Syndromic Craniofacial Dysostosis
- From Genotype to Phenotype.

Studies of FGFR Gene Expression
in Human Craniofacial Development & Craniosynostosis

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Abstract

Fibroblast growth factor receptors (FGFRs) are a subset of receptor tyrosine kinases. Receptor diversity results from the differential splicing of mRNA to generate membrane bound and secreted proteins. FGFR1, 2, and 3 signalling pathways appear fundamental to human skeletogenesis; as illustrated by a range of cranioskeletal and dwarfing dysplasias that occur as a result of activating FGFR mutations. These phenotypes include the syndromic craniofacial dysostoses, which are related human developmental disorders combining variable craniofacial anomaly with extracranial manifestations. Common to these syndromes is craniosynostosis, in which the fibrous cranial suture is prematurely replaced by bone. Mutations in FGFR3 additionally cause the dwarfing chondrodysplasias, which may also feature craniosynostosis and basicranial dysplasia.

Analysis of the expression of FGFR 1-3, and the ligands FGF2, FGF4, and FGF7, has been undertaken in human craniofacial tissues representing early foetal, late foetal, and infant stages of development. All tissue was collected and studied within strict ethical guidelines. FGFR transcripts were detected using isoform specific probes in in-situ hybridisation studies, whilst protein products were detected by immuno-histochemistry.

FGFR1, 2, and 3 are differentially expressed throughout human cranioskeletal development and human craniosynostosis. In infant sagittal suture fusion, FGFR1 is a marker of pre-osseous proliferative and early osseous differentiative stages, whereas the FGFR2 – IgIIc (BEK) isoform is a marker of later osseous differentiation. Differential expression of FGFR1–3 in human craniofacial development and infant craniosynostosis correlates with the clinical craniofacial dysmorphism of the related syndromes. Comparison of mutant FGFR2 – C278F and mFGFR2 - P253R human embryo-foetal cranial skeletogenesis to age – matched wild – type, suggests that FGFR2 functional gain results in negative - autoregulation of FGFR2 expression in-situ. Furthermore, the isoform – specific expression of FGFR2 and ligands in human palatogenesis predicts a dominant – negative role for FGFR2 – IgIIa/b (KGFR) in the pathogenesis of Apert cleft palate.
Dedications

This thesis is dedicated to Professor Peter Thorogood, who as an experienced mountaineer, died in an Alpine climbing accident on 25th August 1998. Peter was supervisor, mentor, confidant, and friend. His elevation onto the road to Deanship of the Institute of Child Health, by the vote of his peers and the first non-clinician to be so honoured, is a tribute more eloquent than any I might offer.

That this work was completed at all was within the gift of Shyamala, who met me (again!) as I entered into the ups and downs of laboratory research, and married me anyway! Labours such as this, often completed when back in the clinical arena, are a most selfish passtime. I will be forever indebted to my wife for her support, and the occasional reminder that the quality of life is also measured by its breadth!

Declaration

The studies presented in this thesis represent original work undertaken entirely by the author, under the laboratory supervision of Professor Peter Thorogood (†).
Acknowledgements

The period of research which resulted in this thesis coincided with two extremely happy years in both professional and personal terms. That they should have ended with the untimely death of Professor Peter Thorogood is poignant indeed and the cause of great sadness. Peter was one of those rare scientists whose personal charm and general worldliness made his scientific authority accessible to many outside his chosen field of expertise. In particular, he was able to communicate the value of scientific method and technique to clinicians, and he enthused me with this philosophy; one which has informed my view of craniofacial pathologies and emphasised the value of contemporary developmental biology to congenital clinical presentations.

The clinical service which gave the life to these studies was established by Barry Jones and Richard Hayward, and I am greatly indebted to both. Their craniofacial practice based at Great Ormond Street Hospital for Children in London was necessary for much of the pioneering genetic investigation of Willie Reardon, Robin Winter, and Sue Malcolm. Richard Hayward has elucidated the multiple pathologies of raised intracranial pressure in these patients, and provided and encouraged several opportunities to publish clinical studies. Barry Jones has always understood the relative pressures of combining a clinical and laboratory commitment, and I am immensely grateful for his support as this work has been presented in various parts of the globe. That support has ensured a warm welcome in many arenas, at a precocious stage of my surgical career and despite the scepticism that many surgeons may hold for some aspects of laboratory science! I am also grateful for his surgical tutorship, generous hospitality, and many and varied a conversation; ranging from the finer points of fronto-facial bipartition, to the effortless skills of his ‘namesake’ E.C, and the digital dexterity of Martin Taylor. All three men are virtuosi.

Particular thanks are extended to Rob Evans, who has provided great company, and an understated touch of guidance through this work. Perhaps above all, Rob has understood the frustrations of failing lab - techniques, incomprehensible reviewers, and the difficult period following August 1998, when he took over as Supervisor. I am grateful for the unswerving support, and to Rob, Richard Hayward, and Barry Jones for their patience in the ‘writing - up’ period.
Particular thanks are also due to Andy Copp, Sue Malcolm, and Robin Winter, who have each provided valuable comment upon various parts of this work. Andy, whose desk began to creak to the point of collapse, agreed to read thorough the work despite the various pressures and I am most grateful for that.

Many of my contemporaries in the laboratory have since moved on; but thanks are due to Rachel Hunt and Joe Chan, who were very patient in teaching laboratory techniques to this junior surgeon! Thanks to them and to all who were a part of the highs and lows of getting married, job applications, and weekends in the laboratory! In the clinical environment, my thanks go out to Jeanette Karaphillides, and many of the Tiger Ward staff, who made Mondays and Thursdays such a great pleasure, and a reminder of what its all about!
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The studies contained within this thesis are principally concerned with the roles of FGFR proteins and their ligands in human craniofacial skeletogenesis and palatal shelf fusion. In the General Introduction, therefore, an opportunity is taken to review relevant aspects of FGFR biology to these the themes. A detailed review of the human craniofacial FGFR phenotypes is given, in concert with their non-skeletal and extracranial manifestations. The purpose of this (S-1.3) is to illustrate the breadth of the clinical phenotypes in their completeness, and therefore the range of the functional sequela of activating FGFR mutation. Genotype–phenotype correlations reflect both gene expression and the functional nature of the mutation, and, therefore, a clinico-pathological classification of FGFR mutations is given (S-1.4). The clinical relevance of the studies contained within this thesis is subsequently discussed against this information. Complete Materials and Methods are given (S-2), with Appendices for specific details where appropriate.

The Results section is divided into 4 parts (S-3.1 – 3.4). Each part may be read independently, thus a brief introduction and methodology are repeated with specifics for each set of studies. Each set of studies is discussed separately, with an overall concluding Discussion (S-4) and considerations for future work. This approach to the organisation of this thesis is taken in order to facilitate its reading. Some repetition of information in various sections has been necessary, but kept to a minimum, and it is hoped that the aim of clarity has been achieved.

In-text references are given in bracketed italics. Hence, an in-text reference in the General Introduction to a later section is given as (S-3.3) for information contained in Section 3.3.
The Figures in this thesis are labelled according to numerical order within the relevant section of the text. Hence Figure 1.1 is the first figure in Section 1; and Figure 3.1-5 is the fifth figure in Section 3.1. Figures and their legends are placed at the end of each section. Table data is included, where relevant, inter-currently in the text.

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1. General Introduction

1.1 Human skeletal dysplasias are caused by FGFR mutations

Over recent years several human skeletal dysplasias have been linked to a series of mutations in the FGFR genes 1-3. The clinical presentation of these conditions can be broadly classified into two groups:

1. the dwarfing chondrodysplasias, which primarily affect the appendicular skeleton and include hypochondroplasia (HCH), achondroplasia (ACH), and thanatophoric dysplasia (TD). TD is subdivided into TD1 and TDII on clinical grounds. A further severe form of skeletal dysplasia incorporates achondroplasia, developmental delay, and acanthosis nigricans, a corrugating dysplasia of the skin (SADDAN).

2. the syndromic craniofacial dysostoses (including the acrocephalosyndactylies), which are a group of congenital anomalies of craniofacial growth and development that feature craniosynostosis, the premature fusion of the cranial sutures; facial dysmorphism, and extracranial manifestations of variable phenotypic severity.

All of the individual syndromes that are encoded by FGFR mutations are inherited in an autosomal dominant manner, and by mutations that occur with some of the highest frequencies in the human genome. The chondrodysplasias are caused by a series of mutations in FGFR3, and, in displaying a dwarfing phenotype, implicate this gene in the regulation of the endochondral ossification of long bones. These phenotypes may also include craniofacial manifestations; such as a short skull base, macrocephaly, and craniosynostosis (including pan-synostosis causing ‘clover-leaf’ skull). These phenotypic features implicate the FGFR3 gene product in basicranial endochondral ossification, but also suggest that it plays a contributory role in the membranous ossification of the neurocranium and the regulation of cranial sutural morphogenesis.

Craniosynostosis is the premature fusion of the cranial sutures, which are the adaptive joints between the calvarial bones. In normal growth and development these sutures allow passive cranial molding, a process essential to birth by normal vaginal delivery and one which gives
the calvarium some flexibility to respond to brain growth. Programmed human cranial suture closure begins within weeks of birth with the closure of the posterior fontanelle and ends in mid-adult life. Before this time the cranial sutures may also act as the primary regulatory site of active cranial growth, allowing increases in cranial volume secondary to regulated and directional osteogenesis at the interface between suture and dura mater.

The majority of the FGFR-associate syndromic craniofacial dysostoses feature craniosynostosis as part of a wider craniofacial phenotype. The craniofacial dysmorphism presents a number of recognisable traits within a very broad phenotypic spectrum. Unicoronal synostosis, or fusion of one coronal suture, causes the plagiocephaly phenotype and is commonly accompanied by facial scoliosis and vertical dystopia (Fig 1.1-1). These features are considered a secondary phenomenon to the uni-sutural fusion (S-3.3). By contrast, whilst the facial involvement in 'non-syndromic' bicoronal synostosis (Fig 1.1-2) may be minimal, the midfacial retrusion of syndromes such as those of Apert and Pfeiffer with a bicoronal synostosis is often very severe (S-1.3). Associated craniofacial anomalies such as hypertelorism, pterional growth arrest, and retrusion of the supra-orbital region also feature to greater or lesser degree. The phenotypic overlap of many of these clinical features between eponymous named syndromes, which may change in any one individual with time, may cause diagnostic confusion even amongst experienced clinicians (Pulley et al, 1996; Steinberger et al, 1996; Muenke et al, 1997).

A range of mutations in FGFR1, FGFR2, and FGFR3 are causative to these related cranioskeletal dysplasias, which were eponymously named for those clinicians who first recognised the recurrent clusters of commonly associated craniofacial and extracranial features in the phenotypes. The extracranial manifestations of the most severe phenotypes include skeletal and soft tissue anomalies of the limbs, and developmental anomalies of the respiratory tract and viscera (S-1.3). The extent of the human clinical phenotypes suggests that the genes FGFR1-3 play a broad and functionally overlapping role in human development that extends beyond their role in skeletogenesis. In particular, however, in displaying similar ranges of cranial and limb dysplasia, the FGFR-mutant clinical phenotypes demonstrate that common molecular control mechanisms regulate human skull and limb morphogenesis. A fundamental role for FGFR signalling in development is further indicated by the fact that members of the FGFR protein family display great sequence homology, and are highly conserved amongst a range of species including C.elegans, Drosophila, chicken, and higher mammalian vertebrates (Johnson and Williams, 1993; Park et al, 1995a). Human FGFR mutations have been demonstrated to affect a range of different sites throughout the receptor protein. Certain non-random, high frequency sites of pathologic
mutation within the protein structure are common amongst the three FGFRs, however, and these predict common functional effects upon receptor dimerisation and signal transduction (S-1.4). These, in turn, generate common phenotypic effects, which are expected to reflect the pattern of FGFR expression during human development.

The variable nature of the craniofacial phenotype indicates that the role of FGFR signalling in basicranial, facial, and skull development is functionally important, and modified to variable extent by epigenetic influences and/or other signalling pathways. The clinical phenotype will therefore reflect the functional effects each specific FGFR mutation, the tissue – specific expression of the mutant genes, the extent of co - regulation by modifier signalling pathways, and environmental influences. The variability of cranioskeletal presentation within and between these related syndromes, and often between affected members of the same family (Jackson et al, 1976; Baraitser et al, 1980; Reardon and Winter, 1994), suggests that there is great plasticity in the genotype – phenotype relationship. Over 100 human disease phenotypes feature craniosynostosis (Reardon and Winter, 1995). In general, these can be divided into those where the craniosynostosis has traditionally been considered a secondary phenomenon, and those where the sutural fusion is considered a primary event in the disease process. Examples of secondary craniosynostosis include the sutural fusion of mucopolysacharidosis diseases such as Hurler and Morquio syndrome (Gorlin et al, 1990). There are many other conditions featuring secondary or occasional craniosynostosis including haematological disorders such as thalassaemias, metabolic anomalies including hyperthyroidism and idiopathic hypercalcaemia; and iatrogenic conditions such as shunted hydrocephalus (Gorlin et al, 1990). The interaction of the breech position in - utero and FGFR2 - genotype has been recently considered in the pathogenesis of unicoronal synostosis (Johnson et al, 2000); and it may be that the genetic environment which predisposes to 'secondary craniosynostosis' will reflect as yet undefined mutations in genes encoding constituents of the FGFR signalling pathway.

Of the 'primary' syndromic forms of craniosynostosis, approximately 30 are considered to be single gene disorders (Reardon et al, 1994). The FGFR genes were originally considered as candidate causative genes for human skeletal dysplasias following a number of corroborative observations. Crouzon and Jackson – Weiss pedigrees had been mapped by linkage studies to the chromosome regions 10q2, and 10q23 – q26 respectively (Preston et al, 1994; Li et al, 1994; Ma et al, 1995), having been excluded from a Saethre Chotzen syndrome locus at chromosome 7p (Brueton et al, 1992; van Herwerden et al, 1994). Human FGFR2 had been mapped to the same region 10q25 – q26 (Johnson and Williams, 1993), and initial studies in the mouse had defined expression domains for the bek / IgIIIc isoform of fgfr2 in the frontal

Pfeiffer syndrome was linked to the peri-centromic region of chromosome 8 in 5/11 affected pedigrees in 1994 (Robin et al, 1994), although genetic heterogeneity was implied by exclusion of linkage criteria in a further 6 families. Human FGFR1 maps to 8p11.2 – p12 (Johnson and Williams, 1993); was thus considered a strong candidate; and mis-sense mutations in the linker – sequence of the FGFR1 protein were reported in Pfeiffer syndrome in 1994 (Muenke et al, 1994). Linkage of a further three Pfeiffer kindreds to chromosome 10q was reported in 1995, in whom a number of mutations in FGFR2 were identified (Schell et al, 1995). The paucity of Apert kindreds (only eleven cases of vertical transmission had been recorded by 1995) had precluded classical genetic linkage studies in Apert syndrome.

However, the similarity of aspects of the craniofacial and limb phenotypes to those of Crouzon and Pfeiffer syndromes resulted in FGFR1 and FGFR2 being strong potential candidates. FGFR2 mutations were found to be causal in 40 unrelated Apert cases in 1995 (Wilkie et al, 1995a). Acondroplasia, inherited in an autosomal dominant fashion, was linked to chromosome 4p16 (Francomano et al, 1994; Le Merrer et al, 1994; Velinov et al, 1994), the same position to which FGFR3 had been localised (Johnson and Williams, 1993). In – situ hybridisation studies in the mouse model show that fgfr3 is strongly expressed throughout endochondral ossification (Peters et al, 1993), and therefore presented FGFR3 as a candidate for achondroplasia. FGFR3 mutations causing achondroplasia were first reported in 1994 (Shiang et al, 1994).

The following sections of this general introduction review the molecular biology of those members of the FGFR gene family that cause human skeletal dysplasia (S-1.2) and the FGFR mutant clinical phenotypes, introduced according to their mutational basis (S-1.3; S-1.4). Detailed emphasis is placed upon the craniofacial phenotypes, with note of the historical background of the original phenotypic classifications where appropriate. The introduction is completed by a clinico – pathological review of the various classes of human FGFR mutation (S-1.4).
1.2 FGFRs demonstrate bio-functional diversity.

1.2.1 The general structure of FGFR

The FGFRs form a sub-family of the receptor tyrosine kinases. The FGF – FGFR interaction is facilitated by heparin and heparin sulphate proteoglycan, and there is evidence that the HSPG may act as an independently activating ligand in some circumstances (J.

1.2.3). The FGFs also interact, in a heparin-independent manner, with a cysteine-rich receptor lacking in a tyrosine kinase domain (Johnson and Williams, 1993). This receptor is highly conserved amongst vertebrates, which suggests an important as yet undefined developmental role. The human FGFR family of genes encodes a group of structurally related protein receptors of the tyrosine kinase subclass IV which are predominantly membrane-bound and transduce ligand interaction to intracellular signal cascades. Four FGFR gene loci are identified, FGFR1 on chromosome 8p; FGFR2 on chromosome 10q; FGFR3 on chromosome 4p; and FGFR4 on chromosome 5q (Johnson and Williams, 1993).

The full-length mammalian receptors share basic structure consisting of an extracellular domain, a transmembrane (TM) domain, and an intracellular region; incorporating a juxtamembraneous (JM) domain and a split tyrosine kinase (TK) domain (Givol and Yayon, 1992; Johnson and Williams, 1993). The extracellular domain incorporates a number (usually three) immunoglobulin-like loops (IgI – III), named for their similarity in structure to immunoglobulins, which contain a similar distribution of intra-molecular Cys-Cys disulphide bonds (Fig. 1.2 – 1). The different human FGFR homologues show great sequence conservation, in particular within the IgII – loop, the IgII – III linker sequence, and the IgIIIc – loop isoforms of the extracellular domain. The intracellular TK domains 1&2 also display ~80% sequence conservation. Comparison of the full-length proteins (the IgIIIc isoforms, Table 1) encoded by the four human genes indicates that FGFR1 and FGFR2 have a 72% sequence identity and are the most closely related members of the receptor family. The FGFR1 and FGFR4 proteins share only 55% sequence identity (Johnson and Williams, 1993). A table of human FGFR sequence homology is shown in the table below:
Table: The degree of amino acid identity between the three Ig–loop forms (IgIIIc isoforms) of the receptor (Johnson and Williams, 1993).

<table>
<thead>
<tr>
<th></th>
<th>FGFR2</th>
<th>FGFR3</th>
<th>FGFR4</th>
</tr>
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<tbody>
<tr>
<td>FGFR1</td>
<td>72%</td>
<td>62%</td>
<td>55%</td>
</tr>
<tr>
<td>FGFR2</td>
<td>66%</td>
<td></td>
<td>57%</td>
</tr>
<tr>
<td>FGFR3</td>
<td></td>
<td></td>
<td>61%</td>
</tr>
</tbody>
</table>

Of the three Ig–loops of the extracellular domain, the Ig–I loop displays the least degree of sequence conservation between species and also has a lower sequence identity with IgI and IgII loops of the same protein. Similarly, there is reduced sequence conservation of the signal peptide region and TM domains between species – specific FGFR homologues.

A continuous stretch of 4-8 amino acids, high in Asp and Glu content, known as the acid box, lies between the IgI and IgII domains and is variously considered to play a discrete role in receptor function (Johnson and Williams, 1993). An additional sequence of ~20 amino acids characterises the region between the IgI and IgII – loops and displays a high level of homology to the cell adhesion molecules (CAMs); L1, NCAM (neuronal cell adhesion molecule), and N-cadherin (Williams et al, 1994). This region, the ‘CAM homology domain’ suggests that the FGFR signalling, which is associated with neuronal differentiation and neurite outgrowth (Chao, 1992), is linked to co – activation by NCAM (Williams et al, 1994). There is evidence that FGFRs may be activated by matrix molecules such as heparin sulphate proteoglycan (S-1.2.3). The structural homology between CAMs and FGFRs is indirect evidence for common binding motifs and possible functional interdependence. Evidence from the crystal structure of FGFR1, however, indicates that the His-166 and Val-168 residues, which may contribute to intermolecular bonds, are folded within the hydrophobic core of the CAM homology domain. This suggests that they are important to the folding of the loop and may be unable to directly interact with CAM (Plotnikov et al, 1999).

Despite the high level of FGFR sequence homology, a high degree of receptor diversity results from mRNA splice events that generate protein receptors with variable sequence composition within the extracellular domain. Splice variance may generate extracellular domains variously lacking the IgI – loop, the acid box, and CAM homology domain (Johnson and Williams, 1993). The ‘short – form’ receptors retain their ligand – binding ability, suggesting that the interaction between FGFR and ligand FGF is predominantly a function of the IgII and IgIII loops and the intervening linker region amino acid sequence (Hou et al,
In the membrane-bound FGFRs, the transmembrane domain anchors the protein to the hydrophobic cell membrane and is accompanied by a relatively long juxtamembranous region (Johnson and Williams, 1993). The split TK domain of the FGFR group contains a 13–14 sequence insert, which defines the receptor family amongst the receptor tyrosine kinases (Johnson and Williams, 1993; Green et al, 1996). It consists of a number of consensus tyrosine residues that may act as phosphate acceptors for autophosphorylation. The kinase insert sequence separates the two parts of the tyrosine kinase domain into two nearly equal halves. The intracellular TK domain adjoins the intracellular COOH tail of the receptor, which contains tyrosine residues that may be phosphorylated upon receptor activation (Johnson and Williams, 1993).

**FGFR splice variance & ligand - receptor specificity**

FGFR variants are generated by alternative splicing of mRNA (Champion Arnaud et al, 1991; Johnson et al, 1991), to deliver a degree of receptor diversity that is unprecedented by any other growth factor receptor family (Johnson and Williams, 1993). The generic structure of the FGFR genes provides a basis for the number and types of splice variant observed. FGFR1 protein is encoded by 19 exons (Johnson et al, 1991). The extracellular domain is encoded by 9 exons. Exon 1 encodes a non-translatable sequence and exon 2 encodes the leader sequence. The IgI-loop sequence and acid box are each encoded by a single exon, whereas the Ig-loops II and III are each encoded by two exons. The IgIII-loop, at its NH2-terminal half (IIIA) is encoded by a single ‘constant’ exon 7, but the COOH half varies by an equal length amino acid sequence in a mutually exclusive manner. The IIIB isoform is encoded by exon 8, and the IIIc isoform is encoded by exon 9. Extended to FGFR2, which has a similar exon arrangement, the IgIIIa/c isoform (exons 7/9) is translated to the BEK receptor, and the IgIIIa/b isoform (exons 7/8) forms the KGFR protein in both humans and mice (Werner et al, 1992; Yayon et al, 1992; Miki et al, 1992). Hence two functional receptor proteins are generated from a single gene, which differ within a confined internal amino acid sequence that occupies a critical loop within the extracellular ligand–binding domain.

A hierarchy of mRNA splicing events thus determines a wide range of receptor protein variants (observed in FGFR1, 2, and 3) that differ in:

1. the number of Ig-like loops of the extracellular domain of the receptor
2. the presence of the acid-box and the -COOH terminal sequence in forms of the short-form receptor
3. the presence of a TM domain, absence of which correlates with various secreted forms of FGFR
4. the presence and structural integrity of the the TK domain

The receptor isoforms may be broadly divided into secreted forms, a cytosolic form, and membrane bound forms. The secreted forms lack a TM domain, but have variable parts of the extracellular domain intact, and may display the IgIIIa sub-domain spliced to the TK domains. Those variants with an intact IgII-III structure will bind ligand in the extracellular environment, and may act to regulate the availability of free ligand to the membrane-bound receptor. FGFR1 and 2 may be generated as 'short-form' receptors with a truncated extracellular domain, lacking the Ig-I loop, but retaining ligand binding ability (Johnson and Williams, 1993). The short-form receptors may be co-expressed with the full extracellular domain (IgI-III loop) form in a variety of cells but the functional role of these receptors is unknown. The lack of Ig-I loop and acid box may confer subtle alterations of cofactor-ligand-receptor binding affinity and signal transduction in isoform-heterodimers (Wang et al, 1995a), or alternately confer differential properties of cellular internalisation and nuclear localisation of the activated receptor (Prudovsky et al, 1996).

The IgIIIb and IgIIIc isoforms of FGFR2 differ by a sequence of approximately 50 residues (Johnson and Williams, 1993). The IgIIIb form dictates ligand specificity via the linker-region and a few residues into each neighbouring (IgII and IgIII) loop (Wang et al, 1995b). Replacement of the BEK/IgIIIc sequence with that of KGFR/IgIIIb donates the chimeric construct the high affinity binding for FGF7 of the KGFR-wild-type, and abrogates its affinity for FGF2 (Yayon et al, 1992). Similarly, a chimera of FGFR1 with the IgIIIb sequence of the KGFR protein increases FGF7 binding, the affinity of which may be increased further by substituting the BEK/IgIIIc subdomain into the complex chimera (Givol and Yayon, 1992). FGFR3 also demonstrates splice variance of the IgIII-loop, with IIIb and IIIc isoforms that differ in their ligand affinity. The FGFR3-IgIIIc isoform is the more commonly expressed, and has a wider profile of ligand binding, being activated by FGF1 and FGF4, but FGF2 to a lesser extent. FGFR3-IgIIIb has a restricted expression pattern and is activated by FGF1 exclusively, although affinity for FGF7 can be engineered in a chimera with the IgIIIb-hemi-loop of FGFR2 (Avivi et al, 1993;Chellaiah et al, 1994).

Table: Ligand Affinity of FGFR isoforms
Values represent % incorporation of {3H}-thymidine in standardised assay compared to response to FGF1 (Kannan and Givol, 2000; De Moerlooze et al, 2000)

<table>
<thead>
<tr>
<th>FGFR1</th>
<th>FGF1</th>
<th>FGF2</th>
<th>FGF3</th>
<th>FGF4</th>
<th>FGF5</th>
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<th>FGF8</th>
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<tr>
<td>-IIIC</td>
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<td>100</td>
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<td>20</td>
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<tr>
<td>FGFR1</td>
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<td>-IIIB</td>
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<td>25</td>
<td>60</td>
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<td>16</td>
<td>90</td>
</tr>
<tr>
<td>FGFR2</td>
<td>100</td>
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<td>45</td>
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<td>12</td>
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<td>40</td>
<td>95</td>
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<td>FGFR3</td>
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<td>75</td>
</tr>
</tbody>
</table>

Splice variance also controls for soluble, secreted forms of the FGFR, which lack a TM or TK domain, and display variable lengths of the extracellular Ig – loop (Johnson and Williams, 1993). Secreted forms of the receptor which bear an intact IgII – IgIII sequence are able to bind ligand and may thus have a functional role in the extracellular regulation of ligand bioavailability to membrane-bound receptor protein. Several other truncated forms may be generated including a truncated kinase form, lacking the potential for self-phosphorylation, with the replacement of the second kinase domain by a terminal-COOH. Such a receptor could theoretically play a modulatory functional role in the membrane by acting as a dominant negative in heterodimers with active receptors.

The gene structure of FGFR is therefore demonstrated to allow for several forms of the receptor, generated by alternative splicing, splice site skipping, and polyadenylation - which allows transcription of intronic sites (Givol and Yayon, 1992). The low – threshold for splice variation in the transcription of the FGFR genes is also demonstrated by clinical FGFR mutations in human skeletal dysplasias. Aberrant splicing of exon 7 in FGFR2 is proposed to account for a subgroup of mutant human FGFR2 – phenotypes, recognised as Pfeiffer, Crouzon, and Jackson – Weiss syndromes (Li et al, 1995; Schell et al, 1995; Lajeunie et al,
1995a). These mutations cause abnormal splice events in FGFR2 which yield stable transcript RNA and stable altered IgIIc receptor.

Receptor diversity makes for complexity of signalling. There is marked overlap of ligand affinity between some of the receptors, yet others, such as the IgIIb isoform of FGFR2, demonstrate great selectivity for ligand. Many of the splice – variants are expressed in the same cells. The stomach cancer cell line Kato-III co – expresses both IgIIb and IgIIc isoforms of FGFR2, the membrane – bound short form of the receptor, two truncated secreted forms, and a secreted form with tyrosine – kinase activity (Johnson and Williams, 1993). In these cases the net effect of signal transduction across the cell membrane will presumably reflect the net availability of ligand to membrane bound receptor, and the specificity and/or commonality of downstream signalling pathways. These factors are important in considering the relationship between mutant genotype and the functional effect in the phenotype of FGFR mutations causing human skeletal disease (S-1,3).

1.2.2 Differential expression of FGFR homologues & splice - variants

The degree of structural homology and overlapping ligand sensitivities between the FGFR homologous proteins serves to generate a high potential for functional redundancy between the receptors. A number of studies demonstrate that a tissue – specific control of FGFR signalling is achieved by the differential expression of FGFR homologues in chicken, mouse, and human. The independent regulation of the FGFR homologues is consistent with the lack of sequence homology within their promoter regions (Avivi et al, 1992). In the chicken the FGFR genes, (cek 1,3, and 2) are expressed in common tissues, but in cell – specific manner (Patstone et al, 1993; Wilke et al, 1997). Differential expression of the cek gene transcripts is noted in the skin/feather primordia, pancreas, brain, muscle, lung and intestine. Differential expression is also noted in skeletogenic tissues. In the vertebral column, whereas cek1 (the chicken homologue of FGFR1) is expressed in the intervertebral discs, cek3 (chicken homologue of FGFR2) is expressed in the perichondrium and peristeum and cek2 (chicken homologue of FGFR3) is expressed in chondrocytes up until the stage of hypertrophy; whereupon its expression is downregulated. In cranial membrane bone, however, the cek2 (FGFR3) transcript is barely detectable compared to expression of the cek3 (FGFR2) transcripts in undifferentiated mesenchyme and peristeal domains, and cek1 (FGFR1) in osteoblasts (Patstone et al, 1993). The preferential expression of cek2 (FGFR3) in chondrogenic, as opposed to membranous, osseous differentiation has potential relevance to human FGFR3 — phenotypes, which predominantly reflect chondrodysplasia (S-1,3). Further,
though limited, correlations may be drawn with human \textit{FGFR} - cranioskeletal dysplasias. \textit{Cek3 (FGFR2)} is expressed in the frontonasal mass, which is associated with rapid growth to form the midfacial structures, but neither \textit{cek2} nor \textit{cek3} transcripts are expressed in the chicken maxilla (Wilke et al, 1997), which correlates with a lack of growth response to chicken maxillary mesenchyme to FGFR2 in vitro (Richman and Crosby, 1990). Lack of \textit{cek3 (FGFR2)} expression in the chicken midface, however, provides a poor correlate for human \textit{FGFR2} - skeletal mutants (S-3.3; S-4).

The developmental expression of murine \textit{fgfr1} and \textit{fgfr2} is first seen in the primitive ectoderm of egg-cylinder-stage embryos, and both genes are actively transcribed both in the mesoderm and neuroectoderm during later somitogenesis and embryogenesis. Whilst \textit{fgfr2} is expressed in the surface ectoderm and in various epithelia of the developing skin and internal organs (E9.5 - E16.5), \textit{fgfr1} expression is restricted mainly to the mesenchyme (Orr-Urtreger et al, 1991; Peters et al, 1992). This differential expression includes the murine trachea (E9.5 - 16.5), and correlates with a tissue specific role for FGF/FGFR signalling in tracheal morphogenesis; which, when disrupted by activating mutations of \textit{FGFR1} and \textit{FGFR2}, cause human tracheal anomaly (S-1.3). The wide distribution of differential \textit{fgfr} expression in murine organogenesis (digestive, cardiovascular, nephro-ureteric and neuro-endocrine systems) also correlates with a range of sporadic visceral anomalies that characterise a range of human mutant \textit{FGFR} - phenotypes. Whilst tracheal anomaly is often declared by clinical sequelae, many of these wider anomalies may have subclinical effects and become noted post-mortem (S-1.3). It is likely that the phenotypes of human activating \textit{FGFR} - mutations extend beyond those skeletogenic effects that are immediately obvious and clinically stimulating.

Despite the wide expression of \textit{fgfr1-2} during stages E9 - E16 of mouse organogenesis, \textit{fgfr3} is not detected in most epithelia or mesenchyme (Peters et al, 1993). It is expressed, in company with \textit{fgfr1-2} in the germinal epithelium of the neural tube at 9.5-16.5 days post-conception. \textit{Fgfr3} is also expressed at high levels in differentiating hair cells of the cochlear duct, and is expressed in highest concentration in the cartilage primordia of developing bone, thus correlating with the data from the chicken (Patstone et al, 1993). A specific role for murine \textit{fgfr3} in the regulation of endochondral ossification is suggested by the observation that it is expressed exclusively in resting cartilage, in a distinct pattern from \textit{fgfr1} and \textit{fgfr2}, which are widely expressed in the pre-osseous cartilage domains (Peters et al, 1993). \textit{Fgfr3} is not expressed in the hypertrophic cartilage domain. By one day postpartum and in the adult brain, \textit{fgfr3} is diffusely expressed in glial - type cells, unlike the neuronal expression pattern of the \textit{fgfr1} homologue (Peters et al, 1993). \textit{Fgfr3} is expressed in adult kidney, skin and lung, at
lower levels than \textit{fgfr1}, which is also expressed in heart and skeletal muscle unlike the \textit{fgfr2} homologue.

There is a paucity of data indicating whether tissue or differentiation stage – specific developmental regulation of the individual splice - variants of FGFRs occurs in non – transformed cells \textit{in – situ}. Many of the FGFR2 mutations causing human craniofacial dysostosis will be predicted to affect both major functional splice – variants of FGFR2 protein, and thus may allow genotype – phenotype correlation by means of their differential tissue expression. In the murine model, the \textit{kgfr/Iglllb} and \textit{bek/Iglllc} isoforms show differential expression throughout gastrulation and organogenesis (Orr-Urtreger et al, 1993). Whilst expression is diffuse in the gastrula, patterns become more established later during organogenesis, when \textit{kgfr} transcripts are evident mainly in epithelia, whereas \textit{bek} is present in the corresponding mesenchymes. There is minimal overlap of the expression of \textit{kgfr} and \textit{bek} transcripts in the skin, mammary gland, and hair follicles, establishing a spatial specificity for the isoform expression of \textit{fgfr2}. The expression of \textit{bek} greatly exceeds that of \textit{kgfr} in the osseous development of the murine limbs, ribs, skull, and facial skeleton (Orr-Urtreger et al, 1993), which might suggest that the \textit{bek} isoform is dominant in human skeletogenesis also, and is primarily affected by human FGFR2 skeletal dysplasia mutations (see S-3). \textit{Kgf/ kgfr} expression may provide a paradigm for mechanisms of epithelio – mesenchymal co – ordination in development. Whereas \textit{kgfr} is predominantly expressed in murine (E14.5 – E16.5) epithelia, \textit{kgf} (FGF7) is expressed in the subjacent mesenchyme, and may provide the mitogenic ligand activation across these tissue boundaries (Finch et al, 1995). \textit{Kgf/ kgfr} complementary domains are also noted in murine perichondrium, cartilage, and muscle domains (Mason et al, 1994;Finch et al, 1995). FGFR3 also demonstrates splice variance of the IgIII – loop; whereas the IgIIIc receptor is expressed in brain and cartilage, the IgIIIb isoform is predominant in skin and has a very restricted ligand affinity profile (Chellaiah et al, 1994).

The degree of mutual exclusivity of the expression domains of murine \textit{fgfr1}, the splice – isoforms of \textit{fgfr2}, and \textit{fgfr3} suggests that modulatory mechanisms operate at the levels of both transcriptional activation and splice – control. It remains to be seen whether the splicing factors that control the isoform – specific expression of murine \textit{fgfr2} operate in similar fashion in human tissue. Sequence conservation between the murine and human FGFR – genes would suggest that intronic splice signals are conserved, generating isoform – specific expression in human tissues as well. Such mechanisms have the potential to be disrupted by human FGFR mutations, creating novel splice sites and proteins, which may then be functionally activating in human dysmorphogenesis. FGFR expression has been investigated
in wild-type human development. Northern analyses of human mRNAs from various 17–18 week tissues indicate that \textit{FGFR1} is expressed in the brain, skin, and growth plates of bones; \textit{FGFR2} is expressed in the choroid plexus, skin, lung, kidney and temporal lobe; \textit{FGFR3} is abundant in the skin, lung, kidney, and bone growth plates; and \textit{FGFR4} is expressed in adrenal, lung, kidney and liver (Partanen et al, 1991). These studies, in common with later emerging data from the mouse and human clinical genetics, provided an incentive to demonstrate FGFR expression in human development \textit{in situ}. These studies (Delezoide et al, 1998; Chan and Thorogood, 1999) are extended by those presented in this thesis with which they contrasted below (S-3.2, S-3.3, S-4).

1.2.3 FGFR activation: the role of ligand and matrix co-factors

The fibroblast growth factor proteins (FGFs) provide the major ligands for FGFR activation. FGFs were first discovered in the 1970s as heparin binding factors that were mitogenic to NIH3T3 fibroblast cell lines (Johnson and Williams, 1993). FGF1 and FGF2 were originally purified from bovine pituitary gland, and have since been isolated from a variety of tissues including bone, cartilage, adrenal gland, corpus luteum, hypothalamus, kidney, liver, prostate, retina, testis and thymus. In increasing number of FGF ligands are identified, which act in a very wide range of developmental and physiological roles. The FGFs are structurally related and highly conserved in vertebrate evolution. Human and bovine FGF1 differ by 12 amino acids, and the FGF2 proteins differ by 2 amino acids. The identification of amphibian homologues of FGF2, FGF3, and FGF4, and additional homologues in \textit{Drosophila} suggest that FGFs are probably derived from a common ancestral gene (Johnson and Williams, 1993; Wilkie et al, 1995b).

The FGF genes encode proteins with a molecular mass of 20–30 kDa, which are secreted into the environmental matrix. Many of the FGFs including FGF1 and FGF2, but not FGF5 or FGF7, however, lack classic ‘secretory peptides’ or ‘leader sequences’, which have been considered necessary for the secretion of the ligand from the parent cell. This suggests that novel secretory pathways have evolved for individual FGFs within the ligand family (Mason, 1994). FGF1 and FGF2 consist of a \( \beta \)-trefoil structure, made up of 12 interlinked strands. FGF2 displays a heparin-binding site between \( \beta \) strands 10–11, and a spatially separated receptor binding site (\( \beta 8-9 \) region) (Plotnikov et al, 1999). Human FGFs show high level of sequence conservation (Givol and Yayon, 1992), suggesting that this three-dimensional structure is likely to be a common theme within the group (FGF4 and FGF2 have \( \sim 40\% \) sequence homology, FGF5 and FGF2 have \( \sim 50\% \) homology, but FGF7 has only \( \sim 30\% \) homology with FGF2). This homogeneity of structure would indicate a high degree of
functional redundancy within the group, and commonality of control mechanisms for ligand bioavailability and receptor signalling.

FGF diversity is generated by alternative splicing for different ligand isoforms, alternative translation events; and post - translational modifications including glycosylation, methylation, phosphorylation and nucleotidylation (Mason, 1994). FGFs are expressed throughout mammalian development and maturity (Johnson and Williams, 1993) in an, often overlapping, but tissue specific manner. Individual FGFs are implicated in a number of physiological processes in vivo, including many facets of embryo - foetal development, neovascularisation and responses to wound healing (Johnson and Williams, 1993;Mason, 1994;Green et al, 1996).

A number of studies correlate FGF/FGFR interaction with a limiting requirement for heparin or heparin sulphate proteoglycan. Heparin sulphate proteoglycans (HSPG) are a core constituent of many tissue matrices. The basic HSPG structure consists of a core protein to which are covalently attached several linear heparan sulphate chains. The polysaccharide chains are composed of hexuronic and D glucosamine dissacharides, which are substituted to variable extent by -N and -O linked sulphate moieties and N-acetyl groups (Mason, 1994;Ornitz, 2000). HSPGs include transmembrane proteins (eg syndecans 1-4), GPI (glycosylphosphatidylinositol) – anchored proteins (eg glypican and cerebroglycan); and extracellular matrix glycoproteins (eg perlecan) in which the glycosaminoglycan (GAG) chains are extracellular, and the core protein spans the basal lamina. Perlecan has a high affinity for FGF2 amongst the HSPGs, and can promote its angiogenic and mitogenic effects by facilitating FGF/FGFR binding (Aviezer et al, 1994a). A non - redundant functional role for perlecan in FGF/FGFR interactions in chondrogenic skeletogenesis is also implied by the perlecan – null mouse, which provides a phenocopy of human activating FGFR3 – mutations (Arikawa-Hirasawa et al, 1999). The influence by HSPG upon FGFR activation may be both inhibitory and facilitatory (Mason, 1994;Aviezer et al, 1994b;Guimond and Turnbull, 1999), and may reflect the degree of sulphation of the complex matrix glyco – proteins (Aviezer et al, 1994b;Ornitz, 2000). This raises the possibility that individual tissue matrices will exert regulatory control upon FGFR activation by means of their HSPG composition. The mechanisms of such control may be by means of the introduction of ligand, the stabilisation of the ligand – HSPG – receptor complex, or by a more fundamental functional role in signal transduction.

Many in – vitro studies implicate HSPG with a physiological role in FGF/FGFR interactions. HSPG – FGF complexes are stable to heat and proteolytic perturbation, and HSPG may
promote or limit the diffusion of FGF in the interstitium, thus providing a means for controlled release of FGF. A CHO hamster—ovary cell line, defective in GAG synthesis, cannot bind FGF2 to FGFR, which is restored by exogenous heparin or heparan—sulphate (Yayon et al, 1991). However, similar studies investigating the binding of radiolabelled FGF to FGFR1 indicate that HSPG/heparin act to facilitate the FGF/FGFR binding, rather than in a limiting capacity (Roghani et al, 1994). Subtle differences in the composition and sulphation of the HSPG have been shown to have different effects upon the degree of mitogenicity of FGF and FGFR combinations in a proliferative bio—assay system (Guimond and Turnbull, 1999). Thus different conclusions regarding the functional dependence of the FGF/FGFR interaction upon heparin may reflect the experimental circumstances and particular ligand—receptor combinations under study (Spivak-Kroizman et al, 1994; Gao and Goldfarb, 1995).

The Drosophila model provides evidence of a physiological role for HSPGs in FGF/FGFR signalling. The Drosophila htl and btl genes encode proteins with sequence homology to vertebrate FGFR. The null mutants of these genes share phenotypic features with the null mutants of the genes sgl (sugarless) and sfl (sulphateless). Drosophila sgl is essential to heparin biosynthesis, whereas the sfl gene encodes a protein that catalyses the sulphation of heparan to heparan sulphate—thus facilitating the activation of FGF (Ornitz, 2000). This evidence of a limiting functional role for HSPG components in FGF/FGFR signalling, which is generally highly conserved, correlates with the diversity of human FGFR—phenotypes. It is likely that activating human FGFR mutations, set against a variable background of matrix composition, will generate variable phenotypic effects. Human diastrophic dysplasia, caused by a mutation in a gene encoding a putative sulphate transporter, exhibits a severely malformed skeleton, progressive joint disease, and dwarfism resulting from defects in bone and cartilage matrix (Ornitz, 2000). Phenotypic features such as dwarfism, broad thumbs, and broad great toes are reminiscent of human FGFR—phenotypes (achondroplasia, Pfeiffer syndrome, Jackson—Weiss syndrome, see 5.1.3); and provide circumstantial evidence that defects in the matrix composition will have functional effects upon FGFR signalling.

