 by cranial tissues.

The other interesting finding to emerge from these experiments is that addition of antiFGF-2 soaked beads has no discernible effect on limb patterning, unlike the skull where they have a profound effect on differentiation (see Chapter 8). One possible theory is that rate-limiting factors are different in the limb and skull and as such, FGF-2 may have differing consequences for morphogenesis when added to/removed from these systems. This will be discussed at length later in the Thesis.
Chapter Eight: CAM-grafting (2) – Effect of local release of antiFGF-2 on cranial development

8.1 Introduction

Since increasing endogenous levels of FGF2 appeared to have no effect on osteogenic differentiation, the next logical step must be to use a neutralising antibody to block FGF-2 in CAM-grafts. Historically, changes in local concentrations of growth factors have always been increased by the addition of heparin-coated acrylic beads. These beads are particularly efficient at binding growth factors that require heparin as a co-factor for receptor binding and activation *in vivo*. However, this means that they are unlikely to bind antibodies with such avidity. Other mechanisms by which antibodies could be delivered to tissues have usually involved directly injecting the substance into a small area or infecting the tissue with an antibody-expressing viral construct (e.g. Savage et al. 1993). The major constraint of both these methods is that the extent of tissue affected cannot be defined and tends to be highly variable, making comparisons difficult.

It was noted that several recent studies using bead-implantation techniques in the chick had used agarose not heparin-coated beads (Kim et al. 1998; Vahtokari et al. 1996). Affi-Gel™ Blue consists of a beaded cross-linked agarose gel normally used to purify proteins by affinity chromatography. The beads can be isolated from the slurry, washed and soaked in solutions before transferring with forceps to tissue. Up until now, these beads have only been soaked in growth factors such as FGF-2, FGF-4 and BMP-7. The mechanism by which these proteins are released is not clear, but it seems likely that agarose beads soak up a solution, rather like a sponge, until they reach equilibrium. They then release their contents when implanted into a tissue where the equilibrium is shifted. If
this was the case, then these beads would be ideal for the delivery of a blocking antibody to a defined region of tissue.

A neutralising antibody to basic FGF (FGF-2) raised against the bovine FGF-2 used in previous experiments (see Chapter 6) was therefore used to block endogenous FGF-2 in CAM-grafted cranial tissue. This antibody has previously been tested and does not cross-react against a huge range of growth factors and cytokines (Rizzino et al. 1988). Implantation of different numbers of antiFGF-2 beads resulted in a distinct concentration-dependent response, the nature of which will be discussed in this Chapter.

8.2 Results

8.2.1 Grafts implanted with a single antiFGF-2 bead

Cranial tissue was dissected out at 10 days and grafted as described in Chapter 6. Agarose beads were soaked in 10 mg/ml antiFGF-2 neutralising antibody for 1 hour before implanting into different locations in the tissue. Initially, a single bead was implanted and the graft allowed to develop in ovo until 16 days. It was evident even before the recovered grafts were stained for bone and cartilage that addition of a single antiFGF-2 bead results in a massive increase in graft size. Unstained grafts fixed in 4% PFA clearly show that the graft in which endogenous FGF-2 has been blocked is at least 3 times larger than an equivalent control graft implanted with a pre-immune serum or PBS soaked bead (Fig. 8.1). Bone formation appears normal in these grafts, in terms of patterning and size of the skeletal elements (Fig. 8.2, A,B) and the increase in size appears to be globally distributed. Arrows on each panel mark the site of bead implantation and the expansion of tissue is not localised around this region but spread evenly throughout the graft. A proportion of these grafts (Fig 8.2, C) also show a reduction in the amount of bone formed, a phenotype seen more frequently when multiple beads are implanted (see section 8.2.2).
Figure 8.1 Implantation of a single antiFGF-2 bead results in a massive increase in graft size

Cranial tissue was implanted with either a single antiFGF-2 bead (on the left) or a single PBS bead (right), grafted for 6 days and then fixed in 4% PFA. Grafts treated with an antiFGF-2 bead show a 2-3 fold increase in size when compared with an equivalent control. Bar=850μm.
Figure 8.2 Whole mount CAM-grafts stained for bone and cartilage

Implantation of a single antiFGF-2 bead results in an overall increase in graft size and skeletal elements but generally, osteogenic differentiation is normal (A,B). The increased growth is likely to result mainly from mesenchymal proliferation. In addition, the effect is not localised around the site of bead implantation (arrows) but is globally distributed. In a proportion of grafts (C), this increase in size is accompanied by a reduction in bone formation. Control grafts implanted with a single PBS bead (D,E) display normal osteogenic differentiation and are shown at the same magnification for comparison. Bar=2mm.
AntiFGF-2 1 bead; PBS 1 bead.
8.2.1.1 **PCNA staining in grafts treated with a single antiFGF-2 bead**

It seems likely that the increase in graft size may be due to an increase in proliferation of mesenchymal cells. To examine this further, representative grafts were embedded, sectioned and stained for Proliferating Cell Nuclear Antigen (PCNA) protein. PCNA, an auxiliary factor of DNA polymerase δ, is synthesised in early G1 and S phases of the cell cycle and its expression and activity is tightly co-ordinated with that of cyclin D1 (Driscoll et al. 1999; Liu et al. 1999). At late S phase, it is prominent in nuclear regions undergoing DNA synthesis and can therefore be used as a marker for cell proliferation.