FGF:FGFR:HSPG binding relationships may be dynamic, such that short—term signal induction may be heparin/HSPG independent (Roghani et al, 1994), but sustained FGFR signal transduction requires a stable FGFR—dimerisation and activation, which itself requires HSPG. The crystal structure of FGF-FGFR complexes has been modelled from FGF1 and FGF2 complexed with the IgII— and IgIII—loops of FGFR1 and FGFR2 (Plotnikov et al, 1999; Plotnikov et al, 2000). The models predict that 2 complexes of 1:1 FGF:FGFR combine to form the activating FGFR—dimer. FGF binds both the IgII and IgIII linker, which
explains how alternative splicing of the IgIII – loop generates different FGF binding affinities. The dimeric complex is stabilised by direct FGFR interactions, and by interactions between the FGF in one 1:1 complex and the FGFR in the partner complex, such that FGF molecules do not directly interact. The FGF:FGFR complex dimer forms a positively charged ‘canyon’, that acts as a putative heparin binding site, created principally by the IgII – loops in each FGFR monomer (Kannan and Givol, 2000). The heparin moiety is thus proposed to act like a ‘seat – belt’ to hold the complex FGF:FGFR dimer stable (Plotnikov et al, 1999;Kannan and Givol, 2000). It is therefore proposed that an initial low – affinity dimeric complex of FGF – FGFR forms under high ambient concentrations of FGF to initiate a short – period of signal transduction. This complex is stabilised in the presence of heparin or a suitable HSPG, such that sustained signal transduction is maintained. Clearly, human mutations affecting the IgII – loop will differentially affect the charge distribution of the heparin – binding ‘canyon’ and may have downstream functional effects as a result of this.

1.2.4 Downstream consequences of FGFR activation – mechanisms for phenotypic diversity

Activation of FGFRs in a variety of cell types may initiate processes as disparate as proliferation, differentiation, migration, apoptosis and neurite outgrowth at different stages of maturity in variety of cell types. Furthermore, activating FGFR mutations may result in accelerated membranous osseous differentiation but inhibition of chondrogenesis (S-3.3). This suggests that the mechanisms of activation of FGFR are multiple and may be cell – type specific. Treatment of cells with FGF results in increased intracellular pH and Ca2+ flux, increased phosphorylation of a number of intracellular proteins, and increased transcription of various genes including c-myc and c-fos (Wilson, 1994). Activation of mutant – FGFR induces dimerisation, and the phosphorylation of intracellular downstream proteins (Neilson and Friesel, 1995;Naski et al, 1996;Neilson and Friesel, 1996). Several intracellular pathways may be activated by FGFRs, involving Ras and Raf proteins, mitogen activated protein kinase (MAPK), phosphatidyl – inositol 3 kinase (PI -3K), protein tyrosine phosphatases, and phospholipase C1 (Wilson, 1994). Ras and MAPK are involved in a linear signalling pathway, which maintains the level of MAPK activation above a threshold level to enable phosphorylation of target molecules, including transcription factors, which may then modify gene expression. Dominant negative Ras proteins and MAPK - inhibitors may be used to successfully inhibit FGFR activation in models of human mutations (Neilson and Friesel, 1995;Naski et al, 1996;Neilson and Friesel, 1996). Similarly, dominantly active signalling by Ras can induce vertebrate neuronal differentiation in a manner similar to FGFR, which is
reversed in a dominant - negative Ras model (Chao, 1992). Linear activation of the Ras - MAPK pathway can be achieved by the alternate/combined use of intracellular cascades involving phospholipase C and protein kinase C; and increased complexity is generated by the utilisation of different molecular variants of individual proteins in these pathways (Wilson, 1994).

Examination of the phenotypes of loss - of - function models in Drosophila, where sequential components of the homologues of the Ras - MAPK pathway are modified, reveals many similarities to different Drosophila receptor tyrosine kinase (RTK) loss - of - function mutants. This suggests that the Ras - MAPK pathway is common, highly conserved, and that its major steps are non - redundant, amongst a number of RTK activation cascades in Drosophila (Wilson, 1994). Genetic modelling in Drosophila also indicates that gain of function mutant homologues of the Ras - MAPK pathway can induce phenotypes consistent with a range of Drosophila - RTK activation pathways; including the induction of embryonic tracheal cell migration by Drosophila FGFR1 (Reichman Fried et al, 1994). This raises the possibility that in certain systems the absolute level of MAPK activation is the major prerequisite for a phenotypic response, and that the specific activating ligand - receptor event is at least partially redundant. If this was the case in vertebrate skeletogenesis in vivo, one might expect gain - of - function human mutant FGFR signalling in cells of osteogenic lineage to be modified by parallel pathways reducing MAPK activation in a variable dominant - negative fashion. Phenotypic response may depend on the degree of MAPK activation, and might explain why, for example, epidermal growth factor (EGF) causes proliferation in PC12 cells, whereas FGF and nerve growth factor (NGF), acting via the same pathway, produce a differentiative effect and neurite outgrowth (Chao, 1992). Differential lengths of time of sustained MAPK activation by independent RTKs may additionally cause differential phenotypic in different systems (Mason, 1994; Wilson, 1994), and this is an additional potential mechanism generating phenotypic diversity amongst human FGFR - phenotypes.

Further mechanisms that may induce the expression of a range of modifying genes, and thus exert effect upon the phenotype, include the internalisation of FGF and its direct effect upon the nucleus. This may occur by FGFR-HSPG dependent or independent pathways (Mason, 1994; Prudovsky et al, 1996). The nuclear translocation of FGFR in human chondrogenic osseous differentiation under the control of activating FGFR3 mutations has been demonstrated, and raises the possibility that such mutations confer constitutive nuclear translocation of the receptor and are activating in pathways independent of Ras - MAPK (Legeai-Mallet et al, 1998). The various potential modes of activation of mutant FGFR in human disease, and their various downstream consequences, will affect the phenotypic
outcome of these mutations as a function of their expression in human development. These themes are explored further in Sections 3 and 4 of this thesis.
1.3 FGFR genotype – phenotype relationships: A review

1.3.1 Phenotypes associated with FGFR1 mutations

1.3.1.1 Pfeiffer Syndrome – locus heterogeneity with mutations in FGFR1 and FGFR2

Pfeiffer syndrome is an autosomal dominant craniosynostosis syndrome, featuring patterns of sutural fusion varying from a mild bicoronal synostosis to pan-synostosis and clover-leaf skull. The craniofacial features, detailed below, exhibit a range of severity; and the syndrome was originally defined as an entity in 1964 by the association of the craniofacial dysostosis with characteristic limb anomalies (Pfeiffer, 1964). Eight affected individuals across 3 generations were reported with 2 instances of male-to-male transmission. In addition to the craniofacial features, consistent limb anomalies were noted to include broad, short thumbs and big toes, with the proximal phalanx of the thumb often triangulated to cause a pre-axial deviation of the thumb. Fusions between thumb phalanges (sympalangism) were also noted, together with partial soft tissue syndactylies of thumb and fingers. The recognition of this constellation of limb anomalies in conjunction with the craniofacial phenotype remains the major criterion for the clinical diagnosis and eponymous label (Cohen, Jr., 1993).

Pfeiffer syndrome was initially causally associated with a linker-region mutation in FGFR1 in 1994, in all the affected members of 5 unrelated Pfeiffer syndrome families (Muenke et al, 1994). The same mutation, a C755G transversion, was subsequently noted in an additional sporadic case and one family (Schell et al, 1995), two additional cases (Meyers et al, 1996), and one of a review of five Pfeiffer syndrome patients (Passos-Bueno et al, 1998a). The mutation has also been reported in a single female patient with the diagnostic label of Jackson-Weiss syndrome (5.1.3.2), presenting with a mild craniofacial and limb phenotype (Roscioli et al, 2000). The similarity of phenotype between Pfeiffer and Jackson-Weiss syndromes has been previously noted (Cohen, Jr., 1993). Phenotypic overlap in these human FGFR1 mutants demonstrates a common theme amongst the FGFR1 and FGFR2 - associated skeletal dysplasias; that eponymous syndromes represent the peak frequency clusters of a spectrum of phenotypic features.

The C755G transversion mutation in FGFR1, which is common to all these reported phenotypes, is an exon 5 mutation which predicts a P252R mis-sense substitution in the
linker-region sequence between the IgII and IgIII – loops of the FGFR1 protein. The proline to arginine substitution is not encoded by any other nucleotide change (Muenke et al., 1994), and this suggests that the substitution of arginine at this position will have specific functional sequelae for the mutant receptor - despite the observed range of the phenotype. Exon 5 of FGFR1 is common to all its demonstrated splice – variants (Johnson and Williams, 1993), and the P252R mutation in FGFR1 can therefore be predicted to exert functional effects through all the naturally occurring isoforms of the membrane – bound receptor protein. The variable effects of the mutant FGFR1 protein upon human skeletal development might be explained by the variable net effect of signalling via these heterogeneous mutant receptor isoforms co-expressed \textit{in vivo}, in the presence of differential concentrations of matrix cofactors and ligands (S-3, S-4).

Locus heterogeneity of Pfeiffer syndrome is demonstrated in that several mutations throughout the FGFR2 gene are also causative in sporadic and familial cases (Lajeunie et al., 1995a;Passos-Bueno et al., 1998a;Gripp et al., 1998a;Cornejo-Roldan et al., 1999;Glaser et al., 2000). In a review of 50 patients presenting with a variety of syndromic craniofacial dysostosis syndromes, mutations in the FGFR1 gene accounted for only 2% of cases in comparison to 93% causation by the mutant forms of FGFR2 (Passos-Bueno et al., 1998a). It is a point of note that despite the many pathological mutations affecting the IgIII – loop of FGFR2, and the >70% sequence homology between the IgIIIc isoforms of FGFR1 and FGFR2, no IgIIIc mutations of FGFR1 have been reported to cause human skeletal dysplasias. There is, however, a demonstrable increased mutation rate affecting the homologous position in the linker – sequence between the IgII – and IgIII loops of all the FGFR1, FGFR2 and FGFR3 proteins. Where the Pro252Arg mutation in FGFR1 causes Pfeiffer syndrome, the Pro253Arg mutation in FGFR2 causes Apert syndrome and the Pro250Arg mutation causes a broad range of coronal synostoses (S-1.3.2, S-1.3.3, S-1.4). One report (Bellus et al., 1996) assigns the Pfeiffer phenotype to the Pro250Arg mutation in FGFR3, however, it seems likely that the phenotype is more correctly assigned to the ‘coronal synostosis’ group.
1.3.1.2 Pfeiffer Syndrome: craniofacial and extracranial manifestations

The pattern of cranial sutural synostosis in Pfeiffer syndrome is variable, although the coronal and sagittal sutures are most commonly involved. Cranial suture fusions combine to cause turribrachycephaly (tower-shaped skull), the most common Pfeiffer craniofacial phenotype (Fig 1.3-1). In severe cases, the syndrome is associated with the 'clover-leaf skull' or kleeblattschadel (Fig 1.3-2), in which there is severe biparietal protrusion of the skull, turribrachycephaly, and a shallow posterior fossa (Cohen, Jr., 1993). The cranial base is short in company with the retruded midface. True hypertelorism (increased inter-orbital distance) is less common than in Apert syndrome (S-1.3.2.), and there is a much milder 'anti-mongoloid' cant to the face, with laterally down-slaning palpebral fissures. The ears are typically low set. The dysmorphogenesis of the cranial base is also manifest in the auditory mechanism. In a study of 9 patients with Pfeiffer syndrome, CT findings consisted of stenosis and/or atresia of the external auditory canal, hypoplasia of the middle ear cavity, and an enlarged middle ear cavity (Vallino-Napoli, 1996). The ossicles may be hypoplastic, and ossicular synostosis has also been reported (Cremers, 1981). These anomalies are associated with moderate to severe conductive or mixed hearing loss in most patients, despite the observation that inner ear anatomy is predominantly normal (Vallino-Napoli, 1996).

The midfacial retrusion of Pfeiffer syndrome is accompanied by ocular proptosis and lagophthalmos (failure of corneal cover by the upper eyelid), which may progress to cause exposure keratitis in severe cases. Severe midfacial retrusion may cause subluxation of the entire globe onto the cheek. Ocular strabismus has been reported (Van Dyke et al, 1983), which may reflect the dysmorphogenesis of the bony orbit and secondary mal-insertions of the peri-orbital musculature. The midfacial retrusion results in a relative mandibular prognathism, beaked nose, and class III skeletal and dental malocclusion, which is progressive through childhood and adolescence to skeletal maturity. The maxillary dentition is crowded and malaligned, and there is delayed eruption of the secondary dentition. The palate is high arched, in common with Apert syndrome, but cleft palate does not occur. The nasopharynx is of restricted volume, and choanal stenosis, or less commonly choanal atresia, may occur. Clinical problems of the upper airway are therefore common, and lower airway compromise may also be noted. Primary tracheal anomalies have been reported in both Pfeiffer and Crouzon (Devine et al, 1984) syndromes, including replacement of the cartilaginous rings by a solid cartilaginous plate extending the full length of the trachea and beyond the carina (Stone et al, 1990). The tracheal anomalies and basicranial growth dysplasia specifically implicate FGFR1 and FGFR2 signalling in human chondrogenesis.
The limb phenotype commonly consists of brachydactyly, primarily but non-exclusively affecting the thumb or great toe (Cohen, Jr., 1993; Anderson et al, 1998a). Broad thumbs are the obligate diagnostic clue, and preaxial deviation of the first digit, caused by a triangulated or trapezoidal first phalanx of thumb or toe is common. A "longitudinally bracketed epiphysis" or delta—phalanx may occur. Subclinical tarsal and metacarpal fusions and dysplasias of the feet are also common, particularly affecting the first ray, and may be progressive. The feet may range from normal to those resembling Apert syndrome with preaxial deviation of the first ray and multidigit syndactyly (Anderson et al, 1998a). Symmetrical ankyloses, radiographic, and clinical anomalies of the elbow (Anderson et al, 1998b) and knee have been reported, and bear some correlation with the global severity of the phenotype (Cohen, Jr., 1993; Anderson et al, 1997a).

Spinal dysplasia is a common finding, affecting the vertebral bodies and posterior articular elements. The severity of fusion, which ranges from minor fusions of the facet joint to block fusion of the vertebrae at several levels, also correlates with global severity of the phenotype. The most severe spinal phenotype has been correlated with the clover-leaf skull (Moore et al, 1995a). The upper cervical spine, at C2-C3 level, is most commonly involved (Moore et al, 1995a; Anderson et al, 1996a), a finding in common with Crouzon syndrome (Anderson et al, 1997b) which displays allelic homogeneity with the Pfeiffer phenotype via the FGFR2 gene (S1.3.2.1). Radiological abnormalities in a series of 22 Pfeiffer patients included hypoplasia of the neural arches, hemivertebrae, and a "butterfly" vertebra, as well as vertebral fusion (Anderson et al, 1996a). Evidence of vertebral fusion was present in 16 (73%) of cases, and most commonly involved the vertebral bodies and posterior elements, a finding common to Apert syndrome. Analysis of sequential radiographs in 11 patients revealed evidence of progressive severity in eight patients, again in common with the Apert phenotype (Kreiborg et al, 1992; Thompson et al, 1996).

Developmental intelligence in Pfeiffer children is usually within normal limits, though some cases are mildly intellectually impaired. Poor intellectual performance co-segregates with the clover-leaf variant (Cohen, Jr., 1993), though a favourable prognosis in some of these cases is the author's experience and has been reported in response to aggressive multidisciplinary clinical management, (Moore et al, 1995b; Robin et al, 1998). Delayed and impaired intellectual development also accompanies neurodevelopmental anomalies which occur in Pfeiffer syndrome, including, hydrocephalus, ventriculomegaly, tonsillar herniation, and intracranial venous sinus anomaly (Cohen, Jr., 1993; Taylor et al, 2000). Untreated raised intracranial pressure may result in visual impairment by retinal damage, and intellectual compromise.
Visceral anomaly in Pfeiffer syndrome is sporadic, and includes anal stenosis and cryptorchidism (Cohen, Jr., 1993). FGFR1 and FGFR2 isoforms are expressed in various epithelia, and the inclusion in the phenotype of developmental anomalies in systems characterised by epithelial fusion and dissolution is therefore not unexpected (S-1.2.2).

1.3.1.3 Pfeiffer syndrome encompasses a variable phenotype

Various reports attest to the clinical variability of the Pfeiffer phenotype, which may range within families from a mild expression in the hallux and partial syndactyly in several generations to the full craniofacial and limb presentation (Baraitser et al, 1980). The range of intra-familial variability includes the presentation of clover-leaf skull in the affected child of a mildly affected mother (Soekarman et al, 1992). In a 1993 review, Cohen analysed 7 previously published Pfeiffer syndrome pedigrees (three 3-generation and four 2-generation) and a number of sporadic cases. The variability of the clinical presentation of both the limb and craniofacial phenotypes in each generation of each family describes an autosomal dominant syndrome with complete penetrance but very variable expressivity, particularly with reference to the limb phenotype (Cohen, Jr., 1993). The presence of digital syndactyly, in particular, was highly variable, but was reported to most commonly involve the 2/3 web, a finding which has been latterly disputed in a series of 22 Pfeiffer syndrome patients, in which the digital syndactyly is wide ranging (Anderson et al, 1997c).
Three sub – groups of Pfeiffer syndrome, Types 1 – 3, have been described by Cohen (Cohen, Jr., 1993), and tabulated by Cohen and Barone (Cohen, Jr. and Barone, 1994). These classifications are combined and updated from other published reports as below:

<table>
<thead>
<tr>
<th>Major characteristics</th>
<th>Associated anomaly</th>
<th>Functional outcome</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type 1</strong></td>
<td>Low frequency anomalies of viscera, cervical spine fusions</td>
<td>Survive to adulthood</td>
<td>Sporadic; Autosomal dominant</td>
</tr>
<tr>
<td>Turribrachycephaly, midface retrusion, broad thumbs and great toe, brachydactyly, variable syndactyly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type 2</strong></td>
<td>More severe limb and spinal phenotypes, elbow synostoses. Intellectual compromise Intracranial and neurodevelopmental anomaly</td>
<td>Poor prognosis</td>
<td>Usually sporadic</td>
</tr>
<tr>
<td>Cloverleaf skull, ocular proptosis, and severe midface retrusion Digital anomaly as T1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Type 3</strong></td>
<td>Intellectual compromise Intracranial and neurodevelopmental anomaly, Elbow and knee synostoses Visceral anomaly</td>
<td>Poor prognosis</td>
<td>Usually sporadic</td>
</tr>
<tr>
<td>Severe midface retrusion without clover - leaf skull, ocular proptosis, CIII malocclusion. Distal limb features as T1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

An example of the clover – leaf cranial deformity is shown in Figure 1.3 - 2.

Some authors have suggested that the milder forms of Pfeiffer phenotype can be correlated with the FGFR1 mutation, whereas phenotypic severity correlates with mutations in FGFR2 (Rutland et al, 1995;Schell et al, 1995;Muenke and Schell, 1995). These observations have correlated a less severe thumb phenotype and lower apparent incidence of clover - leaf skull with the FGFR1 mutation. The available data is from small numbers of patients, however, and has yet to be subjected to a stringent analysis of age – matched cases. Furthermore, clover – leaf skull accompanies a broad range of phenotypes, encompassing many mutations within FGFR2 and FGFR3 (S-3). The variability of the Pfeiffer phenotype, in conjunction with the similar phenotypic range of the Jackson – Weiss and Crouzon syndromes, describes a continuous distribution of craniofacial, skeletal, and soft tissue anomalies. The molecular pathogenesis of this range of related phenotypes is considered further below (S-3, S-4).
1.3.2 Phenotypes associated with FGFR2 mutations

1.3.2.1 Introduction

Mutations within FGFR2 account for the majority of the craniosynostosis phenotypes (Passos-Bueno et al, 1998a). These phenotypes all have craniofacial anomalies, and the majority of these include coronal synostosis, with a variable forehead, ocular, and midface presentation. This may vary from minimal dysplasia in forms of Saethre - Chotzen and Jackson – Weiss syndromes to severe midface retrusion, with ocular proptosis and airway compromise; hypertelorism, pterional indrawing, and palatal clefting in Apert syndrome. There is also great variation in the extracranial phenotype displayed by the range of FGFR2 – human mutants. For example, the Apert syndrome is characterised by severe forms of complex complete syndactyly with anomalies of the proximal joints. By contrast, the Pfeiffer, Jackson – Weiss, Crouzon, and Saethre – Chotzen syndromes display progressively clinically less morbid limb phenotypes, ranging from elbow anomalies and first ray digital brachydactyly with preaxial deviation in Pfeiffer syndrome, to subclinical radiographic anomaly in Crouzon and Saethre – Chotzen syndromes. The spectrum of human FGFR2 – mutants can be considered in three broad clinical groups; the Apert syndrome, the ‘Crouzon - Pfeiffer’ group, and the syndrome of Beare – Stevenson cutis gyrata.

The Apert syndrome phenotype has a remarkably consistent set of clinical features, which bear only limited overlap in a minority of cases with related FGFR2 mutants. The phenotype, which is caused in ~98% of cases by one of two point mutations in a linker – region dipeptide of FGFR2, is described in detail below (S-1.3.2.2).

The ‘Crouzon – Pfeiffer’ group are particularly interesting. The original eponymous labels are attributed to reports in 1912 (Crouzon) and 1964 (Pfeiffer) detailing the craniofacial and limb anomalies which consistently co – segregate. A re – examination of the phenotypes by several authors and correlation with their FGFR2 genotypes indicates that the phenotypic spectrum is very wide and overlapping. This group of syndromes includes that of Jackson – Weiss, and variants of the Saethre – Chotzen and Antley – Bixler syndromes. A further single - kindred of FGFR2 craniofacial mutants is ‘unclassifiable’ according to eponymous criteria, despite sharing the G1044A transition at codon 344 of FGFR2 (Steinberger et al, 1996) with previously reported cases of Crouzon syndrome (Reardon et al, 1994;Jabs et al, 1994). Numerous examples of multiple phenotypes arising from the same mutations in
FGFR2 exist. The Cys342Tyr substitution causes Crouzon (Reardon et al, 1994) and Pfeiffer (Rutland et al, 1995) syndromes. Cys342Arg has similarly been associated with Crouzon (Reardon et al, 1994), Pfeiffer (Rutland et al, 1995), and Jackson – Weiss syndromes (Park et al, 1995b). These examples demonstrate that Cys342 as a ‘hotspot’ for mutations affecting the amino acid sequence, and that there is a divergence of phenotype generated by certain specific mutations. An explanation for this phenomenon has yet to emerge, but indicates potentially important roles for modifier – sequences, variable ligand – receptor activation, co – activation of downstream pathways by parallel receptor systems, and the modification of mutant receptor signalling by matrix co – factors. Whilst there is convergence of different phenotypes upon specific mutations within FGFR2, great variability amongst the ‘Crouzon – Pfeiffer’ group of syndromes also correlates with the wide range of their causative mutations, which predominantly affect the IgIII – loop of extracellular domain. Genotype - phenotype convergence and variance, and the possible mechanisms for this, are discussed further in subsequent sections of this thesis.

The Beare – Stevenson cutis gyrata syndrome is a distinct entity of craniofacial dysostosis, including craniosynostosis, with the cutaneous anomalies of acanthosis nigricans and cutis gyrata. It is caused by a subset of transmembrane and carboxy – terminal IgIII – loop mutations in FGFR2, and is described below.

In considering the range of phenotypes generated by the range of mutations in the FGFR2 gene the classic ‘one gene – one disease’ model is not tenable in this series of skeletal dysplasias (Mulvihill, 1995). Instead the FGFR2 – mutant phenotypes present a series of developmental anomalies which co – segregate with variable frequency, within which certain high frequency combinations are eponymously labelled. The causative mutations also lie within a range, which demonstrates high frequency sites or ‘hotspots’. With the exception of Apert syndrome, these ‘hotspots’ generate phenotypic diversity. However, despite various crossovers, classes of FGFR2 mutation may be associated with trends in the syndromic phenotypes to ascertain how specific amino – acid deletions, substitutions, insertions and aberrant splicing events in different domains of the receptor protein can affect the role of FGFR signalling in human skeletal development. These correlations, in conjunction with independent in – vitro studies, and the in - vivo studies presented in this thesis, begin to unravel the pathways between gene mutation and clinical pathology.
1.3.2.2 Apert Syndrome

Apert syndrome is characterised by turribrachycephaly, midfacial retrusion with a variable degree of hypertelorism, and symmetrical complex complete syndactylies. The first description of Apert syndrome is eponymously attributed to 1906 (Apert, 1906), though previous post-mortem cases had been described (Wheaton, 1894). Apert reported a fifteen-month old girl presenting in 1896 at the L'Hopital des Enfants-Malades in Paris with a high brachycephalic head, severe syndactyly of all four limbs and a cleft palate. Ten years later, he had collected eight more cases from the literature and coined the term "acrocephalosyndactyly", to describe the appearance of a flattened occiput, tall forehead and the syndactyly deformity. A case series was reported in 1920 (Park and Powers, 1920), and in 1960 a collection of 54 United Kingdom cases were reported and sub-classified (Blank, 1960). Blank distinguished two clinical categories: (1) 'typical' acrocephalosyndactyly, the group to which Apert's name now commonly applied; and (2) other forms, comprising phenotypes now otherwise attributed, grouped as 'atypical' acrocephalosyndactyly. The 'true Apert' was distinguished by the mid-digital hand mass, or 'spatulate hand', with a single nail common to digits 2-4, found in Apert syndrome and lacking in the non-Apert group (see below). Thirty-nine of the 54 in Blank's series were of the 'true Apert' type.

Apert syndrome is the second commonest craniofacial syndrome, after Crouzon syndrome, accounting for 4.5% of all craniosynostosis cases (Cohen et al, 1992). Estimates of the frequency of Apert syndrome vary. Pooled data from 7 geographic areas (across North America, Denmark, Spain and Italy) gives a birth prevalence of 15.5 per 1,000,000 births (Cohen et al, 1992). Racial variation in incidence has been reported in a Californian series (Tolarova et al, 1997). Birth prevalence was calculated at 12.4 cases per million live births (confidence interval [CI] 8.6,17.9), and the calculated mutation rate was 6.2x10^-6 per gene per generation (compared to 7.8x10^-6 per gene per generation as reported by Cohen and Kreiborg; 1992). Asians had the highest prevalence (22.3 per million live births; CI 7.1,61.3) and Hispanics the lowest (7.6 per million, CI 3.3-16.4). Most cases of Apert syndrome are sporadic, though reports of vertical transmission suggest an autosomal dominant inheritance (Blank, 1960; Lewanda et al, 1993) consistent with the subsequent demonstration of causal activating mutations of FGFR2. Apert syndrome has a particularly restricted genotype amongst the FGFR2-related craniofacial dysostosis syndromes, in that ~99% cases result from two closely related mutations in the extracellular domain of the receptor (Wilkie et al, 1995a; Oldridge et al, 1999). These cytosine to guanine transversions (C934G and C937G in exon IIIa/U/7) predict the neighbouring amino acid mis-sense substitutions Ser252Trp and
Pro253Arg respectively in the ‘linker — region’ sequence between the IgII and IgIII loops which form the putative ligand binding region (§7.4).

In the first series report of 40 unrelated patients, Wilkie (1995) found that the Ser252Trp and Pro253Arg substitutions occur in 63% and 37% of patients, respectively. In a similar independent report of 36 Apert patients in the same year, S252W and P253R were given frequencies of 71% and 26%, respectively (Park et al, 1995c). Similar frequencies have subsequently been reported by a number of authors (Moloney et al, 1996; Passos-Bueno et al, 1998a; Lajeunie et al, 1999; von Gernet et al, 2000). Moloney et al (1996) in studying 118 patients with new mutations, in combination with an independent series of 48 patients, estimated germline mutation rates for C934G (Ser252Trp) and C937G (Pro253Arg) to be $5 \times 10^{-6}$ and $2.7 \times 10^{-6}$ respectively, which are the highest transversion rates in the human genome. Previous observations of a paternal age effect in the genesis of Apert FGFR2 mutations were confirmed in 55 sporadic cases. The authors conclude that within 95% confidence limits, ‘most or all cases of Apert syndrome arise by paternal mutation’, and that the mutation arises in spermatogenesis (Moloney et al, 1996).

The narrow genotype, and relatively restricted craniofacial and limb phenotype amongst Apert patients suggests that the tryptophan and arginine substitutions in the linker region have particularly specific effects upon receptor signalling in Apert skeletogenesis. The Apert phenotype has, however, been linked to other FGFR2 mutations in rare cases. Three further types of mutation of the FGFR2 — linker have been described, although not all are exclusive to Apert syndrome. A C—T mutation that predicts a Ser252Leu substitution correlates with both mild Crouzon syndrome (craniosynostosis with normal limbs) and a normal phenotype in members of the same family. The Ser252Phe substitution, resulting from a double nucleotide substitution (CG to TT) of residues 934 and 935, generates the Apert phenotype, (Oldridge et al, 1997)(Lajeunie et al, 1999). However, a complex CGC-->TCT mutation, predicting a double amino acid substitution of both Ser252Phe combined with Pro253Ser causes a Pfeiffer syndrome variant with mild craniosynostosis, broad thumbs and big toes, fixed extension of several digits, and only minimal cutaneous syndactyly (Oldridge et al, 1997). It therefore appears, that whilst the substitution of wild — type serine for phenylalanine at position 252 will encode Apert syndrome, the relationship is exquisitely sensitive. The effects of the Ser252Phe mutation are modified (with a decreased severity of the limb phenotype) by the substitution of proline by serine at neighbouring position 253. Furthermore the Ser252Leu mutation at the same site generates either a normal appearance or mild craniofacial dysostosis; and in addition Ser252Trp, whilst most frequently correlated with Apert syndrome, has been noted to generate a Pfeiffer — like presentation (Passos-Bueno et al,
The relationship of these mutations to receptor function and phenotypic outcome are further considered below (5.1.4).

Given this data, it might be supposed that the Apert phenotype would be restricted to specific amino acid substitutions in the FGFR2 – linker. However, in rare cases, the Apert phenotype does not appear to be exclusive to mutations in exon 7/IIIa of FGFR2. Atypical Apert syndrome has been linked to a ‘Pfeiffer mutation’ (Schell et al, 1995; Lajeunie et al, 1995a); a nucleotide substitution, 1119 – 2A—G, at the 3’ splice site upstream of exon 9/IIIc (Passos-Bueno et al, 1997). The patient displayed a craniosynostosis phenotype with complete digital syndactyly of the feet and 3-4 syndactyly of the hands. Furthermore, in a study of 260 Apert patients, whilst 258 had causative mutations in the established dipeptide of the linker region, the remaining 2 patients had Alu-element insertions in or near exon 9, which was correlated in one patient to an upregulation of the Igllb isoform of FGFR2 (Oldride et al, 1999). Apert syndrome thus demonstrates a complex and subtle relationship with the range of causative mutations. The subsequent sections of this thesis review the Apert phenotype and the current information available from which to draw genotype – phenotype correlations.

The Craniofacial Phenotype

Craniofacial features include a wide turri - brachycephaly, which tapers down from the parietal to the supraorbital region (Fig 1.3-3). There is a steeply inclined, flattened forehead, and shortened, shallow posterior fossa with short, asymmetric cranial base. The anterior cranial fossa and tuberculum sellae, pituitary fossa, dorsum sellae and clivus are all shortened and widened. There is reduced orbital volume and significant proptosis, with hypertelorism, and an expanded, dysplastic ethmoidal labyrinth. The lesser wings of the sphenoid bone, forming the posterior wall of the orbit and upper border of the superior orbital fissure are angled obliquely up and laterally producing a characteristic radiographic sign. The summary effect is that of the skull base being foreshortened and angled, such that there is an increasing lateral angulation of the cranial fossae above.

The cranial sutures are affected in a broadly consistent fashion. There is a wide - open calvarial defect from the root of the nose to the posterior fontanelle in midsagittal plane, encompassing the territory of the metopic and sagittal sutures and the anterior fontanelle. Progression to bony fusion in this mid – sagittal plane is by the formation of islands of bone in the presumptive mesenchymal connective tissue, which coalesce to bony fusion by 36 months without the sutures ever having been truly formed (Kreiborg and Cohen, Jr., 1990; Kreiborg and Cohen, Jr., 1991; Cohen and Kreiborg, 1994). True frontal encephalocele probably does not occur but reflects protrusion of the frontal fossa anteriorly via the calvarial
defect. The intracranial volume is consistently greater than controls (Cohen and Kreiborg, 1994; Gosain et al, 1995) and the brain is large within the skull with an expanded head height, although the head circumference normalises at birth and slows thereafter (Cohen and Kreiborg, 1994). In contrast, true fusion of the coronal suture in most cases begins in-utero, commencing at the cranial base and extending cephalad (Kreiborg and Cohen, Jr., 1990). Clover-leaf skull has been reported in association with Apert syndrome (Gosain et al, 1997), but occurs in a minority (4%) of cases (Cohen, Jr. and Kreiborg, 1994).

Reports of open coronal sutures in Apert syndrome may reflect variance in age of onset (Cohen, Jr. and Kreiborg, 1994; Lajeunie et al, 1999), but are consistent with reports of persistently open synchondroses at the Apert skull base (Kreiborg et al, 1993). The variability in pathology of both the skull base chondrocranium and membranous calvarium suggests that there are heterogeneous effects of Apert FGFR mutations upon human modes of ossification, and this is explored further in subsequent sections of this thesis.

Apert syndrome patients demonstrate consistent and typical facial features. There is severe midface retrusion and secondary ocular proptosis with lagophthalmos. Ocular subluxation of the globe onto the cheek may occur, in conjunction with the retruded midface and marked protrusion of the greater wing of the sphenoid, with elevation of the lesser wing (Kreiborg et al, 1999). Maxillary and nasal height is reduced (Kreiborg et al, 1999). The nasal root is depressed, such that the nasal bridge is humped and beak-like. Deviated nasal septum is common. Hypertelorism, and down slanting palpebral fissures (the anti-mongoloid cant), are common features (Cohen and Kreiborg, 1996). A deeply wrinkled forehead skin is common. The retruded midfacial phenotype gives rise to a skeletal and dental class III malocclusion and relative pseudo-mandibular prognathia, though the mandible itself remains within control parameters (Kreiborg et al, 1999). The upper dental arch is crowded and V-shaped, with an anterior open bite and posterior cross bite. Dental anomalies include severely delayed eruption, ectopic eruption, and shovel-shaped incisors. Malocclusion is severe with a mandibular overjet, anterior and posterior crossbites, and severe crowding of teeth (Kreiborg and Cohen, 1992).

The Apert craniofacial morphology (n=12) has been compared to that of Crouzon syndrome (n=19) in an age-banded series of cases studied with serial 3D-CT scans. Whilst the operative history of some cases make the interpretation of the upper calvaria data problematic, the study currently provides the best longitudinal analysis of the comparative cranial and skull base morphology of the two syndromic groups (Kreiborg et al, 1993). The comparative morphology is tabulated below:
Cranial and skull base features of Apert and Crouzon syndromes
(Kreiborg et al, 1993):

<table>
<thead>
<tr>
<th></th>
<th>Apert</th>
<th>Crouzon</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Calvaria</strong></td>
<td>Coronal synostosis</td>
<td>Synostosis of coronal, sagittal, metopic, and squamosal sutures.</td>
</tr>
<tr>
<td><strong>Aged 0-1 year</strong></td>
<td>Median sagittal diastema, including median plane fontanelles, with no midline sutures formed.</td>
<td>Fontanelles close early.</td>
</tr>
<tr>
<td></td>
<td>Open squamosal and lambdoid sutures.</td>
<td>Lambdoid suture may remain unfused in early infancy</td>
</tr>
<tr>
<td></td>
<td>Thin calvarial bone</td>
<td>Thin calvarial bone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>No median plane diastema</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increased digital markings</td>
</tr>
<tr>
<td><strong>Calvaria</strong></td>
<td>Delayed closure of fontanelles and median diastema by coalescing islands of bone formation. No median plane sutures form. Digital markings appear late</td>
<td>Pan – synostosis and progressive closure of fontanelles</td>
</tr>
<tr>
<td><strong>Aged 1-4 years</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Skull Base</strong></td>
<td>Open synchondroses in early infancy(^1), which fuse late (&gt;1yr), with delayed closure of the sphenop–occipital synchondrosis. Enlarged sella turcica. Widened ethmoid from birth with a depressed cribriform plate. V–shaped anterior cranial fossa.</td>
<td>Synchondroses close consistently early (&lt;1yr) in progressive manner. Enlarged sella turcica with narrow floor. Ethmoid not widened, and cribriform plate undisplaced. Thin clivus and tendency for basilar kyphosis.</td>
</tr>
</tbody>
</table>

\(^1\) also reported by Holten and Kreiborg (Holten, 1994)(Kreiborg et al, 1976).

Of note is the tendency for the sutures and synchondroses in the Crouzon phenotype to fuse early, compared to their delayed closure in Apert syndrome. The Apert phenotype displays
the pathognomonic unossified median diastema, which is not seen in Crouzon syndrome; and also develops a widened ethmoid with V-shaped anterior fossa. This is clinically manifest as hypertelorism, which is also not a major feature of the Crouzon phenotype. The Crouzon cases, in company with early closure of sutures and synchondroses, manifest early radiological signs of raised intracranial pressure such as a widened sella turcica and digital markings (S-4). Pan-sutural closure, correlated with raised intracranial pressure and herniation of the cerebellar tonsils through the foramen magnum, has been associated with the Crouzon phenotype to greater extent than the Apert phenotype in a corroborative independent study (Cinalli et al, 1995). The genotype – phenotype correlations which may account for these differences are discussed in Sections 3 and 4 of this thesis.

Ophthalmic manifestations include reported absence of the superior rectus muscle (Cuttone et al, 1979; Morax and Pascal, 1982) and intrinsic extraocular muscle dysplasia (Margolis et al, 1977a). Clinical sequelae of this include a consistent divergent upgaze and esotropic downgaze (Cohen and Kreiborg, 1996). Ocular albinoid findings (Margolis et al, 1977b), congenital glaucoma, keratoconus and ectopic lens are rare observations. Secondary ophthalmic concerns include corneal exposure keratitis and ocular subluxation. Visual loss and ocular atrophy are serious concerns and reflect untreated, sustained rises in intracranial pressure (Thompson et al, 1995a; Taylor et al, 2000).

Abnormalities of palatal morphology are common. Cleft palate is a consistent finding, and in the remaining population the palate is highly arched, with a deep median groove or 'pseudocleft' created by swelling in the upper arch mucosa (Peterson and Pruzansky, 1974). Gorlin has postulated that the high arched and laterally swollen palate results from compression of the upper dental arch during Apert craniofacial morphogenesis, and is secondary to the midfacial retrusion and hypertelorism (Gorlin et al, 1990). However, similar palatal morphology in Crouzon syndrome without the incidence of cleft palate (Peterson and Pruzansky, 1974) suggests that the Apert mutations modulate a particularly sensitive role for FGFR2 in human palatogenesis (S-3.4). Furthermore, whilst the palates of both Apert and Crouzon patients are thicker and shorter than documented norms, no correlation between such parameters and palatal clefting can be demonstrated (Peterson and Pruzansky, 1974), thereby suggesting a molecular, rather than morphological, aetiology for Apert cleft palate.

Where it occurs, palatal clefting affects the secondary palate in variable severity from complete clefts of the hard palate, to soft palatal clefts or simply bifid uvula. Reported frequencies of palatal clefting vary from 17% (N=54) (Holten, 1994) to 43% (Peterson and Pruzansky, 1974). The accuracy of reporting reflects the inclusion of milder phenotypes such
as bifid uvula and occult cleft palate, where reported frequencies rise to 75% (Kreiborg and Cohen, 1992). The non-cleft hard palate is short, reflecting the reduced anteroposterior dimensions of the skull base. Nasopharyngeal height and depth are reduced (Peterson-Falzone et al, 1981). There is no association with cleft lip, the Apert upper lip being characteristically intact in an open mouth, hood-like position at rest (Cohen and Kreiborg, 1996), reflecting an anterior open bite beneath. The relationship of cleft palate to Eustachian tube malfunction may reflect the 71% incidence of middle ear effusions in Apert children (Park et al, 1995c). Conductive hearing loss occurs in 40% of Apert children (Park et al, 1995c) and may reflect middle ear ossicular dysplasias revealed on computerised tomography of the middle temporal fossa (see also Pfeiffer syndrome, S1.3.1.2).

Nasopharyngeal anomalies (Peterson-Falzone et al, 1981) reflect the expected reduction in nasopharyngeal volume predicted by the cranioskeletal dysplasia. In a recent study of 13 Apert and 27 Crouzon patients, 40% of patients with severe phenotypes demonstrated upper airway obstruction, caused by midface hypoplasia, tonsillar and adenoid hypertrophy, and choanal stenosis (Lo and Chen, 1999). Choanal atresia/stenosis, which presents a surgical emergency, features in 23% of Apert cases (Park et al, 1995c).

The extracranial skeletal phenotype

Following a German-language study of the Apert foot in 80 patients in 1978 (Blauth and von Torne, 1978), the most comprehensive contemporary analyses of the limb phenotype are those of Upton (1991) and Cohen and Kreiborg (1995). The common features of the Apert hand are a short, radially deviated thumb; a complex, complete syndactyly and symbrachyphalangism which affects the 2-3-4 rays to form a mid-digital hand mass with single nail (synonychia); and a fifth ray simple syndactyly (Fig 1.3-4). More severe forms completely incorporate the little finger (though only via soft tissue syndactyly) and thumb rays. The bony anomalies include a delta phalanx (longitudinally bracketed epiphysis) in the thumb ray; radial clinodactyly of the thumb, symphalangism (fused proximal and middle phalanges); 4/5 metaphyseal fusions of the metacarpals; and epiphyseal anomalies. Carpal fusions are common. The soft tissue anomalies include abnormal skin furrows and pilosebaceous activity, syndactyly, ectopic chondrification, and synonychia (Upton, 1991; Cohen, Jr. and Kreiborg, 1995a; Holten et al, 1997). Anomalous findings such as reduplications have also been reported (Anderson et al, 1996b). Multiple anomalies of neurovascular and tendon anatomy are common (Upton, 1991). More proximally, elbow fusions and elbow and shoulder joint anomalies have been commonly associated with the Apert limb phenotype (Upton, 1991; Cohen, Jr. and Kreiborg, 1993a; Anderson et al, 1998b). The
classification of Upton remains the most common reference for the analysis of the Apert hand malformations and is tabulated below (see also Fig 1.3-4):

**Upton's classification of the Apert Hand** (Upton, 1991)

<table>
<thead>
<tr>
<th>Deformity</th>
<th>Type 1 (n=28)</th>
<th>Type 2 (n=24) ‘Mitten hand’</th>
<th>Type 3 (n=16) ‘Hoof hand’</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thumb radial clinodactyly</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Index radial clinodactyly</td>
<td>Absent</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>First web syndactyly</td>
<td>Simple (non - osseous)</td>
<td>Simple (non - osseous)</td>
<td>Complex (osseous)</td>
</tr>
<tr>
<td>Complex 2-3-4 syndactyly &amp; symbrachy - phalangism</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>4-5 syndactyly</td>
<td>Simple, incomplete</td>
<td>Simple, incomplete</td>
<td>Simple, complete</td>
</tr>
</tbody>
</table>

The Type 1 hand, the most common form (Upton, 1991; Cohen, Jr. and Kreiborg, 1995a), has a thumb separated from the mid – digital hand mass by a shallow web. The mid – digital hand mass, a 2-3-4 syndactyly, displays bone fusions of the distal phalanx at the metaphyseal and interphalangeal joint levels. There may be proximal osseous or cartilagenous fusion of the second and third rays. The fifth ray is joined by a variable soft tissue syndactyly only, and the hand position can present a flat palm.

The Type 2 hand has the thumb joined to the mid – digital hand mass by a simple soft tissue syndactyly, but the nail is separate and the distal phalangeal segment is radially deviated. The mid – digital hand mass features a severe fusion of the 2-3-4 distal phalanges; such that the proximal phalanges and metacarpals are splayed apart to create a ‘mitten’ or ‘spoon’ appearance with central concavity. The fifth ray is joined by a complete soft tissue syndactyly.
The Type 3 or ‘hoof hand’ is the most severe and least common variant (Upton, 1991; Cohen, Jr. and Kreiborg, 1995a). The 1-4 rays are joined by osseous or cartilagenous fusion, with a complex synonychia, such that the thumb and mid – digital hand mass are indistinguishable. The palm is deeply concave. The fifth ray is joined by a simple, complete syndactyly. Carpal and metacarpal coalitions are common.

The bony fusions of the Apert distal limb are progressive, beginning as cartilage ankyloses and ossifying with increasing maturity. Schauerte and St-Aubin (1966) pointed out that progressive synostosis occurs in the feet, hands, carpus, tarsus, and cervical vertebrae (Schauerte and St Aubin, 1966); and the progressive nature has been documented by several authors (Upton, 1991; Cohen, Jr. and Kreiborg, 1995a; Anderson et al, 1998b). Cohen and Kreiborg (1995) studied 44 pairs of hands and 37 pairs of feet in Apert syndrome, using clinical and radiographic methods. In common with Upton (1991) they noted a more severe phenotype in the upper than lower limb. Upton was unable to correlate upper and lower limb severity, but parallels were drawn by Cohen and Kreiborg (1995) and the severity of upper and lower limb phenotypes has been significantly positively correlated more recently (Slaney et al, 1996).

The foot deformity is also characteristic. Progressive synostosis occurs upon an unsegmented cartilaginous template. The first ray shortens with medial deviation of the great toe, secondary to growth abnormality and a delta phalanx (Mah et al, 1991). The two phalanx digits characteristically go on to fusion, with maintenance of minimal motion at the metatarso - phalangeal joints. There is a 2-3-4 mid - digital mass analogous to that of the hand, with more severe forms incorporating progressively four, or all five toes in the syndactyly (Cohen, Jr. and Kreiborg, 1995a; Slaney et al, 1996). Progressive tarsal and metatarsal fusions occur and the midfoot and hindfoot fuse in a supinated position. There is prominence of the fifth metatarsal with callosities under the fifth and third metatarsal heads in all patients.

Kreiborg et al. (1992) found fusion of cervical vertebrae in 68% of patients with Apert syndrome: single fusions in 37% and multiple fusions in 31%. The C5-C6 level was most commonly involved. Similar figures have been observed independently (n=59 patients) and progressive fusion was noted in 10/17 patients in whom longitudinal radiographs were available (Thompson et al, 1996). Complete bony fusion across the intervertebral disc space has been noted, and fusions most commonly involve the vertebral bodies and posterior elements in an apparent antero – posterior vector. The spinal canal is not compromised radiologically or clinically (Thompson et al, 1996). In contrast, cervical fusion occurs in 18 - 25% of patients with the Crouzon phenotype, which commonly involves C2-C3 (Kreiborg et
al, 1992; Anderson et al, 1997b), the level also most commonly fused in the Pfeiffer phenotype (Anderson et al, 1996a). Kreiborg et al. (1992) concluded that when fusions are present, C5-C6 involvement in the Apert syndrome and C2-C3 involvement in Crouzon syndrome separate the 2 conditions in most cases.

**Intellectual Performance & the CNS phenotype**

Apert patients generally have a decreased intellectual capability, though individuals with normal intelligence have been reported (Park and Powers, 1920). In a review of 29 patients (aged 8-35 years) over a time period from 1952 to 1980, 14 (48%) had a normal or borderline IQ, of which group 6 of 7 school leavers were in vocational training or full time employment (Patton et al, 1988). Nine patients had mild learning difficulties (IQ, 50-70), 4 were moderately compromised (IQ, 35-49), and 2 (7%) were severely intellectually compromised (IQ less than 35). None had an IQ of greater than 100. Early cranietomy, within the first year of life, did not appear to have improved intellectual outcome in this patient group, a conclusion supported by Cohen and Kreiborg (Cohen, Jr. and Kreiborg, 1990), who document a range of CNS anomalies, with malformations of the corpus callosum, the limbus, the hippocampus, the gyri, and pyramidal tracts. An absent or defective septum pellucidum was also noted. The authors observed that intracranial hypertension in the Apert group, given the tendency to increased intracranial volume (Kreiborg and Cohen, Jr., 1990; Cohen, Jr. and Kreiborg, 1994), would be unlikely to be a major contributor to the intellectual deficit. Early surgery for cranial vault decompression would therefore be unlikely to have a therapeutic effect upon poor intellectual capacity in the Apert group, which would most likely reflect neuro-developmental abnormality. Evidence supporting a primarily neuro-developmental cause for poor Apert intellectual performance is given by a series of 20 Apert children, assessed on psychometric scales, who displayed verbal skills that were consistently lower than motor skills, despite the upper limb anomalies in the phenotype. Mean IQ was 73.6 (range 52 - 89), all children having undergone early cranial vault surgery (Lefebvre et al, 1986). Lower verbal than motor scores were also noted by Holten in seven Apert infants (Holten, 1994).

Persistent raised intracranial pressure (RICP) is less common in Apert patients than the 'Crouzon – Pfeiffer' group (Cinalli et al, 1995; Thompson et al, 1995a). This may represent differences in cranial vault volume, but such volume/pressure relationships have been shown to be too simplistic (Gault et al, 1992). The Crouzon – Pfeiffer group of patients demonstrate higher profiles of RICP, in concert with earlier patterns of synchondrosal and sutural fusion, than the Apert group (S1.3.2.2). This suggests that the Apert genotype, whilst related to a higher incidence of neuro-developmental anomaly, segregates with a lower RICP profile that
is independent of intracranial volume. Anomalies of intracranial dural venous sinus diameter have been implicated in the restriction of drainage of intracranial blood, thus causing intracranial hypertension (Cinalli et al, 1995; Martinez-Perez et al, 1996). A recent series demonstrates that progressive anomalous venous drainage may characterise the FGFR – associated craniosynostosis syndromes, and contribute to RICP in this patient group (Taylor et al, 2000). The finding that FGF1 and FGF2 promote the endothelialisation of PTFE grafts, (Gray et al, 1994) suggests that FGFR activating mutations may accelerate endothelial hyperplasia in the intracranial phenotype. The subsequent reduction of dural venous sinus diameter and restriction of flow would account for persistent RICP independent of intracranial volume. However, the lack of clinical evidence for hyper – endothelialisation elsewhere in the phenotype has yet to be explained (5–4).

Progressive hydrocephalus has been observed to be uncommon in the Apert group (Cohen, Jr. and Kreiborg, 1990), and in comparison to the Crouzon phenotype, in which RICP, pansynostosis, and herniation of the cerebellar tonsils through the foramen magnum co – segregate (Cinalli et al, 1995). Nevertheless, early craniectomy to improve intellectual outcome in Apert syndrome is advocated from a review of IQ in Apert 70 children. An IQ greater than 70 was documented in 50% of the children who had a skull decompression before 1 year of age versus only 7.1% in those operated on later in life (Renier et al, 1996). Malformations of the corpus callosum (found in 12% of cases in the series of Park et al, 1995) and ventricular size did not correlate with the final IQ. Anomalies of the septum pellucidum had a significant negative effect, however, with the proportion of patients with an IQ over 70 increasing more than twofold in patients with a normal septum compared with patients with septal anomalies (p < 0.04). In concert with findings from the general population, the third significant factor in intellectual achievement was the setting in which the children were brought up. Only 12.5% of institutionalized children had a normal IQ, compared to 39.3% of normal IQ cases living with their families.

Holten has reported CT data on 40 Apert patients of mean age 4.1 years and median age of twelve months (Holten, 1994). Ninety-eight percent of patients had some form of ventriculomegaly, predominantly of the lateral (93%) and third (68%) ventricles, with mild to moderate dilatation. No particular pattern of dysmorphology could be established with-in or between each ventricle. Despite ventriculomegaly being present in thirty-nine of the forty cases, only eight of these thirty-nine (20%) had CT evidence of raised intracranial pressure and craniostenosis, with eleven cases demonstrating wide subarachnoid spaces and basal cisterns. Holten reports thirteen anomalies of the septum pellucidum (32%) in the Australian series of 40 cases, but was unable to demonstrate a particular effect of this anomaly upon the
formal Wechsler IQ testing undertaken in only seven patients of the total group. Agenesis of the corpus callosum was noted in three cases. Other anomalies noted were: cerebellar hypoplasia, heterotopic grey matter, abnormal gyral patterns and schizogyria. The severity of the CNS phenotype may be related to the severity of the skeletal phenotype, but no objective correlating data is given.

The summary data therefore suggests that developmental impairment, in the form of a verbal skill deficit greater than the motor skill deficit, is a more common association than not in Apert syndrome. Intellectual deficit appears to correlate with primary neuro-developmental malformation rather than raised intracranial pressure per se, and the incidence of which is in keeping with the role of FGF/FGFR signalling in neural differentiation (Chao, 1992)

**Visceral and cutaneous anomalies**

Reports of visceral anomalies in Apert syndrome indicate a range of sporadic malformations affecting many systems. Blank reported half of his autopsy group of 12 cases had non-identical visceral anomalies (Blank, 1960). Anal atresia in company with small bowel malrotation has also been reported (Park et al, 1995c). Cohen and Kreiborg, in a review of 136 patients (Cohen, Jr. and Kreiborg, 1993b) of which 12 were postmortem autopsies, concluded a high rate of minor anomalies. The cardiovascular and genitourinary systems were most commonly affected (10% and 9.6% respectively). A cardiovascular anomaly rate of 17% has been independently reported, and urogenital abnormalities (hydronephrosis, nephrocalcinosis, hydrocele, cryptorchidism) in 19% cases of the same series (Park et al, 1995c). In Holten's series of forty-eight Apert syndrome patients, nine (19%) had clinically detectable visceral anomalies (Holten, 1994). The genito-urinary (14.6%), cardiovascular (10.4%) and gastro-intestinal (10.4%) were the commonest systems affected. A respiratory system anomaly was diagnosed in one patient (2.1%), which may have contributed to early death. Autopsy showed tracheal cartilage dysplasia with calcification of the whole trachea and bronchi and tracheal stenosis. This patient also demonstrated ectopic cartilage formation and calcification in the kidneys. Tracheal ‘tube’ cartilage in eight post-mortem Apert cases has been reviewed (Cohen and Kreiborg, 1992a) and this highly morbid anomaly has been reported in both Pfeiffer (Stone et al, 1990) and Crouzon (Devine et al, 1984; Sagehashi, 1992) syndromes.

Cohen and Kreiborg (1995) commented on the cutaneous manifestations in a series of 136 cases of Apert syndrome (Cohen, Jr. and Kreiborg, 1995b). Hyperhidrosis is found in all patients, and a predilection for oily skin with cystic acne eruptions persists from adolescent to
maturity. Apert skin biopsy data shows expanded sebaceous gland tissue, and the acneiform lesions, which are very resistant to treatment (Henderson et al, 1995), are particularly prevalent on the face, chest, back, and upper arms. The skin of the hand and forearm shows hyperhydrosis and excess pilosebaceous activity with a distribution of forearm acne that is characteristic of the syndrome (Solomon et al, 1971; Upton, 1991; Cohen, Jr. and Kreiborg, 1995a). The localisation of 'acneiform eruptions' to the Apert phenotype, and their lack of correlation with the 'Crouzon – Pfeiffer' group of phenotypes suggests that Apert mutations within FGFR2 modulate specific functional effect. The two predominant Apert linker region mutations, S252W and P253R will exert mutant effects via both the IgIIIc/Bek and IgIIIb/KGFR receptor isoforms. The KGFR isoform is predominantly expressed in cells of epithelial lineage including the skin (Rubin et al, 1989; Miki et al, 1992; Finch et al, 1995), whereas Bek is predominantly mesenchymal (Orr-Urtreger et al, 1993; Oldridge et al, 1999). A relative minority of the mutations causing the 'Crouzon – Pfeiffer' group of syndromes are expressed through both the IgIIIc/Bek and IgIIIb/KGFR isoforms, which perhaps provides a functional correlation with the low incidence of cutaneous manifestations in these syndromes. However, the Beare Stevenson cutis gyrata syndrome, which invariably features the skin pathologies of cutis gyrata and acanthosis nigricans, is also caused by mutations affecting both the KGFR and Bek isoforms of FGFR2 (Przylepa et al, 1996). The variable pathology of the skin phenotype in those FGFR – mutants expressed through both KGFR and Bek suggests that there is a scale of severity of mutant gain-of function modulated through KGFR. A functional role for KGFR in skin physiology is suggested by the report that dominant-negative targeting of FGFR in skin causes epidermal hyper thickening and an aberrant expression of keratin 6 (Werner et al, 1993). Furthermore a patient with forearm acne, reminiscent of that seen in the Apert phenotype, has been demonstrated to express the Ser252Trp Apert mutation within lesional skin. Control skin from the same patient failed to demonstrate the Ser252Trp mutation, suggesting that activating somatic FGFR mutations, expressed in the skin, may demonstrate the cutaneous pathology normally seen as part of the congenital syndrome encoded by the germline mutation (Munro and Wilkie, 1998).

**Phenotypic variability and genotype correlations**

The Apert phenotype demonstrates a relatively narrow group of consistent features within the broad phenotype range demonstrated by the FGFR – associated syndromic craniofacial dysostoses. Nevertheless, the range of syndactylous phenotypes within the Apert limb (Upton, 1991); and the variable incidence of cleft palate (Kreiborg and Cohen, 1992), neurodevelopmental, vertebral and visceral anomaly suggests that phenotype correlations with the established narrow genotype range might be drawn. A systematic analysis in a group of 87
patients (S252W n=42; P253R n=24, undetermined genotype n=21) was reported in 1996 in a broadly age-matched population (Slaney et al, 1996). Severity of craniofacial phenotype (five features scored collectively as 0-5) was associated with the S252W mutation, although this did not reach significance. The correlation of cleft palate and the S252W mutation was statistically significant. In contrast, the severity of limb phenotype significantly correlated with the P253R mutation, thereby suggesting potentially differential effects for the two mutations upon craniofacial and limb morphogenesis. A highly specific role for FGFR2/FGF signalling in palatal shelf fusion is also implied, given that the S252W Apert mutation selectively perturbs palatogenesis in 75% cases (Kreiborg and Cohen, 1992), but the FGFR2 mutations of the 'Crouzon – Pfeiffer' group do not (despite broadly similar craniofacial morphology). Severity of syndactyly has since been independently positively correlated with the P253R mutation in a group of 21 Apert patients, in whom the corresponding lack of severity of craniofacial phenotype correlated with good surgical outcome by a single surgeon (von Gernet et al, 2000). These data contrast with that of Park et al. (1995) who were unable to demonstrate significant genotype – phenotype correlations using a single stringent statistical analysis in 36 Apert patients with a skewed, severe proportion of hand anomaly (by Upton's classification) that was not consistent with the major published series (Blauth and von Torne, 1978; Upton, 1991; Cohen, Jr. and Kreiborg, 1995a). Park (1995) were also unable to demonstrate a significant correlation of cleft palate with the S252W mutation, although combination of the two groups reported by Park et al. and Slaney et al. confirm the association (Slaney et al, 1996).

That the association of P253R with the severity of Apert syndactyly was not demonstrable by Park (1995), yet is demonstrated by Slaney (1996) and von Gernet (2000), may reflect differences in statistical methodology and severity of phenotype in each patient series. The conflicting data does, however, imply that the relationship may be a subtle one. Indeed the neighbouring S252W linker – region mutation, normally causal to Apert syndrome with the characteristic 2-3-4 mid-digital hand mass, has been found in a Pfeiffer – like patient without the severe Apert - limb phenotype (Passos-Bueno et al, 1998b). Furthermore, an analysis of 2 rare non-linker Apert mutations in and upstream of the exon 9 of FGFR2 suggests that the generation of stable splice variants of FGFR2, particularly the IgIIIb/KGFR isoform, may specifically correlate with the pathogenesis of syndactyly (Oldridge et al, 1999). If the preliminary data correlating KGFR upregulation with the syndactyly phenotype are substantiated, then it may be that P253R has a greater functional effect when acting via KGFR than does the S252W mutation. Correspondingly, S252W may exert a greater effect than P253R in skeletogenesis via the IgIIIc/bek isoform of the receptor.
The association of phenotypic severity with particular types of mutation may begin to provide a molecular explanation for case reports of intermediate ‘Apert – Crouzon’ patients. Many of these cases lack severe syndactyly, or combine syndactyly with mild craniofacial dysostosis (Vogt, 1933; Nager and de Reynier, 1948; Temtamy and McKusick, 1969) - quoted in (Slaney et al, 1996).

1.3.2.3 Syndromic Craniofacial Dysostosis & mutations of the FGFR2 extracellular domain.

This is a wide group of ‘syndromic craniosynostosis’ phenotypes. The craniofacial dysostosis ranges in severity from severe pan-synostosis and clover-leaf skull; with severe midfacial retrusion and exorbitism, to mild bicoronal synostosis and brachycephaly. The limb anomalies similarly occupy a broad range of severity from clinically innocuous radiographic changes to severe thumb and toe deformity requiring surgical intervention. Named eponymously for those clinicians who first documented their ‘peak frequency’ combinations of co-segregating phenotypic features; these syndromes, including Crouzon, Pfeiffer, Jackson – Weiss, and Saethre – Chotzen are now increasingly recognised to span a clinical continuum. The Antley – Bixler phenotype may be considered to be part of this group and this is considered further below.

This ‘Crouzon – Pfeiffer’ group of FGFR2 – mutations are most commonly missense, and located in exons IIIa/7 and IIIc/9 (Passos-Bueno et al, 1999). Those mutations within exon IIIa will affect both IgIIa/b and IgIIa/c protein isoforms of FGFR2, whereas those mutations within exon IIIc exert effect through the IgIIa/c expression domain alone. FGFR2 - mutations causing craniosynostosis phenotypes are less common in the IgI – loop of the extracellular domain, juxtamembranous or transmembrane region, however, these mutations also occur and may generate diagnostic confusion (Pulley et al, 1996). The heterogeneity of mutational base within this group of phenotypes is likely to increase still further, as 50 – 60% of Crouzon cases are not successfully genotyped (Park et al, 1995b; Passos-Bueno et al, 1998a), and may reflect mutations in different regions of the receptor, or in modifying genes.

Great debate has characterised the literature regarding these phenotypes in recent years as their associated molecular pathology has been elucidated. There is no doubt that the wide phenotypic cross-over has exercised the diagnostic skills of expert clinicians in attempting to apply eponymous labels to individual patients (Cohen, 1995). Some cases with craniofacial
phenotypes suggesting eponymous labels lack craniosynostosis (Reddy et al, 1990; Cohen et al, 1993; Park et al, 1995b), which may be a delayed and progressive feature (Puley et al, 1996). Many patients fall outside the expected 'phenotype clusters' by which the eponymous syndromes are recognised (Vogt, 1933; Nager and de Reynier, 1948; Temtamy and McKusick, 1969; Tsukahara et al, 1985; Maroteaux and Fonfria, 1987; Cantrell et al, 1994).

The range of causative FGFR mutations may well provide the explanation for these observations. Heterogeneity is demonstrated in the various numbers of substitutions, insertions, deletions and aberrant splicing events within FGFR2 that generate common phenotypes within this range. It emerges that there is great crossover between causative genotype and resultant phenotype. Certain high frequency co-segregating phenotypic features show a high degree of correlation with specific mutations: the most obvious example of which is Apert syndrome, which has been separately considered (§1.3.2.2). However, even within the 'narrow' Apert phenotypic range, there is diversity of genotype (Passos-Bueno et al, 1997; Oldridge et al, 1999), and not all 'Apert mutations' generate Apert phenotypes (Passos-Bueno et al, 1998b). This principle is extended by examination of the genotype-phenotype crossovers exhibited by the 'Crouzon - Pfeiffer' group of phenotypes in association with FGFR2 mutations in the extracellular domain. Numerous specific mutations have been demonstrated to generate more than one eponymous syndrome within the range (Passos-Bueno et al, 1999). Many of these (non - Apert) sites are 'mutation hotspots', codons of high - frequency mutation rate, such as Cys278, for which substitution of phenylalanine generates Crouzon, Pfeiffer and Jackson - Weiss syndromes. The Cys342 site is also highly mutable, but for a range of amino acids. Arginine, tryptophan and tyrosine are most commonly, but non - exclusively, substituted, and it seems that it is the substitution of the cysteine residue that delivers the common functional effect, and the pathogenesis of the syndromes of Pfeiffer, Crouzon, and Jackson - Weiss.

Phenotypic diversity generated from single point mutation may reflect the genetic background of the individual or maternal environmental influences, for example. There is precedent for the same gene mutation to generate wide phenotypic range, such as in the example of the adenomatous polyposis coli mutation quoted by Rutland (1995). The same mutation that causes colonic polyposis in some members of a pedigree may cause additional non - bowel tumours (Gardner's syndrome) in other members of the same family. Analogous examples in syndromic craniofacial dysostosis include the wide phenotype range of Jackson - Weiss syndrome (Jackson et al, 1976), and the very broad ranging pedigree reported by Steinberger (1996) and resulting from a splicing mutation at codon 344. This latter pedigree ranges in phenotype from minor anomalies such as slight hypertelorism and maxillary hypoplasia to
severe skull dysplasia from multiple suture synostosis. The silent A344A (1032G—A) mutation, which creates a stable splice variant (Li et al, 1995), also characterises Crouzon syndrome in independent cases (Park et al, 1995b;Steinberger et al, 1996;Passos-Bueno et al, 1999). This example of phenotype diversity from a single mutation, as displayed by mutations substituting the cysteine residues at positions 278 and 342 in FGFR2, also has precedent in other forms of human molecular pathology. The Arp178Asn mutation in the prion protein gene, PRI, causes the non-overlapping phenotypes of familial Creutzfeld - Jacob disease and fatal familial insomnia. The phenotype is determined by a sequence polymorphism in the intronic codon 129 (Goldfarb et al, 1992) quoted in Rutland, 1995). The plasticity of the genotype-phenotype relationship in syndromic craniofacial dysostosis is well documented by many observers, and further potential for confusion results from the tendency of the phenotype to change with increasing maturity (Pulleyn et al, 1996).

Although attempts at such correlations have been made (Park et al, 1995b), it is noteworthy that there is a lack of consistent difference between the phenotypes of mutations within the IgIIIA and IgIIIC domains of FGFR2 (Oldridge et al, 1995;Meyers et al, 1996). The first group of mutations would be expected to affect both Bek/IgIIIC and KGRF/IgIIIB isoforms of the receptor protein, and the latter exert effect though the IgIIIC isoform only. In this case, it might be expected that the IgIIIA-phenotypes within this group would display both mesenchymal and 'epithelial' manifestations, analogous to the acne of Apert syndrome. In fact, whilst 'atypical features' of Crouzon syndrome such as anal and ear anomalies (Park et al, 1995b) in two Crouzon family members have been associated with the W290G mutation in FGFR2 (affecting both IgIIIB and IgIIIC isoforms); no definitive and consistent associations have been made across a large patient group. The lack of such correlation perhaps suggests that the activating mutation has a different phenotypic effect upon cells of different lineage, or that specific mutations in the IgIIIA or transmembrane regions, for example, have differential effects upon the mutant isoforms. Mutations in the FGFR2 - transmembrane domain, functionally affecting both BEK and KGR, do invariably result in the cutaneous manifestations of Beare - Stevenson cutis gyrata (S-1.3.2.5). This apparent anomaly may reflect the type and degree of functional activation of the mutant receptor, as well as the pattern of expression of both receptor and ligand. It is possible, for example, that the FGFR2 - transmembrane mutations that introduce cysteine result in a covalently activating S=S dimer without disruption of ligand kinetics, whereas the 'cysteine - mutations' of the IgIIIA - hemiloop; whilst activating, abrogate ligand binding by the disruption of the loop (Hou et al, 1992). The transmembrane mutations would then theoretically be susceptible to 'ligand overdrive', and greater functional gain. Such a mechanism might account for the high incidence of clover-leaf skull and cutaneous malformation in the Beare - Stevenson cutis
gyrata phenotype compared to that group of ‘Crouzon – Pfeiffer’ phenotypes from extracellular FGFR2 mutations that may act via both Bek and KGF expression domains. The genotype – phenotype correlations will additionally depend upon the expression of the various FGFR homologues in human craniofacial development and craniosynostosis (S-3).

The various FGFR2-associated phenotypes that lie within the ‘Crouzon – Pfeiffer’ group are described below. Genotype – phenotype correlations, and investigations of the functional effects of various mutations are considered in Sections 3 and 4 of this thesis. It is of interest that a Crouzon phenotype with the cutaneous condition acanthosis nigricans is caused by a single transmembrane mutation in FGFR3 (S-1.3.3), and FGFR3 mutations also cause Saethre – Chotzen syndrome and coronal synostoses (S-1.3.3). Pfeiffer syndrome is associated with a specific linker-region mutation in FGFR1 (S-1.3.1). The pathogenetic heterogeneity of these syndromes thus extends beyond FGFR2 to its homologues, and implies that the high sequence homology of the resultant receptors is susceptible to similar mutational alteration with similar functional effect. The functional similarity and cross-over demonstrated by mutations in these genes suggests that they occupy overlapping expression domains, with the potential for functional redundancy in human skeletal development. This theme is explored below (S-3, S-4).

1.3.2.4 Crouzon Syndrome – craniofacial dysostosis associated with a range of mutations within FGFR2 and FGFR3

Crouzon syndrome is named for the author of the first published cases in 1912 in the French language literature (Crouzon, 1912). This report describes craniosynostosis, maxillary hypoplasia with exorbitism and Class III malocclusion, and hypertelorism. The Crouzon phenotype does not have as significant an extracranial skeletal manifestation as the related Apert and Pfeiffer phenotypes. However, a re-appraisal of limb radiographs in a series of Crouzon patients indicates that whilst the limbs in many cases may be clinically silent of obvious abnormality, subtle anomalies occur. The phenotype is described in detail below.

Cohen and Kreiborg (1992) estimated that Crouzon syndrome represents approximately 4.8% of cases of craniosynostosis at birth, compared to Apert syndrome, estimated at 4.5% cases (Cohen et al, 1992; Cohen and Kreiborg, 1992b). The birth prevalence was estimated to be 16.5 per million births. There is a relatively high rate of vertical transmission (familial cases = 44%; sporadic cases = 56%) and a significant influence of paternal age amongst new sporadic cases (Glaser et al, 2000).
Phenotypic variability between generations is a common observation. The craniofacial variability includes the severity of the midface retraction and exorbitism, and variable features such as supra-orbital recession. Craniosynostosis may be absent or delayed (Reddy et al, 1990; Pulley et al, 1996). Cohen (1993) reported the insidious and late onset of familial nonsyndromic craniosynostosis, which may have been labelled with Crouzon syndrome. A mother, son, and daughter were described in whom serial photographs documented an insidious and late onset of exorbitism and midfacial retraction. Intracranial hypertension in the absence of a severe craniofacial phenotype was also observed, and if unchecked this may progress to optic nerve damage, retinal damage and visual loss (Cohen et al, 1993).

The Craniofacial phenotype

The craniofacial manifestations of the ‘Crouzon – Pfeiffer’ group of syndromes (S-1.3.1.1) are comparatively variable with respect to the more consistent Apert craniofacial phenotype (Fig 1.3-5). At the most severe end of the spectrum, clover-leaf skull has been reported (Hall et al, 1972), and a wide range of less severe craniosynostosis phenotypes commonly occurs. The most useful published data (Kreiborg et al, 1993) relates to work done on Crouzon patients in comparison to the Apert phenotype (S-1.3.2.2). Reports of phenotype variability include cases of Crouzon syndrome without an apparent craniosynostosis, but a marked midfacial retraction and the secondary ocular and occlusal problems thereof. Early closure of the basicranial synchondroses, contrasting with later closure in Apert syndrome, is a commonly reported feature (Kreiborg et al, 1993; Cinalli et al, 1995), and is thought to contribute to the shallow posterior fossa, cerebellar tonsillar herniation (Cinalli et al, 1995), and raised intracranial pressure (Thompson et al, 1995a) observed in these patients.

The commonest cranial phenotype of this group is brachycephaly from a bicoronal synostosis, and combinations of sutural fusion invariably include the coronal suture. The sagittal and lambdoid sutures are also commonly involved (Kreiborg et al, 1993; Cinalli et al, 1995). Median time to closure of various sutures in a series of Apert (n=65) and Crouzon (n=68) patients, as measured by plain radiography using uniform criteria, has been reported (Cinalli et al, 1995). Despite the inaccuracies inherent in using plain radiography as a measure, the trends are for similar time to closure of the coronal suture (Apert: 5 months, Crouzon: 8 months). However, accelerated closure of both sagittal and lambdoid sutures correlates with Crouzon syndrome (Sagittal suture - Crouzon: 6 months, Apert by default ossification: 51 months. Lambdoid suture - Crouzon: 20 months, Apert: 60 months). Comparison of the
cranial features of the Crouzon—type syndromes and Apert phenotype is given in Section 1.3.2.2.

Midface retrusion in the ‘Crouzon—Pfeiffer’ group is not accompanied by a gross hypertelorism or the ‘anti—mongoloid’ cant of Apert syndrome (Kreiborg et al, 1993; Cohen and Kreiborg, 1996). The midfacial retrusion is accompanied by the dental and ocular problems encountered in other craniofacial dysostosis syndromes of the group. Ocular problems include strabismus and problems of exposure keratitis secondary to lagophthalmos. Visual loss secondary to unrecognised raised intracranial pressure is a constant clinical concern (Thompson et al, 1995a; Taylor et al, 2000). There is also evidence for the primary involvement of FGFR2 in the development of the anterior chamber of the ocular globe. The FGFR2 Ser351Cys (1231C to G) mutation in association with Crouzon and Pfeiffer syndrome causes ocular anomaly, including opaque corneae, thickened irides and ciliary bodies, and shallow anterior chambers with occluded angles (Okajima et al, 1999).

Cleft lip and or palate are not commonly associated, though in Kreiborg’s series rates of 2% and 3% were reported respectively (n=61), and these may have been unrelated chance occurrences (Kreiborg and Cohen, 1992). The palate is high arched and short, reflecting the maxillary hypoplasia and short cranial base (Peterson and Pruzansky, 1974). In 3/13 Crouzon palates, the short high arched palatal morphology with lateral soft tissue swelling of the upper arch mucosa was indistinguishable from the Apert palate; but in the majority of cases the palatal morphology is nearer normal (Peterson and Pruzansky, 1974). Nasopharyngeal volume is restricted, and both the anterior and posterior cranial base are short—features which worsen with age (Peterson-Falzone et al, 1981).

Intellectual Performance & the CNS Phenotype

The Crouzon population generally has normal intelligence. Marked intellectual compromise was manifest in only 3% of Kreiborg’s series (n=61) (Kreiborg, 1981). Central nervous system anomalies are not as common as in the Pfeiffer or Apert group. Agenesis of the corpus callosum has been reported in single cases (Kreiborg, 1981), but the commonest malformation is herniation of the cerebellar tonsils (CTH) from the posterior fossa via the foramen magnum in association with lambdoid synostosis (Cinalli et al, 1995). Cinalli et al reviewed 44 patients with Crouzon syndrome and 51 with Apert syndrome, and showed a 72.7% incidence in Crouzon syndrome versus 1.9% in the Apert group. All the patients with Crouzon syndrome and progressive hydrocephalus had CTH, but of 32 individuals with Apert syndrome and CTH, only 15 had progressive hydrocephalus. This is consistent with the
observation that intracranial hypertension is more common in Crouzon syndrome (>15mmHg in 63% cases; (Ma et al, 1995) than in Apert syndrome; with an increased risk of visual loss, papilloedema, and optic atrophy.

The Extracranial skeletal phenotype

Crouzon syndrome shares many of the extracranial features observed in Pfeiffer syndrome, which correlates with the crossover of their causative mutations. The most consistent difference with Pfeiffer syndrome relates to the limb phenotypes. Whilst Apert, Pfeiffer and Crouzon syndromes share the finding of progressive elbow anomalies and synostoses, elbow pathology is more common in the Apert (n=33, 67%) and Pfeiffer (n=16, 68%) phenotypes than in Crouzon syndrome (n=22, 36%). The common findings are synostoses, subluxation of the radial head and humero–ulnar joint, and epiphyseal anomalies (Anderson et al, 1998b). Such anomalies emphasise the role of FGFR signalling in both endochondral ossification, such as in the skull base and appendicular skeleton, as well as the membranous ossification of the calvarium. Why the observed range of anomalies should be less severely manifest against the background of the Crouzon phenotype, compared to that of the Pfeiffer phenotype remains unclear, but it is a consistent finding in the distal limb also. Pfeiffer syndrome is so defined by the broad and radially deviated thumb, and range of simple syndactyly. Crouzon syndrome, despite the similar genotype range, lacks a clinically morbid phenotype in the hands. However, radiographic examination of the Crouzon hand does reveal subclinical anomalies of the metacarpal and carpal bones, and clinodactyly and brachydactyly have also been reported (Proudm an et al, 1994;Anderson et al, 1997d) {Anderson & Evans 1998 21 /id}. Furthermore, the Crouzon phenotype has been reported to demonstrate subtle sub–clinical anomalies of the feet (n=18), as well as normal feet in cases sharing a ‘Jackson – Weiss’ mutation (Anderson et al, 1997c). This data provides subtle evidence that the ‘Crouzon – Pfeiffer’ group of mutations generate syndromes that exhibit a linear range of severity in the limb phenotype with cross – over of phenotypic features between syndromes.

The range of severity in the craniofacial phenotype is less easy to define. Whilst Apert syndrome and cleft palate co–segregate, the sutural fusion phenotype of the ‘Crouzon – Pfeiffer’ group might be considered to be ‘worse’ according to the pattern and number of suture's involved and the incidence of the ‘clover – leaf’ skull. Range of phenotypic severity amongst the related syndromes, however, is evident in the cervical spine, where progressive fusions and anomalies occur in 44 (18%) of patients, commonly at the C2-3 level (Anderson et al, 1997b), compared to the higher incidence in Pfeiffer and Apert syndromes (S-1.3.1.1, S-1.3.2.2).
Other Phenotypic Features, Crouzon Syndrome & Acanthosis Nigricans

Kreiborg (Kreiborg, 1981) reported a number of further features, including hearing deficits in 55%, and structural ear anomalies. Calcification of the stylohyoid ligament was also reported in 88% patients (n=50), compared to 50% of Proudman's series (Proudman et al, 1994). Tracheal cartilage anomalies occur, in company with the Apert and Pfeiffer phenotypes (S-1.3.1.1, S-1.3.2.2). Visceral anomalies are less common than in the Pfeiffer or Apert phenotypes, but may sporadically occur (7% of Proudman's series, n=59). Anal and ear anomalies have been correlated with mutations in the IgIIIA - hemiloops of FGFR2 acting via the KGFRIgIIIA/b isoform expressed in epithelia, but a definitive association has yet to be demonstrated (Park et al, 1995b).

The association of Crouzon syndrome with skin pathology has been variously reported, including a sporadic association with multiple congenital naevi (Gines et al, 1996). A number of case reports relate the skin condition acanthosis nigricans to Crouzon syndrome (Reddy et al, 1985; Suslak et al, 1985; Breitbart et al, 1989; Koizumi et al, 1992). The skin phenotype consists of rugated skin with thickening and hyperpigmentation, particularly affecting the flexure creases. The syndrome is associated with an FGFR3 mutation and is considered further below (S-1.3.3).

Jackson – Weiss Syndrome

Jackson et al. originally reported this syndrome (JWS) in a large Amish kindred. The craniofacial phenotype is similar to that of Crouzon syndrome, but milder, with reduced midface retrusion and proptosis. Some members of the large original kindred have no craniofacial anomaly at all, and the most consistent feature of the phenotype is a broad and medially deviated big toe with tarso–metatarsal coalition (Jackson et al, 1976; Jabs et al, 1994). Calcaneo– cuboid fusions also occur. The broad thumb by which Pfeiffer syndrome is defined was not present in 130 JWS cases, however, mild 2/3 syndactyly was noted in one case. Eighty-eight affected cases were studied in the original kindred, and another 50 were reliably reported to be affected, with a range of phenotypes encompassing almost the entire spectrum of the dominantly inherited craniofacial dysostoses and acrocephalosyndactylies. Intellectual impairment is noted by in a branch of the family (Cross and Opitz, 1969), but is not considered to be a major feature.
Independent cases of Jackson – Weiss syndrome have been reported (van Herwerden et al, 1994; Park et al, 1995b), and it originally seemed as if the phenotype defined a distinct and independent group (Lewanda et al, 1994a). It is likely, however, that the phenotypic variability of many other FGFR2 - craniosynostoses would also be manifest across a putative single kindred of equal size, and that there would be wide phenotypic crossover with Jackson – Weiss syndrome. Examples of this in small pedigrees have been reported (Meyers et al, 1996), despite the frequent assertion that the various eponymous syndromes 'breed true' without generational cross- over of phenotype. In this light, Winter and Reardon (1996) proposed that the designation Jackson-Weiss syndrome should 'be reserved for large pedigrees showing extreme intrafamilial variability of craniosynostosis phenotypes encompassing features of Crouzon, Pfeiffer, and Apert syndromes (Winter and Reardon, 1996).

**Antley Bixler syndrome**

The Antley Bixler phenotype (Antley and Bixler, 1975) is characterised by brachycephalic craniosynostosis with fronto- parietal bossing, and a combination of extra- craniofacial anomalies that include elbow synostoses and uro- genital anomalies. The phenotype has been reviewed by a number of observers (HasseU and Butler, 1994; Reardon et al, 2000). The craniofacial features include coronal craniosynostosis with brachycephaly, high incidence of choanal stenosis or atresia, midfacial retrusion with exorbitism, depressed nasal bridge, and low set dysplastic pinnae. Choanal stenosis and airway compromise result in clinical urgency. Early death is usually due to respiratory complications, and occurred in 54% of the reported cases of Hassell and Butler (Hassell and Butler, 1994). The oldest patient was 10 years old at the time of follow-up, and was reported to be of normal intelligence, though impaired intellectual performance was documented in patients from the same series.

There is obligate radio- humeral synostosis (lack of this feature in a newborn probably predates its onset - (Chabchoub et al, 1998), which may also effect the radio- ulnar and ulna- humeral joints. There are joint contractures at hip and knee, talipes equino varus (Crisponi et al, 1997) and ulnar and femoral bowing. Long bone fractures occur. Anomalies of the fingers may occur, including camptodactyly and MP joint anomalies. Arachnodactyly, with long slender fingers, is also reported. Urogenital abnormalities are commonly reported and include vaginal atresia, fused labia, cliteromegaly, and reduplication of the kidney. Renal agenesis and malformations are commonly associated, and cardiac anomaly has been reported. Imperforate anus is an uncommon association (LeHeup et al, 1995; Bottero et al,
1997). Defects of steroidogenesis have been reported (Reardon et al, 2000; Roth et al, 2000) and may be the cause of the abnormal urogenital manifestations, which are more common in females (Reardon et al, 2000).

The apparently disparate phenotype represents the severe end of a range of FGFR2—mutants, in which the predominant skeletal features overlap with Pfeiffer syndrome (craniosynostosis, elbow ankylosis) and with thanatophoric dysplasia (femoral bowing). The co-segregation of skeletal and urogenital anomaly has been postulated to result from a digenic inheritance, whereby the skeletal phenotype results from mutations in FGFR2, acting against a genetic background of defective steroidogenesis (Reardon et al, 2000). The FGFR2 mutations include Ser351Cys, which is common to a severe craniosynostosis and elbow synostosis (Pulley et al, 1996); and Trp290Cys, which is recorded in Pfeiffer syndrome and a non-classified craniosynostosis and elbow synostosis (Tartaglia et al, 1997a; Schaefer et al, 1998). Cysteine substitutions are a common theme in FGFR2—craniosynostoses and correlate with constitutive activation of the mutant receptor.

_Crouzon, Jackson—Weiss, & Pfeiffer syndromes: a distribution of phenotypic diversity which includes the Kleeblattschaedel Anomaly (Clover—leaf skull)_

An analysis of the phenotypes of the non-Apert FGFR2 related syndromic craniofacial dysostoses indicates that they lie within a distribution of severity. The common craniofacial features, as described above, vary in severity around the common themes of hypertelorism, midface retrusion and patterns of craniosynostosis. The limb anomalies range from radiographic subclinical anomaly in Crouzon syndrome, to medial deviation of the big toe and radiographic tarsal—metatarsal fusion in Jackson—Weiss syndrome (JWS), to clinically morbid soft tissue and skeletal anomalies of hand and foot in Pfeiffer syndrome. The scale of the limb anomalies, which is inclusive of the non—classifiable families and individuals that pepper the literature, suggests that limb morphogenesis is subject to control by functional dose—dependency of FGFR2 isoforms. The scale of skeletal and soft tissue limb anomaly, which is not easy to quantify in the craniofacial skeleton, implies that mutant FGFR2 'gain of function' also demonstrates a scale of activation, dependent upon the type of mutation and modifying influences (S-1.4). 

The degree of severity of the craniofacial dysostosis is perhaps more difficult to quantify in linear terms and relate to graded mutant functional gain. This is because the growth of the viscerocranium and the neurocranium are complex and related by multiple factors, including
the growth of the brain, deformational constraints, and the morphogenesis of the
chondrocranial skull base (Thorogood, 1993). The 'degree' of craniosynostosis is difficult to
standardise and quantify from multiple independent studies, which use combinations of plain
radiography, CT and MRI to make the diagnosis. Evolving technology in imaging technique
makes standardisation of imaging protocols across different clinical centres difficult.

The Kleeblattschaedel anomaly, or clover-leaf skull, is an uncommon and severe craniofacial
phenotype resulting from multisutural fusion involving both coronal sutures and a
combination of the metopic, lambdoid and sagittal sutures. The result is a broad bi-temporal
and bi-parietal diameter, with a turricephalic vertex and steep forehead (Fig 1.3-2). The head
has a flattened, trilobular configuration, caused by hydrocephalus in combination with
extensive craniosynostosis. The midface is retruded with the secondary ocular and
dentoalveolar problems elsewhere described (S-1.3.2.2; S-1.3.1.2). There is a shallow posterior
fossa, and herniation of the cerebellar tonsils via the foramen magnum is a common
accompaniment. The clinical prognosis is often poor, but improved by aggressive surgical
management.

Clover-leaf skull has been reported in conjunction with a number of FGFR related
phenotypes, including 'Crouzonoid' non-specific craniosynostosis (Pulleyn et al, 1996),
Pfeiffer syndrome (Soekarman et al, 1992; Meyers et al, 1996), Crouzon syndrome (Hall et al,
1972; Cohen, Jr., 1975), Apert syndrome (Cohen, Jr. and Kreiborg, 1994; Gosain et al, 1997),
Beare - Stevenson cutis gyrata (Hall et al, 1992; Przylepa et al, 1996), and thanatophoric
dysplasia (Langer et al, 1987; Machin, 1992). The severe craniosynostosis phenotype may
reflect particularly activating mutations in FGFR2 or FGFR3. However, the relationship of
genotype to severe craniosynostosis phenotype is not a simple one. The Cys342Ser mutation
'hotspot' in FGFR2 causes cloverleaf in association with Pfeiffer syndrome (Meyers et al,
1996), but not invariably, and not in association with Crouzon or JWS cases. Cloverleaf in
association with FGFR3 phenotypes is restricted to thanatophoric dysplasia (TD).
Furthermore, cloverleaf skull has been reported in association with Carpenter syndrome (not
associated with FGFR mutations) and Boston - type craniosynostosis, which is caused by
mutations in Msx2 (Warman et al, 1993). The sutural phenotype of these syndromes
therefore appears to have a complex relationship with their causative mutations (S-3,S-4).
1.3.2.5 Beare — Stevenson cutis gyrata

This syndrome is eponymously attributed to Beare in 1969, and Stevenson in 1978 (Beare et al, 1969; Stevenson et al, 1978); and six collected cases were reviewed and the syndrome delineated in 1992 (Hall et al, 1992).