In grafts implanted with a single antiFGF-2 bead (Fig. 8.3 A-D), almost all mesenchymal cells are proliferating whereas in PBS bead implanted grafts (Fig. 8.3, E-G), only a low level of proliferation can be detected. Since these cells constitute the majority of the tissue in these grafts, the increase in graft size can therefore be attributed to an increase in mesenchymal cell proliferation. To ensure that normal levels of PCNA could be detected in tissues known to be proliferating at this stage, regions of epidermis, bone and cartilage were also analysed in PBS treated grafts. All these tissues displayed high levels of proliferation (Fig. 8.3, F,G) in contrast to the relatively low level of staining seen in mesenchyme (Fig. 8.3, E). In grafts implanted with an antiFGF-2 bead, PCNA staining appeared higher in epidermis and bone than in control (PBS bead) grafts (Fig. 8.3, compare B and C with F and G), suggesting that lowering levels of FGF-2 promotes proliferation of other cell types, not just of mesenchyme. Negative control sections treated with pre-immune serum (Fig. 8.3, H) did not show non-specific staining.
Figure 8.3 antiFGF-2 treated CAM-grafts show enhanced cell proliferation

Representative CAM-grafts were sectioned and stained for PCNA protein. In grafts implanted with a single antiFGF-2 bead (A-D), almost all mesenchymal cells are proliferating (A). Other tissues, including epidermis (B, e) and bone (C,D, b) show higher than normal levels of cell proliferation. At the site of bead implantation (be) PCNA staining is particularly enhanced (C,D). In contrast, grafts implanted with a single PBS bead (E-G) have only a low level of mesenchymal proliferation. Tissues which are normally dividing at this stage, epidermis (F), bone and cartilage (G) show elevated levels of proliferation although not as high as those seen in antiFGF-2 treated grafts. Negative control sections stained with pre-immune serum in place of the primary antibody do not show non-specific staining (H). b: bone; be: bead; c: cartilage; e: epidermis. Bar = 100μm in A, D, E, and H; 50μm in B, F, and G and 250μm in C.
antiFGF-2
1 bead

A
PBS
1 bead

E

B

C

D
Control

F

G

H
bead
### 8.2.2 Grafts implanted with multiple antiFGF-2 beads

Given that addition of only a single antiFGF-2 bead has such a dramatic effect on cell proliferation, it was interesting to investigate whether this level of proliferation could be enhanced further when multiple beads were implanted. However, even before staining, it became clear that addition of more beads had the opposite effect (Fig. 8.4). Comparison of the size of grafts implanted with a single or 3 beads suggests that the latter is not significantly different in size from PBS bead treated grafts (compare with Fig. 8.1). When these grafts are stained for bone and cartilage, although chondrogenesis appears normal, there is a reduction in the level of osteogenesis. The severity of bone reduction varies from graft to graft and representative examples are illustrated in Fig. 8.5. Grafts which have been implanted with 2 or 3 antiFGF-2 beads develop only the most anterior portion of the frontal bones (Fig. 8.5, A-C). Frontal bone deposition normally proceeds from anterior to posterior and at the time of grafting (10 days), the most anterior tip of the frontal bones would have already commenced differentiation. Again, this bone reduction tends to be evenly distributed throughout the graft, although in some cases, a greater reduction is seen around the site of bead implantation (Fig. 8.5, C, arrow). Generally, these grafts are of equivalent size to those implanted with PBS beads (Fig. 8.5, E,F), but there is a degree of overlap between categories. A few grafts implanted with 3 beads display both increased cell proliferation and reduced osteogenesis (Fig. 8.5, D) and the same is true of grafts implanted with a single antiFGF-2 bead (see Fig. 8.2, C for an example).

This is particularly well illustrated by Fig 8.6 where data from all CAM-grafting experiments are displayed. Grafts treated with a single antiFGF-2 bead are generally much larger than either control (PBS or rabbit serum) or FGF-2 implanted grafts. The dimensions of each graft were measured by taking the mean of the greatest length and the greatest width and then converting to surface
Figure 8.4 Implantation of multiple antiFGF-2 beads does not affect CAM-graft size

Cranial tissue was implanted with either a single antiFGF-2 bead (on the left) or 3 antiFGF-2 beads (right), grafted for 6 days and then fixed in 4% PFA. Grafts treated with 3 antiFGF-2 beads are of equivalent size to control grafts. Bar=850μm.
Figure 8.5 Implantation of multiple antiFGF-2 beads prevents osteogenesis

When 2 (A) or 3 (B-D) antiFGF-2 beads are implanted into cranial tissue, osteogenesis is blocked. Generally, this effect is manifest over the entire graft and not localised to the site of bead implantation (as indicated by arrows). However, a few grafts do show a further decrease in osteogenesis around the beads (C). Occasionally, grafts displayed a phenotype characteristic of treatments with both single and multiple antiFGF-2 beads (D) with both reduced osteogenesis and increased proliferation. The size of grafts implanted with multiple antiFGF-2 beads does not appear significantly different from those grafts treated with 3 PBS beads (E,F). Arrows indicate the site of bead implantation. Bar=2mm.
Figure 8.6 Combined graph illustrating the effects of different numbers of antiFGF-2 beads on graft size and differentiation.

This graph displays the percentage of grafts that show a reduction in bone differentiation (pink bars) as opposed to normal bone formation (blue bars) and the average surface area of grafts in each treatment (line). The response seen is broadly dependent on the number of beads implanted although there is some overlap between treatments. The addition of PBS, rabbit serum or FGF-2 coated beads has no significant effect on osteogenesis or proliferation. Implantation of a single antiFGF-2 bead results in a massive increase in CAM-graft surface area whereas addition of two or more beads prevents further bone formation.
Table 8.1 Statistical analysis of the effect of blocking endogenous FGF-2 protein on CAM-graft size

The surface area of CAM-grafts in each treatment were measured and compared statistically using Student’s t-test. The table above illustrates the number of samples and mean surface area±SE in each category on the left, and the level of significance on the right. There are significant differences in the size of grafts treated with a single FGF-2 bead when compared with PBS-, serum- or FGF-2 treated grafts. SE-standard error; ns=not significant.
### Table 8.2 Statistical analysis of the effect of blocking endogenous FGF-2 protein on osteogenic differentiation

The percentage of CAM-grafts displaying reduced bone formation in each treatment was measured and compared statistically using the G-test for independence. The table above illustrates the number of samples and percentage bone reduction in each category on the left, and the level of significance on the right. There are significant differences in the number of grafts with reduced bone formation treated with antiFGF-2 beads when compared with PBS-, serum- or FGF-2 treated grafts. ns=not significant.