There is craniosynostosis documented in 5 of 7 known cases, with cloverleaf skull in 3 of Hall’s cases; although association with clover-leaf skull has since been reported (Bratanic et al, 1994; Ito et al, 1996), and in association with the cerebellar tonsillar herniation of high incidence in Crouzon syndrome (Cinalli et al, 1995; Ito et al, 1996). Hydrocephalus and agenesis of the corpus callosum have been reported. Hypertelorism, midface retrusion and ocular proptosis occur, with downsloping palpebral fissures and a broad flat nasal bridge. Ear anomalies of cartilage size and shape are common. Choanal atresia has been reported in 5/6 patients (Hall et al, 1992).

Cutaneous features include cutis gyrata, furrowed and corrugated skin, which particularly affects the forehead and occiput; and also the face, preauricular area, neck, trunk, hands, and feet. Cutis gyrata has been noted in Apert cases (Hall et al, 1992), and may also account for the typical Apert furrowed brow. Acanthosis nigricans, which features verrucous hyperplasia, hypertrophy and hyperpigmentation of the skin, is evident from birth in the palms, soles, lumbosacral region and labia majora (Hall et al, 1992; Bratanic et al, 1994). There is increased keratin deposition, and increased melanocyte numbers in representative skin biopsies. Anogenital anomalies such as bifid cleft scrotum and undescended testis, also occur. There are anomalous skin tags and a prominent umbilical stump. There are no specific limb anomalies, though notched metatarsals are reported. The palate is narrow, although cleft palate and bifid uvula are reported in one case each and may be a random association (Hall et al, 1992). Most patients die in early infancy or childhood, the longest survival has been 13 years (Przylepa et al, 1996).

Beare — Stevenson cutis gyrata is associated with the Tyr375Cys mutation at the N-terminal end of the transmembrane region of FGFR2 (n=3); and the neighbouring Ser372Cys mutation (n=1) in the extracellular — transmembrane junction (Przylepa et al, 1996; Krepelova et al, 1998). Both these mutations, by adding a Cys residue, increase the potential for inter — molecular covalent disulphide bonding and would be predicted to be activating. Mutations in two further cases were not identified (Przylepa et al, 1996).
The patient with the peri-transmembrane mutation Ser372Cys did not have a clover-skull, but the Tyr375Cys mutation was associated with clover-skull in 2 cases. Both mutations, lying in exon 10 of FGFR2, would be expected to alter both the IgIllc and IgIllb forms of the receptor protein. The extent of cutaneous anomaly and severity of craniosynostosis reflects the probability that the mutations are highly activating and act via the KGFR/IgIllb expression domain in skin and the IgIllc/Bek expression domain in mesenchyme (Orr-Urtreger et al, 1993). The preliminary evidence from these genotyped cases is that the transmembrane Tyr375Cys, in generating a more severe craniosynostosis phenotype, is more highly activating; however, the possibility that Ser372Cys is modified by other means remains. The Ser351Cys mutation in the juxta-membranous region of FGFR2 has also been associated with clover-skull and elbow synostosis without cutaneous pathology, but the Gly384Arg mutation of the transmembrane region displayed mild coronal and sagittal synostosis (Pulleyen et al, 1996). The introduction of an unpaired cysteine to the transmembrane region may define a particularly activating mutation of FGFR2 (S-1.4).

The incidence of craniosynostosis and acanthosis nigricans is also evident in a form of Crouzon syndrome associated with a C-terminal transmembrane mutation in FGFR3 (Meyers et al, 1995). The association of acanthosis nigricans with mutations at opposite ends of the transmembrane region of two homologous genes is interesting. Both mutations would be expected to affect both IgIllb and IgIllc isoforms of FGFR2 and FGFR3 respectively. The IgIllb isoforms of each predominate in the skin, where FGFR2 - IgIllb has demonstrated function (Werner et al, 1994). It may be that the transmembrane mutations which introduce cysteine (note; Gly384Arg has no cutaneous anomaly) permit heterodimerisation of mutant FGFR3 in skin (Chellaiah et al, 1994) to enrol the established cutaneous expression domain of FGFR2 - IgIllb (Rubin et al, 1989; Miki et al, 1992; Finch et al, 1995). These mutations would presumably be differentially activating to generate the skin pathologies that are not seen in the IgIlla - 'Crouzon - Pfeiffer' mutants, which are also encoded in the FGFR2-IgIllb domain.
1.3.3 Phenotypes associated with FGFR3 mutations

1.3.3.1 FGFR3 – associated craniosynostosis describes a wide phenotypic range

Coronal synostosis – variability of phenotype defined by the P250R mutation in FGFR3

'Non syndromic' coronal synostosis is associated with a variety of cranial phenotypes that are distinct from the eponymous syndromes associated with mutations in FGFR2 and FGFR1. The common cranial phenotypes are plagiocephaly associated with a unicoronal synostosis, and brachycephaly, associated with bicornal synostosis. The combined incidence of these phenotypes is calculated as 1 in 2,100 - 2,500 (Lajeunie et al, 1995b). The plagiocephaly phenotype consists of a ridged, fused suture in the coronal territory on one side causing a flattened forehead on the same side. There is elevation of the sphenoid on that side at its articulation with the coronal ring. The orbit is therefore also elevated in a vertical dystopia, and there is a resultant facial scoliosis. On the opposite side of the skull there is frontal bossing, and the orbit is lower than its partner. The coronal, orbital, and skull base asymmetry may extend to involve the maxillary arch and create a skeletal and dental cross-bite. The bicornal synostosis phenotype is brachycephalic with a wide flat forehead, which may be bossed forwards above the supraorbital plane. There is no dystopia and the midface is not recessed as seen in the 'Crouzon – Pfeiffer' group of FGFR2 related syndromes. There are no specifically related dental problems. 'Non – syndromic' synostosis, or 'simple synostosis' has a low incidence of intracranial anomaly. Intracranial hypertension occurs in 17% (n=74; including a proportion of sagittal and metopic cases) of single suture synostoses (Thompson et al, 1995b).

The involvement of the coronal suture in many of the craniosynostosis syndromes, and the recognised mutability of the Pro252 site in FGFR1 and the Pro253 site in FGFR2 resulted in the examination of the homologous site in FGFR3 for mutations causing craniosynostosis (Bellus et al, 1996). The FGFR3 – P250R mutation was demonstrated in two families with phenotypes reminiscent of Pfeiffer syndrome in whom there was no linkage to chromosomes 8 or 10. Subsequent analysis of 65 unrelated cases with craniosynostosis and non – classifiable limb anomaly revealed the FGFR3 – P250R mutation in 10 individuals. The affected patients showed great intra- and interfamilial variation and craniosynostosis was not consistently present. The limb phenotypes were predominantly normal, but displayed radiographic evidence of short middle phalanges.
The retrospective analysis of a further 61 cases from 20 unrelated families revealed the point mutation 749C—G, encoding the Pro250Arg substitution in a range of phenotypes with coronal synostosis (Muenke et al, 1997). The group included a kindred previously reported as linking to chromosome 4p (Hollway et al, 1995) and named the Adelaide type, and a kindred previously described as brachydactyly – craniosynostosis (Glass et al, 1994). The phenotype defined by the FGFR3 - Pro250Arg mutation is very broad. It includes uni- and bi- coronal synostosis, although normocephalic and macrocephalic individuals without craniosynostosis were also noted; and in these all had abnormal radiographic findings in the extremities. A minority of cases with craniofacial features had no radiographic limb anomaly. Facial findings included mild midface hypoplasia, high arched (non – cleft) palate, mildly down – slanting palpebral fissures, and ptosis (Muenke et al, 1997). In a subsequent report, no association of FGFR3 – P250R with other forms of single suture synostosis or anterior plagiocephaly without coronal synostosis was found, in an otherwise wide cranial phenotype (Gripp et al, 1998b), which includes pansynostosis in one rare case (Golla et al, 1997). The analysis of family members of probands with coronal synostosis and the FGFR3 – P250R mutation indicates that the mutation may be associated with very mild cranial phenotypes and subtle limb phenotypes (Moloney et al, 1997). It is likely that the coronal synostoses present a heterogeneous group of conditions, in that only a minority (~40%) test positive for the FGFR3 – P250R mutation (Moloney et al, 1997;Gripp et al, 1998b).

Although the limb phenotype is clinically normal in most cases, there is a range of subclinical radiographic anomaly. This includes thimble – like middle phalanges, coned epiphyses and carpal and tarsal bone fusions (Moloney et al, 1997). The tarso – metatarsal fusions characteristic of Jackson – Weiss syndrome are not seen (Muenke et al, 1997). Brachydactyly, but not syndactyly, is noted in some patients, together with short, but non – deviated great toes. The phenotypic range is wide and subtle in both the limb and craniofacial skeleton, and there is no apparent correlation between limb and craniofacial phenotype (Moloney et al, 1997;Muenke et al, 1997;Reardon et al, 1997). Developmental delay is noted in a minority of patients with the Pro250Arg mutation (Muenke et al, 1997;Reardon et al, 1997). Sensorineural hearing loss is also identified in some patients.

It is of note that none of the defining skeletal manifestations of the dwarfism dysplasias are present in the craniosynostosis FGFR3 – P250R group. This suggests that the activating mechanism of the mutation is discrete, and independent of the range of mutations causing achondroplasia, thanatophoric dysplasia and hypochondroplasia. FGFR3 – P250R, in company with the dwarfism mutations in FGFR3, is likely to be an activating mutation, as patients with the 4p - deletion syndrome lack any of the features of skeletal dwarfism or
craniosynostosis. The FGFR3 – P250R mutation defines a genetic syndrome in which the phenotype is so variable as to make clinical diagnosis and genetic counselling difficult. The phenotypic crossover between similar phenotypes without the mutation (in the majority of cases) is very great. Furthermore, phenotypes previously described as Saethre – Chotzen have linked to chromosome 4p and subsequently demonstrated the Pro250Arg mutation (Muenke et al, 1997;Golla et al, 1997). The potential mechanisms that may define the various FGFR3 phenotypes are considered in Sections 3 and 4 of this thesis.

Saethre – Chotzen Syndrome: a variable phenotype of FGFR and TWIST mutations

The Saethre Chotzen syndrome (SCS) is eponymously named following initial reports in the early 1930s. In the family described by Saethre (1931), a mother and two daughters showed mild craniosynsostosis, facial asymmetry, low frontal hairline, brachydactyly and partial soft tissue syndactyly of index and middle fingers and the third and fourth toes (Saethre, 1931). Chotzen (1932) described similar malformations in a father and two sons who also had hypertelorism, short stature, deafness and intellectual compromise (Chotzen, 1932). The variability in the phenotype has been emphasised in many reports (Pantke et al, 1975;Marini et al, 1991;Niemann-Seyde et al, 1991;Reardon and Winter, 1994).

Characterizing features include; mild and asymmetrical craniosynostosis, low-set frontal hairline, parrot-beaked nose with deviated septum, ptosis of the eyelids, strabismus, refractive error, tear duct stenosis, dystopia canthorum, brachydactyly and abnormal dermatoglyphic patterns. Muenke has suggested that minimal diagnostic criteria should include tear duct abnormalities, palatal anomalies including cleft palate, parietal foramina, brachydactyly, 2/3 simple syndactyly of hands, and bifid hallux with a lateral deviation (Muenke et al, 1997). Complete hallucal reduplication has been noted, and probably brings the syndrome of Robinow and Sorauf (Robinow and Sorauf, 1975) into the Saethre Chotzen group (Reardon and Winter, 1994), an observation which has latterly gained molecular credibility (Kunz et al, 1999).

The craniosynostosis phenotype is variable, and may include coronal, metopic, lambdoid and sagittal sutures in variable patterns (Hunter et al, 1976;Reardon and Winter, 1994). Cranial defects include parietal foramina and late-closing fontanelles. Vertebral fusion is uncommon. Auricular malformations are common including a long and prominent ear crus, reported as a consistent sign (Carter et al, 1982). A small pinna, with short columella, mild simple syndactyly and craniosynostosis has been described (Aase and Smith, 1970;Kopysc et al, 1980;Kurczynski and Casperson, 1988;Legius et al, 1989), and other nasal anomalies, such
as long thin nose also occur (Aase and Smith, 1970). Extracranial skeletal manifestations have been further catalogued by Anderson (Anderson et al, 1996c; Anderson et al, 1998b). Elbow synostosis, noted rarely by Reardon and Winter (1994), was not seen in this series, and only 3/15 cases had mild 2/3 syndactyly. The hand phenotype included radiographic evidence of anomalies of the thumbs, fingers, metacarpals, and the radius. Clinodactyly was a common finding. Epiphyseal anomalies of the distal phalanx of the thumb were noted in 7 of 15 patients. There was a variable delay of the bone age.

Saethre Chotzen syndrome is predominantly familial, and of such great variability and subtlety of phenotype that figures of incidence are probably unreliable (Reardon and Winter, 1994), although 1 in 25,000 to 1 in 50,000 has been suggested (Howard et al, 1997). The heterogeneity of the phenotype, with variable anomalies of both osseous and epithelial development, suggests that the causative gene is multifunctional in the control of the development of head mesenchyme, and that its effect is dose dependent or modified by variable control mechanisms.

Linkage studies in familial SCS (Brueton et al, 1992; van Herwerden et al, 1994; Lewanda et al, 1994b) lead to association with markers in chromosome 7p21. The TWIST gene, broadly expressed in murine head mesenchyme (Stoetzel et al, 1995), maps to the same region and presented a candidate (Bourgeois et al, 1997). Furthermore, whilst TWIST knock out mice are embryonic lethal and fail with cranial neural tube closure, the heterozygotes survive and present with subtle craniofacial and limb anomalies (El Ghouzzi et al, 1997; Bourgeois et al, 1998).

A range of mis-sense, non-sense, deletion, and 21bp duplication mutations in the TWIST gene has been demonstrated in familial and sporadic SCS (El Ghouzzi et al, 1997; Howard et al, 1997; Rose et al, 1997) and is reviewed by Rose and Malcom (1997). Complete TWIST deletions are also associated with SCS, and correlate with the presence of learning difficulties (Johnson et al, 1998), suggesting the involvement of neighbouring genes in intellectual development. Balanced translocations in 7p21 in SCS patients that do not interrupt the coding sequence of TWIST have been interpreted to exert deleterious effects upon TWIST expression by positional effect (Rose et al, 1997).

Description of the SCS phenotype in patients with 7p deletions (Chotai et al, 1994) suggested that the TWIST human phenotypes would result from haplo - insufficiency, and this is corroborated by the wide range and type (deletion, non-sense, insertion) of causative mutation, affecting various functional domains of the protein. The similarity of the mouse
TWIST null heterozygote phenotype to human SCS also supports this contention. TWIST mutations affecting the alpha − helix are predicted to disrupt dimerisation, which by analogy with experimental evidence in the related E47 bHLH, leads to loss of function (Rose et al, 1997). TWIST mutations affecting the loop − sequence are predicted to have the same effect, and the termination mutations, in affecting a severely truncated protein, would also comply with this molecular model of TWIST haploinsufficiency in SCS.

The variability in the SCS phenotype suggests that the developmental pathogenesis of the TWIST mutant is dose − dependent. There is some evidence that the nonsense mutations resulting in a severely truncated transcription factor correlate with severity of phenotype (Howard et al, 1997), and that microdeletions of the TWIST gene also result in severe intellectual compromise (Johnson et al, 1998). The range of mutations is wide, however, and no firm genotype − phenotype correlations have been drawn from a large number of patients. The TWIST +/− mouse displays similar heterogeneity of phenotype, and was initially considered phenotypically normal. There is hindleg reduplication of digits in 50%, which may involve variable extents of the digit ray, and is seen in the hallux of SCS patients. Squamosal and interparietal bone dysplasias are noted, together with accelerated calvarial ossification (El Ghouzzi et al, 1997; Bourgeois et al, 1998).

There is considerable overlap of the phenotypes of SCS and phenotypes of FGFR3 − P250R (Muenke et al, 1997), and the Pro250Arg mutation in FGFR3 has been identified in SCS phenotypes without TWIST mutations (Rose et al, 1997; Paznekas et al, 1998). Furthermore, the VV269 − 270 deletion in FGFR2 was also noted in a single sporadic SCS case, identified by the same phenotypic criteria (Paznekas et al, 1998). This suggests that FGFR and TWIST mutations occupy the same signalling pathway. Evidence for this comes from Drosophila, which shares 68% twist sequence conservation with Xenopus, mouse and man, rising to complete conservation in the loop − region (Howard et al, 1997). The DFR1 and DFR2 genes in Drosophila, which are related to the vertebrate FGFR family are dependent upon the twist protein to function in the induction of Drosophila mesoderm and endoderm layers (Shishido et al, 1993). This suggests that the TWIST gene is an upstream regulator of FGFR expression. It remains to be seen, however, whether vertebrate TWIST differentially regulates FGFR expression in membranous and endochondral ossification, as the human TWIST phenotypes do not display a dwarving dysplasia.
Acanthosis nigricans is a velvety hyperplasia of the skin, which commonly affects flexure creases. There is hyperpigmentation and accentuation of the skin markings, distributed in the neck axillae and other flexural areas. An hereditary autosomal dominant form exists, and the condition may also be associated with endocrine problems, drug complications, or occult malignancy (Koizumi et al, 1992).

Acanthosis nigricans in association with Crouzon syndrome may affect unusual distributions, such as eyelids and face, as well as chest abdomen and breasts. It takes onset in childhood and always by puberty. Histologically, it is similar to other forms of the disease, consisting of marked papillomatosis and a thin hyperpigmented epidermis (Meyers et al, 1995). It may be associated with multiple melanocytic naevi of the face and neck (Meyers et al, 1995; Wilkes et al, 1996; Gines et al, 1996). A female preponderance is noted (Breitbart et al, 1989; Koizumi et al, 1992; Meyers et al, 1995), which may reflect co-genetic or hormonal influence. There is a high association with choanal atresia, which is sporadic in the FGFR2-related Crouzon phenotype (Breitbart et al, 1989; Meyers et al, 1995).

The Crouzon - Acanthosis nigricans (Cr - AN) variant is associated with the Ala391Glu mutation in the transmembrane region of FGFR3 (Meyers et al, 1995). An acidic, bulky, glutamic acid residue is substituted for a neutral hydrophobic alanine, and the mutation is specific to the Cr-AN phenotype, but lies within a few residues from the two mutations encoding achondroplasia (ACH). These mutations, also specific to the phenotype, substitute neutral for neutral residues (Gly375Cys) or neutral glycine for basic, bulky, arginine (Gly380Arg) in the most common case. The specific mechanism of functional activation of these mutant receptors may generate the great differences in their phenotypes. It is likely that the Cr-AN mutation preferentially recruits FGFR isoforms expressed in the skin and membranous bones, whereas the ACH mutations specifically influence chondrogenic differentiation. The genotype – phenotype relationship for FGFR-associated acanthosis nigricans is further complicated by its association with the SADDAN dwarfism phenotype and its Lys650Met mutation in FGFR3, which lies in a different functional domain of the receptor (S-1.4).
1.3.3.2 The skeletal dwarfism syndromes: Achondroplasia, Hypochondroplasia, and Thanatophoric Dysplasia are caused by mutations in FGFR3

Achondroplasia (ACH) is the commonest dwarfing dysplasia, inherited as an autosomal dominant syndrome with maximal penetrance. It has an estimated frequency of 1/26,000 – 1/15,000 live births and is predominantly (80 - 90% cases) sporadic (Rousseau et al, 1994; Shiang et al, 1994). Consistent mapping of the phenotype to chromosome 4p (Francomano et al, 1994; Le Merrer et al, 1994; Velinov et al, 1994), and the recognition that 4p deletions have no features of dwarfism, predicted mutant gain – of – function of a gene affecting chondrogenesis as a more likely disease mechanism than haplo – insufficiency (Shiang et al, 1994). The FGFR3 gene had been mapped to the same region (Thompson et al, 1991), and was considered a candidate gene because of its expression in chondrogenic ossification in mice (Peters et al, 1993), its mediation of the mitogenic effect of FGF2 upon chondrocytes (Iwamoto et al, 1991), and its inhibitory effect on chondrocyte terminal differentiation (Kato and Iwamoto, 1990a).

DNA studies revealed point mutations in the FGFR3 gene in both ACH heterozygotes and homozygotes. The mutation in 15 of 16 ACH-affected chromosomes was the same: a G-to-A transition at nucleotide 1138 of the cDNA, and the remaining case demonstrated a 1138 G—C transition. Both mutations predicted a Gly380Arg substitution in the juxtamembranous - transmembrane domain of FGFR3 (Shiang et al, 1994). In a parallel independent study, all 23 of the familial and sporadic cases that were studied bore Gly380Arg mutations (Rousseau et al, 1994), and the highly restricted mutational base of ACH has since been confirmed by studies across many populations.

The phenotype of achondroplasia is highly constant and therefore reflects its restricted genotype. The clinical features include dwarfism, relative macrocephaly with a short skull base, and an exaggerated lumbar lordosis. There is reduced chondrocyte proliferation and a reduced zone of hypertrophy (Stanescu et al, 1990) with premature closure of the long bone epiphyses, such that the tibiae are short; but there is increased fibular length. There may be focal severe changes in various epiphyseal plates including clusterlike, enlarged chondrocytes; vacuolization; premature calcification, and fibrosis of the cartilagenous matrix with membranous ossification (Briner et al, 1991). The iliac wings are small and dysplastic, and the femoral neck is short. There is metaphyseal flaring. The vertebrae are short and interpedicular distance is reduced. The rib cage is shortened and barrel – like, with respiratory compromise. Brachydactyly is common with shortened metacarpals and phalanges. Motor development
may be delayed, but intellectual performance is usually normal. ACH homozygotes have a much more severe phenotype and rarely survive into late childhood, succumbing to respiratory difficulties from a very restricted thorax or the sequelae of cerebellar tonsillar herniation at the craniocervical junction. Whilst the genotype FGFR3- Gly380Arg is the predominant ACH mutation, a cysteine substitution at Gly375 has been noted (Ikegawa et al, 1995; Superti-Furga et al, 1995), and the introduction of the unpaired cysteine may be significant for the activation of the mutant receptor, via disulphide bonds forming receptor dimers at the juxtamembranous - transmembrane junction (S-1.4).

Thanatophoric dysplasia (TD) is the commonest neonatal – lethal skeletal dysplasia, with a frequency of 1/20,000 live births. It is associated with severe narrowing of the thorax, as seen in ACH homozygotes, and on the basis of radiological and criteria is separated into TD type 1 (short bowed femurs plus or minus cloverleaf skull) and TD type 2 (straight femurs and cloverleaf) (Tavormina et al, 1995a). The TD1 and TD2 phenotypes derive from specific non- overlapping mutations in the FGFR3 gene. Tavormina et al (1995) demonstrated that 16 cases of TD2 were all attributable to the Lys650Glu mutation caused by the same 1948A—G transversion, which has been recently independently corroborated (Wilcox et al, 1998). The distinguishing phenotypic feature is straight femora; the clover–leaf skull affects both types of TD, and accompanies mutations characteristic of both.

In contrast, TD1 (Tavormina et al, 1995a), which has a more heterogeneous craniosynostosis phenotype (n=23/29 cases), results from a range of mutations affecting many functional domains of FGFR3 (Passos-Bueno et al, 1999). Various sites have been reported, and the most frequent of these introduce cysteine to the IgII – IgIII linker sequence (Ser249Cys, Arg248cys), or the juxtamembranous – transmembrane junction (Tyr373Cys, Gly370Cys, Ser371cys). Additionally a series of TD1 mutations cause a base change in the translational stop codon 807, resulting in a 141 AA extension of the carboxyl terminus of the protein (Rousseau et al, 1995; Tavormina et al, 1995a; Tavormina et al, 1995b; Rousseau et al, 1996a).

It is noteworthy that the TM (Gly380Arg) mutation in ACH accounts for ~99% of the recorded ACH genotypes, and that the ACH phenotype is not variable. The TD phenotypes resemble homozygous ACH Gly380Arg FGFR3 mutants in their phenotypes. Thus heterozygous Lys650Glu causing TD2; and a number of cysteine – introducing heterozygous mutations of the extracellular domain causing TD1, are functionally expressed in the phenotype as approximate to the homozygous ACH mutation. Despite the fact that these mutations affect different discrete regions of the receptor protein, the genotype - phenotype relationship suggests that the ACH mutation is the 'less – functionally activating' of these
dwarfism FGFR3 mutations according to the phenotypes they generate. This theme has a further clinical correlate in the hypochondroplasia (HCH) phenotype, which is another, related autosomal dominant skeletal dysplasia with a phenotype of milder severity than ACH. There is a relative macrocephaly, with shortening of the long bones and interpedicular distance between spinal vertebrae, but the condition is often not diagnosed at birth. Furthermore, the mixed HCH/ACH heterozygote has an intermediate phenotype (McKusick et al, 1973). Hypochondroplasia is caused in the majority of cases by FGFR3 mutations which substitute the asparagine residue at position 540 for lysine (Asn540Lys). This mutation might be considered a semi-conservative one, where the strong base, lysine, substitutes the weak base asparagine (Prinos et al, 1995; Bellus et al, 1995a; Rousseau et al, 1996b; Prinster et al, 1998). Its functional effect, therefore, might be considered to be slight in comparison to the activating mutations of FGFR3 causing the more severe phenotypes of ACH and TD. The HCH phenotype has recently been reported in association with mutations at the Lys650 codon. Whereas Lys650Glu is associated with TD II exclusively, Lys650Asn and Lys650Gln generate HCH, with a milder phenotype than HCH from the Asn540Lys (Bellus et al, 2000).

The graded severity of the dwarfism phenotypes, and the functional ‘dose effect’ which suggests that the homozygous Gly380Arg (ACH) functionally approximates heterozygous TD mutations, indicate that a linear graded severity of activating mutation may be causative. The theory is supported by the observation that HCH, ACH, TD1, and TD2 do not result from the same mutation in any instance, which is unlike the phenotypic crossover related to craniosynostosis mutations in FGFR2. Where the Cys342 site in FGFR2 may generate three different related phenotypes, there is no direct equivalent in FGFR3 related to the linear severity of the dwarfism dysplasias.

There is, however, limited phenotypic diversity for several mutations causing TD1, which also cause SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans), or PLSD – SD (platyspondyl lethal skeletal dysplasia – San Dieo type). In the case of SADDAN, the single causative mutation is the Lys650Met substitution in the tyrosine kinase activation loop (the alternative Lys650Glu causes TD2 exclusively). The SADDAN phenotype is a severe achondroplasia, with acanthosis nigricans that develops in early childhood. There is profound developmental delay, but intensive care in early life allows survival into later childhood (n=3/4). The skeletal phenotype is similar to TD1 in that there is tibial/fibular bowing, spinal curvature and a constricted thorax, but differ in the presence of acanthosis nigricans (Kitoh et al, 1998). TD1 is also homogeneic with the PLSD – SD at several sites within FGFR3. The phenotype is closely related to the TD variants, and the distinguishing feature was said to have been the presence of large rough endoplasmic
reticulum (rER) inclusion bodies within chondrocytes, though some crossover exits (Brodie et al, 1999). It appears, therefore, that the SADDAN, PLSD – SD and TD1, whilst variable in aspects of their phenotypes, can nevertheless be considered part of a continuum generated by differentially activating mutations within FGFR3. This possibility has been put to the experimental test and is considered further in Section 3.3. It may be that the various variants of the 'PLSD – TD – like' conditions will yet demonstrate genetic heterogeneity beyond the FGFR3 gene (Brodie et al, 1999). So far, however, only the HCH phenotype suggests a further as yet un-identified pathogenesis. Although the great majority of successfully genotyped HCH cases involve the Asn540Lys substitution (Passos-Bueno et al, 1999), a proportion of HCH cases (~35%) are not successfully genotyped in FGFR3. Some familial cases are not linked to the FGFR3 locus on 4p, and it has been suggested that these represent a milder phenotypic sub-group on skeletal criteria (Rousseau et al, 1996b). The genotype-phenotype relationships for FGFR3 mutants are considered further below (§3.3).
1.4 A clinico-pathological classification of FGFR mutations: ‘gain – of – function’ & phenotype correlations

In general, the syndromic craniofacial dysostoses are primarily characterised by the presence of craniosynostosis and are caused by mutations of the extracellular domain of FGFR2. Exceptions include the predeliction for proline to arginine substitutions in the IgII – IgIII linker, which occur in FGFR1 (Pro252Arg causing Pfeiffer syndrome), FGFR2 (Pro253Arg causing Apert syndrome) and FGFR3 (Pro250Arg defining a non – specific coronal synostosis syndrome). Furthermore, transmembrane mutations cause non – specific craniosynostosis (Pulleyen et al, 1996), Beare – Stevenson cutis gyrata (Przylepa et al, 1996), and Couzon syndrome (Passos-Bueno et al, 1999) in FGFR2; and Crouzon – acanthosis nigricans in FGFR3 (Meyers et al, 1995; Wilkes et al, 1996). The phenotypes generated by these mutations implicate FGFR1, 2, and 3 proteins in both membranous (craniosynostosis, midface retrusion), and endochondral (skull base, tracheal anomalies, digital anomalies) ossification, as well as the developmental mechanisms of epithlio – mesenchymal signalling.

By contrast, the dwarfing dysplasias are caused by mutations in FGFR3. The specific phenotypes of achondroplasia and thanatophoric dysplasia (TD) type II result from a very narrow genotype range. Associated hypochondroplasia and TD I, with wider phenotypic range, result from a wider range of mutations within FGFR3. The dwarfing dysplasias all feature premature closure of the cartilage epiphysis of the long bones, and thus specifically implicate FGFR3 in chondrogenic differentiation. The FGFR3 – P250R mutation, in lacking the dwarfism phenotype, appears to exert a differential functional effect upon the receptor that affects membranous and basicranial ossification, but not the long – bone epiphyses.

Most of the FGFR mutations causing human skeletal dysplasias are mis-sense, with a smaller number of splicing mutations, small insertions, and deletions. The mutations remain in -frame, and there are no non -sense or frame - shift mutations which might associate with loss – of - function. In contrast, mutations of the TWIST gene causing Saethre Chotzen syndrome are predominantly non - sense and 21 bp duplications; and are predicted, by analogy with experimental evidence in the analogous E47 transcription factor (Voronova and Baltimore, 1990) to cause functional loss. The Saethre Chotzen phenotype is also caused by the FGFR3 – P250R mutation, and has considerable phenotypic overlap with other phenotypes of this mutation, which implies that TWIST and FGFR occupy the same signalling pathway.
There is considerable evidence that FGFR mutations will convey functional gain upon the receptor, whilst TWIST mutations result in haplo-insufficiency. All the FGFR mutations described thus far are dominantly acting. The mutant protein acts in the cellular environment containing wild type allele and endogenous mechanisms for in built functional redundancy. Whereas the TWIST null - heterozygote mouse has a craniofacial and limb phenotype that is reminiscent of the human heterozygote, models of FGFR loss of function do not model human skeletal dysplasia. Studies in *Xenopus*, chicken and mouse, using dominant - negative receptor constructs expressed with tissue - specific promoters, imply non - redundant roles for FGFR in a variety of fundamental processes. The loss of function determined by a potential dominant - negative mechanism of FGFR mutants in human disease would therefore suggest in – utero lethality. Various 'knock – out' models demonstrate fgfr dose reduction and support this contention whilst demonstrating the fundamental role of fgfr - signalling which appears highly conserved throughout development. Disruption of the *Drosophila* homolog of fgfr1 indicates fundamental roles in branching morphogenesis and cell migration. The mutants, termed breathless (btl) have shortened tracheae with no branching, and failure of cell migration of the posterior glia (Klambt et al, 1992). Expression of a dominant - negative truncated fgfr1 protein in *Xenopus* induces failure of mesoderm formation, and defects in gastrulation and posterior development that are 'rescued' by over – expression of the wild – type receptor (Amaya et al, 1991). Later defects include loss of notochord and muscle morphogenesis (Amaya et al, 1993). Fgfr1 is also essential to early mammalian development. Mutant mouse embryos may gastrulate but began to display early growth defects. The nascent mesoderm of fgfr1 homozygous mutant embryos differentiates into diverse mesodermal subtypes, but with defective mesodermal patterning and failure to form somites (Yamaguchi et al, 1994). The role of fgfr1 in cell migration, axial organisation and patterning has been independently confirmed, in fgfr1 deficient mice embryos which failed to survive gastrulation but formed non – axial tissues and were severely growth retarded (Deng et al, 1994). A proportion of mouse embryos that survive gastrulation after injection with fgfr1- deficient embryonic stem cells display malformations of posterior structures at later stages of embryogenesis. There is limb bud malformation, partial duplication of the neural tube, tail distortion, and spina bifida caused by disorganised amplification of neural tissue in the posterior portion of the spinal cord (Deng et al, 1997); and deficiencies of neural tube and mesoderm development in homozygous fgfr1 mouse mutants are independently reported (Ciruna et al, 1997).

Targeted disruption of fgfr2 in the mouse model also donates embryonic lethality. Preimplantation development is normal until the blastocyst stage, and homozygous mutant
embryos fail a few hours after implantation, and at a random position in the uterine crypt (Arman et al, 1998). Murine fgfr2 is essential for chorioallantoic fusion and proliferation of trophoblast cells, and the mutants do not form limb buds, with a down - regulation of the expression of fgf8 and fgf10 in the presumptive limb ectoderm and underlying mesoderm respectively (Xu et al, 1998). The application of dominant - negative techniques allows the targeted investigation of fgfr loss - of - function in particular tissues in later stages of development. The introduction of a truncated fgfr2 - IglIIb isoform, with loss of the functional tyrosine kinase domain, targeted to murine lung epithelia under the control of a surfactant promoter results in a viable mouse pup with undifferentiated and unbranched epithelial tubes below the level of the trachea (Peters et al, 1994). Expression of a dominant - negative fgfr in murine skin, targeted to suprabasal keratinocytes using a keratin 10 promoter, results in epidermal hyper thickening and aberrant expression of keratin 6 (Werner et al, 1993). Such studies, whilst providing useful information about the role of FGFR signalling in various organ systems, do not model human FGFR1 and 2 - skeletal dysplasia syndromes, for which gain - of - function is therefore implied. The relative specificity of the site (non - random), and type (predominantly non - sense and in - frame) of clinical FGFR mutation corroborate this; however, the range of mutations across different functional domains of the receptor suggests heterogeneous mechanisms of receptor activation.

Mouse fgfr3 knock - out models survive, and have been relatively useful indicators of the role of vertebrate fgfr3 - signalling in skeletogenesis. Homozygous fgfr3 (-/-) mice exhibit kyphosis, scoliosis, crooked tails and curvature and overgrowth of the long bones and vertebrae as well as inner ear defects (Colvin et al, 1996; Deng et al, 1996). Heterozygote (fgfr+/-) mice are phenotypically normal, as are the fgfr3 -/- embryos at E12.5 to E18.5 (Deng et al, 1996). The vertebral and long bone epiphyses show an expansion of the zone of hypertrophic chondrocytes (in contrast to the contraction of the hypertrophic zone seen in the dwarfism dysplasias) with high level of PCNA positivity denoting proliferating cells, and alkaline phosphatase activity (denoting osteoblastic infiltration) (Deng et al, 1996). The calvarial bones at all ages are normal. Such observations are consistent with the role of wild - type FGFR3 as a negative regulator of chondrogenic ossification whilst playing a functionally redundant role in membranous ossification. Mutant FGFR3 functional gain might therefore be predicted to promote dyschondrogenesis by affecting the regulation of chondrocyte proliferation and hypertrophy, and this theory has been investigated in a number of mouse transgenic models (S-3.3). Such models allow the investigation of functional gain in FGFR signalling pathways, which, in conjunction with information from in - vitro systems, may yeild
useful information relevant to human clinical pathogenesis. These are discussed in clinical context in subsequent sections (S-3.1; S3.3)

1.4.1 Pathological FGFR mutations follow non-random 'themes'

The extensive crossover between FGFR gene mutation and syndromic craniofacial dysostosis phenotype has been alluded to in preceding sections. Many mutations in FGFR1 and FGFR2 generate phenotypic heterogeneity. Mutations in FGFR3 generate less heterogeneity of dysplastic dwarfism phenotype, yet specific mutations generate craniofacial dysostoses without dwarfism. Wilkie has described this group of mutations, which include some of the highest incidence germline mutations in the human genome (Bellus et al, 1995b;Moloney et al, 1996; Moloney et al, 1997), as 'a remarkable series in human genetics for genotype – phenotype correlations' (Wilkie, 1997). Despite the various complexities, human FGFR mutations follow non-random 'themes', and may be usefully divided into various groups according to their position within the highly conserved receptor structure, and/or the effect which they are predicted to impose upon receptor function in-vivo. In the subsequent sections, therefore, an attempt is made to group these mutations according to certain common features, by which some mutations may unavoidably fall into more than one class. An understanding of the mechanisms of human FGFR mutant function provides a basis for the studies described in Section 3.

1.4.2 Mutations that characterise paralogous positions in FGFR1, FGFR2 & FGFR3

1.4.2.1 Proline – Arginine substitutions in the IgII – IgIII linker

The FGFR proteins demonstrate a high degree of sequence homology, particularly within the IgII – loop, the linker sequence, and the amino terminal half of the IgIII – loop (Johnson and Williams, 1993; Plotnikov et al, 2000). Substitution of the conserved proline by arginine in the IgII – IgIII linker (Pro-252 in FGFR1) is common to the three FGFRs associated with human skeletal dysplasia (Bellus et al, 1996). The substitution of arginine introduces a bulky side chain amino acid and creates a change of orientation in the linker. The wild-type proline forms part of a conserved 'hydrophobic pocket' in the linker, which envelopes and enhances a hydrogen – bond between ligand FGF2 and the FGFR protein (Plotnikov et al, 2000). This is one of a series of such bonds between conserved linker sequence residues and residues on the ligand which when altered by specific mutagenesis reduce ligand affinity (Zhu
et al, 1997; Plotnikov et al, 2000). The P-R substitution is not encoded by any other nucleotide change, and is predicted to create aberrant interactions between ligand FGF and FGFR. A study of the affinity of the Apert linker - region for ligand indicates that both the Pro253Arg and the Ser252Trp linker - region mutations display selectively altered binding affinity (Anderson et al, 1998c). Although there is no difference between ligand association kinetics, the rate of dissociation of excess FGF2 from Apert mutants is less than wild - type. The affinity of FGF2 for Ser252Trp is greater than that for Pro253Arg, which is greater than that for wild - type. The effect is quantitatively less for FGF1, and no different from wild - type with FGF4, thus providing functional evidence that the hydrogen - bonds formed by the FGFR linker sequence and ligand sequences are exquisitely sensitive (Plotnikov et al, 2000). Recent evidence suggests that the Apert Ser252Trp and Pro253Arg mutations in FGFR2 cause loss of ligand specificity of the IgIIIc/BEK and IgIIib/FGFR isoforms for FGF2, and FGF7 or FGF10, respectively (Yu et al, 2000), further illustrating the functional sensitivity of the linker - sequence. The substitution of specific residues in the linker has specific phenotypic consequences (S-1.3.2.2), which are thus likely to reflect specific ligand - receptor kinetics. Given the sequence conservation, it seems likely that the P-R mutations of FGFR1 and FGFR3 will have similar functional effect upon ligand kinetics, which will be conducted via both IgIIib and IgIIIc forms of the receptor protein. (The Ser252 substitution is not observed in FGFR1, and this may be because the homologous FGFR1 serine residue is encoded by a different sequence - TCC, and not TCG as in FGFR2; commonly mutated to TGG; Ser252Trp.)

The functional effect of the Pro252Arg mutation in FGFR1 (Pfeiffer syndrome) has been modelled in Xenopus. As might be predicted from the studies of Anderson et al (1998), the analogous P160R mutation in Xenopus (xfgfr1) consistently binds more radiolabelled FGF1 and FGF2 than the wild - type receptor. However, the xfgfr1 - P160R mutant, in comparison to wild - type receptor, failed to show increases in tyrosine phosphorylation or elongation of animal pole ectoderm, despite such increases displayed by Xenopus models of other classes of activating FGFR mutation (Neilson and Friesel, 1996). This raises the possibility that the ligand - dependent mutations of the linker - region of the extracellular domain of the FGFRs may be quantitatively less activating than certain cysteine altering mutations (S-1.4.3) or transmembrane mutations (S-1.4.2, S-1.4.4) examined in the same system. The different phenotypes of the linker - region P-R mutations in each of FGFR1, 2, and 3 (Pfeiffer; Apert; 'non - syndromic' craniosynostosis, respectively) will, therefore, reflect the specifics of ligand - receptor activation as well as their relative bioavailability in human skeletal development (S-3).
1.4.2.2 Paralogous transmembrane region mutations in FGFR2 and FGFR3

Glycine — Arginine mis-sense mutations

Paralogous glycine — to — arginine mutations in the transmembrane regions of FGFR2 and FGFR3 cause very different syndromes. Glycine is substituted by arginine at Gly384 in FGFR2 to cause an unclassified craniosynostosis (Gly384Arg), featuring coronal and sagittal synostosis and choanal stenosis (Pulleyn et al, 1996). By contrast, the Gly380Arg mutation in FGFR3 causes the relatively invariable achondroplasia phenotype with one of the highest mutation rates in the human genome. The neutral glycine residue is replaced by a basic, bulky, arginine in each case, and given the sequence homology between the receptors, could be expected to affect the same functional change. It was originally thought that the disruption would hinder the movement of the receptor through the membrane, and also disrupt receptor dimerisation. Given also that the net physiological and developmental effect of the mutation is inhibition of chondrogenesis, it was an attractive idea that the mutant receptor acted in a dominant - negative manner to disrupt normal cartilage differentiation (Rousseau et al, 1994). However, the introduction of a charged, basic, molecule into the TM region is analogous to the activating Val664Glu mutation in rat Neu, which encodes a membrane bound receptor tyrosine kinase (Bargmann et al, 1986;Bargmann and Weinberg, 1988), the activation of which is proposed to involve hydrogen bond formation and stabilisation of an active dimer (Sternberg and Gullick, 1989;Webster and Donoghue, 1996).

Receptor chimeras of Neu with the transmembrane domain of wild — type or achondroplasia mutant FGFR3 were subsequently observed to generate different tyrosine kinase activity via the Neu - TK domain in NIH3T3 - fibroblast cell lines (Webster and Donoghue, 1996). The Gly380Arg construct showed a ligand - independent, twenty — five fold increase in kinase activity over the wild — type, and successfully transformed the NIH3T3 cells, an effect which was corroborated in full — length FGFR3. The Gly380Arg mutation, in a Xenopus two — Ig — loop receptor model, significantly increased receptor tyrosine phosphorylation over control, with constitutive induction of mesodermal muscle — specific actin transcript, and the elongation of Xenopus cap mesoderm (Neilson and Friesel, 1996). Receptor chimeras of the murine fgfr3 - Gly380Arg receptor with a substituted fgfr1 - tyrosine — kinase domain, did not constitutively stimulate kinase activity however, despite being moderately mitogenic in a BaF3 transfected cell line, (Naski et al, 1996). This was in contrast to the strongly mitogenic Arg248Cys mutation, modelling thanatophoric dysplasia type 1, which did drive constitutive kinase activity (Naski et al, 1996).
The sequence homology of the TM region of FGFR2 and FGFR3 suggests that the Gly—Arg mis-sense substitution will have the same functional effect in the two receptors, which will be enacted through all the natural extracellular—domain isoforms of each. Both paralogous mutations generate relatively mild phenotypes within the expected range, achondroplasia compared to severe thanatophoric dysplasia; and mild craniosynostosis compared to the Crouzon—Pfeiffer phenotypes. In each case the phenotype may reflect the relatively weak mutant receptor dimerisation, via putative hydrogen bonds created by the arginine substitution (Webster and Donoghue, 1996). In comparison, the mutations causing thanatophoric dysplasia (Webster et al, 1996;Naski et al, 1996), and Crouzon—type phenotypes (Neilson and Friesel, 1995;Neilson and Friesel, 1996;Robertson et al, 1998) are highly activating. Graded activation of the receptor by classes of activating receptor may thus reflect severity of phenotype in both human mutant FGFR2 and FGFR3 syndromes. Furthermore, the difference between the craniofacial phenotypes of achondroplasia (FGFR3) and craniosynostosis (FGFR2) will reflect, in part, the differential expression of the receptors in human craniofacial development (S-J).

Cysteine substitutions in the juxtamembranous—transmembrane domain

Cysteine substitutions in the transmembrane regions of FGFR2 and FGFR3 also result in different skeletal dysplasia phenotypes. The Ser372Cys and Tyr375Cys mutations in FGFR2 result in Beare—Stevenson cutis gyrata, whereas the analogous Tyr373Cys and Gly375Cys mutations cause thanatophoric dysplasia type 1 and achondroplasia respectively. The introduction of cysteine residues to the transmembrane domain has been shown to be activating in independent systems. The Gly375Cys mutation in FGFR3, the less common of the two achondroplasia mutations, is activating in a chimera with the extracellular domain of the platelet derived growth factor receptor (PDGF) (Thompson et al, 1997). Chimeras of the PDGF extracellular domain and the transmembrane and tyrosine kinase domains of either wild—type human FGFR3 or G375C—FGFR3, activate neurite differentiation in a stable PC12 cell line (which naturally lacks the PDGF receptor) on stimulation with PDGF. Neurite differentiation was associated with PDGF—dependent receptor auto—phosphorylation and the phosphorylation of the intracellular signalling molecules MAPK, phospholipase C, and Shc. Nerve growth factor elicits the same cascade in the induction of the differentiation of natural PC12 cells (Thompson et al, 1997). The G375C—FGFR3 chimera, in particular, was more rapidly responsive to PDGF ligand with less sustained MAPK response, suggesting that the achondroplasia mutation confers ‘priming’ of the construct to activation by reduced concentration of ligand.
The Tyr373Cys mutation in thanatophoric dysplasia type 1 is also shown to be activating, causing dimerisation and increased kinase activity in a COS cell line (D'Avis et al, 1998). It is interesting that the two FGFR3 mutations, both cysteine introducing at positions 373 and 375 should result in similar phenotypes but of different severity, thanatophoric dysplasia type 1 and achondroplasia respectively. It may be that the Gly373Cys mutation, in most closely abutting the membrane, is relatively restricted in its ability to form free disulphide bonds with other receptor monomers. In contrast the two analogous mutations Ser372Cys and Tyr375Cys in FGFR2 both result in Beare – Stevenson cutis gyrata from similar positions in the juxtamembranous – transmembrane region. The relative rarity of these FGFR2 mutants denies definitive phenotypic comparison between them, however, the skeletal phenotype of the condition is severe, and reflects the activating nature of the mutations. It is intriguing that the FGFR2 phenotype has severe cutaneous manifestations, probably via the activation of the IgIIIb isoform, yet the FGFR3 phenotypes lack cutaneous manifestations despite the expression of FGFR3 – IgIIIb in skin (§1.2.2). Severe cranial phenotypes are seen in both sets of phenotypes in the form of clover-leaf skull (Beare – Stevenson cutis gyrata, and thanatophoric dysplasia), and reflect the potential expression of FGFR2 and FGFR3 in human craniofacial development (§3).

1.4.3 FGFR mutations which alter the functional availability of cysteine residues in the extracellular domain

The introduction or replacement of cysteine residues is a common theme of mutations in the extracellular domain of FGFR2 and FGFR3. The Cys278 and Cys342 in FGFR2 are very commonly mutated (Park et al, 1995b; Passos-Bueno et al, 1999), and the non – specificity of the mis – sense replacement predicts that functional gain is delivered by the removal of the cysteine residue. Both these positions are crucial to the structure of the IgIII – loop of the protein. The cysteine residues form intra – molecular covalent disulphide bonds and are thus integral to the structural integrity of the protein; a feature which is common to members of the immunoglobulin superfamily which share the structure (Jones et al, 1993). The removal of one of the cysteine residues would be predicted to leave unfurl the IgIII – loop and leave the partner residue free to form intra – molecular covalent bonds. Candidate molecules for such bonds include other mutant alleles within the membrane, and create the opportunity for mutant receptor dimerisation in the absence of ligand. The Cys278 and Cys342 residues are highly conserved between the members of the FGFR family, and mutations in the equivalent positions in FGFR1 have been shown to abolish ligand binding (Hou et al, 1992). The loss of ligand – binding by such mutants suggests that the structural integrity of the IgIII domain is
functionally limiting. The interface between ligand FGF and the IgIII loop is mostly formed from polar hydrogen bonds, which are sensitive to amino acid substitution by site-directed mutagenesis in the ligand (Zhu et al, 1997; Plotnikov et al, 2000). These bonds depend upon the polarity of specific amino acids in FGFR2, many of which are directly or indirectly disrupted by the IgIII mutations observed in the Crouzon–Pfeiffer group of syndromes. For example, a conserved Gln-285 in FGFR2 creates a bond with Glu-96 in FGF2, which would be prone to disruption by mutations at positions 276, 278, 289, and 290 in FGFR2. Furthermore, the Asp-321 and Ser-347 residues in FGFR2, responsible for 3 and 2 hydrogen bonds respectively in binding with FGF2, are directly mutated to alanine and cysteine in Pfeiffer and Crouzon cases, respectively (Passos-Bueno et al, 1999). These data provide the molecular basis for observations that certain FGFR2 IgIII–mutations in the Crouzon–Pfeiffer group of syndromes abrogate ligand binding and are constitutively activating (Neilson and Friesel, 1995; Neilson and Friesel, 1996).

Constitutive activation of the receptor by mutations that directly or indirectly alter the functional availability of cysteine has been demonstrated in a number of systems. The Cys342Tyr mutation, modelled in Xenopus, induces ligand independent FGFR–dimerisation, tyrosine kinase activity, expression of the mesodermal markers Xbra and muscle alpha actin; and the induction of mesodermal elongation (Neilson and Friesel, 1995). The response is dose–dependent upon the amount of mutant receptor micro–injected into the Xenopus blastomere, and provides a possible explanation for the range of phenotype severity in these syndromes, which may reflect the degree of activation established by each individual mutation. Further studies in the same system examine the Xenopus analogues of the common clinical mutation FGFR2 Cys-278; an xfgfr1-Cys249Tyr mutation which also results in an unpaired cysteine; and a double FGFR2 mutation that disrupts the IgIII–loop without the generation of an unpaired cysteine residue (xfgfr2-c268F/C332Y). All three mutations abrogated the binding of radiolabelled FGF1 and FGF2 and resulted in tyrosine kinase activation; however, only the mutations creating an unpaired Cys resulted in mesodermal induction (Neilson and Friesel, 1996). Similarly, only the mutations resulting in an unpaired Cys residue migrated in electropheresis studies as dimeric forms, suggesting that it is the availability of Cys that potentiates ligand–independent dimerisation and activation of the mutant receptor (Neilson and Friesel, 1996). Similar negative results for the activating potential of ‘non–free–cysteine creating’ double mutations are independently reported (Robertson et al, 1998).

The FGFR2 - Cys342Tyr and has been modelled in a chimera of the Neu–transmembrane and tyrosine kinase domains. The mutant extracellular domain of the FGFR2 was able to
induce signal transduction via the Neu chimera and induce focus formation in the NIH3T3 fibroblast cell line. Similar results were obtained for the Cys342Arg and Cys342Ser mutations, as well as the Tyr340His, and Ser254Cys mutations (Galvin et al, 1996). All of the mutations induced tyrosine kinase activity and formed disulphide bonded dimers, thus raising the question that non – cysteine mutations causing the Crouzon – Pfeiffer phenotypes can modulate similar effects to those directly creating free cysteine residues. The Trp290Gly and Thr341Pro mutations cause Crouzon (Park et al, 1995b) and Pfeiffer (Rutland et al, 1995) syndromes, respectively, and each neighbours a conserved IgIII – loop dependent cysteine residue (Cys278 & Cys342) in the 3D structure (Plotnikov et al, 2000). When expressed as FGFR2/Neu chimeras, both mutations cause transformation of NIH3T3 cells compared to the mis-sense substitutions Cys278Phe and Cys342Tyr acting as positive controls (Robertson et al, 1998). Transforming activity was lost when these non – cysteine mutations were combined with double mutation constructs replacing the Cys278 and Cys342 with alanine, thereby suggesting that the non – cysteine mutations confer activation by virtue of their intact cysteine neighbour. In addition, as full - length FGFR2 mutants, Trp290Gly and Thr341Pro induced receptor dimerisation and elevated levels of tyrosine kinase activity, which was lost in the absence of an intact cysteine neighbour (Robertson et al, 1998). The data suggests that clinical mutations, which, by their proximity to conserved cysteine residues disrupt the IgIII – loop Cys=Cys disulphide bond, activate the receptor when inter – molecular disulphide bonds are created by default (Plotnikov et al, 2000). Such a mechanism might explain the observation that the FGFR2 - Ser267Pro Crouzon - mutant receptors migrate as a dimer in polyacrylamide gel electrophoresis studies, despite abrogation of FGF2 ligand binding (Anderson et al, 1998c).

1.4.3.1 Phenotypic diversity generated by cysteine – related, and other mutations in FGFR2

Studies in Xenopus blastomeres indicate that the induction of mesoderm by the micro – injection of FGFR2 – Cys342Tyr analogue is dose dependent (Neilson and Friesel, 1995). It may be that the range of craniofacial dysostosis phenotypes observed clinically reflects not only epigenetic influences and the effect of modifier genes, but also the degree of functional gain. Epigenetic influences, such as the effect of the growing brain on the neurocranium, or influences in – utero may influence the range of phenotypes generated by specific mutations.

Alternatively, Rutland et al (1995) cite the example of the variability of phenotypes generated by the Asp178Asn mutation in the prion protein gene as a potential model mechanism in
generating phenotypic diversity in craniofacial dysostosis. The Asp178Asn mutation results in familial fatal insomnia when the amino acid at position 129 is methionine, and in Creutzfeldt-Jakob disease when the amino acid at position 129 is valine (Rutland et al, 1995). Such, as yet unidentified modifier sequences may exist that influence the Crouzon – Pfeiffer group of phenotypes generated by the extracellular – domain mutations in FGFR2 (Passos-Bueno et al, 1999). The variable genetic background of the individual may explain this phenomenon and others such as the variability of phenotype in the original FGFR2 – A334G Jackson – Weiss kindred (Winter and Reardon, 1996), the phenotypes from the splicing mutation G1044A at codon 344 of FGFR2 (Steinberger et al, 1996), and the FGFR3 – P250R group of phenotypes.

1.4.4 Amino acid substitutions with sensitive effects upon the phenotype

1.4.4.1 Linker – region substitutions in FGFR2 and FGFR3

The degree of conservation of the IgII – IgIII linker – region sequence, and the demonstration of the exquisite specificity and functional sensitivity of the region to disruption (Anderson et al, 1998c; Plotnikov et al, 2000), provides a basis for the observation that the phenotypes generated by mutations in the linker are highly specific. Ser252Trp and Ser252Phe result in Apert syndrome (S-1.3.2.2), whereas Ser252Leu generates both mild Crouzon and normal phenotypes in the same family (Oldridge et al, 1997). The Ser252Leu mutation demonstrates normal FGF dissociation kinetics under experimental conditions compared to the selective increased ligand affinity displayed by Ser252Trp and Pro253Arg (Anderson et al, 1998c). It may be that the Ser252Leu mutation facilitates the genetic background for epigenetic influences to propagate a mild craniofacial dysostosis phenotype. A similar theory has been advanced to explain unicoronal synostosis resulting from the FGFR2 - Ala315Ser mutation and breech presentation (Johnson et al, 2000). Further evidence of the genotype – phenotype specificity demonstrated by the linker region mis – sense substitutions is given by the rare double mutation Ser252Phe and Pro253Ser, which generates a Pfeiffer phenotypic variant, with mild craniosynostosis, broad thumbs and toes, digital joint anomalies but minimal syndactyly. It may be that certain 'Apert' phenotypic features which demonstrate a range of expressivity, such as syndactyly or cleft palate (in only 70% of Apert cases, S-1.3.3.2), become manifest under variable conditions of specific ligand – mutant receptor kinetics (S-3.4, S-4).
The sensitivity of the phenotype to specific linker–region mutations is demonstrated by the Arg248Cys mutation and the Pro250Arg neighbouring substitution in FGFR3, which cause the mutually exclusive phenotypes of thanatophoric dysplasia (TD) I (5.1.3.3.2) and 'Muenke' non–syndromic craniosynostosis (5.1.3.3.1), respectively. The FGFR3 - Pro250Arg, as discussed (1.4.2), probably causes a selective increase in ligand affinity and is moderately activating by analogy with the *Xenopus xfgfr1* mutant (Neilson and Friesel, 1996). In contrast, the Arg248Cys mutation, modelled in the BaF3 transfected cell–line, is highly activating; demonstrating maximal mitogenicity against positive and negative controls (Naski et al, 1996). The introduction of the cysteine confers constitutive dimerisation and tyrosine phosphorylation, which can be further stimulated by ligand FGF1 to higher levels of kinase activity (Naski et al, 1996). The substitution of the cysteine residue appears to be critical to the activating effect of the mutation. FGFR3 - Arg248Cys, and the neighbouring Ser249Cys mutation which is also causative to thanatophoric dysplasia type 1, are dimerising and comparatively highly activating as Neu/FGFR3 chimeras in NIH3T3 fibroblasts, which are driven to focus formation by the mutant constructs (D’Avis et al, 1998). In contrast, the control Arg248Ala mutation and wild–type FGFR3/Neu chimeras were non–dimerising and non–transforming. Human FGFR mutations which alter the functional availability of cysteine residues are considered further in (1.4.3).

The differential activation conferred by the two mutations has a parallel in their phenotypes. TD1, the result of the constitutive Arg248Cys mutation, has a particularly severe skeletogenic phenotype, whereas the Pro250Arg, presumptively a ligand–dependent mutation, generates a variable phenotype with no dwarfism. The different craniofacial phenotypes of the two mutations may reflect their differential activation and the expression of FGFR3 in human craniofacial development (5.3.3).

### 1.4.4.2 Transmembrane substitutions in FGFR3; Crouzon – acanthosis nigricans and achondroplasia

Neighbouring substitutions in FGFR3 result in very different phenotypes. The Gly380Arg, Gly375Cys substitutions, which are predicted to cause activation by dimerisation via hydrogen bonds and disulphide bonds, respectively, give rise to achondroplasia. The neighbouring Ala391Glu mutation, however, causes Crouzon syndrome with acanthosis nigricans (Cr-AN). The FGFR3 - Ala391Glu mutation is activating in an FGFR3 – Neu chimera system (Webster and Donoghue, 1997a), yet surprisingly does not feature a dysplastic long bone phenotype. The relative degree of activation of Gly380 and Gly375 compared to Ala391,
which is buried deeper within the membrane, will reflect the specific mis-sense substitution as well as their relative 3-dimensional position. Furthermore, the association of cutaneous pathology with craniosynostosis also features in the Beare - Stevenson cutis gyrata syndrome, and it may be that the Cr-AN mutation signals by dimerisation with FGFR2 isoforms in human skin and craniofacial development (S-4).

1.4.4.3 Tyrosine kinase substitutions in FGFR3 - a range of dwarfism dysplasias from a range of activating mutations

The FGFR3 Lys650 codon is located within a critical region of the intracellular domain in the tyrosine kinase activation loop. Two mis-sense mutations generate three skeletal dysplasias. The thanatophoric dysplasia type II (TDII) is exclusively caused by a Lys650Glu substitution (S-1.3.3.2), whereas the Lys650Met mutation causes thanatophoric dysplasia type I (TD1) and SADDAN (severe achondroplasia with developmental delay and acanthosis nigricans). Furthermore, Lys650Asn and Lys650Gln cause the hypochondroplasia (HCH) phenotype (Bellus et al, 2000). The Lys650 site thus appears to be a relative 'hotspot' in the protein for mis-sense substitutions generating different phenotypes.

The TDII Lys650Glu mutation is more activating in a FGFR3 - induced transcription - assay than the TD1 linker - region (Cys-introducing) mutations (D'Avis et al, 1998), but mutant receptor activation does not result from covalent dimerisation (Naski et al, 1996; D'Avis et al, 1998). In concert with this, the truncated Lys650Glu tyrosine kinase domain is activating at the plasma membrane in the absence of the extracellular moiety (Webster and Donoghue, 1997b). Furthermore, whilst the two - loop Xenopus fgfr1 analogue of human FGFR3 - Lys650Glu causes constitutive activation, and molecular and morphological induction of mesoderm; this effect cannot be blocked by a dominant - negative receptor lacking the TK domain (Neilson and Friesel, 1996). This suggests that the mutation very specifically alters the tyrosine - kinase domain independently of the rest of the receptor. Furthermore, the Lys650Glu mutation is mitogenic when transfected into a BaF3 cell line as the full - length receptor, and cellular proliferation can be 'over - driven' in the presence of ligand FGF1 (Naski et al, 1996). This assay provides a phenotype - genotype correlation in that the TDII Lys650Glu mutation has the maximal activating potential of a group comprising the achondroplasia mutation (Gly380Arg) and a TD1 mutation (Arg248Cys); being partly ligand - independent, but capable of further ligand drive and mitogenic effect. The degree of constitutive tyrosine autophosphorylation of the Lys650Glu mutant receptor has been shown to be mis-sense specific, such that the Gln mutation (causing the less severe HCH) is less
activating (Webster et al., 1996; Bellus et al., 2000). These data show that the apparent linear severity of phenotype displayed by hypochondroplasia, achondroplasia and thanatophoric dysplasia has a molecular correlate in the degree of activation bestowed by each type of mutation.

The mechanism of activation of the tyrosine kinase mis-sense mutations reflects the crystal structure (Mohammadi et al., 1996). The FGFR3 - Lys650Glu mutation lies within the kinase loop and appears to be directly activating, by mimicking the activating conformational changes that normally accompany autophosphorylation at conserved Tyr residues within the activation loop (Webster et al., 1996). The degree of activation reflects the specific substitution at that site, such that the Lys650Asn mutation causes the less severe hypochondroplasia phenotype, whereas the Lys650Met mutation, which has a threefold greater kinase activity than Lys650Glu (Wilcox et al., 1998; Tavormina et al., 1999) causes the severe SADDAN phenotype.

1.4.5 FGFR mutations which create stable splice - variants

Several mutations causing craniosynostosis phenotypes are proposed to cause aberrant splicing of FGFR2 (Reardon et al., 1994; Li et al., 1995; Schell et al., 1995; Lajeunie et al., 1995a; Meyers et al., 1996; Steinberger et al., 1996). These mutations are projected to yield various stable transcript RNAs and stable altered receptor proteins. Similar mutations add amino acid sequences to the carboxy – terminal of FGFR3 in thanatophoric dysplasia (Rousseau et al., 1995).

An interesting set of splicing mutations may correlate with the pathogenesis of variable syndactyly in the limb phenotype of FGFR2 – craniofacial dysostosis syndromes. These mutations are Alu insertions upstream and within in exon 9 in Apert syndrome (Oldridge et al., 1999), and either 1119 – 2A – G at the 3′ splice site upstream of exon 9 in both atypical Apert (Passos-Bueno et al., 1997) and Pfeiffer syndrome (Schell et al., 1995; Lajeunie et al., 1995a; Oldridge et al., 1999), or the 1119 – 2A-T substitution of the same nucleotide in Pfeiffer syndrome (Anderson et al., 1998a; Oldridge et al., 1999). In the Pfeiffer cases, the limb phenotypes are noted to be particularly severe; and the 1119 – 2A – G mutation has, in fact, also been ascribed to the Apert phenotype. Oldridge et al (1999) correlated the site of these mutations with the probability that they create stable splice variants of FGFR2 transcripts, and demonstrated in 3 patients an upregulation of the IgIIIb/KGFR mRNA in fibroblast cell lines derived from skin. The authors’ attempt to quantify the relative upregulation of KGFR
as a percentage of the expression of both IgIIIc/BEK and KGFR can be criticised, and the resulting conclusions cannot be applied unchallenged to the \textit{in vivo} model. The quantification assumes an equal level of BEK expression in the cultured fibroblasts of all 3 patients. The method also assumes that the fibroblasts in cultured cell lines share the \textit{in vivo} phenotype. Furthermore, the study was applied to only 3 non-age matched patients against 3 non-uniform controls. However, within these parameters, the preliminary evidence indicates that a number of aberrant splice variants, including stable KGFR mRNA were ectopically upregulated in fibroblasts of both Apert and Pfeiffer patients, in proportion to the severity of the digital phenotype (Oldridge et al, 1999). The ‘dose – dependency’ of aspects of the FGFR2-related phenotypes upon KGFR suggests a novel pathogenic mechanism (S-3.1; S-3.3).
1.5 Aims of this thesis

The craniofacial dysostoses and human chondroskeletal dysplasias exemplify the theme of phenotypic crossover and genetic heterogeneity within a common gene family. As a result of clinical genetic studies, members of the FGFR gene family have been recognised to play a major role in human membranous and endochondral ossification. The functional effects of FGFR signalling have been studied in animal models in vitro and in vivo. The aim of the studies described in subsequent sections of this thesis is to present an analysis of the expression of FGFR homologues and the clinically significant isoforms of FGFR2 in human craniofacial development in situ.

Studies have therefore been undertaken to:

1. Describe the expression of FGFR 1, 2, and 3; and the FGF ligand proteins 2, 4, and 7 in normal endochondral and membranous ossification during human craniofacial development
2. Assess the differential expression of FGFR homologues in human infant calvariae, and investigate the effect of human infant sagittal craniosynostosis upon the regulation of sutural FGFR expression in vivo
3. Assess the effect of mutant FGFR gain-of-function upon FGFR expression in human membranous ossification in an Apert (P253R), and Pfeiffer (C278F) fetus
4. Describe the expression of FGFR1, 2, and 3; and the ligand proteins FGF2, FGF4, and FGF7 in palatal epithelia during human palatal shelf fusion

The purpose of these investigations in human tissue is to provide data that might then be used to validate and qualify that of various models, including the mouse. Studies in transgenic mice will undoubtedly provide a platform for unravelling the pathogenesis of craniosynostosis and the wider phenotype of these syndromes. It is hoped that these descriptive studies in human tissue in situ, in which experimental methodology is not appropriate, will provide a comparative data—set by which to evaluate experimental manipulations and emerging therapeutic interventions in the transgenic mouse.
Section 1 - Figures and Legends

Figure 1.1-1

Unicoronal synostosis with ridged palpable coronal suture on the right side in conjunction with a flat forehead. There is contralateral frontal bossing. There is facial scoliosis, with a raised right orbit (vertical dystopia). The ears, nose, and mouth are normal, with no midface retrusion or airway obstruction. The hands are normal.

Figure 1.1-2

Bicoronal synostosis. There is symmetrical frontal bossing and supraorbital ridging. The face is symmetrical, with no retrusion or scoliosis. The ears, mouth and nose are symmetrical. The hands are normal.

Figure 1.2-1

Schematic of FGFR structure and interactions (simplified). The FGFR monomer consists of an extracellular domain, characterised by two or three immunoglobulin – like loops (above transverse white line = cell membrane). The Ig – like loops are maintained by consistently positioned, covalent, disulphide bonds (C-C), which also define the spatial relationship of the functional receptor dimer, and the FGF - ligand binding affinity of the receptors. Between the first and second Ig – like loops lies an 'acid - box' sequence of amino – acids (AB). The trans – membrane domain (TM) traverses the cell membrane, and is in continuity with a split tyrosine kinase domain (TK) by means of the juxtamembranous region of amino acid sequence.

Various human pathogenic FGFR mutations influence receptor dimerisation to cause ligand independent functional gain. Mechanisms include the promotion of intermolecular covalent bonds (C-C in green) or hydrogen bonds (H-H) which promote dimerisation. Mutations in the TK – domain may facilitate autophosphorylation (P) and promote constitutive gain of function (see S-1.4 for details).
Plagiocephaly; unicoronal synostosis phenotype
Brachycephaly; Bicoronal synostosis phenotype
Mutated FGFRs

Normal FGFRs

Mutated FGFRs
Figure 1.3-1
Craniofacial features of the Pfeiffer syndrome include turribrachycephaly, with a high flat forehead, short skull base and shallow posterior fossa. The ears are low set. The midface is retruded with exophthalmos and lagophthalmos (see text). There is mild hypertelorism and a mild antimongoloid cant to the face.

Figure 1.3-2
Pfeiffer syndrome – ‘clover – leaf’ variant. There is a trilobar skull with pan – sutural synostosis. The ears are low – set. There is midface retrusion with hypertelorism, and exophthalmos and lagophthalmos. Anti – mongoloid cant of the face is not severe in this example.

Figure 1.3-3
The craniofacial features of Apert syndrome. There is a high slanting forehead with supraorbital retrusion and pterional indrawing. There is marked hypertelorism (not easily appreciable in lateral view). The ears are low – set and there is a shortened skull base and shallow posterior fossa. The nose is ‘beaked’ and the mouth is shaped in a ‘trapezoidal’ manner, which reflects the anterior open bite beneath. There is midface retrusion and shallow orbit, causing exophthalmos and lagophthalmos. The hand, seen in partial, lateral, view; displays a complex complete acrosyndactyly.

Figure 1.3-4
The Apert Hand, classification of Upton. For details; see text (S-1.3.2.2).

Figure 1.3-5
The craniofacial features of Crouzon syndrome include a ridged bicornal synostosis and bossed forehead. The sagittal and lambdoid sutures may also be synostotic, and the high sloping turribrachycephalic forehead is an uncommon feature. The midface is retruded and there is exophthalmos and lagophthalmos. Hypertelorism, if present, is mild. The ears are normal or mildly low set, and the open mouth posture results from a restricted nasopharynx and airway compromise.
Pfeiffer Craniofacial phenotype
Pfeiffer Clover - leaf skull
Apert Craniofacial Phenotype
Crouzon Craniofacial phenotype
2. Materials & Methods

2.1 Materials

2.1.1. Human Foetal & Embryonic tissue

Craniofacial tissues from a total of 6 normal human embryo/fetuses were used for the studies described in this thesis. In addition, tissue was obtained from an Apert 14 week fetus, and a 26 week fetus with Pfeiffer syndrome.

Normal Human Embryo Tissue

Embryos were collected under the aegis of the MRC/Wellcome Trust Human Developmental Biology Resource and according to the Polkinghorne guidelines (Polkinghorne et al, 1989). All such tissue was obtained at social termination of pregnancy by either the RU486 or surgical method and introduced immediately into media at 4 °C. The embryos used in these studies ranged from age 8 to 14 weeks gestation, staged according to external morphology.

After staging with the aid of stereomicroscopy, a dissection of the cranioskeletal and midface tissues in tissue media was undertaken, so as to preserve the integrity of the head and face from skull base to vertex. Many of the embryos initially examined under the microscope had undergone surgical trauma and were subsequently excluded.

The embryo material was fixed for 24 – 36 hours in 4% PFA, and then progressively dehydrated in increasing concentrations of ethanol/PBS solution (30%, 50%, 70% for 30 minutes each) to 70% ethanol at 4 °C.

The fixed and dehydrated normal human embryo tissue was then embedded in 70 °C wax, according to the protocols in Appendix A, and stored at 4 °C prior to sectioning at 6 μm onto TESPA treated slides as described in Appendix B.
**Apert Foetal Tissue**

Apert tissue was obtained from surgical termination of the 14 week pregnancy of an Apert mother, after molecular diagnosis from tissue obtained at chorionic villus sampling (FGFR2 – Pro253Arg mutation).

Calvarial tissue was immediately transferred into tissue medium for transfer, and then into 4% PFA at 4 °C for fixation over 36 hours. The tissue was then progressively dehydrated in increasing concentrations of ethanol/PBS solution (30%, 50%, 70% for 30 minutes each) to 70% ethanol at 4 °C.

The stored samples were then embedded in wax block as described in Appendix A, and sectioned to 6 μm onto TESPA subbed slides as described in Appendix B.

**Pfeiffer Foetal Tissue**

Pfeiffer tissue was obtained from the medical termination of a 27 - week Pfeiffer foetus. The diagnosis of this phenotype, arising from a de novo mutation, had been made on the morphological criteria of abnormal skull shape secondary to craniosynostosis, detected by transabdominal ultrasonography at 26 weeks.

The termination was performed by transabdominal transuterine potassium chloride injection to achieve circulatory arrest in the foetus, which was delivered as a stillbirth 18 hours later. There was therefore an extended warm ischaemic time prior to transfer for postmortem examination and fixation of the calvarial tissue used in these studies.

At postmortem, the diagnosis of Pfeiffer syndrome was confirmed on morphological grounds. The skull shape exhibited turribrachycephaly, resulting from coronal, lambdoidal and sagittal synostosis. There was midface retrusion, with proptosis and choanal stenosis. There was no cleft palate evident. There was an obvious digital phenotype, with flattening of the halluces, but no soft tissue syndactyly. Radio - ulnar synostosis was also evident with a fixed flexion deformity of both elbows. Molecular diagnosis was subsequently obtained, and confirmed a Cys278Phe substitution in FGFR2.
Calvarial sutures were harvested and fixed in 4% PFA at 4 °C for 10 days, with sequential
daily changes of fixative. By 27 weeks of gestational development, the calvariae were
appropriately calcified, and had to be decalcified prior to embedding and sectioning, as
indicated below (S-2.1.5). Data from the 27 - week sagittal suture primordia is included in this
report (S-3.2).

2.1.2 Postnatal Suture Material

Human

Redundant tissue samples were collected intraoperatively after local ethical committee
approval. Samples of suture, cranial bone, and periosteum were harvested en bloc with surgical
bone cutters and saws from patients with syndromic craniofacial dysostosis and non -
syndromic craniosynostosis; undergoing cranioplasty for vault expansion or cosmesis. A total
of 16 infant suture samples were collected, of which data from six sagittal sutures, two
coronal sutures, and a lambdoid suture is reported (S-3.1).

The samples were washed with isotonic saline, placed in 4% ice cold PFA, then transferred to
4 °C for variable time periods of up to 8 days with frequent changes to fresh fixative.

Post - fixed postnatal human calvariae were densely calcified, and required extensive
decalciﬁcation as indicated below (S-2.1.3).

Mouse

Mouse calvarial tissue was used as a means of testing and establishing experimental protocols
for the preparation and experimental use of calcified tissues.

CD1 adult female mice were sacrificed and the intact cranial vaults dissected free intact. These
intact crania were washed with isotonic saline and placed in 4% ice cold PFA, then
transferred to 4 °C for 72 hrs.
2.1.3 Dehydration, decalcification, and wax embedding of calcified human and mouse calvariae

After adequate fixation, the calvarial/sutural samples were progressively dehydrated in increasing concentrations of ethanol/PBS solution (30%, 50%, 70% for 30 minutes each) to 70% ethanol at 4 °C.

Mouse samples were then decalcified in 50% 0.5 molar EDTA:50% PBS for variable time periods until suitable for section at the microtome. Human samples were decalcified in 0.5 molar EDTA solution at 37 °C for variable time periods with frequent changes to fresh solution.

Decalcification of this postnatal calcified material proved to be a difficult technical exercise in balancing adequate demineralisation for successful sectioning and in-situ analysis with preservation of tissue architecture and molecular integrity. The development of the protocols for decalcification is discussed in Section 2.2.6.

The samples were then further dehydrated to dryness, and embedded according to the protocols in appendix A, prior to sectioning to 6 μm onto TESPA subbed slides as described in Appendix B.

2.2 Experimental Methods

2.2.1. Introduction

An understanding of the expression patterns of the FGFR genes within the human craniofacial skeleton is an essential prerequisite to understanding the relationship between genotype and phenotype in syndromic craniofacial dysostosis. In these studies FGFR gene expression has been accessed by the techniques of in – situ hybridisation and immunohistochemistry, to study the expression domains of FGFR transcript and FGFR protein respectively.
Radiolabelled, antisense, isoform-specific riboprobes were designed to detect the transcripts of the FGFR1, FGFR2 and osteonectin genes, in *in-situ* hybridisation studies upon human embryo, fetus and postnatal calvarial tissues. In addition, FGFR1, FGFR2, and FGFR3 proteins were accessed by immunohistochemistry, with primary antibodies raised in rabbit against human receptor epitopes. Immunohistochemical techniques were also used to study the expression domains of the FGFR ligands FGF2, FGF4, and FGF7 in these tissues, using primary anti–human antibodies raised in rabbit, goat, and mouse.

The FGFR downstream target STAT 1, a nuclear transcription factor, was also accessed by immunohistochemistry, together with the transforming growth factors β1 and β3, which have been shown to act in synergy with FGF mediated signalling (S-3.3).

### 2.2.2 In-situ hybridisation studies

**Introduction**

*In-situ* hybridisation relies upon the hybridisation of a radiolabelled RNA probe to a specific complementary mRNA sequence in the tissue. In these studies the riboprobes used were antisense and complementary to mRNAs for osteonectin, FGFR1, and the FGFR2 isoforms bek and KGFR.

**Preparation of solutions, glassware and plasticware.**

Gloves were worn at all times and the working area was dust-free. All solutions for RNA work were made, where possible, from solids that had been kept separately from the general laboratory chemicals. Where handling was essential, chemicals were weighed out using baked spatulas. 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. The only aqueous solutions that were not DEPC-treated were those containing TRIS or amines, and those that could not be autoclaved. These solutions were made up in DEPC treated bottles that were then autoclaved and baked.

Organic solvents were filtered where possible. If this was not possible, for example with phenol, then aliquots were kept which were only used for RNA work. All glassware, spatulas,
and homogenisers were baked overnight at 180°C before use. Non-sterile plasticware was immersed in a 0.1% solution of DEPC in water overnight at room temperature. The DEPC solution was then poured off, the plasticware was autoclaved and baked dry at 80°C. Sterile plasticware was assumed to be RNase-free.

*Preparation of the Riboprobes*

*Preparation of DNA plasmid template*

$FGFR1$ exon IIIa, $FGFR2$ exon III$_b$ (KGFR) and $FGFR2$ exon III$_c$ (BEK), were amplified from normal human genomic DNA and cloned into Bluescript II SK$^+$ (Chan and Thorogood, 1999). The identities of the $FGFR$ exons were verified by sequencing. IMAGE Consortium cDNA clone (GenBank No T54933), corresponding to human *osteonectin* was obtained from the Medical Research Council Human Genome Mapping Project Resource Centre (Hinxton, Cambs, UK) and subcloned into Bluescript II SK$^+$.

DNA plasmids were then prepared from colonies of stock attenuated E Coli, containing the specific cloned Bluescript II SK$^+$ vectors. These were stored at -70°C, and aliquots as required were grown overnight in LB broth on an aerating hotplate at 37°C.

The overnight culture was centrifuged at 1500rev for 10 minutes in 50 ml Falcon tubes, and pellets were resuspended in 5 mls Solution 1 (Appendix C) by vortex.

Solution 2 (5 mls, Appendix C) was added, and mixed by inverting to clarity of the resulting homogeneous solution, whereupon Solution 3 (5 mls, Appendix C) was added, and mixed by inverting until a fine white protein precipitate appeared. This precipitate was centrifuged, and the supernatant removed.

An equal volume of isopropanol was added to the supernatant, mixed thoroughly and centrifuged. The resulting pellet (protein and nucleic acid) was resuspended in distilled water and aliquoted into eppendorf tubes.

Equal volumes of phenol: chloroform: resuspension were mixed in solution in the eppendorf tube by vortex, and centrifuged. The upper phase was removed to fresh eppendorf tubes, 1 ml of ice cold 100% ethanol added and the mixture centrifuged.
The pellet (plasmid DNA, bacterial nucleic acid) was washed in 70% ethanol, and resuspended in 50µl TE with 5µl RNase at 10mg/ml.

Lithium chloride 50µl was added, the solution mixed thoroughly, and kept on ice for 10 minutes.

The solution was centrifuged, and the supernatant containing plasmid DNA retained. Two parts 100% ethanol were added, and the solution centrifuged aggressively.

The pellet, containing plasmid DNA, was washed in 70% ethanol, resuspended in 50µl TE and stored at -20°C.

**Estimation of plasmid concentration**

A 1 µl aliquot was serially diluted x10, and concentration was estimated by electrophoresis on a 0.8% agarose gel (Fig 2.2.2-1).

**Linearising Plasmids to form a DNA template**

Plasmid DNA containing the template sequence for riboprobe synthesis was linearised with the appropriate DNA ligating enzyme in a total reaction volume of 500µl as follows:

<table>
<thead>
<tr>
<th>Linearisation mix; Total volume 500µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC H2O</td>
</tr>
<tr>
<td>10x Enzyme Buffer</td>
</tr>
<tr>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>RNA'se (10µg/ml)</td>
</tr>
<tr>
<td>Enzyme: FGFR1 plasmid: Hind III</td>
</tr>
<tr>
<td>Osteonectin plasmid; Hind III</td>
</tr>
<tr>
<td>FGFR2 – bek plasmid; XbaI</td>
</tr>
<tr>
<td>KGFR plasmid; XbaI</td>
</tr>
</tbody>
</table>
The reaction volume was maintained at 37 °C for one hour, after which 10μl of the linearised DNA was quality assessed by gel electrophoresis (Fig 2.2.2-2).

The linearised DNA template was then further subjected to phenol: chloroform extraction, in an equi-volume solution with the linearisation mix.

The DNA: phenol: chloroform solution was vortex mixed and centrifuged. The upper phase, containing the linearised DNA template, was mixed by careful pipette with 2 volumes of 100% ethanol, and 1/10th volume 3 molar sodium acetate.

The resulting solution was mixed thoroughly, and centrifuged, to bring down a DNA pellet, which was resuspended in 20μl DEPC H20 and stored at —20 °C.

**Riboprobe synthesis and purification**

The following procedures were undertaken in a designated laboratory for radioactive substances. All laboratory hardware used for these procedures was rendered 'RNA grade’ by autoclave at 180 °C to destroy ambient RNA'se, and all solutions, where possible, were autoclaved with DEPC to achieve the same effect.

Linearised DNA template and RNA nucleotides (35S-UTP) were introduced with the appropriate RNA polymerase in a total reaction volume of 25μl, and incubated at 37 °C under a mineral oil meniscus:

<table>
<thead>
<tr>
<th>Transcription Reaction volume</th>
<th>25μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC H2O</td>
<td>7.5μl</td>
</tr>
<tr>
<td>5x Transcription buffer</td>
<td>5μl</td>
</tr>
<tr>
<td>DTT 0.1M</td>
<td>2.5μl</td>
</tr>
<tr>
<td>Nucleotide GAC mix: (equivolume amounts of 10mM ATP, 10mM GTP, 10mM CTP)</td>
<td>3μl</td>
</tr>
<tr>
<td>35S - UTP (100μCi)</td>
<td>5μl</td>
</tr>
<tr>
<td>DNA linearised plasmid</td>
<td>1μl (1μg)</td>
</tr>
<tr>
<td>RNA guard</td>
<td>0.5μl</td>
</tr>
<tr>
<td>T7/T3 polymerase</td>
<td>0.5 μl (5-10 units)</td>
</tr>
</tbody>
</table>
The riboprobe was eluted through a swollen sephadex G50 bead column to bring down the transcribed riboprobe. A 1ml drip column was designed in a suitable Pasteur pipette, and rinsed three times with 300µl elution buffer.

The transcription reaction volume was aspirated from under the mineral oil and loaded into the column; then eluted with 150µl elution buffer. The column was further eluted three times with 150 µl elution buffer, allowing each eluent to drain through prior to adding the next, and this first combined eluent was discarded.

A series of eppendorf tubes was set up in a rack, and the elution column - Pasteur pipette moved to the first of these. 150µl elution buffer was added to the top of the column and allowed to drain through it completely, to be collected in the first eppendorf tube. After it had completely drained, the column was moved to the second eppendorf, and a second elution repeated.

The column was eluted a total of six times, such that the eluted probe – transcription reaction was collected in a series of six eppendorf tubes in series.

1µl of each eluted fraction was placed in a series of wells to which were added 40 µl scintillation fluid. The beta - radioactive emission was counted on a calibrated Wallac scinticounter and displayed graphically and numerically using Microbeta software (Fig 2.2.2-3).

The optimum spectrum is a peak emission of the two ‘best’ fractions at the apex of a bell - shape distribution. The two ‘best’ fractions represent the fractions containing the synthesised riboprobe. The remaining samples represent beta emissions from probe fragments and scattered nucleotides and were discarded.

The ‘best’ fractions of 150µl each were combined, and 2 µl (20µg) of yeast RNA (10mg/ml) was added. To this solution, 30µl 3M lithium chloride was added and the solution precipitated with 900 µl ethanol, after mixing thoroughly and incubation over dry ice for 20 minutes.

The pellet (riboprobe) was washed in 70% ethanol, and dissolved in 50 ml hybridisation mix, with the solution then made up to 10'5 cpm/µl and used immediately or stored at −20 ºC.
Preparation of slides and in-situ hybridisation

Sections, mounted on TESPA subbed slides (Appendix B), were dewaxed by immersion in two fresh sequential Histoclear (Fissons) solutions for 10 minutes each, and then dried in 100% ethanol by sequential immersion in two fresh solutions for 10 minutes each.

Slides were then rehydrated by transfer through a sequentially less concentrated ethanol series for 2-3 minutes each (Ethanol/DEPC water 95% - 85% - 70% - 50% - 30%)

Slides were sequentially washed in saline, then PBS, for 5 minutes each, and prefixed in 4% PFA at room temperature for 20 minutes. Two further PBS washes were performed for 2 minutes each.

Slides were treated with 10μg/ml Proteinase K in PBS for 5-10 mins only, and washed in in PBS for 5 minutes. Slides were then post fixed in 4% PFA for 5 minutes, and washed in DEPC water for a further 5 minutes.

Slides were treated with acetic anhydride in a fume cupboard. 400 mls of 0.1M triethanolamine were brought to pH 8 with sodium hydroxide, into which the slides were immersed, and acetic acid added dropwise to a constantly stirred solution.

Slides were again washed in PBS and saline for 5 minutes each, then dehydrated by transferring through an alcohol series (Ethanol 30% - 50% - 70% - 85% - 95% - 100%-100%; 2-3 minutes each).

Slides were then air dried in a fume cupboard and the probe introduced for hybridisation on the same day.

Hybridisation

The hybridisation steps were conducted in a specially designated and separated laboratory for radioactive chemicals.

The probe in hybridisation mix was brought to 80 °C for 2 minutes on a preheated hot plate, then cooled to ambient temperature.
Hybridisation mix was applied to each section/set of sections on the prepared slides according to the volume required for adequate cover (15 μl of hybridisation mix per 22/mm²), and so as to avoid bubbles under the silicone cover slip.

The hybridising sections on each slide were placed horizontally in a black hybridisation box together with a piece of tissue soaked in 10 ml 50% formamide and 5x SSC in the back/base to prevent slides drying out.