<table>
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<tr>
<th>Treatment</th>
<th>Number of Specimens</th>
<th>% Bone Reduction</th>
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<th>AntiFGF-2 2 beads</th>
<th>AntiFGF2 3 beads</th>
</tr>
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<tbody>
<tr>
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<td>4.3</td>
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<td>p&lt;0.001</td>
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<td>p&lt;0.001</td>
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<td>-</td>
<td>p&lt;0.05</td>
<td>ns</td>
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<td>p&lt;0.05</td>
<td>-</td>
<td>ns</td>
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<tr>
<td>AntiFGF-2 3 beads</td>
<td>n=21</td>
<td>71.4</td>
<td>ns</td>
<td>p&lt;0.001</td>
<td>-</td>
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area using the formula $\pi r^2$. Grafts implanted with 2 or 3 antiFGF-2 beads have the highest level of bone reduction but 50% of grafts treated with a single antiFGF-2 bead also show some degree of reduction in osteogenesis. Grafts treated with PBS, rabbit serum or FGF-2 do not show significant bone reduction.

**8.2.2.1 PCNA staining in grafts treated with multiple antiFGF-2 beads**

To assess the level of proliferation after treatment with multiple antiFGF-2 beads, representative grafts were sectioned and stained for PCNA as before (Fig. 8.7). The level of mesenchymal proliferation is barely detectable and is clearly lower than that seen in grafts implanted with 3 PBS beads (Fig. 8.7, E-G). Individual PCNA-positive cells are indicated by arrows. Around the bead itself (Fig. 8.7, B), staining is absent (compare with Fig. 8.3, D) and very few proliferating cells can be seen in the remaining bone (Fig. 8.7, C) whereas in PBS treated grafts a high level of proliferation can be observed in this tissue (Fig. 8.7, E). Similarly, at this stage, chondroctyes are rapidly proliferating in control tissue (Fig. 8.7, F) but not in grafts implanted with 3 antiFGF-2 beads. Sections treated with pre-immune serum (negative controls) do not display non-specific staining (Fig. 8.7, H).

**8.2.2.2 Statistical analysis of cell proliferation in CAM-grafts**

To quantitatively assess the proportion of dividing cells in each graft, positive and negative cell nuclei stained with PCNA were counted in the following areas: 5 fields of mesenchyme from one section on each of 5 slides from each graft, making a total of 25 counts per graft. Five grafts, for each of four treatments, (antiFGF-2 1 and 3 beads, PBS 1 and 3 beads) were assessed in this way. Equivalent fields of bone, cartilage and epidermis were counted in each section and used as positive controls. For the purpose of measuring proliferation, osteogenic cells were defined as those contained within the periosteum. All
Figure 8.7 Further blocking of endogenous FGF-2 in CAM-grafts results in a reduction in cell proliferation

CAM-grafts were sectioned and stained for PCNA protein. Grafts treated with 3 antiFGF-2 beads (A-D) have a reduced level of proliferation in all tissues analysed when compared with control grafts implanted with 3 PBS beads (E-G). A few PCNA-positive mesenchymal (A), osteogenic (C) and chondrogenic (D) cells are identified with arrows. Proliferation around the implanted bead is almost non-existent. Equivalent control (PBS) grafts have high levels of proliferation in bone (E), cartilage (F) and developing feather germs (G). Sections treated with pre-immune serum do not show specific staining (H). Arrows point to PCNA-positive cells. b: bone; be: bead; c: cartilage; fg: feather germ. Bar=50μm.
Figure 8.8 Effect of blocking FGF-2 on cell proliferation

PCNA-positive and negative cell nuclei were counted randomly and blind in 5 fields in 5 grafts in each category. The mean PCNA index (the percentage of PCNA-positive cells) for each treatment is plotted on the graph above.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>PCNA index (%)</th>
<th>AntiFGF-2 1 bead</th>
<th>AntiFGF-2 3 beads</th>
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<tr>
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<td>p&lt;0.001</td>
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<td>1.12</td>
<td>p&lt;0.001</td>
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Table 8.3 Effect of antiFGF-2 on mesenchymal cell proliferation
(see legend below for explanation)
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<th>PCNA index (%)</th>
<th>AntiFGF-2 1 bead</th>
<th>AntiFGF-2 3 beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS 1 bead</td>
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<td>84.8</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
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<tr>
<td>PBS 3 beads</td>
<td>n=25</td>
<td>71.20</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
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<tr>
<td>AntiFGF-2 1 bead</td>
<td>n=25</td>
<td>92.53</td>
<td>p&lt;0.001</td>
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<tr>
<td>AntiFGF-2 3 beads</td>
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<td>40.95</td>
<td>p&lt;0.001</td>
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Table 8.4 Effect of antiFGF-2 on epidermal cell proliferation

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<th>AntiFGF-2 3 beads</th>
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<td>91.99</td>
<td>p&lt;0.001</td>
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<tr>
<td>AntiFGF-2 3 beads</td>
<td>n=25</td>
<td>15.31</td>
<td>p&lt;0.001</td>
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Table 8.5 Effect of antiFGF-2 on osteogenic cell proliferation

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<th>PCNA index (%)</th>
<th>AntiFGF-2 1 bead</th>
<th>AntiFGF-2 3 beads</th>
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<tbody>
<tr>
<td>PBS 1 bead</td>
<td>n=25</td>
<td>84.8</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>PBS 3 beads</td>
<td>n=25</td>
<td>71.20</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
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<tr>
<td>AntiFGF-2 1 bead</td>
<td>n=25</td>
<td>92.53</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>AntiFGF-2 3 beads</td>
<td>n=25</td>
<td>40.95</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Table 8.6 Effect of antiFGF-2 on chondrogenic cell proliferation

The mean level of cell proliferation was measured for each treatment in mesenchymal, epidermal, osteogenic and chondrogenic cells and compared statistically using the G-test for independence. The tables above (8.3-8.6) illustrate the number of samples and PCNA index in each category on the left, and the level of significance on the right.
counts were carried out blind. The results are displayed graphically in Fig. 8.8 and in Tables 8.3-8.7. Grafts implanted with a single antiFGF-2 bead have an elevated level of cell proliferation in all tissues analysed, although this is most striking in mesenchymal cells. Treatment with 3 antiFGF-2 beads has the converse effect and cell proliferation is markedly reduced in all tissues, particularly in tissues which are normally dividing at this stage – epidermis, bone and cartilage.