The hybridisation box was sealed with tape, placed in plastic bag, and sections left to hybridise overnight, weighed down in a 55-60 °C water bath.

Post hybridisation treatment & slide washing

Racked slides were soaked in 5x SSC and 10 mM DTT at 55°C for 30 minutes, and the cover slips encouraged to separate by gentle agitation. Studies involving suture material were conducted with particular care. This wash was repeated.

Slides were then washed in formamide wash solution at 65 °C for 30 mins.

Slides were washed x3 in TEN buffer at 37 °C for 10 minutes, to wash away formamide. The slides were then treated with 10μg/ml RNA'se A in TEN buffer at 37 °C for 30 minutes to digest remaining unhybridised probe.

Slides were then washed with TEN buffer at 37 °C for 15 minutes.

Slides were washed again in formamide wash at 65 °C for 30 minutes.

Slides were then sequentially washed with 2x SSC, and 0.1x SSC for 15 minutes.

Slides were then dehydrated by sequential washing through an alchohol/0.3M ammonium acetate series (30% - 60% - 80% - 95%) and finally in absolute alchohol, prior to air drying in a fume cupboard.

Autoradiography

The following steps were undertaken in a dark room under safety light.
Blue silica gel was wrapped in tissue paper and taped to the inside of a dark box, to act as a dessicant environment.

7.5 ml emulsion (K4, Ilford Company) was melted at 43 °C in a slide mailer, and an equal volume of prewarmed 2% glycerol was added.

Slides were dipped into the emulsion mix, laid out to dry, and placed in the box containing dessicant. The box was closed tightly, and wrapped in tin foil. Slides were left to expose for 3-7 days, depending upon the probe in use and the tissue type, incubated at 4 °C. Test slides were developed prior to developing the whole series to assess optimum time for incubation.

The incubation period of the hybridised sections proved to be a function of the age and type of the probe. 'Newer' probes were those made closer to the activity date of the $^{35}$S isotope and required shorter incubation periods. The osteonectin probe had the shortest exposure times, and the FGFR probe exposure times were variable. In practice, test slides were developed for each series of studies at time periods from 3 – 7 days. All probes were optimally developed on the tissue sections by this time.

*Developing and staining of slides*

The slide box was removed from 4 °C and brought to room temperature.

Slides were removed from the box in dark room environment, and passed through developing solution (2 mins), 1% acetic acid/1% glycerol (1 min), and fixative solution (2 mins).

Slides were then washed in water for 10 minutes, subsequently stained with toluidine blue for 10 minutes, and rinsed twice in water prior to destaining.

Slides were destained in an alcohol/0.3M ammonium acetate series of increasing concentration (30% - 50% - 70% - 85%) then in alcohol (95% - 100%).

Cover slips (washed in 100% alcohol) were mounted on the sections using DPX mountant, taking care to exclude bubbles. The slides were left to dry prior to dark field microscopy.
2.2.3 Immunohistochemistry for protein detection in - situ

Introduction

In parallel to the analysis of FGFR transcript expression by the in - situ hybridisation technique, the detection of the FGFR protein product was undertaken by immunohistochemistry. Comparison of the data achieved by each technique substantiates the other and may give an insight into molecular events between transcript expression and the detection of the protein product in - situ.

Immunohistochemistry is a routine histochemical technique. For conventional immunohistochemistry using tissue sections, tissues are first fixed to preserve morphology, then dehydrated, embedded in a wax block and cut into thin (3-6 μm) slices. These are placed on a glass slide, which has been pre-coated to facilitate adhesion. Sections are then rehydrated, may be pre-treated to increase penetration of the antibodies as necessary, and then 'blocked' with serum and/or albumin. This prevents non-specific antibody binding. Optimal blocking may be obtained with serum of the same species as the secondary antibody, but good results are obtained in most reactions with foetal calf serum.

Primary antibodies are then applied. In direct immunohistochemistry, primary antibodies are directly conjugated to a detection system. Although this eliminates the non-specific background signal generated by secondary antibodies, it is less sensitive than indirect techniques. Indirect techniques have been used throughout this thesis. In these studies, the primary antibody is unconjugated, and is detected by a conjugated antibody raised against the primary host species.

The detection system used in this thesis is the avidin - biotin reaction to a biotinylated secondary antibody. This method is based on the ability of the egg-white glycoprotein avidin to non-immunologically bind four molecules of the vitamin biotin, conjugated to the secondary antibody.

Multiple copies of the biotinylated secondary antibody bind with the unlabelled primary antibody (bound to the tissue antigen of choice). The section is then treated with the avidin-biotin peroxidase complex, which again binds multiple sites, such that the signal is greatly amplified. Primary antibodies can therefore be used at a high dilution, which reduces the false - positive background staining.
Avidin - biotin peroxidase activity is then detected by a brown colour change on exposure with DAB. A brown precipitate is produced by the peroxidase in the streptavidin-biotin mixture, and this can be monitored by direct vision or microscopically. Appropriate counterstaining allows accurate assessment of the protein expression domains within the histological preparation.

The primary antibodies used in this thesis were anti-human, raised in a variety of host animals (rabbit, mouse, and goat) to injected recombinant human antigens. Secondary antibodies used in this thesis were biotin-conjugated and raised in a variety of secondary animal hosts. Using modifications of this double-antibody labelling technique, protein expression data was obtained for the cell surface receptors FGFR1, FGFR2, and FGFR3 in a variety of embryonic, foetal, and postnatal tissues.

In addition, immuno-histochemistry was also undertaken to detect the FGF ligands FGF2, FGF4, and FGF7; the FGFR target transcription factor Stat1, and the growth factors TGFβ1, and TGFβ3.

**Primary Antibody reaction**

Human embryonic, foetal, and calvarial tissue sections were sectioned and prepared on subbed slides as described in Appendices A & B of this thesis. Mouse calvarial sections were similarly prepared.

Sections were dewaxed by immersion in two sequential Histoclear (Fissons) solutions for 10 minutes each.

Sections were rehydrated by transfer through sequentially less concentrated ethanol solutions for 2-3 minutes each (Ethanol 100% - 100% - 95% - 85% - 70% - 50% - 30%) to water.

The epitope was unmasked by treating the sections with 100 μl 0.1% trypsin (30 mins, 37 °C) under plastic cover slips, and then rinsed with PBS (5 mins), and the excess PBS dried from the slide. This has the effect of increasing primary antibody penetration.

Sections were then treated with 3% hydrogen peroxide in PBS (15 mins, room temperature) to block endogenous peroxidases, and then washed in PBS x2, and the excess PBS dried from
the slide taking care to protect each section. This had the effect of reducing background false positivity from the avidin biotin reaction, which detects peroxidase activity.

Sections were then treated with ‘Block’ solution (1% BSA, 10% FCS, PBS) for 30 mins, at room temperature; to reduce non-specific binding of the primary antibody.

Slides were washed in PBS, 5mins x3, and excess PBS around the sections dried carefully from the slide.

Sections were divided into control and test slides. Test slides were incubated with 75 – 100 μl per section (enough to cover each section beneath a plastic cover slip) of primary antibody in ‘block’ solution overnight at 4 °C. Optimal dilution of each primary antibody was determined by a series of preliminary experiments on each tissue type. Control slides were incubated with pre-immune serum in ‘block’ solution under similar conditions.

The overnight 4 °C incubation was undertaken in covered plastic trays humidified by lining with PBS soaked tissue.

Primary antibody reactions were undertaken at a range of concentrations in preliminary studies to determine optimum dilution for immuno-histochemistry. Antibody specificities are as supplied by the manufacturer, and according to various independent reports of use in immuno-histochemistry and Western blots. The details of each primary antibody, its dilution factor and target antigen, are tabulated below:
<table>
<thead>
<tr>
<th>Anti - human Antibody</th>
<th>Host</th>
<th>Concentration @ supply</th>
<th>Dilution</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR1</td>
<td>Rabbit polyclonal IgG</td>
<td>100 µg/ml</td>
<td>1/50</td>
<td>Carboxy – terminal of receptor</td>
</tr>
<tr>
<td>FGFR2</td>
<td>Rabbit polyclonal IgG</td>
<td>100 µg/ml</td>
<td>1/50</td>
<td>Carboxy – terminal of receptor</td>
</tr>
<tr>
<td>FGFR3</td>
<td>Rabbit polyclonal IgG</td>
<td>100 µg/ml</td>
<td>1/50</td>
<td>Carboxy – terminal of receptor</td>
</tr>
<tr>
<td>Stat 1 G 16930</td>
<td>Rabbit, polyclonal</td>
<td>100µg/ml</td>
<td>1/50</td>
<td>Amino – terminal of the ISGF3/STAT1 protein complex</td>
</tr>
<tr>
<td>FGF2 #AB 33 NA</td>
<td>Rabbit IgG polyclonal</td>
<td>8.8 mg/ml used as stock 50 µg/ml</td>
<td>1/200</td>
<td>Recombinant FGF4 as expressed in E.coli</td>
</tr>
<tr>
<td>FGF7 Cat MAB251</td>
<td>Mouse, IgG1 monoclonal</td>
<td>50 µg/100 µl</td>
<td>1/250</td>
<td>Recombinant FGF4 as expressed in E.coli</td>
</tr>
<tr>
<td>FGF4 Cat MAB235</td>
<td>Goat</td>
<td>20µg/µl</td>
<td>1/500</td>
<td>Recombinant FGF4 as expressed in E.coli</td>
</tr>
<tr>
<td>TGF β 3</td>
<td>Rabbit Polyclonal</td>
<td>200 µg/ml</td>
<td>1/100</td>
<td>Carboxy terminus of TGFβ1 (mouse/human precursor)</td>
</tr>
<tr>
<td>TGF β 1</td>
<td>Rabbit polyclonal</td>
<td>200 µg/ml</td>
<td>1/100</td>
<td>Carboxy terminus of TGFβ1 (mouse/human precursor)</td>
</tr>
</tbody>
</table>
Secondary Antibody reaction

In the secondary antibody reaction, biotinylated anti-rabbit, anti-goat and anti-mouse antibodies were detected by reaction with an Avidin complex (Sigma) and DAB reaction (Sigma). For antibodies raised in mouse, the secondary anti-mouse antibody was detected with a strepavidin complex (DAKO) and DAB reaction (Sigma).

For Primary Antibodies raised in Rabbit

After incubation with the primary antibody overnight, slides were washed in 1% TritonX/PBS solution (x3, 5 min), and rinsed in PBS.

Sections were incubated with 100µl secondary antibody (biotinylated anti-rabbit, 30 min, room temperature) under plastic coverslip.

Slides were washed in TritonX/MilliQ, (x3, 5 min).

Sections were incubated with 100µl Avidin peroxidase (30 min, room temperature), under plastic cover slip, and then lavaged x2 with PBS.

For Primary Antibodies raised in Goat

After incubation with the primary antibody overnight, slides were washed in 1% TritonX/PBS solution (x3, 5 min), and rinsed in PBS.

Sections were incubated with 100µl secondary antibody (biotinylated anti-goat, 30 min, room temperature) under plastic coverslip.

Slides were washed in TritonX/MilliQ, (x3, 5 min).

Sections were incubated with 100µl Avidin peroxidase (30 min, room temperature), under plastic cover slip, and then lavaged x2 with PBS.
For Primary Antibodies raised in Mouse

After incubation with the primary antibody overnight, slides were washed in 1% TritonX/PBS solution (x3, 5 min), and rinsed in PBS.

Sections were incubated with 100μl secondary antibody (biotinylated anti-mouse, 30 min, room temperature) under plastic coverslip.

At the same time, the streptavidin-biotin mix was made from 1:100 dilutions of the two reagents (DAKO), and maintained over ice during the incubation period of the secondary antibody.

Slides were then rinsed and washed in PBS x3, and the streptavidin mixture applied (30 min, at room temperature).

Slides were then washed in PBS x2.

Detection & Counterstaining

Sections were incubated with 300-500μl DAB (0.5 mg/ml DAB with 0.03% hydrogen peroxide) per slide for up to ten minutes. For sections turning rapidly brown before this time, the reaction was stopped by washing with PBS/MilliQ.

Sections were counterstained by immersion in 0.5% methyl green in 0.1M sodium acetate (pH 4.0) for 5 to 10 minutes. Methyl green stains nuclei blue, contrasting to the brown DAB stain.

Slides were then washed three times in deionised water by dipping them 10 times in the first and second wash and then 30 seconds in the third. This procedure was repeated with three changes of butan-1-ol, under cover of a fume hood.

Slides were then washed in Histoclear for 10 mins, allowed to air-dry, and then mounted under cover slip with dextropropoxyphene (DPX) mountant.
2.2.4 Histo-chemical Methods

Staining studies with durazol red & alcian blue

A series of sections from human sagittal sutures showing progressive bony replacement was subjected to staining with durazol red and alcian blue to stain for sutural bone collagens.

Serial sections of sagittal sutures previously embedded in paraffin wax were dewaxed by immersion in two sequential histoclear baths. The sections were rehydrated by immersion in graded alcohols of decreasing concentrations to water.

The sections were stained in haematoxylin for 15 minutes, and placed in water for a further 15 minutes.

The sections were then stained in alcian blue for 10 minutes, and washed in distilled water by dipping for 3 seconds.

The sections were placed in phosphomolybdic acid for 10 minutes, and rinsed again by dipping in distilled water for 3 seconds.

The sections were stained in durazol red for 5 – 7 minutes, and rinsed by dipping in distilled water for 3 seconds.

The sections were dehydrated by immersion in sequential increasing concentrations of alcohol to dryness, mounted in DPX mountant, and covered with glass coverslips.

2.2.5 Image documentation

Slides were examined using a Olympus BH2 microscope, and images were captured electronically using a Kontron ProgRes3012 digital camera, version 2 of the associated software, and stored as Adobe Photoshop v5.0 files.
2.2.6 Practical Problems encountered

The successful manipulation of calcified calvariae was fraught with difficulty. The principal problems encountered were the adequate fixation of calcified tissue, particularly the human postnatal calvariae, and the subsequent decalcification of the fixed specimens. Once decalcified, these tissues were sectioned to present thin ribbon-like slivers of bone which were secured on TESPA—subbed slides. The subsequent treatment of these slides for in-situ hybridisation studies involved many sequential changes of solution, and despite frequent change of strategy, often resulted in section loss. Strategies for overcoming each problem are indicated below.

Initial protocols for mouse calvariae

Initial experiments were conducted using mouse calvariae obtained from euthanased female CD1 mice. The fresh calvariae were cut into thin strips with a hand held scalpel, rinsed in fresh PBS and passed into 4% PFA for fixation for a time periods ranging from 2 to 7 days, with regular changes of fixative.

Quality of fixation was assessed by light microscopic examination of cellular architecture, which could only be adequately observed after appropriate sectioning, post decalcification.

Decalcification of postfixed samples was achieved by initial immersion in sequential drying solutions of ethanol/PBS in increasing concentrations of ethanol to 70%, and then via PBS/ethanol washes to an EDTA/PBS decalcification solution.

The samples were then divided into two groups, each equally containing samples from all time periods of fixation. Group A samples were placed in EDTA 0.5 molar solution, diluted to a 50% solution with PBS at room temperature. Group B was placed in the same solution at 37°C.

Samples within each group were maintained in 50% 0.5 molar EDTA:50% PBS for a range of time periods from one to four weeks, with changes of solution every two days, and then paraffin wax embedded prior to sectioning as in Appendices (A & B).

Sectioning of all samples was undertaken to 6 μm as per Appendix A&B. Only samples which had been incubated in decalcifying solution for longer than two weeks were easily sectioned.
into sequential ribbon-like strips, those with shorter incubation periods would not cut easily on application of a fresh microtome blade. The temperature of incubation with the EDTA/PBS solutions did not appear to be an important variable for these sections.

The adult sectioned mouse calvariae were then stained for routine histological examination with haematoxylin and eosin, and immunostained for distribution of FGFR proteins, in order to establish optimal antibody dilutions and incubation periods for the human calvarial studies to follow.

It became clear that small sections with longer fixation periods had greater fixed tissue quality. The quality of fixation was generally good for mouse calvariae, and differences between groups were subtle. On the basis of these observations it was decided to progress to human calcified calvariae, incubating the samples with 4% PFA fixative for 4–7 days.

Decalcification in 50% EDTA 0.5 molar, 50% PBS at room temperature was undertaken for as long as required to achieve unperturbed hand–held sectioning, prior to embedding and sectioning the samples at the microtome.

Decalcification of Human Foetal & Postnatal sutures.

Despite the observations made in the preparation of adult mouse calvariae, the preparation of the human late foetal and postnatal cranial sutures and cranial bone proved to be a difficult process. Each sample of redundant patient calvarium/suture from the operating theatre was washed in sterile warmed saline at time of harvest and placed directly into 4% PFA fixative. The specimens were cut into en-bloc samples, each of which presented a large flattened surface area for the penetration of immersion solutions (endocranial and pericranial respectively) and two cut transverse ends (transverse section across suture and parasutural fields).

These samples were transferred over ice to the laboratory and maintained at 4 °C for 4–7 days, after which they were sequentially dehydrated in an ascending alchohol series, and initially transferred to 50% EDTA 0.5 molar; 50% PBS at room temperature. The solution was changed daily, and the specimens were tested for adequate decalcification with a hand held scalpel blade periodically. It became clear that a more aggressive decalcification protocol was necessary.
Post fixation human calvariae were therefore subjected to two separate concentrations of
EDTA 0.5 molar/PBS solution changed daily, at both room temperature and 37 °C. Each
patient sample was divided in the operating theatre into subsets of small specimens in order
to present as large a surface area to fixative and decalcificant as possible, whilst maintaining
landmarks for identification. The range of specimens of a subset was fixed in 4% PFA,
changed daily, for a range of times from 4 -7 days.

Each specimen was then decalcified in either 0.5 molar EDTA, or 75% 0.5 molar EDTA;
25% PBS at room temperature or 37 °C. At this point it was still not clear whether the EDTA
concentration would adversely affect the process of in – situ hybridisation, or whether
potentially prolonged periods of decalcification at temperatures higher than 37 °C would
cause protein or transcript degradation. Because each bony sample and integral suture was
different in architecture, density and volume size, it became impossible to identify a single
protocol for fixation and decalcification for all bony samples. The 50% EDTA 0.5 molar;
50% PBS concentration at 37 °C became the preferred method, with frequent changes of
solution, and hand testing of the sample with a scalpel blade until successful hand sectioning.
At this point a small area of each sample was embedded and sectioned, and the rest returned
to EDTA/PBS.

Once fixed, decalcified, embedded, and sectioned; most of the sections of each bony en – bloc
sample exhibited a range of qualities of fixation. Adjacent sections from the same sample, and
therefore identically treated, showed variable fixation quality. This variability extended to
regional fields within the same section, and in order to generate an interpretable data set,
many sections from each suture were analysed, with a high redundancy rate.

Well – fixed sections exhibited plump osteoblast cell bodies in continuous array around bony
lacunae, and in areas of osteoid new bone formation (5-3.1). Osteocytes within established
bone matrix were plump, and filled their spaces within the matrix, sending out dendritic
complexes visible at high power into the matrix in overlapping territorial fields (5-3.1). Well
fixed sutural cells were also defineable as plump oval or round cells within a fibrous matrix.
Sutural cell density increases with progression of sutural fusion, as assessed histo – chemically
and microscopically (5-3.1).
Technical problems with in – situ hybridisation of de-calcified calvariae

A further problem, encountered with both mouse and human calvariae, was that of section loss during the multiple washes of the in – situ hybridisation process. Each gentle change of solution resulted in a percentage loss of the sections into the wash. The problem was particularly evident immediately post - hybridisation, after washing in SSC at 60 °C for 30 minutes. Often, when removing the slide rack from the solution, the appearance remained of tiny 'plankton – like' slivers of sections swirling in the SSC bath. A similar problem was encountered after formamide washing.

A variety of manoeuvres was attempted to overcome this problem. All the TESPA subbed slides were changed for freshly subbed slides, and sections were floated upon the new slides and allowed to evaporate to dryness for prolonged periods of 2 - 3 days at 37 °C. Sections were cut to a variety of thicknesses (6 μm, 8 μm, and 10 μm) to see whether this made for greater adherence to the subbed slide during processing without adversely affecting probe penetration in hybridisation. Furthermore, the temperature and duration of the SSC washes was reduced to 20 minutes at 55 °C for two experiments to assess the affect of these variables on section – slide adherence. None of the above strategies appeared to significantly improve upon the problem. Eventually, all sections were trimmed to include the suture and a limited amount of flanking cranial bone on each side to minimise the available 'lifting – edge' of the sections. This had the effect of reducing the section loss from about 50% to 30% in human studies.

Various reports in the literature since these studies were undertaken have attempted to access mRNA transcripts from bony tissue. Most centre upon studies relating to mouse tissues. Kim et al and Liu et al have studied neonatal murine calvariae with in – situ hybridisation techniques, using similar decalcification protocols with 0.5 molar EDTA in volume dilutions with PFA (Kim et al, 1998; Liu et al, 1999). Decalcification times with these neonatal tissues (to P6) were 4 – 7 days. There is a paucity of data or guidance in the literature with respect to protocols for decalcification for eventual riboprobe analysis, though osteoblast cultures ex – vivo have been successfully subjected to in – situ hybridisation studies (Lomri et al, 1998). In general, the tissue density and extent of calcification in human infant calvariae renders the tissue very resistant to successful in – situ hybridisation by the radiolabelled 35S technique, and a high ratio of tissue loss is to be expected. Further studies on human tissue may be rewarding, however (5-4). Alternative techniques, such as digoxigenin – labelled in – situ hybridisation which involves fewer washes, may be a better option.
Section 2 - Figures and Legends

Figure 2.2.2-1

Gel electrophoresis of the re-suspended FGFR1 plasmid DNA, containing the exon IIIa sequence cloned into Bluescript II SK+, and prepared from stock - attenuated E. Coli. A 1μl aliquot was serially diluted x10. Linearised DNA for osteonectin has been run in the single outside lane on the gel.

Figure 2.2.2-2

Gel electrophoresis of FGFR1 - plasmid DNA (Lane 1, left), and two examples of FGFR1 linearised DNA (Lanes 2&3). Plasmid DNA containing sequence for osteonectin has been run in the inside right-hand lane, against a sample of linearised osteonectin DNA (outside right-hand lane) which is undetectable in this assay.

Figure 2.2.2-3

Scinticount of the eluted transcription reaction to synthesise FGFR1 riboprobe. Fractions 2 and 3 from the sephadex column give the greatest counts, indicating the highest concentrations of synthesised radioactive riboprobe.
9/22/96

FRIKS-1 cut per preceding gels + the clean-up:
* mixed with equal vols (1:1:1) of phenol: chloroform
  upper phase mixed with 1/10 3.0 M Na Acetate (50 μl)
  + 2 vols 100% EtOH.
  \[\text{had mixed, spun 15,000 x g 5 min.}\]
  \[\text{pellet washed 70% EtOH; dried in air.}\]
  \[\text{disolved in 20 μl D.I. H}_{2}O;\]
  \[\text{kept at -20°C.}\]

11/22/96. FRIKS-1 d 0NP.

- Centrifuge 50 μl 37°C 1 hr
  1) dH_{2}O 150 μl
  2) 10×5′ 20 μl
  3) phosh 20 μl (20 μg)
  4) RNase 
     (10 μg/mL)
  5) RNase 5 μl (50 μg)
  \[\text{then dried up as above.}\]

\[\text{FRIKS-1 cut 1 = (mg)}\]
\[\text{cut 2 = 0.4 μg/μl.}\]
Counting protocol no: 97
Name: PAUL H S-35
PM normalization protocol no: 6

** DETECTORS NOT NORMALIZED

Experiment made: High probe incubation
37°C for 2-5 hours (no. 6) and 0-20 overnight (flow)
from probe diluted per PM protocol using dilution buffer
in PM protocol

- Counts below: peaks in Table 2.3, then combined
- pH in LiCl 4.8 N for 5C protocol 0.20 mg yeast RNA
- Pellet washed in 70°C. chilled 14°C.
- Diluted to 330 μL high protein 70% or 70% count
- 1x10^5 μL probe concentration.

<table>
<thead>
<tr>
<th>Pos</th>
<th>CCPM1</th>
<th>CCPM1%</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6895.4</td>
<td>2.4</td>
</tr>
<tr>
<td>002</td>
<td>134902.9</td>
<td>0.5</td>
</tr>
<tr>
<td>003</td>
<td>86322.7</td>
<td>0.7</td>
</tr>
<tr>
<td>004</td>
<td>40677.6</td>
<td>1.0</td>
</tr>
<tr>
<td>005</td>
<td>14749.9</td>
<td>1.6</td>
</tr>
<tr>
<td>006</td>
<td>3.0</td>
<td>115.5</td>
</tr>
</tbody>
</table>

Dilution: CCPM1 1:4

Combined: cpm = 22 x 10^4.
3. Results:

Studies in FGFR gene expression in human craniofacial development and craniosynostosis
3.1 The expression of FGFR1, FGFR3, and the BEK and KGFR isoforms of FGFR2 co-regulates with human calvarial osteoblast maturity and premature cranial suture fusion in situ.

3.1.1 Abstract

Human craniosynostosis may affect one or a number of cranial sutures. Isolated sagittal synostosis is the commonest 'non-syndromic' clinical presentation, whereas coronal synostosis characterises a range of skeletal dysplasia syndromes. Studies in clinical genetics implicate members of the FGFR—gene family in the pathogenesis of human craniofacial dysmorphogenesis and various forms of craniosynostosis. Analysis of the isoform—specific expression of FGFR genes has therefore been undertaken in human calvarial and meningeal development at ten weeks, infant parietal calvariae, and infant age—matched fusing and unfused sutures.

Human calvarial osteogenesis and sagittal sutural synostosis are consistently characterised by a differentiation stage—specific expression of FGFR genes. FGFR1 characterises the pre—osteoblast and osteoblast phases, whereas FGFR2 is expressed as BEK in osteoblasts and osteocytes. KGFR is weakly expressed in calvarial osteoblasts. Whereas BEK is expressed in the unfused suture, the expression domain is negatively regulated as suture fusion commences. As the pathologic ‘osteogenic front’ develops in the fusing suture, both KGFR and BEK are strongly expressed in the presence of FGF2, but not FGF4 or FGF7. FGF2 and FGF4 are, however, co—expressed with the FGFR1—3 in human calvarial osseous development at 10 weeks, and in the underlying meninges.

The differential expression of FGFR genes characterises different phases of human infant calvarial osseous differentiation. FGFR1 characterises proliferation in the early osteogenic pathway, FGFR2 a later, differentiative, phenotype. Furthermore, the differential expression of FGFR1—3 in human calvariae correlates with the phenotypes of their activating mutations. These observations are contrasted with that obtained in various mouse models, in which experimental manipulation is expected to be a prelude to novel therapeutic approaches.
3.1.2 Introduction

The molecular signalling pathways which regulate mammalian cranial sutural morphogenesis are complex, and are accompanied by the interaction of the dura mater, cranial mesenchyme, and cells of the approaching cranial bone fronts (Albright and Byrd, 1981; Johansen and Hall, 1982; Opperman et al, 1998; Levine et al, 1998; Kim et al, 1998). Various genes have been implicated in calvarial and sutural morphogenesis; including the homeobox gene MSX2 (Jabs et al, 1993; Liu et al, 1999), sonic hedgehog (Ming et al, 1998), and TGFβ isoforms (Opperman et al, 1997; Most et al, 1998; Downs et al, 1999). Furthermore, studies in clinical genetics implicate human mutations in TWIST (Rose and Malcolm, 1997), MSX2 (Jabs et al, 1993), and members of the fibroblast growth factor receptor (FGFR) gene family in craniosynostosis, the pathologic premature replacement of human cranial sutures by bone. A range of mutations in the FGFR 1, 2, and 3 genes is causal in many craniosynostosis syndromes (Wilkie, 1997; Passos-Bueno et al, 1999). FGFR3 mutations additionally cause related dysplasias of the appendicular skeleton (Rousseau et al, 1994; Shiang et al, 1994; Tavormina et al, 1995a) some of which feature pansynostosis (Angle et al, 1998).

FGFR mutations causing human cranioskeletal dysplasias confer functional gain upon the mutant receptor (Wilkie, 1997; Webster and Donoghue, 1997a; Burke et al, 1998), which has a variable effect upon human calvarial osteoblasts (Lomri et al, 1998; Fragale et al, 1999). The behavioural phenotype of cells of both the human (Debiais et al, 1998) and rat (Pitaru et al, 1993) osteoblast lineage in response to ligand FGF is maturation specific, and fgfr - activation in murine calvarial osteoblasts has proliferation/differentiation stage - specific effects (Iseki et al, 1999; Mansukhani et al, 2000). The possibility that the variable behavioural phenotype of human calvarial osteoblasts to FGFR activation reflects a differential, maturity - dependent, FGFR expression in – situ, however, remains to be established.

The FGFR gene family encodes a series of 4 structurally related tyrosine kinase receptors of which only FGFR1, 2, and 3 have been implicated in human craniosynostoses (Gaudenz et al, 1998). Each receptor may exist as at least two isoforms, which display different ligand binding properties via the alternative splicing of the exons IIIb and IIIc (Kannan and Givol, 2000). Mutations in FGFR2 account for the majority of craniosynostoses where the molecular pathogenesis is identified, and many of these are predicted to exert functional effect via both the KGFR (IgIIIa/b) and BEK (IgIIIa/c) splice - variants (Passos-Bueno et al, 1999). The kgr isoform of murine fgfr2 is expressed in epithelia, and the bek isoform is predominantly expressed in mesenchymal lineages (Orr-Urtreger et al, 1993). The regulation of receptor isoform expression, therefore, provides a mechanism for both the molecular control of normal vertebrate development, as well as the correlation of human mutant
\textit{FGFR} - genotype with clinical phenotype. Coronal synostosis, in isolation or as part of a wider sutural fusion phenotype, is the most common \textit{FGFR} - associated craniosynostosis. Reconstructive craniofacial surgery for this range of phenotypes requires the extensive re-modelling of infant calvariae without deference to intact coronal suturectomy. In contrast, isolated human sagittal synostosis causes familial or sporadic scaphocephaly (Lajeunie et al, 1996). Surgery for scaphocephaly at this centre involves excision of the entire length of the sagittal suture with a margin of parietal bone, with subsequent bi-parietal plication for skull vault reconstruction. The entire redundant sagittal suture, in serial transverse section, allows the \textit{in-situ} analysis of gene expression in the 'unfused' human fibrocellular suture as it undergoes progressive pathologic replacement by osteoid bone. When completely 'fused', the sutural domain consists of trabecular bone that is morphologically indistinguishable from the adjoining parietal calvarium (Fig 3.1-1). The current investigation asks whether the differential expression of \textit{FGFR}s accompanies the maturation of human infant cranial osteoblasts \textit{in-situ}, whether splice - variance of \textit{FGFR2} is osteoblast - differentiation stage - specific; and whether the co-regulation of \textit{FGF/FGFR} expression accompanies human craniosynostosis. Observations in the sagittal suture, where the entire suturectomy favours controlled molecular investigation, are compared with those in isolated, rarely available, age-matched coronal and lambdoid sutures. The presented data provide a means by which to evaluate experimental work in the mouse model, and further inform the development of novel therapeutic strategies.

\textbf{3.1.3 Materials & Methods}

\textit{Preparation of infant suture material}

Infant cranial suture samples were obtained with prior ethical approval at elective cranioplasty. Redundant sutures and their cranial bone margins were retrieved from sixteen patients of the Craniofacial Centre at Great Ormond Street Hospital, of which nine cases are reported. In no case was a specific molecular diagnosis sought. The \textit{in-situ} analysis of \textit{FGF/FGFR} gene expression in six sagittal, two coronal, and one lambdoid sutures is reported (\textit{Tables} below). All sutures were removed \textit{en-bloc} with adjacent membrane bone, cut into serial longitudinal samples and fixed, dehydrated, decalcified, embedded, and serially sectioned at 6 \textmu m onto TESPA subbed slides; as described in 5.2.1.2.
Tables of sutures

Table: Unfused 'control' suture and parietal trabecular bone

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Phenotype</th>
<th>Suture</th>
<th>Analysis presented</th>
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<td>RC</td>
<td>11 months</td>
<td>Scaphocephaly</td>
<td>Unfused sagittal</td>
<td>Infant cranial bone (Fig 3.1-2, iii-iv)</td>
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<td></td>
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<td>Unfused 'control' suture (Fig 3.1-4)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Trabecular bone (Fig 3.1-3)</td>
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<tr>
<td>LP</td>
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<td>Parietal bone</td>
<td>Trabecular bone (Fig 3.1-3)</td>
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<td>Unfused sagittal</td>
<td>Unfused 'control' suture (Fig 3.1-5)</td>
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<tr>
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<td>Unfused sagittal</td>
<td>Unfused 'control' suture (Fig 3.1-9; i&amp; ii)</td>
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<tr>
<td>Patient</td>
<td>Age</td>
<td>Phenotype</td>
<td>Suture</td>
<td>Analysis presented</td>
</tr>
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<tr>
<td>RC</td>
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<td>Fusing sagittal</td>
<td>Durazol/alcian histochemistry (Fig 3.1-6, i-iii)</td>
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<td>FGFR1, FGFR2-IgIIIc (BEK) and <em>osteonectin</em> transcripts (Fig 3.1-9, iv - vi)</td>
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<td>Fusing sagittal</td>
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<td>FGFR2-IgIIIb (KGFR) transcript (Fig 3.1-10, iv)</td>
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<td>FGF4 protein (Fig 3.1-13)</td>
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<tr>
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<td>Fusing coronal</td>
<td>FGFR1 and FGFR2-IgIIIc (BEK) transcript (Fig 3.1-11)</td>
</tr>
</tbody>
</table>
Preparation of Human embryo-foetal material

Sections of human craniofacial development were obtained and prepared as described in Section 2.1.1. Calvarial and dental FGF and FGFR gene expression data is used as control material in this series of studies.

Studies of FGFR and allied gene expression

FGFR1, osteonectin, and the BEK and KGFR isoforms of FGFR2 mRNA transcripts were detected by in-situ hybridisation as described in S-2.2.

The protein receptors FGFR1, FGFR2, and FGFR3; and the FGFs 2, 4, and 7 protein products were detected by immunohistochemistry using commercially available specific antibodies (S-2.2.3). The anti-receptor antibodies have epitopes corresponding to the carboxy-terminal sequences of the receptor protein, and therefore label both IgIIIc and IgIIIb isoforms in immunohistochemistry studies.

Durazol red & alcian blue histochemistry

Prepared sections of infant suture in various states of progressive fusion stained were stained with alcian blue and durazol red as previously described (see S-2.2.4).
3.1.4 Results

The expression of FGFR1 and 2 is differentiation stage – specific in human calvarial osteogenesis in - situ.

Infant cranial osteoblasts are plump, rounded cells which predominantly populate the periosteum, immature parietal bone matrix, and mature trabecular parietal bone edge (Fig. 3.1-2). Osteocytes are flattened cells, with multiple dendritic processes, and represent a mature, differentiated phenotype. Osteocytes densely populate mature calvarial bone matrix, and maintain intercellular contact by means of gap junctions between their network of dendrites (Fig. 3.1-2). FGFR2 is expressed as protein in the osteoblasts lining the bone matrix, and in the osteocyte cell bodies and dendritic processes (Fig. 3.1-2, iii). FGFR1 protein labels very strongly in the osteoblast cell bodies, but signal is weak in the osteocyte cell bodies and dendrites (Fig. 3.1-2, iv). Corroborative data are obtained by in – situ hybridisation for transcript RNA. FGFR1 is strongly expressed in the osteoblasts of the periosteum and lining trabecular bone, but signal is comparatively weak from osteocytes of the parietal bone matrix (Fig. 3.1-2, ii, iii). FGFR2 is expressed as the BEK transcript in matrix osteocytes, their dendrites, and the osteoblasts lining parietal trabeculae (Fig. 3.1-2, iv, v). However, the KGFR isoform of FGFR2, whilst being expressed at low level in osteoblasts, is not expressed in the osteocytes of the trabecular bone matrix (Fig. 3.1-2, vi).

The expression of FGF and FGFR in human sagittal sutural fusion

FGFR immunolabelling in unfused (control) sutures

The expression of various FGF and FGFR was examined in fused and unfused (control) sagittal sutures from infants undergoing reconstructive cranioplasty (n=6; median age 10 month, range 10-12 months). Control FGFR protein data from two cases is presented in Figures 3.1-4 and 3.1-5 (see also Fig. 3.1-9). The unfused sagittal suture is a simple ankylosis between two parietal membrane bones, the central part of which consists of undifferentiated sutural cells and sparse blood vessels in a fibrous stroma. The suture is flanked symmetrically by narrow zones of osteoid stroma which line the opposing parietal bone edges. The suture and trabecular bone are lined on the ectocranial surface by periosteum and endocranially by dura mater in – vivo. FGFR1 and FGFR2 are each uniformly expressed as protein in the central fibro-cellular suture, and in the osteoblasts of the periosteum and osteoid stroma (Fig.s 3.1-4, 3.1-5). In contrast, FGFR3 protein is not uniformly detected (see Fig. 3.1-5, iii) in the
osteoblasts of the periosteum and osteoid stroma, and labels comparatively poorly in the central sutural domain.

**FGFR immunolabelling in fusing sutures**

Sagittal sutural fusion is accompanied by the accumulation of bone matrix proteins at the endocranial aspect of the central fibro-cellular suture (Fig. 3.1-6, i). Progression to sutural fusion is characterised by an increased cell density in the osteoid lining the suture (Fig. 3.1-6, ii) and in the endocranial central sutural domain (Fig. 3.1-6, iv – vi). These cells may be appropriately termed 'pre-osteoblasts' (Iseki et al, 1997; Opperman et al, 1998; Liu et al, 1999). With progression of suture fusion, the density of bone matrix protein increases, and the pre-osteoblasts become enveloped in advancing osteoid stroma, forming an 'osteogenic front' populated by osteoblasts (Fig. 3.1-6, iv – vi). This pathologic process, in the sagittal suture, progresses in an endocranial to pericranial direction in an analogous manner to the programmed sutural fusion of the mouse/rat posterior-frontal suture (Bradley et al, 1996; Roth et al, 1997). The osteoid stroma of the osteogenic front subsequently mineralises and the sutural domain gains a trabecular bone structure populated by osteocytes (Fig. 3.1-1, Fig. 3.1-11). FGFR1 is expressed as protein across the central fibro-cellular (pre-osteoblast) domain, and in the osteoblasts of the osteoid stroma of the osteogenic front. FGFR2 protein, however, labels weakly in the pre-osteoblast domain compared to the neighbouring osteoid (Fig. 3.1-6, iv – vi). Morphological and histochemical evidence of the commencement of sagittal sutural fusion is accompanied by the in-situ immunolocalisation of FGFR1 in the absence of FGFR2 in the fibro-cellular (pre-osteoblast) sutural domain in all the sutures studied (see Figs 3.1-6 - 3.1-8 for examples).

**The in-situ expression of FGFR transcript mRNA in human craniosynostotic sutures**

A study of the expression of FGFR1 and isoform-specific FGFR2 mRNAs was additionally undertaken and contrasted with that of osteonectin, an early marker of osteogenesis in cells of osteogenic fate (Jundt et al, 1987; Nakase et al, 1994). The osteonectin transcript is not expressed in the central unfused suture compared to positive control in the periosteum of the neighbouring parietal bone (Fig. 3.1-9, i). The FGFR2- BEK transcript is expressed across the central, fibro-cellular, domain of the unfused suture and in the periosteum (Fig. 3.1-9, ii). As the suture fuses, however, BEK expression is restricted to the endocranial osteoid of the osteogenic front, and there is negative regulation of the BEK transcript in the adjacent pre-osteoblast domain (Fig. 3.1-9, iii). The progression of sutural fusion is accompanied by the
expression of osteonectin and FGFR1 transcripts in the 'pre - osteoblast' domain, but BEK is not expressed in these cells compared to neighbouring positive control domains in the parasutural parietal bone (Fig. 3.1-9, iv - vi). The onset of human sagittal sutural fusion is thus characterised by the expression of osteonectin and FGFR1 in pre - osteoblasts, where there is a negative regulation of BEK transcript compared to its expression throughout the unfused suture (Fig. 3.1-9, ii).

Sagittal sutural fusion is characterised by the gradual replacement of the fibro - cellular zone by pathologic osteoid as sutural pre - osteoblasts differentiate into osteoblasts within the osteogenic front. BEK transcript is strongly expressed in the osteoblasts of the osteogenic front (Fig. 3.1-9, iii; and see protein data Fig. 3.1-6 - 3.1-8), where the KGFR isoform of FGFR2 is equally co - expressed (Fig. 3.1-10). KGFR is, however, not expressed in the fibro - cellular sutural domain.

The requirements of reconstructive surgical technique in the majority of cases involving lambdoid and coronal suture fusion deny the opportunity to access these sutures for gene expression studies. Suture - specific controlled studies are, therefore, not available for human lambdoid and coronal synostosis. Single isolated cases of lambdoid and coronal suture fusion, however, demonstrate similar themes of FGF/FGFR co-regulation as observed in sagittal synostosis (Fig. 3.1-11).

The expression of FGF2, 4, and 7 protein in human craniosynostosis

Human suture synostosis is accompanied by the expression of FGF2 as protein, which is immuno - localised to all sutural domains throughout stages of progressive fusion (Fig. 3.1-12, 3.1-11, and 3.1-8). FGF2 is a high affinity ligand for the BEK receptor, but binds KGFR with weak affinity. The protein ligands FGF4 and FGF7 (KGF), which have high affinity for KGFR, were therefore investigated in sagittal sutural fusion in - situ (Fig. 3.1-12, Fig. 3.1-13). At no time was either ligand detected in suture or parietal bone, compared to positive controls in human 10 - week dermis (FGF7) or tooth germ (FGF4).

FGF4 is, however, expressed in 10 - week cranial membranous ossification in the osteoblasts closely applied to the nascent osteoid matrix (Fig. 3.1-13), whereas FGF2 is more widely detected (Fig. 3.1-14). This data suggests that FGF4 has a role in embryonic calvarial osseous differentiation, which is down - regulated in foetal and infant stages of development (FGF4 is not expressed in 14 - week human calvariae; S-3.2); alternatively the expressed ligand may be functionally redundant. FGF7, present in the human 10 - week embryonic cranial dermis (Fig.
3.1-12), is not expressed in the underlying cranial mesenchyme or osteoid. FGF2 and FGF4, but not FGF7, are co-expressed as protein in the embryonic meninges with FGFR1, 2, and 3 (Fig. 3.1-13). FGFR3 is weakly expressed, which is consistent with its comparatively weak pattern of expression in the infant cranial membrane bone.

### 3.1.5 Discussion

**FGFR1 and BEK as markers of osteogenic proliferation and differentiation in human cranial ossification**

The differential expression of FGFR1 and the BEK isoform of FGFR2 in human calvarial osteogenesis suggests that these two genes have specific roles in the differentiation and cellular function of osteoblasts and osteocytes. Furthermore, whilst KGFR is expressed at low levels in osteoblasts, it is not expressed in matrix osteocytes, an observation consistent with the transient expression of kgfr in murine embryonic calvarial ossification (Rice et al, 2000). The regulation of the isoform-specific expression of FGFR2 in normal human calvarial osseous differentiation thus appears to be maturity dependent.