These data are corroborated by statistical analyses using the G-test for independence. Individual comparisons between treatments indicate in all tissues that there are significantly different levels of cell proliferation in grafts treated with antiFGF-2 beads from those implanted with PBS beads (Tables 8.3-8.6). In addition, there is a statistically significant difference in the PCNA index between grafts treated with different numbers of antiFGF-2 beads. This verifies that addition of a single antiFGF-2 bead promotes cell proliferation whereas addition of multiple antiFGF-2 beads suppresses both proliferation and differentiation.

8.3 Discussion

Implantation of agarose beads is a highly effective technique for delivering proteins to a defined region of embryonic tissue. By implanting only one antiFGF-2 bead, huge increases in graft size occur as a result of enhanced mesenchymal cell proliferation. These increases in proliferation, and hence growth, are not restricted to the site of bead implantation but are observed throughout the graft. Given that the size of the grafted tissue can be quite large (several mm) and the diameter of the implanted beads is only 50-100 µm, either the protein is able to diffuse across large distances or the phenotype is a result of the activation of downstream target genes.

Implantation of multiple beads to further reduce the level of endogenous FGF-2 results in a distinct phenotype. Such grafts are of similar size to PBS bead
treated tissues. However, when they are stained for bone and cartilage it is revealed that osteogenesis is aberrant. Frontal bone formation is arrested at the point of grafting and only the most anterior portion of these bones forms. Cell proliferation in these grafts is drastically reduced, not only in mesenchyme but also in epidermis, cartilage and remaining bone. Addition of more beads therefore appears to increase the concentration of antiFGF-2 delivered and hence the amount of endogenous FGF-2 blocked.

However, there may be another explanation. The different responses may simply be a consequence of increasing the diffusion or extent of tissue which is exposed to a given concentration of antiFGF-2. To confirm that adding more beads does indeed increase the concentration of antiFGF-2 delivered to grafted tissue, qualitative and quantitative methods were next used to map the release of antiFGF-2 from different numbers of beads. These data are the subject of the next Chapter.
Chapter Nine: The kinetics of antiFGF-2 diffusion from agarose beads

9.1 Introduction

In the previous Chapter, it emerged that implantation of different numbers of antiFGF-2 soaked beads evoked distinct phenotypic responses. The most logical explanation for this phenomenon is that addition of more beads results in an increase in the level of endogenous FGF-2 blocked. Hence, the cellular response and resulting phenotype is concentration-dependent. To verify that this is indeed the case, some form of assessment of the release of antiFGF-2 from agarose beads was necessary.

Several studies have analysed the release of growth factors from beads – mostly from heparin beads (Edelman et al. 1991; Eichele et al. 1984). The release dynamics are different for each growth factor (due to the size of the protein and its mobility) so it is difficult to extrapolate such data. In any case, agarose beads have yet to be widely used in experimental embryology. However, one study has assessed the uptake and release of radiolabelled FGF-2 from agarose beads and it appears that these beads soak up very small amounts of FGF-2 before slowly releasing the protein over 4 days (Hayek et al. 1987; Riley et al. 1993).

In this Chapter, two methods, one qualitative and the other quantitative, have been used to assess the level of antiFGF-2 released from different numbers of agarose beads over time. Whole mount immunohistochemistry on CAM-grafts pictorially illustrates the level and rate of diffusion of antiFGF-2 from beads in vivo. ELISA enables the quantification of these data by measuring the actual concentration of antibody released by different numbers of beads into solution.
9.2 Results

9.2.1 Whole mount immunohistochemistry

Using a whole mount immunostaining protocol, the amount of antibody released and the extent to which it had diffused over time were measured in vivo.
A method previously used by Wheatley and co-workers (1993) was modified for these experiments. Cranial tissues were prepared as before (see Chapter 3) and either 1 or 3 antiFGF-2 or PBS soaked beads implanted. The presumptive grafts were then incubated for specific periods of time before fixing. The primary antibody therefore is already present in the tissue and the relative levels and distance over which it has diffused can be detected by the addition of a HRP-conjugated secondary antibody and a colorimetric reaction.

The result of these experiments is conclusive: addition of more antiFGF-2 beads raises the level of antiFGF-2 in a given region of tissue and also increases the distance over which this antibody can be detected (Fig. 9.1). At all time points analysed, there appears to be a higher concentration of antiFGF-2 (stained black) detectable in grafts implanted with 3 beads than in those implanted with a single bead (compare grafts on the left side of Fig. 9.1 with those on the right). In addition, the distance over which the antibody had diffused was greater in the former category. By 12 hours after implantation, antiFGF-2 was detectable within at least 2/3 of the graft (Fig. 9.1, H), perhaps explaining why the observed effects on proliferation and differentiation are global. By 24 hours, protein can no longer be detected in grafts implanted with a single antiFGF-2 bead whereas distinct staining can still be seen in grafts implanted with 3 beads (Fig. 9.1, compare G and H). PBS bead implanted tissues show no coloration, confirming that the staining in antiFGF-2 implanted grafts is specific (Fig. 9.1, K,L).
Figure 9.1 Whole mount immunohistochemistry to detect the level of antiFGF-2 released in vivo

Grafts were implanted with a single bead coated in antiFGF-2 (A, C, E, G, I) or with 3 antiFGF-2 beads (B, D, F, H, J). Control grafts were implanted either with a single PBS coated bead (K) or with 3 PBS beads (L). Grafts were fixed after 15 minutes (A, B); 2 hours (C, D); 6 hours (E, F) 12 hours (G, H) or 24 hours (I-L). The level of antiFGF-2 protein is indicated by the black staining. The level of antiFGF-2 released from 3 beads (B) is clearly higher only 15 minutes after implantation than in grafts implanted with a single antiFGF-2 bead (A). This remains the case at 2 hours although the difference is less marked (compare C with D). AntiFGF-2 is detectable at 6 and 12 hours after implantation of 3 beads, whereas it is only weakly found at these timepoints in grafts implanted with a single bead (compare E with F and G with H). 24 hours after implantation, antiFGF-2 is no longer detectable in grafts implanted with a single bead whereas weak staining is still present in grafts implanted with 3 beads (compare I with J). Control grafts at all time points show no staining (K, L). The site of implanted beads is marked with arrows. Bar=1mm.
It appears that agarose beads continue to release antiFGF-2 for up to 12 hours following bead implantation since the staining increases until this point. In addition, the level of antiFGF-2 protein does not start decreasing until around 18 hours post-implantation and remains detectable for at least 24 hours (the longest time point used). This makes these beads ideal for widespread use in the sustained release of other growth factors/antibodies.

9.2.2 Enzyme-linked immunosorbent assay (ELISA)

To confirm these data quantitatively, we used an ELISA assay to measure the level of antiFGF-2 released from different numbers of beads in solution. Agarose beads were first soaked in PBS or antiFGF-2 at concentrations of 10 mg/ml, 1 mg/ml and 100 μg/ml for 1 hour. Either 1 or 3 beads were then removed and placed in a microfuge tube containing PBS and the contents of the beads allowed to diffuse out (without mixing) for defined time points. The supernatant was removed and added to a microtitre plate coated with FGF-2. The addition of an AP-conjugated secondary antibody, and the subsequent level of colour reaction, then enabled antiFGF-2 concentration to be determined.

Known concentrations of antiFGF-2 were added to each plate and from the resulting optical densities (OD), a standard sigmoid curve was plotted of OD against antiFGF-2 concentration (Fig. 9.2). The lower limit of the assay was defined as the point at which the optical density of the standard curve stopped falling at the same rate as the fall in dilution. Using this curve, the ODs from the test solutions can be converted into actual antiFGF-2 concentrations. However, it became clear that many of the ODs fell within a small range making accurate conversion difficult. To overcome this, the ODs were logged \[\log_{10}\] and the graph replotted (Fig. 9.3) illustrating the predicted straight line and enabling a much easier transformation of the OD’s into known antibody concentrations.
Clearly, there is an increased concentration of antiFGF-2 released in the first hour from 3 beads compared with 1 bead (Fig 9.4). Although by 3 hours of incubation the level of antibody released from 3 beads has dropped, it remains higher than that measured from 1 bead and is maintained for a longer period of time. This verifies the data from whole mount immunohistochemistry experiments despite the former being \textit{in vitro} and the latter \textit{in vivo}. Indeed, one reason why the level of antiFGF-2 drops after 3 hours \textit{in vitro} may be due to a lack of restraint by tissues or the absence of FGF-antiFGF binding \textit{in vitro}. Single beads soaked in lower concentrations of antiFGF-2 (1 mg/ml and 100 \(\mu g/ml\)) release reduced levels of antiFGF-2 into solution.

![Figure 9.2 Standard curve of known concentrations of antiFGF-2 measured by ELISA](image)

Serial dilutions of antiFGF-2 were measured by ELISA with each plate and the resulting optical densities plotted on the graph above.
Figure 9.3 Transformed curve of known concentrations of antiFGF-2 measured by ELISA plotted on a logarithmic scale

For easier conversion of optical densities of test solutions to known antiFGF-2 concentrations, the optical densities of the known antiFGF-2 concentrations were logged \([\text{Log}_{10}]\) and the standard curve replotted. A predicted straight-line results and the OD’s of the unknown solutions can then be converted to antiFGF-2 concentration as shown by the example superimposed onto the graph (red line).
Figure 9.4 Concentration of antiFGF-2 released by different numbers of beads as measured by ELISA.

At all time points, the concentration of antiFGF-2 released from 3 beads (soaked in 10 mg/ml) was higher than that released from a single bead. Single beads soaked in lower concentrations of antiFGF-2 (1 mg/ml and 100 μg/ml) released very low detectable levels of antibody. Bars represent mean values from at least 3 experiments with SEM measurements shown on top.
9.3 Discussion

The release of antiFGF-2 from agarose beads has now been measured qualitatively \textit{in vivo} and quantitatively \textit{in vitro}. Using whole mount immunohistochemistry, the rate of antiFGF-2 diffusion over time has been mapped. This showed that the antibody is able to diffuse across large cellular distances and can be detected in over 2/3 of the graft, providing one explanation for the global phenotypic response. By their nature, mesenchymal cells are loosely and randomly orientated and this may facilitate the diffusion of the antibody over larger distances than would otherwise be possible. In addition, antiFGF-2 persists for at least 24 hours following bead implantation, long enough for the activation of most signalling events or to effect a change in cell commitment. As well as increasing the persistence and rate of diffusion of antiFGF-2, implantation of multiple numbers of beads also increases the concentration of antibody in a given region of tissue. Conclusive data for this is provided quantitatively by ELISA.