This is consistent with the differential pattern of FGFR gene expression observed during human sagittal suture fusion in vivo, compared to the unfused control suture. Sagittal sutural fusion is characterised by the accumulation of bone matrix protein and gradual replacement of the suture by bone. This process is accompanied by a contraction of BEK expression and an expansion of FGFR1 expression in cells of the central fibro-cellular fusing suture, which may be considered ‘pre-osteoblasts’ (Iseki et al, 1997) and are consistent with a proliferative role (Opperman et al, 1998; Liu et al, 1999). These cells express osteonectin, which although not exclusively expressed in bone (Mundlos et al, 1992), is an early osteogenic marker in cells of osteogenic fate (Jundt et al, 1987; Nakase et al, 1994). Thus the expression of osteonectin and FGFR1 characterises a proliferative phase, and the expression of FGFR2 determines a subsequent differentiative phase of human calvarial osteogenesis (Fig. 3.1-15). This data is consistent with the clinical observation that the FGFR1 - Pfeiffer phenotype is less severe than the FGFR2 - Pfeiffer corollary (Rutland et al, 1995; Schell et al, 1995; Muenke and Schell, 1995). FGFR1 signalling is associated with an early phase of human osseous differentiation, and subsequent down-regulation of the receptor upon osteocyte differentiation. In contrast FGFR2 signalling pathways are maintained by osteoblasts of the human fusing suture and calvarial bone, as well as calvarial matrix
osteocytes, which would account for the severity of some FGFR2 craniofacial dysostosis phenotypes (Reardon et al, 1994; Rutland et al, 1995; Wilkie et al, 1995a; Przylepa et al, 1996).

Contrast may be drawn between these data and observations from studies in various mouse models (Fig. 3.1-15). The experimental manipulation of murine embryonic calvarial ossification and sutural gene expression indicates opposing roles in osteogenic differentiation and proliferation for the murine fgfr homologues. At the developmental stages E15 – E18; murine fgfr2 expression co-localises with proliferating calvarial cells, and is mutually exclusive to the expression of the osteopontin gene as a later marker of osseous differentiation than osteonectin (Nakase et al, 1994; Iseki et al, 1997). The ligand FGF2 co-localises with regions of osseous differentiation, but not the proliferating mesenchymal domains of murine fgfr2 (Iseki et al, 1999; Rice et al, 2000). Fgfr1 is expressed in more mature cranial mesenchymal domains and does not correspond to the proliferating zone (Iseki et al, 1999). Addition of exogenous FGF2 (by in-utero bead implantation) results in a shift of the proliferation/differentiation balance towards differentiation, with the downregulation of fgfr2 and the upregulation of fgfr1. FGF1 stimulation causes an anti-differentiative response in murine calvarial cell lines in conjunction with wild type fgfr2 expression (Mansukhani et al, 2000), which is also consistent with a predominantly proliferative role for murine fgfr2 (Iseki et al, 1999). Furthermore, the introduction of the murine homologues of activating FGFR2 mutations (S342W, C342Y) into immortalised murine osteoblast clones causes an increase in DNA synthesis, but down-regulation of mineralisation and alkaline phosphatase expression (Mansukhani et al, 2000). The recent report of a transgenic 'Pfeiffer mouse' is particularly interesting in this context (Zhou et al, 2000). The murine fgfr1- Pro250Arg mutation, analogous to the human FGFR1 – P252R mutation, causes a murine phenocopy of human Pfeiffer syndrome. The fgfr1P250R heterozygote is normal at birth but soon displays frontal, sagittal, and coronal sutural fusions with molecular evidence of accelerated osseous differentiation. The homozygote displays a more severe skeletal phenotype, which suggests that the degree of mutant activation via fgfr1 generates increased severity of osseous phenotype in a ‘dose-dependent’ manner. It remains to be seen whether a murine activated fgfr2 murine model will phenocopy the more common FGFR2 – human craniosynostosis phenotypes. Targeted FGF2 – overexpression in murine cranial sutures, which would activate fgfr1 and fgfr2 isoforms, causes calvarial thickening and macrocephaly, but not sutural fusion (Coffin et al, 1995).

Murine models, by associating fgfr2/fgfr1 with proliferation/differentiation, respectively; contrast with these observations in human sutural fusion; in which the proliferative, pre-osteoblast phase is characterised by the expression of FGFR1 and osteonectin, and the negative
regulation of FGFR2. FGFR2 expression as BEK and KGFR commences with osseous differentiation. The cranial phenotypes of gain-of-function FGFR2 mutations are highly consistent with this observation. Apert foetal and infant cranial osteoblasts in culture demonstrate an increased calcified bone matrix, and an increased expression of molecular markers of osseous differentiation (alkaline phosphatase, osteocalcin, and type 1 collagen) compared to age-matched controls (Lomri et al, 1998). Increases in matrix molecule production are observed in Apert fibroblasts (Bodo et al, 1997) and osteoblasts (Locci et al, 1999), and increased transforming growth factor β1 production and secretion are also observed over control osteoblasts (Locci et al, 1999). No differences are observed in Apert osteoblast growth or the cellular incorporation of tritiated - thymidine, however; suggesting that the activating Apert (Ser252Trp) FGFR2 mutation causes accelerated differentiation, rather than a proliferative effect (Lomri et al, 1998). Consistent with this, activated FGFR2 mutant osteoblasts from Apert (Pro253Arg) and Pfeiffer (Cys342Arg) patients exhibit a lower proliferation rates than control osteoblasts (Fragale et al, 1999), and the heterogeneous phenotypes for osseous differentiation that are observed reflect the maturity differences in the various calvariae examined. Human FGF2 is expressed as protein across the unfused and fusing suture in apparent excess, and across both FGFR1 and FGFR2 domains, unlike endogenous fgf2 in the mouse (Iseki et al, 1997; Iseki et al, 1999). Exogenous FGF2, which has increased affinity for the Apert mutant FGFR2 (Anderson et al, 1998c), stimulates a proliferative response in immature cultured human calvarial cells and a differentiative response in mature cells (Debiais et al, 1998), which is clearly consistent with the data presented here. The differences between the proliferation/differentiation roles for mouse and human FGFRs in calvarial osteogenesis indicate that the results of experimental manipulations in mouse models have only a qualified application to human craniosynostosis.

The differential expression of FGFR genes in the human cranial suture: implications for the pathogenesis of human craniosynostosis.

FGFR3 is the least uniformly expressed FGFR homologue as protein across the mid-sutural, para-sutural, or parietal bone domains of the unfused sagittal suture. Consistently, FGFR3 protein is also weakly expressed in the cranial mesenchyme and dura mater at 10 weeks of human cranial development. This suggests that FGFR3 has a limited, possibly redundant, role in human cranial morphogenesis and sutural molecular signalling pathways. The low expression of fgr3 in mouse calvarial ossification (Iseki et al, 1999), and the lack of obvious sutural dysmorphism phenotype in the fgfr3 (-/-) mouse corroborate this (Colvin et al, 1996; Deng et al, 1996). Nevertheless, human craniosynostosis results from the activating
FGFR3 – Pro250Arg mutation (Moloney et al, 1997; Muenke et al, 1997; Reardon et al, 1997), and pan – synostosis occurs in conjunction with activating mutations of the transmembrane and tyrosine kinase domains (Tavormina et al, 1995a; Wilcox et al, 1998). Given the relative paucity of FGFR3 expression in the human suture, it is likely that these severe synostosis phenotypes are generated by mutant FGFR3 – heterodimers, recruiting FGFR1/2 signalling pathways in the sutural osteogenic precursor. Experimental evidence and FGFR sequence conservation suggest that the P250R mutation may be ligand - dependent and less functionally activating than the transmembrane or tyrosine kinase mutations (Neilson and Friesel, 1996; Mangasarian et al, 1997; Anderson et al, 1998c; Mansukhani et al, 2000), and this is reflected in the variability of the P250R craniosynostosis phenotype. The pathogenesis of severe sutural synostosis in Crouzon - acanthosis nigricans and the thanatophoric dysplasias may thus reflect both the degree of mutant receptor activation and the ability of each mutant to form heterodimers in the induction of sutural osseous differentiation.

Achondroplasia (FGFR3 – Gly375Cys), characterised by a short skull base, but not craniosynostosis, is modelled in the mouse transgenic fgfr3-Gly369Cys (Chen et al, 1999). The murine mutation causes a constitutive activation of fgfr3 which does not abrogate ligand binding, and which does not cause craniosynostosis; despite promoting basicranial ossification and synchondrosal fusion with the upregulated expression of osteocalcin, osteopontin and osteonectin. This suggests that human FGFR3 - mutant craniosynostosis may be generated by heterodimers, the functional and phenotypic consequences of which are specific to the nature of the activating mutation. FGFR - heterodimerisation occurs in the natural state in cells expressing FGFR homologues (Johnson and Williams, 1993), and the use of dominant negative constructs has demonstrated this phenomenon in several experimental models (Ueno et al, 1992; Neilson and Friesel, 1996). There is great variability and cross – over of FGFR – associated craniosynostosis phenotype, which might thus reflect the in – vivo ‘net’ signal transduction in the sutural cell of the mutant FGFR, against a background of signalling by FGFR homologous homo - dimerisation and hetero - dimerisation (Nguyen et al, 1997). The rate of pre - osteoblast proliferation/differentiation would thereby be greatly influenced by the subtleties of differential receptor and ligand expression in the human sutural cell – osteoblast lineage.

**FGFR expression in the fusing sagittal suture: Implications for Coronal suture fusion**

The sagittal suture provides a model by which to study the differential expression of FGFR genes at stages of infant calvarial osseous differentiation in – situ. It is to be noted that
isolated sagittal synostosis currently lacks an identifiable genetic cause, and none of these tissues demonstrate activating FGFR mutations. Likely candidate genes include \( M\Omega X 2 \)
, \( \text{TWIST} \), or other genes encoding proteins involved in FGFR signalling pathways. Parallels, however, may be drawn with other human sutures. Like the coronal suture, which is more commonly (but not exclusively) fused in human FGFR – associated craniosynostosis, the sagittal suture closes early in Crouzon syndrome (Kreiborg et al, 1993), and is variably involved in other FGFR – related craniosynostoses (Puleyn et al, 1996; Moloney et al, 1997). Studies in the mouse model indicate some commonality between the the behaviour of coronal and sagittal sutures to perturbation (Iseki et al, 1999; Zhou et al, 2000), and rare preliminary data from age - matched human lambdoid and coronal sutures (this study), indicates that FGFR co – regulation with suture fusion may be consistent across all human suture types.

**FGFR2 expression in sagittal suture fusion is isoform specific**

\( \text{KGFR} \) is found to be ordinarily transiently expressed in infant cranial osteoblasts, which is consistent with murine kgfr signalling being functionally redundant in cranial membranous ossification (De Moerlooze et al, 2000). \( \text{KGFR} \) expression is upregulated in the pathologic osteoid of sagittal suture fusion. Splicing FGFR2 mutations that cause an ectopic expression of \( \text{KGFR} \) thus have the potential to result in a severe craniosynostosis phenotype, as preliminary evidence suggests in the syndactylous hand (Oldridge et al, 1999). Ectopic \( \text{KGFR} \) expression in the pathologic sutural osteoid allows the recruitment of ligands including FGF3, FGF4, FGF7, and FGF10 from the overlying epithelium or subjacent dura mater; which are both implicated in cranial osteogenesis (Yu et al, 1997), and the control of sutural morphogenesis/programmed fusion in the mouse/rat model (Opperman et al, 1995; Bradley et al, 1997; Opperman et al, 1998; Levine et al, 1998; Kim et al, 1998). FGF7, a high affinity ligand for the KGFR isoform (Orr-Urtreger et al, 1993; Rubin et al, 1995), although expressed in the overlying dermis, is not detected in human embryonic dura mater, cranial osteogenesis, or infant sagittal synostosis. FGF10 (Igarashi et al, 1998; Lu et al, 1999) is a candidate ligand for KGFR – binding in limb morphogenesis (Xu et al, 1998) and in the lung, but is not particularly identified as a bone morphogen. Exogenous FGF4, a high affinity ligand for KGFR (Orr-Urtreger et al, 1993) causes osteogenic differentiation over the osteogenic front of mouse sagittal sutures in culture, but not the midsutural mesenchyme (Kim et al, 1998). FGF4 is expressed in 10 – week human dura mater, and also activates via BEK (Orr-Urtreger et al, 1993) and FGFR3 (Ornitz and Leder, 1992), with which it is co - expressed. FGF4 cannot, however, be demonstrated to be co - expressed with FGFRs in human cranial osteogenesis beyond 13 weeks, thus correlating with data from the murine
model (Kim et al, 1998). The ectopic expression of *fgf3* and *fgf4* in the cranial sutures of a transgenic mouse (*Bulgy-eye, Bey*) correlates with pathologic craniosynostosis and a murine phenocopy of Crouzon syndrome (Carlton et al, 1998). This data suggests that *KGFR* upregulation in human sutural fusion may enlist *FGF3* signalling and promote osseous differentiation.

### 3.1.6 Conclusions

*FGFR* expression in human calvarial osteogenesis is differentiation stage specific. *FGFR3* is non-uniformly expressed in calvarial and periosteal osteoblasts. *FGFR2* expression is isoform specific, *BEK* is expressed in the osteoblast and osteocyte, whereas *KGFR* is weakly expressed in osteoblasts only. *FGFR1* is strongly expressed in osteoblasts, and downregulated in the osteocytes of the trabecular bone matrix. *FGFR* expression differentially co-regulates with infant sagittal craniosynostosis. The cells of the unfused central suture express *FGFR1* and *BEK*. As sutural fusion commences, there is negative regulation of *BEK* in the pre-osteoblast proliferative phase, with continued expression of *FGFR1*. Osteogenic differentiation is characterised by the strong expression of *BEK, KGFR*, and *FGFR1* in the osteogenic front, in the presence of FGF2, but not FGF4 or FGF7. As the suture is completely replaced by trabecular bone, populated by osteocytes, there is a predominance of *BEK* expression, and downregulation of *FGFR1*.

The co-localisation of *FGFR1/FGFR2* expression with proliferation/differentiation roles in calvarial osteogenesis differs from observations in mice, but correlates with genotype-phenotype diversity in *FGFR*-associated craniosynostosis. The finding that *KGFR* is upregulated during sagittal sutural fusion is analogous to genotype/phenotype correlations in *FGFR* associated syndactyly (Oldridge et al, 1999); and provides evidence for novel mechanisms in the pathogenesis of craniosynostosis.
S-3.1 - Figure Legends

Figure 3.1-1

Examples of unfused and fused sagittal sutures, plain photography (x2.5 mag). The unfused sutures (i & ii) are simple vertical structures (s) between opposing parietal bones, comprising a mature trabecular bone matrix (tbm). The suture (s) acts as a dense fibrous ankylosis, flanked by narrow zones of osteoid stroma (os), between the calvarial bones. The trabecular bone structure encloses narrow spaces, filled in life by fat, marrow and blood vessels. These spaces are lined by osteoblasts (Fig 2). The ectocranial surface is lined by periosteum (p), and the endocranial surface by dura mater (d) in – vivo. In the fused sagittal suture (fs; iii and iv) the fibrous ankylosis is completely replaced by bone matrix, which is populated by osteocytes, and has an indistinguishable matrix structure from the neighbouring parietal bone matrix (pbm).

Figure 3.1-2

Human parietal bone, 10 – months, phase microscopy (i, ii); immunohistochemistry (iii, iv). Calvarial osteoblasts (ob) are plump round cells that sparsely populate the immature mineralised bone matrix (i, ii); and line the marrow spaces of mature trabecular bone (iii, iv). Osteocytes (oc) densely populate the trabecular bone matrix, sending a dense network of dendrites (ocd) permeating through the bone. FGFR2 protein is detected in osteoblasts and osteocytes (iii), and strongly labels the osteocyte dendritic processes. FGFR1 protein (iv) labels osteoblast cell bodies very strongly, but is weaker in osteocytes, with a poor signal in the ‘dendrite field’ permeating the parietal bone matrix. Bar = 30 μm.
(i) Parietal bone matrix

(iii) FGFR2 protein

(ii) Parietal bone matrix

(iv) FGFR1 protein

- ocd
- ob
- pcm
- oc
Human parietal bone, 10 – months; *in – situ* hybridisation. *Osteonectin* mRNA (i) is expressed in the osteoblasts (ob) lining the parietal bone matrix (pbm) and periosteum (p); and in the osteocyte (oc) dendrites. *FGFR1* transcript (ii, iii) is strongly expressed in the osteoblasts (ob) lining parietal bone matrix and periosteum (p), but is poorly expressed in the matrix osteocytes and their dendritic processes. *FGFR2* (iv, v) is strongly expressed as *BEK* transcript in the osteoblasts and osteocytes, but relatively weakly expressed as *KGFR* (vi) in the osteoblasts of the periosteum and lining the trabecular bone spaces. *KGFR* is not expressed in the osteocytes of the parietal bone matrix (vi). *Bar* = 30 μm.

**Figures 3.1-4 & 3.1-5**

The unfused sagittal suture is a vertically orientated, fibrous ankylosis between the flat edges of two abutting parietal bones (i – iv); immunohistochemistry. The central suture is predominantly fibrous (white arrows), and populated by undifferentiated sutural cells and blood vessels (fibro – cellular domain, fc). This is symmetrically flanked by thin zones of osteoid stroma (os), populated by osteoblasts, which also densely populate the periosteum (p), forming a continuous ‘cap’ over the suture. Osteoid stroma is laterally flanked by mature parietal bone matrix (pb). FGFR1 and FGFR2 proteins (Fig 3.1-4 & 3.1-5; i, ii) are strongly detected in the fibrocellular suture (fc, white arrows). The signal for FGFR3 (iii) is weak in the suture (fc) compared to the maximal signal for FGFR3 in some of the osteoblasts of the narrow osteoid stroma (os) and periosteum (the black arrow, Fig 3.1-5 iii, is artefact). Signal for FGFR1 and 2 proteins is strong throughout the osteoid stroma (os) and periosteal (p) domains (Fig 3.1-4 & 3.1-5, i & ii). *Bar* = 120 μm.
(i) osteonectin transcript

FGFR1 transcript. (ii) Light-field and (iii) Dark-field

BEK transcript. (iv) Light-field, (v) Dark-field

(vi) KGFR transcript. Dark-field
Figure 3.1-6

Human sagittal suture fusion is accompanied by the accumulation of bone matrix proteins (durazol red stain) at the endocranial aspect (e) of the central fibro-cellular suture (i; fc). Progressive fusion is characterised by the proliferation of sutural pre—osteoblasts (ii; po) in the fibro-cellular suture (fc), which is down—regulated as sutural fusion comes to an end (iii; po). Pre—osteoblasts of the fibro—cellular sutural domain become enveloped in bone matrix proteins (i; see area of inset iv—vi) and differentiate into osteoblasts to form an ‘osteogenic front’ (iv—vi; of) in the advancing osteoid stroma at the endocranial aspect (e) of the suture. FGFR1 protein (v) is detected in the fibro—cellular (fc) and osteogenic front (of) domains, whereas FGFR2 protein (vi) is strongly detected in the osteogenic front (of) domain, with a weak signal in the fibro—cellular (fc) midsuture. Bar = 120 \mu m in (i); 30 \mu m in (ii) and (iii); 60 \mu m in (iv—vi).

Figure 3.1-7

The onset of sagittal suture fusion is accompanied by the proliferation of central sutural pre—osteoblasts (i; po) and increased cell density in the endocranial aspect (e) of the fibro—cellular (fc) suture (i; negative immuno—control). The osteogenic front (ii—v; of) advances from the parietal bone (pb) into the suture. FGFR1 protein (ii, iv) is detected throughout the fibro-cellular (fc) and osteogenic front (of) zones. FGFR2 protein (iii, v) is detected in cells of the osteogenic front (white arrows) but not the fibro—cellular zone (black arrows). Bar = 50 \mu m.
Sagittal suture fusion

Inset ii

Inset iv - vi

Sagittal suture: pre-osteoblasts

(iii)

Sagittal fusion: control

(ii)

FGFR1 protein

(i)

FGFR2 protein

(iv)

(v)

(vi)
Figure 3.1-8

Sagittal suture fusion is characterised by a high density of sutural cells in the fibrocellular (fc) zone of the suture (i, immuno — control). Suture fusion is accompanied by the expression of FGF2 (ii) as protein across the parietal bone (pb), osteogenic front (of), pericranial (p) and fibro — cellular (fc) domains. FGFR1 protein (iii) is maximally detected in the osteoblasts (ob) lining parietal bone (pb) and periosteum (p), and broadly throughout the osteogenic front (of, white arrows). Aggregations of cells in the fibro- cellular domain express FGFR1 protein (fc, white arrows). FGFR2 protein (iv) is maximally detected (white arrows) in the osteoblasts (ob) lining parietal bone (pb), but compared to this domain of high signal density, there is poor detection of FGFR2 (black arrows) in the fibrocellular zone (fc). Bar = 120 µm.

Figure 3.1-9

Sagittal suture; unfused and fusing; in — situ hybridisation. Osteonectin transcript (i) is not expressed in the fibro-cellular domain (fc) of the unfused sagittal suture (s), compared to its expression in the periosteum (p), and osteoblasts lining the parietal bone (pb) (small white arrows = artefact). BEK transcript (ii) is expressed in the periosteum (p) overlying the unfused suture (s), and across the fibro — cellular zone (fc). With the commencement of sutural fusion (iii), BEK expression is restricted to the osteogenic front (of) in the endocranial aspect (e) of the suture, but is negatively regulated in the fibro — cellular, pre — osteoblast domain (fc). Osteonectin expression (iv) upregulates in the pre- osteoblast domain (fc) as sutural fusion progresses (s), and is more densely expressed in the osteoid (o), periosteal (p) and parietal bone (pb) domains. FGFR1 (v) is co — expressed in these domains, whereas BEK (vi) is not expressed in the pre- osteoblasts of the midsuture (fc) compared to expression of BEK in the osteoblasts (ob) of the osteoid (o) and parietal bone (pb) domains. The FGFR1 signal intensity (v) from the osteoblast domains in the parietal bone (pb) is consistently higher than that of BEK (vi), correlating with the protein data (Fig.2, see also Fig3). Bar = 120 µm.
Sagittal suture; negative control

Sagittal suture fusion; FGF2 protein

Sagittal suture fusion
FGFR1 protein

Sagittal suture fusion
FGFR2 protein
Unfused sagittal suture
Osteonectin transcript

Unfused sagittal suture
BEK transcript

Sagittal suture fusion
BEK transcript

Sagittal suture fusion:
Osteonectin transcript

Sagittal suture fusion:
FGFR1 transcript

Sagittal suture fusion:
BEK transcript
Figure 3.1-10

Sagittal sutural fusion (schematic, i) commences at the endocranial aspect (e) of the suture (dm; dura mater in vivo), as osteogenic fronts (of) at the parietal bone edge (pb, black arrows); and extends in a pericranial (p) direction into the suture (s). FGFR2 is expressed as both BEK (iii) and KGFR (ii, iv) in the osteoblasts of the osteogenic fronts of the endocranial aspect of the fusing suture. KGFR transcript signal is strong in the osteogenic front (of) compared to the osteoblasts (ob) of the parietal bone domain (pb), and is equal to that of BEK. Bar = 60 μm

Figure 3.1-11

Age matched lamboid (i – iii) and coronal (iv – vi) sutures in states of fusion; immunohistochemistry. FGF2 (i) is expressed across the fibro-cellular (fc), osteoid (o), and osteogenic front (of) domains of the fusing lambdoid suture. FGFR1 (ii) is strongly expressed as protein in the fibro-cellular (fc), osteogenic front (of) and osteoid (o) domains, whereas FGFR2 protein (iii) is poorly expressed in the fibro-cellular domain (fc), compared to the osteogenic front (of), and FGFR1 (compare white arrow zones). The unfused coronal suture (iv) has a low sutural cell density, and is lined with osteoblasts at the parietal bone (pb) edge expressing FGFR1 transcript (white arrows). Sutural fusion (vi) is characterised by increased cell density in the osteogenic front (of, white arrows) in the suture (s), and the expression of FGFR1. Complete replacement of the suture by bone (vi; fs), is accompanied by the expression of FGFR2 as BEK transcript in the matrix osteocytes (white arrows), and periosteum (p). Bar = 120 μm in (i-iii); and 60 μm in (iv-vi)
Sagittal fusion: KGFR

Sagittal fusion: BEK

Sagittal fusion: KGFR
Lambdoid: FGF2 protein

FGFR1 protein

FGFR2 protein

Unfused coronal suture
FGFR1 transcript

Coronal suture fusion
FGFR1 transcript

Fused coronal suture;
FGFR2-lglllc transcript
Figure 3.1-12

Sagittal suture fusion; ligand immunohistochemistry. FGF7 (i) is expressed as protein in the human cranial embryonic dermis, but not the subjacent cranial mesenchyme (m) or osteoid bone (o). FGF7 protein is not detected in the any domain of the fusing sagittal suture (iii). FGF2 protein (iv) is detected in the osteoblasts (ob), 'pre – osteoblasts' of the fibro - cellular domain of the fusing suture (fc), and parietal bone (pb), compared to negative control (ii). Bar = 30 μm.

Figure 3.1-13

FGF4 detection in foetal cranial ossification, and odontogenesis, but not infant cranial ossification; immunohistochemistry. FGF4 protein (i) is detected in the papillary mesenchyme (p), and enamel epithelium (ee) of the 13 – week human tooth germ, compared to negative control (ii). FGF4 is detected in the osteoblasts (o) of human 10 - week parietal bone osteoid (o), but has a weak signal in the wider cranial mesenchyme (cm). FGF4 protein is not detected (vi) in the infant parietal bone osteoblasts (ob) or osteocytes (oc) or infant suture (not shown). Bar = 30 μm.
FGF4 protein: tooth germ

Tooth germ: negative control

Foetal cranial mesenchyme
FGF4 +ve control

Perisuture osteoblasts,
FGF4
**Figure 3.1-14**

The dura mater (dm), lining the nervous tissue (nt) of the embryonic brain; immunohistochemistry. FGF2 protein (ii) is detected in the human 10-week dura mater (dm, white arrows), and overlying cranial mesenchyme (cm). FGF4 is also detected in the dura (iii), but is only strongly expressed in the condensing cranial mesenchyme of the embryonic osteogenic front, and not the wider mesenchymal domain (see Fig12). FGFR1, FGFR2, and FGFR3 proteins are detected in the dura mater (dm; white arrows), FGFR3 gives the weakest signal (iv - vi). FGFR1, FGFR2 and FGFR3 are all expressed in the 10-week cranial mesenchyme (cm, white arrows); FGFR1 and FGFR2 are strongly detected in the condensing mesenchyme around cranial osteoid bone. Bar = 30 μm.

**Figure 3.1-15**

Schematic diagram (i, ii) indicating FGFR expression in human sagittal synostosis as a model of accelerated calvarial differentiation. FGFR1 and FGFR2 (as the BEK transcript) are expressed across the unfused suture (i), but FGFR3 has a relatively restricted domain in the osteoid stroma (o). KGFR is not expressed. In sutural fusion there is proliferation of pre-osteoblasts (pz), which express FGFR1, but the BEK expression domain is negatively regulated. FGFR2 is expressed as BEK and KGFR in the differentiating osteogenic front (of), which advances into the suture from the endocranial aspect (e) and parietal bone (pb) edge; under the presumptive control of soluble factors from the dura mater (dm). Such factors may include FGF2 and FGF4 in human cranial development, but not FGF7. In the murine suture (iii), fgfr2 co-localises with proliferative domains (pz), and fgfr1 with more differentiated domains (dz). The stimulus to differentiate results in expansion of the fgfr1 domain and contraction of the fgfr2 domain.
(i) [Diagram showing labeled structures and colors]

Key:

- FGFR1
- BEK
- KGFR
- FGFR3

(ii) [Diagram showing labeled structures and colors]

(iii) Murine model

- 'Quiescent suture'
- dz
- Stimulus to differentiate

[Diagram showing labeled structures and colors]
3.2 Negative autoregulation of FGFR2-IgIIIa/c expression characterises cranial development in Apert (P253R) & Pfeiffer (C278F) syndromes, and suggests a basis for differences in their cranial phenotypes.

3.2.1 Abstract

Heterogeneous mutations in the fibroblast growth factor receptor 2 gene (FGFR2) cause a range of craniosynostosis syndromes. The specificity of the Apert cranial phenotype reflects its narrow mutational range, in that 98% of Apert cases result from the Ser252Trp or the Pro253Arg mutations in the FGFR2 – IgIIIa extracellular subdomain. In contrast, a broad range of mutations throughout the extracellular domain of FGFR2 causes the overlapping cranial phenotypes of Pfeiffer, Crouzon, and related craniofacial dysostoses.

The expression of FGFR1, the IgIIIa/c and IgIIIa/b isoforms of FGFR2, and FGFR3 is investigated in Apert (P253R) and Pfeiffer (C278F) foetal cranial tissue and contrasted with normal human controls. FGFR1 and FGFR3 are normally expressed at 14 – weeks in the cells of the periosteum and osteoid, in domains overlapped by that of FGFR2, which widely includes pre – osseous cranial mesenchyme. FGFR2 expression is, however, restricted to domains of advanced osseous differentiation in both Apert and Pfeiffer cranial skeletogenesis, in the presence of FGF2 but not FGF4 or FGF7. Whereas expression of the FGFR2-IgIIIa/b (KGFR) isoform is restricted in normal human cranial osteogenesis, there is preliminary evidence that KGFR is ectopically expressed in Pfeiffer cranial osteogenesis.

Contraction of the FGFR2-IgIIIa/c (BEK) expression domain in cases of Apert and Pfeiffer foetal cranial ossification suggests that the mutant activation of this receptor, by ligand - dependent or ligand - independent means, results in negative - autoregulation. This phenomenon, resulting from different mechanisms in the two syndromes, offers a model by which to explain differences in their cranial phenotypes.
3.2.2 Introduction

A range of mutations in three homologous genes encoding the fibroblast growth factor receptor (FGFR) proteins 1-3 cause related syndromic craniofacial dysostoses. These syndromes, which variably feature craniosynostosis, facial dysmorphism, and extracranial manifestations, were initially identified as frequent phenotypic clusters. Apert syndrome (Apert, 1906) features a narrow range of craniofacial dysmorphism including: pterional indrawing, a markedly foreshortened skull base, turribrachycephaly, severe midfacial retrusion with hypertelorism; and coronal sutural synostosis, with a widely unossified median sagittal diastema in place of metopic and sagittal sutures (Kreiborg and Cohen, Jr., 1990; Kreiborg et al., 1993; Cohen and Kreiborg, 1994). The characteristic Apert cranial phenotype reflects a narrow mutational range within the \textit{FGFR2} gene. Two neighbouring 'linker region' mutations in the IgIIia extracellular subdomain of FGFR2 cause 98% Apert cases (Wilkie et al., 1995a) as a result of ligand-dependent 'gain of function' (Anderson et al., 1998c).

By contrast, the related syndromes of Crouzon, Pfeiffer, and Jackson Weiss show a more variable and overlapping craniofacial dysmorphism. The basicranial sutures generally fuse earlier than in Apert syndrome, and the metopic and sagittal sutures form and fuse without the unossified median diastema (Kreiborg et al., 1993; Cinalli et al., 1995). Furthermore, facial retrusion may range from negligible to severe; and may variably effect the supraorbital or midfacial skeleton, with minimal pterional indrawing. The phenotypic variability within and between these syndromes reflects their mutational base. A wide range of mutations encompassing the extracellular and transmembrane domains of FGFR2 causes the 'Crouzon – Pfeiffer' group of syndromes, with greatest frequency in the IgIIic subdomain of the FGFR2-IgIIia/c (BEK) splice variant (Burke et al., 1998). Many of these mutations result in the creation or removal of an unpaired cysteine residue, or affect a neighbouring site to cause conformational change and confer ligand independent functional gain (Neilson and Friesel, 1995; Neilson and Friesel, 1996; Galvin et al., 1996; Mangasarian et al., 1997; Robertson et al., 1998). Previous investigation of mutant FGFR2 signalling has been undertaken in model systems which lack the profile of cofactors and signalling molecules that is endogenous to human tissue. These studies cannot, therefore, directly inform attempts at clinical genotype/phenotype correlation.

A series of FGFR expression studies in normal human foetal and infant parietal osteogenesis is reported, and contrasted with cases of Apert (FGFR2 – P250R) and Pfeiffer (FGFR2 – C278F) cranial morphogenesis. The two different mutations, previously found by \textit{in-vitro} analyses to have different modes of activation, have similar effects upon the \textit{in-situ}
expression of FGFR2. Taken together, previous in–vitro and the current in–situ analyses in human tissues may provide a molecular basis for their differences in craniofacial form.

3.2.3 Materials & Methods

Preparation of foetal crania

Human fetuses aged 8 -14 weeks (n=6) were provided by the MRC/Wellcome Trust Human Developmental Biology Resource maintained at the Institute of Child Health and University College Hospital, London. The fetuses were obtained and prepared as described above (5.2.1, 2.2). The Apert fetus (n=1) of an Apert mother (P253R) was obtained and prepared as described above (5.2.1).

Preparation of late foetal and infant suture material

Cranial tissue was obtained from the medical termination of a 27 - week Pfeiffer fetus; following ultrasound diagnosis of the craniofacial dysostosis phenotype arising from a de novo mutation (FGFR2 - C278F). The sagittal suture (n=1) was harvested en–bloc with adjacent membrane bone, cut into serial samples and prepared as previously described (5.2.1). Redundant sagittal sutures and their parietal cranial bone margins were retrieved with prior ethical approval at elective cranioplasty from scaphocephalic patients (n=6; median age 10 months) of the Craniofacial Centre at Great Ormond Street Hospital, London. In no case was a specific molecular diagnosis obtained. The sutures/calvariae were prepared as previously described (5.2.1). Representative FGFR gene expression analyses from a single sagittal suture (‘unfused’ v. ‘fusing’) and parietal cranial bone samples are used as positive control data in this report.
Studies of FGFR and allied gene expression

FGFR1, osteonectin, FGFR2-IgIIIa/c (BEK), and FGFR2-IgIIIa/b (KGFR) mRNA transcripts were detected by in situ hybridisation. Probe synthesis and in - situ hybridisation steps are described in S-2.2.

The protein receptors FGFR1, FGFR2, and FGFR3; and the FGFs 2, 4, and 7 protein products were detected by immunohistochemistry using commercially available specific antibodies (S-2.2.3). The anti - receptor antibodies have epitopes corresponding to the carboxy - terminal sequences of the receptor protein, and therefore label both IgIIIc and IgIIIb isoforms in immunohistochemistry studies.

Control Analyses

The purpose of this report is to analyse whether FGFR - activation by Apert/Pfeiffer mutations affects FGFR expression during cranial osteogenesis in – situ. The normal human fetuses (n=6) showed similar patterns of differential FGFR expression in the cranial tissue examined; and only 14 – week data is shown in this report. The 14 – week normal human cranial tissue provides age- and site- matched basicranial parietal control for the 14 – week Apert fetus. The 14 – week and 10 - month normal parietal tissue, and 10 - month sagittal suture provide evidence of the continuity of consistent differential FGFR expression throughout normal human cranial development. The 10 - month fusing sagittal suture provides a model for FGFR – independent human cranial osseous differentiation (sutural cell – osteoblast); and thus an adequate positive control for FGFR – dependent Pfeiffer cranial ossification in the sagittal osteogenic front at 27 weeks (mesenchyme – osteoblast). Normal human 27 – week cranial tissue control is unavailable. However, it is expected from these observations in normal human cranial development from 8-14 weeks (n=6), and in the infant sagittal suture group (n=6), that FGFR expression in normal 27 – week cranial ossification would be consistent with the 14 – week and 10 month control data presented. In general, neither immunohistochemistry nor in – situ hybridisation can be considered quantitative in stand - alone studies. In these analyses, however, the relative expression domains of FGFR homologues/isoforms in control tissue are contrasted to changes in their relative expression in study tissue. Consistent differences are noted at the level of both transcript and protein, in tissues having undergone the same experimental protocols.
3.2.4 Results

Apert (P253R) cranial ossification at 14 weeks demonstrates the negative autoregulation of FGFR2 expression

Serial coronal sections of normal human embryo – foetal crania at 14 weeks indicate that cranial membranous ossification begins in the basicranium and extends to the vertex. Cranial mesenchyme consists of cells in an early stage of pre-osseous differentiation. As these mesenchymal cells differentiate, they form aggregate mesenchymal condensations, and, as osteoblasts, become enveloped in matrix osteoid (Hall and Miyake, 1992; Hall and Miyake, 1995). FGFR1 mRNA is expressed in the normal 14-week parietal osteoid, periosteum and dura mater at the cranial vertex (Fig. 3.2-1). FGFR2 expression is illustrated in the basicranial parietal osteoid (Fig. 3.2-1; ii, iii). FGFR2 is expressed as BEK transcript in the parietal osteoid and a wide area of surrounding cranial mesenchyme in coronal section; whereas the KGFR transcript is weakly expressed in the pericranial aspect of the parietal osteoid only. The differential expression of the transcript isoforms of FGFR2 is maintained at 10–months of normal human parietal bone development. BEK is expressed in the osteoblasts lining the parietal trabecular bone spaces, and in the osteocyte cell bodies and dendrites permeating the trabecular bone matrix (Fig. 3.2-1; iii-vi). KGFR, however, is weakly expressed in the osteoblasts lining the trabecular bone spaces, and is not expressed in the osteocyte cell bodies/dendrites that populate the matrix bone.

To investigate whether the Apert FGFR2 - P253R mutation has an effect upon the expression of FGFR2 in – vivo, in – situ hybridisation for the BEK transcript has been undertaken in 14-week Apert parietal bone development and compared with age- and site-matched normal tissue. Apert basicranial parietal osteoid is shown in transverse section (Fig 3.2-2; i-vi). The BEK transcript is not expressed in the cranial mesenchyme; and is only weakly expressed in the osteoblasts closely applied to parietal osteoid, despite the strong co-expression of the osteonectin gene (Fig 3.2-2; i-civ; ii-civ). This is in contrast with the strong expression of BEK in the age- and site-matched normal human parietal osteoblasts and adjacent cranial mesenchyme (Fig 3.2-1; ii, in coronal section). FGFR1 mRNA is expressed by the osteoblasts of the Apert basicranial parietal osteoid and the cells of the cranial mesenchyme (Fig. 3.2-2; iii-civ). The differential density of the FGFR1 signal in the Apert basicranial mesenchyme compared to that of the cranial vertex in the age-matched normal fetus (Fig 3.2-1; i) probably reflects a site-specific difference in developmental maturity.
Age- and site-matched Apert and normal foetal parietal calvariae were therefore investigated for differences in the expression of FGFR2 as protein. FGFR1, 2, and 3 proteins are consistently and differentially detected in normal basicranial parietal development at 14 weeks in coronal section (Fig 3.2-3). FGFR1 protein is detected in the osteoblasts lining the parietal osteoid and in the mesenchymal condensations, but the signal density is weaker in the cells of the wider cranial mesenchyme. FGFR2 protein is strongly detected in all three calvarial domains, including a high signal density in the cells of the cranial mesenchyme; thus corroborating the BEK transcript data (Fig 3.2-1, ii). FGFR3 protein is detected in all osteoblast and cranial mesenchymal domains. This normative control protein data is in keeping with previously published in-situ hybridisation studies (Delezoide et al, 1998; Chan and Thorogood, 1999). The 14-week Apert membranous parietal basicranium is characterised in transverse section by ‘islands’ of osteoid matrix within the cranial mesenchyme (Fig 3.2-3; v-viii). The parietal bone forms from the aggregation of cranial mesenchymal cells into mesenchymal condensations, which establish an ‘osteogenic front’. The cells of the osteogenic front further differentiate into osteoblasts that eventually generate parietal osteoid matrix. FGFR1 and FGFR3 are expressed as protein in the osteoblasts of the parietal osteoid, the osteogenic front, and cells of the condensing cranial mesenchyme (Fig 3.2-3). FGFR2 protein is maximally detected in the osteoblasts closely applied to the parietal osteoid. The FGFR2 – signal intensity is weak in the cells of the Apert mesenchymal condensations, however, when compared to that of the neighbouring Apert parietal osteoblasts; and the FGFR1 - signal in comparative Apert domains. This is a reversal of the comparative FGFR1/FGFR2 expression in the mesenchymal domains of normal basicranial parietal osteogenesis, where FGFR2 has a wider expression domain than FGFR1 (Fig. 3.2-3; ii, iii). Thus, the Apert P253R mutation is associated with a relative contraction of the expression domain of FGFR2 as protein at 14 – weeks of human basicranial parietal ossification.

The two principal Apert mutations (S252W and P253R in FGFR2) have been shown to confer increased ligand - binding affinity for FGF2 but not FGF4 upon the mutant receptor (Anderson et al, 1998c). Apert 14 – week foetal cranial tissue expresses the ligand FGF2 strongly in both mesenchymal condensations and matrix osteoblasts, but FGF4 and FGF7 are not expressed at 14 weeks, compared to positive controls in tooth germ and cranial dermis respectively (Fig. 3.2-4, i – vi).
**FGFR expression in Pfeiffer cranial osteogenesis**

Human cranial ossification commences at ossification centres corresponding to each of the cranial membrane bones and progresses radially, such that the cranial sutures form under the presumptive molecular control of the dura mater at specific sites where the advancing bone fronts meet (Albright and Byrd, 1981; Johansen and Hall, 1982; Opperman et al, 1998; Levine et al, 1998; Kim et al, 1998). At 27 - weeks the cranial sutures of a Pfeiffer (C278F) fetus display degrees of development approaching infant maturity. The coronal and lambdoid sutures, which radiate from the skull base, show more advanced stages of development than those at the vertex, in keeping with previous observations (Albright and Byrd, 1981). In transverse section, the foetal sutures consist of a wide central area of undifferentiated cranial mesenchyme, flanked by 'osteogenic fronts' (Johansen and Hall, 1982) of osteoblasts and osteoid matrix. FGFR1 and FGFR2 are differentially expressed in the osteogenic fronts approaching the sagittal suture of the 27 - week Pfeiffer fetus (Fig 3.2-5). FGFR1 protein is detected in the cranial mesenchymal cells and in the osteoblasts of the osteogenic front osteoid (Fig 3.2-5; i,e,iii). FGFR2 protein is not detected in the cranial mesenchymal cells, but is present in the osteoblasts closely applied to the osteoid matrix of the advancing osteogenic front (Fig 3.2-5; ii,e,iv). The 27 - week Pfeiffer (C278F) cranial osteoid cannot be precisely age - matched with normal cranial tissue. However, a similarly activating and functionally analogous mutation (FGFR2 - C342R) has been shown to promote the expression of osseous differentiation markers in human osteoblasts in - vitro (Fragale et al, 1999), and infant sagittal synostosis provides positive control for accelerated human calvarial ossification in - situ. FGFR1 and FGFR2 are differentially expressed in the 10 - month fusing sagittal suture compared to unfused sagittal suture control (Fig 3.2-6). The unfused sagittal suture at 10 - months in transverse section presents a fibro-cellular midzone, flanked symmetrically by a narrow osteoid stroma and trabecular parietal bone. FGFR1 and FGFR2 proteins are equally detected in the mid-sutural cells and the osteoblasts of the osteoid stroma (Fig 3.2-6, ii,e,iii). Sutural fusion is accompanied by replacement of the fibro-cellular suture by pathologic osteoid as sutural cells undergo osteogenic differentiation (with the expression of bone matrix proteins and markers of osseous differentiation - not shown, 5.3.1.4). FGFR1 protein is detected in the osteogenic front and fibrocellular domain of the fusing suture (Fig 3.2-6; i); whereas FGFR2 protein is detected in the osteogenic front but lacks expression in the mid-sutural, fibro-cellular domain compared to the unfused sagittal control (Fig 3.2-6; iii, vi). Thus, the osseous differentiation pathway in the 10 - month fusing sagittal suture is characterised by the negative regulation of FGFR2. The gain - of - function C278F mutation in the 27 - week Pfeiffer similarly correlates with a restricted expression of FGFR2 as protein in the osteoblasts of the osteoid, but not the cranial mesenchyme (Fig 3.2-5; ii, iv). It is thus
tempting to speculate that C278F negatively regulates the expression of FGFR2 in Pfeiffer cranial osteogenesis. An investigation of the isoform-specific expression of FGFR2 transcripts in the 27-week Pfeiffer osteogenic front corroborates this theory (Fig 3.2-7). The BEK transcript is poorly expressed in the Pfeiffer osteoblasts surrounding osteoid bone, with no appreciable mesenchymal signal, compared to the strong expression of BEK in both infant (Fig 3.2-1; in, iv) and foetal (Fig 3.2-1; ii) normal human cranial osteogenesis. KGFR transcript, however, is strongly expressed in the 27-week Pfeiffer cranial osteoblasts and mesenchymal cells compared to wild-type infant and foetal stages (Fig 3.2-7; Fig 3.2-1; iii, vi). This provides preliminary evidence that KGFR is ectopically expressed in some forms of cranial ossification in FGFR-associated syndromic craniofacial dysostosis, which correlates with independent evidence of KGFR over-expression in forms of FGFR-associated syndactyly (Oldridge et al, 1999).