Early experiments used biocompatible 200 µm diameter polymeric beads for the release of retinoic acid and discovered that a prolonged release is obtained that persists for more than a day. During this interval, the release is diffusion-controlled, and the total amount of compound released is directly proportional to the amount of the compound that the bead is exposed to during the initial loading step. These beads were then manually implanted into wing buds of stage 20 chick embryos. The release rate obtained was comparable to that found \textit{in vitro}, and a time-dependent accumulation of the released radiolabelled retinoic acid was measured that was confined to the site of implantation (Eichele et al. 1984). Subsequently, numerous studies used these and heparin-acrylic beads as biocompatible implants to manipulate the levels of growth factors \textit{in vivo} (Cohn et al. 1995; Tickle, 1996; Tickle et al. 1985). Heparin-sepharose beads have also been used to stabilise FGF-2, preventing degradation \textit{in vivo}. Conventional matrix polymer-based release devices had previously been used for FGF-2
release, however, 99% of FGF-2 mitogenic activity was lost. Addition of heparinase to the solution increased the release of FGF-2 from 25 to 85%, demonstrating the importance of heparin as a co-factor in FGF binding (Edelman et al. 1991). The presence of the enzyme did not alter the mitogenic activity of released FGF-2 suggesting that this may be a useful mechanism to increase the release of FGF from heparin beads *in vivo*.

The level of FGF-2 required by agarose beads to effect a biological response in the limb has been calculated at 15 μM. Radiolabelling of FGF-2 however, indicates that only 0.5-1 pmol of FGF-2 is taken up during a 1 hour incubation period. This FGF-2 reservoir is then slowly released over 4 days (Hayek et al. 1987; Riley et al. 1993). In the experiments in this Thesis, agarose beads have been soaked in a relatively high concentration of both FGF-2 and antiFGF-2. There is a suggestion therefore that agarose beads are not able to soak up large quantities of growth factor but are able to slowly release their contents *in vivo*. This may explain why implanting more antiFGF-2 loaded beads results in such a distinct phenotype and it would be predicted that increasing the concentration of antiFGF-2 in a single bead would not have such a profound effect. When the concentration of antiFGF-2 is reduced, its release from a single bead *in vitro* is barely detectable above background levels. It may be possible to measure the amount of antiFGF-2 taken up by a single agarose bead simply by measuring the diameter of the bead and the amount of antiFGF-2 released into solution *in vitro*. Whether this would be directly applicable *in vivo* is not clear since it is likely that the site of implantation plays an important role in the level of the substance released.

It is evident that implanting more beads elevates the concentration of antiFGF-2 delivered to cranial tissue. Logically therefore, the amount of endogenous FGF-2 blocked should increase proportionally with each additional bead implanted. What remains unclear however, is whether this concentration-dependent response is due to the blocking of more FGF-2 protein, or the length of time that
the ligand is inactivated and unavailable for receptor binding. This will be discussed in the context of other research in the final Chapter.
Chapter Ten: Final Discussion

10.1 Expression of FGF and FGFR transcripts and proteins during development

FGF and receptor mRNAs are found in a wide variety of tissues during the embryonic development of the chick. Their expression patterns correlate with previously proposed roles in the patterning of the limb and somites, development of the heart, and formation of the facial processes (Grass et al. 1996; Richman et al. 1997; Tickle, 1996; Watkins et al. 1998). At early stages of cranial development (4 days), there is strong expression of FGF-2, -4 and FGFR2 throughout the cranial mesenchyme but not in the overlying ectoderm. This is suggestive of a role for these molecules in osteogenic induction in the skull.

In this Thesis, protein localisation data at later stages of skeletogenesis suggest that, in the chick at least, different isoforms are found in different tissue types. Although only one isoform of FGFR1 and FGFR2 can be detected in the head by Western blotting, there are several isoforms of FGF-2. These different sized proteins may be dimers, the result of post-translational modifications, or produced by the use of different translational initiation sites. Such modifications are thought to regulate some of the intracellular activities of FGFs including the maintenance of differentiated function (Mason, 1994).

The current study indicates that FGF-2 is present at early stages of differentiation within bone and at a higher level in the periosteal layer that surrounds the bone. Interestingly, there seems to be a higher concentration of FGF-2 protein localised to the suture than to the rest of the cranial mesenchyme, although this level is relatively lower than that seen in differentiating bone. FGF-2 may be driving the sutural cells to divide rather than differentiate, thereby preventing sutural closure. In this Thesis, FGFR proteins are found in roughly
the same locations as previously reported in gene expression studies (Chan and Thorogood, 1999; Delezoide et al. 1998; Iseki et al. 1997; Kim et al. 1998). Receptors are expressed by the periosteum, rather than in bone itself, suggesting a role in osteogenesis rather than bone maintenance.

10.2 Cranial tissues respond to reduced levels of FGF-2

The vertebrate skull is derived from two mesenchymal lineages; mesoderm and neural crest. The latter is now known to form the majority of the cranial bones and recent work has demonstrated that the neural crest precursors of the skeletogenic tissues in the embryonic head express FGFRs. When cultured, neural crest cells are capable of responding to FGF-2 in a concentration-dependent way (Sarkar and Thorogood, 1999). The work presented in this Thesis complements these findings by showing that the progeny of cranial neural crest cells in vivo respond to inactivation of endogenous FGF-2 by displaying a switch from skeletogenic differentiation to proliferation. In vivo responses appear to be determined by the degree of inactivation of endogenous FGF-2 and seem, at least crudely, concentration-dependent. Delivery of the neutralising antibody by beads was measured by ELISA and indicates that increasing the number of implanted beads can achieve a two- to three-fold increase in the concentration of antibody delivered.

Implantation of a single antibody-coated bead switched mesenchymal cells into a proliferative mode and produced massively overgrown grafts, although osteogenesis remained normal. Surprisingly, this effect was manifest over the entire graft and not locally around the bead implantation site. Immunolocalisation of the released antibody indicates that it diffuses throughout a wide area of the graft (several millimetres in size), perhaps explaining why the response is global. At this level of FGF-2, bone differentiation is unaffected. However, if more endogenous ligand is blocked, further bone differentiation is
halted completely and mesenchymal cells become quiescent, displaying neither proliferation nor differentiation.

This concentration-dependent response complements the effect seen when neural crest cells are cultured in the presence of increasing levels of exogenous FGF-2. At low levels of FGF-2 (0.1-1 ng/ml), neural crest cells proliferate in vitro whereas at higher levels (10 ng/ml) the cells initially differentiate into cartilage and later form bone (Sarkar and Thorogood, 1999). There appears to be an initial period of 24-48 hours during which neural crest cells must be continuously exposed to FGF-2 for skeletogenesis to occur. Exposure for less than 24 hours results only in cell proliferation (Sarkar and Thorogood, 1999). Therefore, it may be that in addition to the concentration of antibody delivered to a region of tissue, the length of time that endogenous FGF-2 is blocked in that region is also a critical factor. In support of this idea is the finding that implantation of an increased number of beads results in prolongation of the time during which the antibody remains detectable.

Collectively, these results and those of Sarkar and Thorogood (1999) support the contention that the mutations in \textit{FGFR1}, \textit{FGFR2} and \textit{FGFR3} that cause craniosynostosis are activating mutations, conferring ligand independence on the receptor in question and leading to an uncontrolled up-regulation of intracellular signalling.

\textbf{10.2.1 The relationship between FGF dose-dependency and severity of craniosynostosis phenotypes}

It is proposed that maintenance of the open suture requires low levels of FGF-2, thereby minimising FGF signalling and promoting mesenchymal proliferation. Experiments in this Thesis using implanted antiFGF-2 beads demonstrate the proliferative response to reduced FGF-2 concentrations, and \textit{in vivo}, FGF-2 levels are low at sites of open sutures (Iseki et al. 1997). Conversely, treatment of cranial neural crest cells with high concentrations of exogenous FGF-2
(Sarkar and Thorogood, 1999) promotes skeletogenic differentiation and this finding may model the situation in craniosynostosis where mutant FGFRs are thought to be constitutively active.

This suggests that the severity of clinical phenotype may reflect quantitative differences in the degree of genetic disruption and activation of the signalling process. However, assessment of the functional consequences of the various mutations has been by indirect means and, to date, meaningful comparisons cannot be made of quantitative differences in function arising from these mutations. It is however predicted from the present data and from related work (Sarkar and Thorogood, 1999), that a relationship between the level of signalling and the resulting clinical phenotype is likely to emerge.

10.2.2 Are the endogenous levels of FGF-2 rate limiting in craniofacial tissues?

The failure of additional exogenous FGF-2 (at levels of 1-10 mg/ml) to produce any discernible effect on skull form or pattern were quite surprising. This is in sharp contrast to the effects found in the limb where exogenous FGF-2 and FGF-4, placed in the flank can induce ectopic limb buds (Cohn et al. 1995). The biological activity of these beads was therefore assayed by implanting them into early limb buds. This demonstrated that the beads do indeed release FGF-2 which is active and capable of altering the development of the limb skeleton. Work by Coffin and co-workers (1995) showed that over-expression of FGF-2 in transgenic mice results in shortening and flattening of the long bones and moderate macrocephaly. This substantiates the phenotype seen in the current study when exogenous FGF-2 is introduced ectopically into the limb bud. Moreover, the introduction of neutralising antibody into the limb produced no effect, in contrast to its effect on the cranial vault where the antiFGF-2 soaked beads elicited a profound response.
Figure 10.1 Diagrammatic representation of the effect of FGF-2 on cranial and limb development

The top row depicts the cranial phenotype resulting from manipulating FGF-2 levels and hence altering the level of FGFR signalling. The bottom row depicts the complementary phenotype shown by a typical long bone undergoing endochondral ossification at each level of FGF signalling. During normal cranial development (A), it is proposed that low levels of FGF-2 at sutures enhance the proliferation of osteogenic precursors and maintain sutural patency. At the edge of bones, higher levels of FGF-2 promote the differentiation of these precursors into osteoblasts. In the limb, FGF-2 is thought to play a role in outgrowth and osteogenesis. (B) In the chick, addition of ectopic FGF-2 to the developing skull has no effect on skeletal patterning whereas in the limb, it induces truncated and thickened limb elements. In contrast, slightly reduced levels of FGF-2 (C) result in increased mesenchymal proliferation, and further reductions (D) block osteogenesis whereas the limb is unaffected by reductions in endogenous FGF-2. In craniosynostosis and skeletal dysplasias such as achondroplasia (E), it is suggested that increased signalling of FGFRs is responsible for premature ossification of the cranial sutures and premature differentiation of the long bones. The level of ligand \textit{in vivo} can therefore be linked to the level of FGFR signalling with the resulting phenotype reflecting this level of signalling.
Animal model

Normal development

Intramembranous ossification - skull

Bone high FGF-2 = differentiation

Suture low FGF-2 = proliferation

Ectopic FGF-2 FGFR signalling

Reduced FGF-2 FGFR signalling

Blocked FGF-2 FGFR signalling

Mutated FGFR FGFR signalling

Endochondral ossification - limb

A

B

C

D

E

Craniosynostoses/ Skeletal dysplasias
These tissue-specific differences may reflect lineage differences, in that limb mesenchyme is predominantly mesodermal in origin (e.g. Hinchliffe and Johnson, 1980) whereas that of the cranial vault is predominantly neural crest-derived (Couly et al. 1993). However, the reasons are likely to be complicated by factors of ligand redundancy, tissue specific differences in isoform expression and perhaps by the fact that the rate-limiting factors may be different in each tissue. Thus, in the limb mesenchyme, it may be ligand, not receptor availability that is rate limiting; addition of extra ligand would therefore be predicted to have morphogenetic consequences. In craniofacial tissues, a situation of superfluous ligand and limited receptor availability might not be expected to display a response to additional exogenous ligand but will do so when endogenous levels fall below a critical threshold level. This hypothesis is supported by work by Iseki and co-workers (1997), in which changes in local osteopontin and FGFR2 expression could only be induced by the application of FGF-2 at very high doses (400 mg/ml). Hence, an extremely large increase in the local concentration of FGF-2 does not provoke morphogenetic changes and explains why, when FGF-2 availability is reduced (as demonstrated in this study), there is a profound effect on differentiation. The phenotypic effects of manipulating endogenous levels of FGF-2 in both the skull and limb are diagrammatically displayed in Fig. 10.1.

10.3 The complexity of FGF-FGFR interactions

It is interesting to note that although many craniosynostosis syndromes such as Apert and Pfeiffer are accompanied by gross limb defects, Crouzon syndrome is not. However, it has been reported that the same base pair mutation in FGFR1 can cause both Crouzon and Pfeiffer syndromes (Muenke and Schell, 1995). The mechanisms involved are clearly complex and it is possible that only subtle differences in the levels of signalling have profound effects on phenotype. The issue is further clouded by the ability of FGFs to bind to more than one receptor, so called ligand-receptor promiscuity, and by the presence of FGFR isoforms generated as a result of alternative splicing (Green et al. 1996). These splice
variants have different ligand binding capabilities and expression patterns and are often found in complementary locations during craniofacial development (Chan and Thorogood, 1999; Delezoide et al. 1998; Orr-Urtreger et al. 1993). In addition, each ligand is known to have several isoforms produced by the use of alternative translation initiation sites, as well as alternative splicing (Mason, 1994), and it is possible that each will therefore have different receptor binding capabilities. In this Thesis, FGFR proteins show broad overlapping expression patterns in the developing skull. Although subtle differences in these expression patterns are visible, these are changes in levels of expression rather than the presence or absence of a particular receptor. These various elements may each play a role in determining which mutations result in more severe phenotypes.

Given that FGF-2 requires HSPG as a co-factor for receptor binding (McKeehan and Kan, 1994; Spivak-Kroizman et al. 1994), it seems likely that local changes in HSPG expression may be responsible for increased sequestration and activation of FGF and hence increased signalling. Some anecdotal evidence for this is provided by histological staining for Alcian blue since an increase in the level of HSPGs can be seen in the prospective skeletogenic region. The dura mater by virtue of its extracellular matrix may therefore provide a conduit for signalling molecules to the overlying mesenchyme.

Work by Kim et al. (1998) also implicates the dura mater in the control of sutural closure. The culture of embryonic calvaria without dura resulted in an increase in the level of osteogenesis and more rapid sutural synostosis. Other studies have suggested that this more rapid closure will only occur at defined points during development (between late fetal and early postnatal stages) and so the dura mater, although not required for initial suture formation, is essential for its patency (Opperman et al. 1993). In this study, the cranial meninges, from which the dura mater differentiates, were not removed from the skull at the time of grafting. It would therefore be interesting to repeat these experiments with this tissue layer removed and assess its effect on skeletogenesis. Sutural
morphogenesis and maintenance therefore seems to be regulated by interactions between at least three tissue types: cranial mesenchyme, differentiating calvarial bones and the dura mater (with possible secondary mechanical influences emanating from the skull base; Rosenberg et al. 1997).

10.4 Future development of the project

In this Thesis, an experimental technique has been used to investigate the role of FGF-2 in skeletogenic differentiation during chick development. Descriptive data has also been obtained on several members of the FGF family. Although this work goes some way towards investigating the complexity and multiplicity of the effects of FGF-2, it does not mimic the in vivo situation where no one molecule acts in isolation but constantly responds to changes in its environment. Our knowledge of the FGF family is constantly expanding and at the time of writing, 22 FGFs have been identified. Several are known to be expressed during skeletogenesis and it is therefore likely that a high degree of functional redundancy exists within the family. Indeed, FGF-3 and -7 knockout mice do not show a significant phenotype despite their broad expression patterns in vivo. However, mice lacking FGFR2 Ig loop IIIC (KGFR) show a range of phenotypic manifestations. This splice variant is known to bind FGF-3, 7 and 10 with high avidity and the phenotype displayed is almost a combination of those seen in the respective knockout mice (Bradley Spencer-Dene, personal communication). In the future, it is important to assess the effects of other FGF ligands on skeletogenesis and preferably in an in vivo environment.

The nature of chick embryos at late stages of development makes them difficult candidates to operate on in vivo and maintain an efficient recovery rate. A way around this could be to transfect regions of the skull with retroviruses expressing FGF ligands, FGFR splice variants or dominant-negative receptors. This could also provide stronger evidence that FGFRs are constitutively activated in human craniosynostoses.
An important area, too large for the scope of this Thesis, is that of downstream target genes. An increasing number of genes are being identified by their association with FGFs. By analysing the expression of genes such as MSX2, Shh, TGFβ, BMPs and bone markers including osteonectin, osteopontin and alkaline phosphatase, their position in the skeletogenic pathway and their relationship with FGFs can be pinpointed. There is a strong suggestion that extracellular matrix molecules are involved in FGF/R signalling and regulation, particularly since HSPG is required as a co-factor for receptor binding and dimerisation. Precisely which matrix components are important for osteogenesis and sutural maintenance has yet to be discovered.

Finally, it has been mentioned that most FGFs have several isoforms. Currently, commercial antibodies do not enable the differences in expression of these variants to be assessed. The development of mRNA probes and antibodies specific to a single ligand form would allow the role of each FGF to be dissected further and clarify which isoforms are important in cranial differentiation.

10.4 Conclusions

In this Thesis, a novel method has been used to manipulate endogenous FGF levels in embryonic tissues and this has demonstrated a concentration-dependent response of cranial skeletal tissues to reduced levels of FGF-2. These findings provide an in vivo experimental model by which the developmental mechanisms underlying normal sutural growth and morphogenesis can be analysed, and enables the understanding of the pathological early closure of sutures seen clinically. It may be that by further manipulating the levels of other growth factors implicated in cranial morphogenesis, the balance between proliferation and skeletogenesis can be shifted, hence obtaining a clearer understanding of the mechanisms involved in both normal and abnormal cranial differentiation.
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