3.2.5 Discussion

Ninety three percent of syndromic craniofacial dysostosis cases are caused by mutations in the FGFR2 gene (Passos-Bueno et al, 1998a). Those mutations within the IgIIIc domain affect the FGFR2-IgIIIa/c (BEK) isoform, which is dominantly expressed in normal embryo-foetal human osseous differentiation (Delezoide et al, 1998; Chan and Thorogood, 1999) and infant cranial bone (this report). Mutations within the IgIIIa domain affect both the IgIIIa/c and IgIIIa/b (KGFR) isoforms. KGFR is dominantly expressed in epithelia (Orr-Urtreger et al, 1993), and is only weakly expressed in normal human cranial osteogenesis. This study describes the differential expression of FGFR genes in normal human cranial skeletogenesis and contrasts this with that observed in Apert and Pfeiffer cranial ossification. These novel data, in tissues containing the complement of human endogenous receptor splice variants, ligands and signalling cofactors, provide an analysis of the in-situ molecular shifts which generate human craniofacial phenotypes resulting from pathogenic mutations in FGFR2.

Embryonic cranial ossification extends from basicranium to vertex. FGFR homologues are normally consistently differentially expressed in the parietal primordia at 14 weeks. In the normal fetus FGFR2 is strongly expressed as BEK transcript in parietal osteoblasts and a wide area of surrounding mesenchyme. FGFR2 protein is also detected in these tissues, where it demonstrates a wider expression domain in the normal cranial mesenchyme than FGFR1. By contrast, the age- and site-matched Apert tissue demonstrates a contraction of the expression domain of FGFR2 expressed as the BEK transcript in the cranial
mesenchyme around the developing parietal bone. This is accompanied by a contraction of the FGFR2 protein domain; such that in Apert basicranial parietal osteogenesis, FGFR1 protein is detected more widely than FGFR2 in parallel section (unlike control). The Apert FGFR2 - P253R mutation, acting by ligand - dependent homodimerisation (Anderson et al, 1998c), thereby appears to cause a negative autoregulation of the BEK isoform in the presence of FGF2 compared to normal wild - type control. Independent evidence from an in - vitro Apert osteoblast culture system corroborates the observation of FGFR2 down - regulation, in immortalised cells derived from a patient with the S252W Apert mutation (Lemonnier et al, 2000). In this report, the negative - regulation of FGFR2 as both transcript and protein is demonstrated in Apert (P253R) foetal parietal ossification, in the in - situ environment of endogenous co - factors, ligands, and matrix molecules. The data thus permits preliminary genotype – phenotype correlations. Apert mutant 'gain - of - function' (Anderson et al, 1998c), phenotypically represented as premature ossification at the cellular level (Lomri et al, 1998,Fragale et al, 1999), will be maximally demonstrated through a restricted and relatively mature domain of osteoblasts in the basicranial osteoid compared to the pre-osseous mesenchyme towards the cranial vertex. This correlates with the 'classic' clinical phenotype (Fig 8) of the markedly foreshortened Apert basicranium and widely unossified vertical median diastema (Kreiborg and Cohen, Jr., 1990). The frontal and parietal bone - fronts become widely splayed by the disproportionate basicranial ossification, such that initiation of Apert metopic and sagittal sutural morphogenesis (Johansen and Hall, 1982) never occurs, and delayed ossification by coalescing islands of bone results by default (Kreiborg and Cohen, Jr., 1990,Kreiborg et al, 1993). Apert coronal synostosis, however, begins at the sphenoidal skull base. It is, therefore, suggested that Apert gain – of - function mutations, promoting mutant FGFR2 – homodimerisation (Anderson et al, 1998c), 'drive' coronal sutural osteogenesis (Lemonnier et al, 2000), despite normal dural influences to maintain sutural patency (Opperman et al, 1995). The negative - autoregulation of FGFR2 – BEK, and differential FGFR expression relative to osteoblast maturity, also correlate with the marked infant Apert pterional stenosis phenotype. Apert pterional prematurity correlates with regulated FGFR2 expression in the 'pro – differentiated' membranous basicranium, whereas osteogenic precursor cells recruited into the early osteogenic pathway in domains towards the vertex will express FGFR1 predominantly. This process occurs in the presence of FGF2, a preferential ligand in Apert FGFR2 signalling (Anderson et al, 1998c), which has been independently shown to negatively regulate FGFR2 expression in a murine cranial suture model (Iseki et al, 1997). Apert mutant FGFR2 does not display increased affinity for FGF4 ligand (Anderson et al, 1998c), in keeping with its lack of expression in cranial ossification in the normal or Apert mutant at 14 - weeks.
Whereas 98% of Apert cases are caused by ligand-dependent mutations in the extracellular 'linker region' (IgIIia subdomain) of FGFR2 (Wilkie et al, 1995a; Oldridge et al, 1999); Pfeiffer, Crouzon and related phenotypes show greater genetic heterogeneity. Although Pfeiffer syndrome may result from an FGFR1 mutation (Muenke et al, 1994; Schell et al, 1995), and a Crouzon variant arises from FGFR3 mutants (Meyers et al, 1995), the majority of causative mutations are spread throughout the extracellular IgIIIc subdomain of the FGFR2-IgIIia/c (BEK) isoform (Cornejo-Roldan et al, 1999). Furthermore, phenotypes of Jackson-Weiss syndrome (Jabs et al, 1994; Gorry et al, 1995; Park et al, 1995b; Tartaglia et al, 1997b), Beare-Stevenson cutis gyrata (Przylepa et al, 1996), and variants of the Antley–Bixler phenotype (Reardon et al, 2000), have all been attributed to mutations affecting the IgIIIa/c subdomains or adjacent transmembrane region of FGFR2. A variable and non-classifiable craniofacial dysmorphism affecting a large kindred also results from an IgIIIc mutation in FGFR2 (Steinberger et al, 1996). The range of craniofacial phenotypes that arise from non-Apert FGFR2 mutations is therefore wide and overlapping and reflects the genetic heterogeneity. Point mutations in the same locus may result in phenotypes ascribed to different syndromes (Passos-Bueno et al, 1999), which change longitudinally with time (Pulleyn et al, 1996), giving rise to diagnostic confusion (Mulliken et al, 1999). Negative-autoregulation of FGFR2-BEK expression appears to characterise Pfeiffer (C278F) cranial development. The Pfeiffer 27-week sagittal suture primordia display a restricted expression domain for both FGFR2 protein and the BEK transcript in the parasagittal osteogenic front. These tissues, bearing the activating C278F mutation, can be contrasted with consistent differences in both the 14-week and 10-month normal controls. At 14-weeks, there is a wide expression of both BEK transcript and the detected receptor protein in the parietal osteoblasts and cranial mesenchyme. BEK transcript is correspondingly strongly expressed in the 10-month parietal osteoblasts and osteocytes of the parietal bone matrix; and protein FGFR2 is detected in the cells of the unfused sagittal suture and osteoblasts of the parasutural osteoid. FGFR2 protein is negatively regulated, however, in the central sutural, fibro—cellular domain in conjunction with sagittal suture fusion and in comparison to FGFR1 protein (see also §3.1). This pathologic process, unconnected with FGFR mutations, provides a positive control for the relative dysregulation of FGFR2 in human calvarial osseous differentiation at 10-months. Acceleration of osseous differentiation is implied by FGFR2 mutations causing Pfeiffer syndrome (Fragale et al, 1999); and is consistent with negative—autoregulation causing the restricted FGFR2 expression domain observed in the 27-week Pfeiffer sagittal osteogenic front, as compared to the relative expression of FGFR1/FGFR2 in control tissue.
C278F also causes Crouzon and Jackson–Weiss syndromes (Passos-Bueno et al., 1998a). These variable ‘Crouzon – Pfeiffer’ phenotypes, lacking the Apert - type median diastema, markedly shortened skull base, and pterional indrawing, may be explained by the possible effect of ligand - independent FGFR heterodimerisation. In this model, the ‘Crouzon – Pfeiffer’ mutant FGFR2 protein, activated by mutations conferring ligand independence, may form heterodimers (Ueno et al., 1992;Johnson and Williams, 1993) with FGFR1 and FGFR3, with which it is co - expressed in human membranous cranial ossification. A common theme of non - Apert craniosynostosis mutations is the creation or ablation of an unpaired cysteine residue, causing conformational change in the extracellular IgIII subdomains of the receptor. Insertional techniques of mutant constructs into cell lines (Galvin et al., 1996;Mangasarian et al., 1997) and modelling in Xenopus (Neilson and Friesel, 1995;Neilson and Friesel, 1996) suggest that FGFR2 mutants with altered or exposed cysteine residues in the IgIII subdomains form ligand - independent, or ‘constitutive’, activated dimers by means of intermolecular disulphide bonds. Adjacent non-cysteine mutations, by causing intramolecular conformational change to expose cysteine moieties, have the same effect (Robertson et al., 1998). In human tissues where the broad range of receptor homologues and their naturally occurring isoforms are expressed, both homodimerisation and heterodimerisation by non - Apert FGFR2 mutants might be expected to occur with a variable net intracellular signal as a result (Nguyen et al., 1997). The variable osteoblast phenotype that results is in contrast with that of the pro - differentiated Apert osteoblast (Lomri et al., 1998;Fragale et al., 1999). Mutant FGFR2 - heterodimerisation by constitutive mutations causing ‘Crouzon – Pfeiffer’ phenotypes would allow recruitment of the FGFR1 and FGFR3 expression domains. In this case, constitutive heterodimers would recruit the pre – osseous cranial mesenchyme by means of FGFR1 and FGFR3 signalling pathways, to promote cranial osteogenesis beyond the negatively - autoregulated mutant FGFR2 domain. Constitutive receptor heterodimerisation by FGFR2 mutations causing the ‘Crouzon – Pfeiffer’ craniofacial dysostosis syndromes is an attractive model which is supported by in – vitro data (Nguyen et al., 1997) and the demonstrated co – expression of FGFR genes in human calvarial development herein. This model correlates with the ‘Crouzon – Pfeiffer’ phenotypes, which lack the distinctive pterional stenosis of Apert syndrome, by encompassing a wide domain of cranial mesenchyme in the pathogenic process via FGFR1 or FGFR3 heterodimers. Thus there is no median mesenchymal diastema, and a variable pattern of craniosynostosis that may involve all sutures results.

Epithelio – mesenchymal interactions account for a variety of normal processes in craniofacial development (Hall, 1981;Wedden, 1987;Richman and Tickle, 1992;Matovinovic and Richman, 1997;Francis-West et al., 1998). The development of cranial mesenchyme is
dependent upon epidermis (Tyler, 1983), and osseoinduction by the dura mater can be driven by epithelium in heterotopic sites (Yu et al, 1997). The KGFR isoform of FGFR2 is normally poorly expressed in both 14 – week and 10 – month human cranial ossification. KGFR is, however, strongly expressed in the osteoblasts of the para - sagittal osteogenic front of the 27 – week C278F Pfeiffer fetus, and is also expressed in the mesenchymal domain. This preliminary evidence that KGFR is ectopically expressed in forms of cranial ossification correlates with the observation that splicing mutations in FGFR2 may upregulate KGFR and provide a novel pathogenetic mechanism for syndactyly (Oldridge et al, 1999). The ectopic expression of KGFR in craniofacial dysostosis/craniosynostosis would recruit a specific group of high affinity FGF ligands, perhaps made available by the overlying dermis or subjacent dura mater/brain. The FGF4 and FGF7 genes are not co – expressed with KGFR, but other possible ligands include FGF10 (Xu et al, 1998;Igarashi et al, 1998), and FGF3; the over-expression of which causes craniosynostosis and a Crouzon – phenocopy in the murine model (Carlton et al, 1998). The range of human FGFR2 – associated craniofacial dysostosis phenotypes is very broad, and encompasses great variability in the observed pattern of craniosynostosis. The possibility that this may reflect the balance between negative - regulation of the BEK isoform of FGFR2 and ectopic expression of KGFR may now be tested in a variety of model systems.

3.2.6 Conclusions

The data presented in this report provide preliminary evidence, from two syndromic foetal tissue – types, that the mutant activation of FGFR2 in human calvarial osteogenesis causes a negative – autoregulation of FGFR2 expression in - situ. Data from the Apert P253R fetus corroborates and extends similar independent observations from Apert S252W osteoblasts in – vitro. In these cases, contraction of the FGFR2 expression domain occurs in the presence of FGFR1 and 3, co – expressed with the endogenous complement of cofactors and matrix molecules in human cranial membranous ossification in - situ. A model is thus proposed for the differential pathogenesis of craniofacial form in the Apert and 'Crouzon – Pfeiffer' syndromes, based upon the differential mechanisms of activation of their mutant receptors, and the data in these molecular case reports. This model may be further tested under stringent conditions of laboratory control. As the specific biochemical consequences of each subtype of mutation become known, and the expression patterns of the relevant cofactors and downstream cascades are plotted in human tissues, clinicians will be able to predict their specific functional consequences. This in turn will allow increasingly informed prenatal genetic counselling and the development of improved strategies for clinical management.
**S-3.2 Figure Legends**

**Figure 3.2-1**

Coronal sections of normal human parietal cranial ossification at 14 weeks (i-iii), and 10 months (iv - vi); *in situ* hybridisation. *FGFR1* (i) is expressed as transcript in the periosteum (p), osteoblasts of the parietal osteoid (po), and dura mater (dm), but poorly expressed in the cranial mesenchymal domain (cm) at the parietal vertex. *FGFR2* is expressed as *BEK* (ii) in the basicranial parietal osteoblasts (po), at both endocranial and pericranial (p) aspects (white arrows), and in the surrounding cranial mesenchymal domain (cm); for histological correlates see Fig 3.2-3; i-iv). *KGFR* is minimally expressed, in parallel section, in the pericranial (p) domain of the parietal osteoid (po); and is absent in the cranial mesenchyme (cm). The differential expression of *BEK* and *KGFR* is consistent at 10 months in the infant parietal bone (iv - vi). *BEK* (iv, v) is expressed in the osteoblasts (ob) lining the trabecular bone space and in the osteocyte cell bodies and dendrites (oc) of the bone matrix (pbm). *KGFR* (vi) is expressed in the osteoblasts (ob) lining the trabecular bone and periosteum (p), but absent in the osteocytes and dendrites (oc) of the parietal bone matrix (pbm). Bar = 30 μm.

**Figure 3.2-2**

Apert basi-cranial parietal ossification at 14 - weeks in transverse section (i - vi); *in situ* hybridisation. *FGFR2* is minimally expressed as *BEK* (i, iv) in the osteoblasts closely applied to the parietal osteoid (po, white arrows), but is absent in the cranial mesenchymal domain (cm). *Osteonectin* transcript (ii, v) is strongly expressed, however, in these parietal osteoblasts (po, white arrows), but not in the wider cranial mesenchymal domain (cm). *FGFR1* (iii, vi) is expressed in the basicranial mesenchyme (cm), and the parietal osteoblasts (po). Bar = 30 μm.
**FGFR1, 14w parietal vertex**

**BEK; Parietal basicranium**

**KGFR**

**BEK transcript.** (iv). Light-field, (v). Dark-field

10 m infant parietal bone

(vi) **KGFR transcript.** Dark-field
Figure 3.2-3

Normal human and Apert parietal osteogenesis at 14 – weeks; immunohistochemistry. Normal parietal ossification in coronal section (i-iv), demonstrates parietal osteoid (po) lined by osteoblasts; and on the pericranial aspect by mesenchymal condensations (mc), and a wider cranial mesenchyme (cm). FGFR1 protein (ii) is detected in the osteoblasts lining the parietal osteoid (po), and in the mesenchymal condensations (mc). Signal density is weaker in the cranial mesenchymal domain (cm). FGFR2 protein (iii) is detected equally strongly in all these domains, including a high signal density in the cells of the cranial mesenchyme (cm). FGFR3 protein (iv) is also detected in all three domains. FGFR2 protein has a wider expression than FGFR1 in equivalent domains of normal cranial mesenchyme (i&iii; white arrows). Age – matched Apert parietal ossification is shown in transverse section (v- viii). Islands of parietal osteoid (po) are enveloped by an ‘osteogenic front’ (o-f) of cellular condensations of mesenchyme (mc; black arrows), within a wider cranial mesenchyme (cm). FGFR1 protein is detected in the osteoblasts of the parietal osteoid (po), and mesenchymal condensations (mc) of the osteogenic front (o-f). FGFR2 protein (vii) is detected in these domains (po, o-f, mc). However, FGFR2 signal (vii) is weak in the Apert mesenchymal domains (mc, cm) compared to that in the parietal osteoid (po). FGFR3 protein (viii) is co – expressed with FGFR1 in Apert parietal osteogenesis (po, o-f, mc). FGFR1 thus displays a wider domain of expression as protein than FGFR2 in equivalent areas of Apert cranial mesenchyme (vi&vii; white arrows). Bar = 30 μm.

Figure 3.2-4

Expression of FGF ligands in 14 – week Apert parietal ossification in transverse section (i – vi); immunohistochemistry. FGF2 (i) protein is detected in the parietal osteoid (po) and cranial mesenchymal domains (cm) compared to negative control (iv). FGF4 (ii), and FGF7 (iii), are not expressed in parietal osteoid (po), or mesenchymal domains (cm) compared to positive control (v, vi) in tooth papillary mesenchyme (pm) and cranial dermis (d), respectively. Bar = 30 μm.
Wild type control (x4)  Wild type: FGFR1  FGFR2  FGFR3

Apert control (x4)  Apert: FGFR1  FGFR2  FGFR3

Wild type control (x4)  Wild type: FGFR1  FGFR2  FGFR3

Apert control (x4)  Apert: FGFR1  FGFR2  FGFR3
Apert fetus: FGF2

Apert fetus: FGF4

Apert fetus: FGF7

FGF4 in 12 wk human tooth germ: +ve control

FGF7 in 12 wk human cranial epidermis: +ve control
**Figure 3.2-5**

*FGFR expression in a 27-week Pfeiffer sagittal osteogenic front (i-v; coronal section); immunohistochemistry. FGFR1 protein (i, iii) is detected in the cranial mesenchymal domain (m), and in the osteoblasts (white arrows) of the osteoid (o), of the osteogenic front (of), compared to negative control (v). FGFR2 protein (ii, iv) is detected in the osteoblasts (white arrows) of the osteoid (o), but not in the cranial mesenchyme (m; black arrows). Bar = 30 μm.*

**Figure 3.2-6**

*Infant sagittal synostosis provides a positive control for *FGFR* expression in human cranial osseous differentiation (i-vi; coronal section); immunohistochemistry. The unfused sagittal suture at 10– months (i–iii) consists of a fibro-cellular midzone (fc), flanked symmetrically by a narrow osteoid stroma (os), and parietal bone (pb). FGFR1 (ii) and FGFR2 (iii) proteins are equally detected in the mid-sutural osteo-progenitor cells (fc) and the osteoblasts of the osteoid stroma (os). In the process of sutural fusion (iv–vi), pathologic osteoid replaces the fibro-cellular suture as an advancing osteogenic front (of), from the endocranial (e) aspect of the suture, with the early expression of bone matrix proteins (not shown) and eventual maturation into trabecular bone. FGFR1 protein (v) is detected in the osteogenic front (of) and fibrocellular domain (fc); whereas FGFR2 protein (vi) is detected in the osteogenic front but lacks expression in the mid-sutural, fibro-cellular domain. Bar = 60 μm.*
Pfeiffer cranial osteogenesis: FGFR1

FGFR2

FGFR1

FGFR2

Negative control
Unfused suture: control

FGFR1 protein

FGFR2 protein

Sagittal fusion: control

FGFR1 protein

FGFR2 protein
**Figure 3.2-7**

*KGFR* transcript (i, ii) is expressed in the cranial mesenchymal domain (cm) and osteoblasts (white arrows) of the osteoid in 27-week Pfeiffer cranial osteogenesis. Signal is strong in the osteoblast domains. *BEK* transcript (iii, iv) is relatively weakly expressed in the osteoblasts (white arrows) of the cranial osteoid (o), and not in the cranial mesenchyme (cm). *Bar* = 30 μm.

**Figure 3.2-8**

Apert craniofacial skeletal phenotype. There is a wide, mesenchymal, unossified diastema in the midline, in place of sagittal and metopic sutures. There is pterional indrawing and a retruded midface. FGFR2 is negatively regulated by the Apert mutation to differentiated osteoblast domains, and exerts its pro-differentiative effect via the relative maturity of the membranous basicranium, leading to pterional indrawing and splaying of the frontal and parietal bones. A midline diastema results, and midline sutures do not form. The mesenchymal domains of the cranial vertex ossify much later, and thereby correlate with the midline diastema.
Pfeiffer sagittal osteogenic front: KGFR

Pfeiffer sagittal osteogenic front: BEK
FGFR1 in mesenchyme
Midline diastema
FGFR2 negatively regulated in differentiated domains
Pterional indrawing
Midfacial retrusion

CT 3D reconstructions: Apert Infant (FGFR2 P253R)
3.3 From Genotype to Phenotype: the differential expression of FGF, FGFR, and TGFβ genes characterises human cranioskeletal development.

3.3.1 Abstract

Mutations in the fibroblast growth factor receptor (FGFR) genes 1, 2, and 3 are causal in a number of craniofacial dysostosis syndromes featuring craniosynostosis with basicranial and midfacial deformity. Great clinical variability is displayed in the pathologic phenotypes encountered. To investigate the influence of developmental genetics upon clinical diversity in these syndromes, the expression of several genes implicated in their pathology was studied at sequential stages of normal human embryo-foetal cranial base and facial ossification.

At 8 weeks of gestation, FGFR1, 2, and 3 are equally expressed throughout the pre-differentiated mesenchyme of the cranium, the endochondral skull base, and midfacial mesenchyme. Both clinically significant isoforms of FGFR2, IgIIIα/c and IgIIIα/b, are co-expressed in maxillary and basicranial ossification. By 10 - 13 weeks, FGFR1 and 2 are broadly expressed in epithelia, osteogenic and chondrogenic cell lineages. FGFR3, however, is maximally expressed in dental epithelia and proliferating chondrocytes of the skull base; but poorly expressed in the osteogenic tissues of the midface. FGF2 and FGF4, but not FGF7; and TGFβ1 and β3, are expressed throughout both osteogenic and chondrogenic tissues in early human craniofacial skeletogenesis.

FGFR expression in the skull base proposes a pivotal role for syndromic growth dysplasia at this site, and the co-expression of FGFR3 and STAT1 proteins suggests an inhibitory role for FGFR3 in basicranial chondrogenesis. Paucity of FGFR3 expression in human midfacial development correlates with the relatively benign human mutant FGFR3 midfacial phenotypes. The regulation of FGFR expression in human craniofacial skeletogenesis against background excess ligand and selected cofactors may therefore play a profound role in the pathologic craniofacial development of children bearing FGFR mutations.
3.3.2 Introduction

That the co-ordinated growth of the craniofacial skeleton with its soft tissues and specialised organs proceeds so often undisturbed represents a triumph of mammalian development. The mechanisms controlling the interrelated development of the various tissues are complex, and require delicate temporospatial coordination. Perhaps because of this, the investigation of specific aetiology for patterns of craniofacial malformation has proved frustrating. In the last decade, however, advances in molecular genetics have identified a range of single gene defects in the fibroblast growth factor receptor (FGFR) gene family which are causal to a number of craniofacial dysostosis syndromes.

Eponymously recognised for the clinicians who described them, the most well documented of these are Apert syndrome (Apert, 1906), Pfeiffer syndrome (Pfeiffer, 1964), and Crouzon syndrome (Crouzon, 1912). These and related syndromes consist primarily of skeletal abnormalities of the craniofacial complex and appendicular skeleton, thus exhibiting a joint role for FGFR signalling in limb and craniofacial development (Britto et al, 2000a). The phenotypic range of these and other related acrocephalosyndactyly syndromes is wide and overlapping. Whilst in the majority of cases a clinical diagnosis can be made, other phenotypes do not easily conform to eponymous categories (Pulley et al, 1996). The craniofacial phenotype of Apert syndrome reflects its restricted genotype. Pterional indrawing, a foreshortened skull base, turribrachycephaly, severe midfacial retrusion with hypertelorism, and coronal sutural synostosis with a widely unossified median sagittal diastema in place of metopic and sagittal sutures are strongly characteristic (Kreiborg and Cohen, Jr., 1990; Kreiborg et al, 1993; Cohen and Kreiborg, 1994). The narrow phenotypic range reflects the finding that only two closely neighbouring linker region mutations in the IgIIIa extracellular subdomain of FGFR2 cause 98% Apert cases (Oldridge et al, 1999) as a result of ligand dependent 'gain of function' (Anderson et al, 1998c).

The related syndromes of Crouzon, Pfeiffer, Saethre–Chotzen (Saethre, 1931; Chotzen, 1932), and Jackson Weiss (Jackson et al, 1976), however, show a more variable and overlapping craniofacial phenotype. The skull base synchondroses tend to early synostosis, and the metopic and sagittal sutures form and fuse without an unossified median diastema (Kreiborg et al, 1993; Cinalli et al, 1995). Facial retrusion variably effects the supraorbital or midfacial skeleton, with a wide range of severity. Phenotypic variability within and between these syndromes reflects their mutational base. A broad range of mutations with effects upon the extracellular and transmembrane domains of the FGFR1 and 2 proteins are causal, with greatest frequency in the IgIIIc subdomain of the FGFR2-IgIIIa/c isoform (Burke et al, 1998). Many of these mutations result in the creation or removal of an unpaired cysteine
residue, or affect a neighbouring site, to cause conformational change and confer ligand
independent functional gain upon the receptor (Neilson and Friesel, 1995; Neilson and Friesel,
1996; Galvin et al, 1996; Mangasarian et al, 1997; Robertson et al, 1998). In addition, mutations
in FGFR3 cause a range of human phenotypes, which display variable cranial and skull base
dysostosis with or without limb anomaly (Rousseau et al, 1994; Shiang et al, 1994; Prinos et al,
1995; Meyers et al, 1995; Bellus et al, 1995a; Tavormina et al, 1995a; Bellus et al, 1995b; Moloney
et al, 1997; Muenke et al, 1997; Reardon et al, 1997; Paznekas et al, 1998; Angle et al, 1998; Gripp
et al, 1998b). In these syndromes the midfacial appearance appears to be secondary to
basicranial deformity, as displayed by the facial scoliosis which accompanies FGFR3 -
uniconoronal synostosis and appears to ‘hinge’ upon the affected cranial base (Persing et al,
1986).

Molecular factors influencing the genotype – phenotype relationship include the mechanism
of activation of the mutant receptor, and the relative bioavailability of ligand, receptor, and
cofactors in the molecular cascades controlling craniofacial endochondral and
intramembranous ossification. FGFR transcript - mRNA expression studies have been
previously reported in chicken (Wilke et al, 1997) and in human foetal tissues (Delezoide et al,
1998; Chan and Thorogood, 1999). In this study, the expression of FGFR1, the IgIIIa/c and
IgIIIa/b isoforms of FGFR2, and FGFR3 is described in human basicranial and midfacial
skeletogenesis at the level of mRNA transcript and protein in a temporal series of human
embryo - foetal craniofacial tissues. The FGFR expression domains are contrasted with those
of the bone differentiation marker, osteonectin; and the ligand proteins FGF2, FGF4 and
FGF7. In addition, the co – factor cytokines TGFβ1 and TGFβ3, which are implicated in
osseous differentiation, are detected in an attempt to correlate genotype and phenotype in
human FGFR - associated craniofacial dysostosis.
3.3.3 Materials & Methods

Preparation of embryonic & early foetal material

Human embryo – foetal tissue aged 8 to 14 weeks (n=6) was provided by the Human Tissue Resource maintained at the Institute of Child Health and University College Hospital, London. Craniofacial tissue was collected and prepared as described above (S-2.1).

Studies of FGFR and allied gene expression

FGFR1, osteonectin, and the IGI\textsubscript{III}a/c (BEK) and IGI\textsubscript{III}a/b (KGF) isoforms of FGFR2 mRNA transcripts were detected by in situ hybridisation using isoform - specific riboprobes. Probe synthesis and in - situ hybridisation steps are described in S-2.2.

The protein receptors FGFR1, FGFR2, and FGFR3; the ligand FGFs 2, 4, and 7; and the TGF\textbeta isoforms 1 and 3 proteins were detected by immunohistochemistry using commercially available specific antibodies (S-2.2.3). The anti - receptor antibodies have epitopes corresponding to the carboxy – terminal sequences of the receptor protein, and therefore label both IGI\textsubscript{III}c and IGI\textsubscript{III}b isoforms in immunohistochemistry studies.

3.3.4 Results

FGFR expression in human midfacial ossification

FGFR1, 2, and 3 are differentially expressed throughout sequential stages of human midfacial osseous development. At eight weeks, the three genes are equally expressed as protein in the periorbital midfacial mesenchyme and basicranial and paranasal cartilages. Staining for the three protein receptors is marked in the nasal epithelium and perichondrium (Fig 3.3-1). At this eight - week stage, maxillary osteogenesis has commenced with the sequential condensation of premaxillary mesenchyme, and the deposition of maxillary osteoid (Fig 3.3-1, black arrows) by osteoblasts. These cells express osteonectin transcript (Fig 3.3-2), a marker of early osteogenesis (Jundt et al, 1987; Nakase et al, 1994). The maxillary osteoblasts co - express FGFR1 mRNA, and the two transcript isoforms of FGFR2, IGI\textsubscript{III}a/c and IGI\textsubscript{III}a/b.
These three genes are further expressed in the ten week maxillary osteoid, where maximal hybridisation of transcript occurs in the periosteal domain (Fig 3.3-2).

Differences in the expression of FGFR protein in human midfacial osteogenesis emerge at ten weeks. FGFR1, FGFR2, and FGFR3 are strongly expressed as protein in the enamel epithelium of the ten-week human tooth germ (Fig 3.3-3), together with the protein ligand FGF2, which is widely expressed. FGFR1 and FGFR2 proteins are also strongly expressed in the adjacent mesenchymal and osteoid domains of the midface. FGFR3, however, is poorly detected in midfacial osteogenesis compared to its expression in the dental enamel epithelium at this stage (Fig 3.3-3). Whereas FGFR1 and FGFR2 are strongly expressed as protein in the cells of the condensing midfacial mesenchyme and throughout the osteoblast domains surrounding maxillary osteoid (Fig 3.3-4); FGFR3 protein labels very poorly in a domain restricted to a few osteoblasts. Mesenchymal cells remain predominantly unlabelled for FGFR3 protein. The differential expression of FGFR1, 2, and 3 proteins in midfacial osteogenesis remains consistently evident at thirteen weeks (Fig 3.3-4). FGFR2 protein is detected in almost every osteoblast surrounding the maxillary osteoid, and throughout the condensing mesenchyme. By contrast, FGFR3 protein labels in only a few osteoblast aggregates, and the majority of the maxillary osteoidal osteoblasts remain unlabelled. Strong FGFR3 protein expression characterises the nasal epithelial domain of the same microscopic field.

Ligand FGF2 and FGF4 proteins are detected in midfacial osteogenesis (Fig 3.3-3) FGF4 is expressed in the osteoblasts of the maxillary osteoid and the cells of the midfacial mesenchyme. FGF4 is also expressed in the cartilage cells, but is less strongly detected in the perichondrium, where FGF2 is preferentially expressed. FGF2 is also expressed in the condensing midfacial mesenchyme, and the maxillary osteoblasts (Fig 3.3-5).
**FGF, FGFR & TGFβ expression in endochondral basicranial ossification.**

Human cranial base ossification is characterised by the development of a precursor cartilagenous template. The matrix chondrocytes undergo hypertrophy and apoptosis, with subsequent vascularisation, mineralisation, and ossification of the cartilage template. At eight weeks, *FGFR1*, *FGFR2*, and *FGFR3* are expressed as protein in the basicranial chondrocytes. Expression of all three protein receptors is greatest in the perichondrium (*Fig 7*); the source of the proliferating cartilage cells which make up the basicranial template for subsequent ossification.

By thirteen weeks (*Fig 3.3-6*), the endochondral component of the basicranium consists of a well-defined perichondrium, containing layers of flattened chondrocytes. Medially, the cartilage matrix consists of hypertrophic chondrocytes, at the stage prior to eventual ossification to form the wings of the sphenoid bone. Laterally, this matrix abuts a zone of proliferating chondrocytes, 'fanning' out from the perichondrium, which then interfaces more laterally with cranial membrane bone. FGFR1, FGFR2, and FGFR3 proteins are differentially detected in the chondrocytes of the 13-week basicranial cartilage matrix and in the dense perichondrium (*Fig 3.3-6*). FGFR1 and FGFR2 proteins are similarly strongly detected in the perichondrium, the proliferating zone of chondrocytes, and the hypertrophic matrix (*Fig 3.3-6, iii; Fig 3.3-7*). FGFR3, by contrast, is strongly detected in the perichondrium and proliferating zone chondrocytes, but is weak in the hypertrophic chondrocytes and in the peri-ocular matrix. Expression is also relatively weak in the membrane cranial bone and associated mesenchyme (*Fig 3.3-6, iv; Fig 3.3-7, v*). Both the *IgIIla/c* and *IgIIla/b* isoforms of *FGFR2* transcript are similarly expressed in this perichondrial domain at 10-weeks (*Fig 3.3-7*). There is evidence that the regulatory effect of FGFR3 signalling in chondrogenesis is mediated via STAT1 transcription factors (see Discussion, S-3.3.5). STAT1 is expressed in 8-week human chondrogenesis in the cartilage cells of the basicranium. At ten weeks, STAT1 is expressed in the cytosol of perichondrial cells and the nuclei of the hypertrophic chondrocytes (*Fig 3.3-8*).

The ligand FGF2 is widely expressed throughout cranial, basicranial, and midfacial osseous and cartilagenous domains at both ten (*Fig 3.3-3*) and thirteen weeks (*Fig 3.3-6 & 3.3-9*). The ligand FGF4 is also expressed in both endochondral and membranous basicranial ossification, where it is most strongly detected in the membranous pericranial osteoblasts (*Fig 3.3-9*). FGF7 was not demonstrable in any of the tissues undergoing endochondral or intramembranous ossification in skull base or midface compared to positive control in human
foetal dermis. TGFβ1 is detected in chondrogenic and membranous domains of human basicranial development at both ten and thirteen weeks, where it is most strongly expressed in the perichondrium and membranous periosteum (Fig 3.3-9). The TGFβ3 isoform is ubiquitously detected, throughout cranial and peri-orbital domains, and in the basicranial cartilage and perichondrium (Fig 3.3-9).

3.3.5 Discussion

Debate regarding the etiology and pathogenesis of craniofacial form in the syndromic craniofacial dysostoses has traditionally revolved around issues of primacy. It is argued that either primary sutural fusion causes cranial malformation with secondary functional consequences to basicranial and facial form; or that the skull base acts as a pacemaker causing secondary effects upon skull, brain and face. That the number and severity of fused sutures correlates with the general severity of the craniofacial phenotype lends force to the argument that sutural fusion is the initiating pathology. Experimental sutural and synchondrosal restriction in animal models suggests that sutural stenosis will cause basicranial deformity (Persson et al, 1979; Persing et al, 1986; Babler et al, 1987), but also that the skull base may be primarily responsible for patterns of craniofacial dysostosis (Rosenberg et al, 1997). The explosion of molecular genetic information correlating the range of FGFR gene mutations to the craniofacial dysostosis syndromes has provided a new means of investigating their pathogenesis. In this report, the isoform – specific expression of FGFR2 is correlated with ligands specific to each isoform, in a longitudinal series of human embryo – foetal craniofacial tissues, encompassing both endochondral and intramembranous modes of ossification. These expression domains are contrasted with those of the FGFR1 and FGFR3 proteins in an attempt to investigate genotype – phenotype correlation with FGFR – syndromic craniofacial dysostoses.
Differential FGFR3 expression in basicranial development – a basis for skull base stenosis?

The period from 8 to 13 weeks of human craniofacial development illustrates the simultaneous progression of maxillary and cranial membranous ossification, and the endochondral ossification of the basicranium. Cartilagenous foci first appear in the basi-occipital plate by the second month, and chondrification of the sphenoid follows with the pre – sphenoid body, the greater and lesser wings of the sphenoid, and the nasal capsules in sequence (1995). The cartilage template is complete by 12 – 13 weeks. FGFR expression is established at eight weeks, predominantly in the perichondrial domain. By 13 weeks, the sphenoid template exhibits three cartilage zones. The basal perichondrium is a thickened layer of chondrocyte precursor cells, which fans out laterally to form the proliferating zone of chondrocytes. This zone forms a renewable source of chondrocytes for growth of the basicranium. The cells of the proliferating zone exit the cell cycle and secrete a matrix composed of chondroitin – sulphate proteoglycans and type II collagen (Naski and Ornitz, 1998;Li et al, 1999). Within the matrix, the chondrocytes hypertrophy and subsequently express type X collagen and alkaline phosphatase (Delezoide et al, 1997). Hypertrophic chondrocytes undergo a programmed cell death as the surrounding matrix is mineralised and replaced by trabecular bone. FGFR1, FGFR2, and FGFR3 are strongly detected in the perichondrium and the zone of proliferating chondrocytes, whereas FGFR3 is comparatively poorly detected in the hypertrophic cartilage matrix. A similar pattern of differential FGFR expression during ossification at cartilage epiphyses has been reported in the rat (Hamada et al, 1999), and the mouse; where FGFR1 characterised hypertrophic cartilage, and FGFR3 was expressed exclusively in resting (Peters et al, 1993), and proliferating chondrocytes (Deng et al, 1996). The restriction of FGFR3 expression to the resting and proliferating phases of endochondral ossification, with apparent down regulation in the phase of chondrocyte hypertrophy, suggests that FGFR3 plays a specific role in chondrocyte differentiation. Fgfr3 -null mice provide a useful model of FGFR3 signalling in vivo. Targeted disruption of murine fgfr3 results in mice with severe and progressive bone dysplasia resulting from prolonged endochondral bone growth, and is accompanied by an expansion of proliferating and hypertrophic chondrocytes in the epiphysis (Colvin et al, 1996;Deng et al, 1996). Receptor activation via native wild – type FGFR3 would, therefore, be expected to restrict endochondral ossification at the stage prior to chondrocyte hypertrophy. FGF2 and FGF4 are high affinity ligands for FGFR3 (Keegan et al, 1991;Ornitz and Leder, 1992), and are co – expressed in human craniofacial membranous and endochondral ossification from 8 – 13 weeks. Excess ligand FGF2 in a transgenic mouse model does, in fact, cause dwarfism and
diminution of the hypertrophic zone (Coffin et al, 1995) despite expansion of the proliferating zone with increased matrix deposition. This is consistent with demonstrations that FGF2 is a powerful mitogen for chondrocyte proliferation (Kato et al, 1987; Trippel et al, 1993; Wroblewski and Edwall-Arvidsson, 1995), and additional observations in the rabbit epiphyseal chondrocyte pellet model, which show that whilst FGF2 stimulates chondrocyte proliferation, it inhibits terminal chondrocyte differentiation (Kato and Iwamoto, 1990b; Iwamoto et al, 1991). The chondrocytes in pellet culture are less sensitive to FGF2 after differentiation to the hypertrophic stage, correlating with a loss of binding to radio-labelled FGF2 (Iwamoto et al, 1991). It this, therefore, reasonable to suggest that the mitogenic effects of FGF2 on chondrogenesis are mediated by signalling via FGFR3. As FGFR3 down-regulates with the transition to hypertrophic chondrocyte, the inhibitory effect of FGF2 is muted.

The transition of ‘proliferating’ to ‘hypertrophic’ chondrocyte in chondrogenic differentiation is characterised by specific molecular control. The role of FGFR3 in chondrogenesis is not made functionally redundant by FGFR1 or FGFR2 in the murine knock-out; despite their high affinity for the ligands FGF2 and FGF4 (Ornitz et al, 1996), co-expressed in human chondrogenesis. The importance of this zone of cells is further illustrated by its restricted expression of the parathyroid hormone–related protein receptor, despite the widely overlapping expression domain of the parathyroid hormone–related protein (PTHrP) cytokine. The PTHrP gene knock-out mouse model exhibits reduced proliferation and premature differentiation of chondrocytes—and displays a skeleton that is highly ossified at birth (reviewed in (Naski and Ornitz, 1998). The restriction of signalling cofactors such as syndecan 3 to proliferating chondrocytes may be an additional controlling factor (Shimazu et al, 1996). Syndecan 3 is a transmembrane glycosaminoglycan that facilitates FGF/FGFR binding, and it may be that the lack of specific GAGs or HSPGs in hypertrophic chondrocytes functionally limits FGF/FGFR interactions at this stage of chondrogenesis. Perlecan is heparin sulphate proteoglycan, which is detected in basement membranes and is also a co-factor for growth factor–receptor interaction. The perlecan−null mouse displays severe craniofacial anomaly, and the 60% that survive to birth are characterised by additional anomalies including broad and bowed long bones and narrow thoraces (Arikawa-Hirasawa et al, 1999). The defects in endochondral bone development displayed by the perlecan−null include severe disorganisation of cartilage differentiation with reduced proliferation, suggesting that perlecan is a limiting co-factor in chondrogenesis and the FGFR3 signalling pathway. The mouse FGF2−null mutant has no pathogenic skeletal phenotype (Zhou et al, 1998), which suggests that there is functional redundancy of ligand. Alternative candidate ligands include FGF9 (Garofalo et al, 1999), and FGF4, co-expressed with FGF2 and the
FGFRs in human embryo – foetal basicranium. FGF4 is expressed predominantly in the cartilage cells, whereas FGF2 is detected in the perichondrial domain also, and this implies a degree of site-directed functional specificity in human chondrogenesis.

This model whereby FGF/FGFR3 signalling is pivotal to endochondral ossification offers an explanation for the pathogenesis of the craniofacial phenotype of human FGFR3 mutations. These mutations cause variants of Saethre-Chotzen syndrome (Paznekas et al, 1998), coronal synostosis (Moloney et al, 1997;Muenke et al, 1997;Gripp et al, 1998b), and Crouzon syndrome with acanthosis nigricans (Meyers et al, 1995), in which the cranial phenotypes vary include craniosynostosis and skull base stenosis. Midface retrusion, even in the Crouzon variant, is not a severe feature. FGFR3 mutations also cause a range of chondrodysplasias, including achondroplasia, hypochondroplasia, and the lethal thanatophoric dysplasia (TD) (Rousseau et al, 1994;Shiang et al, 1994;Bellus et al, 1995a;Tavormina et al, 1995a;Bellus et al, 1995b). These phenotypes display cranioskeletal anomalies including clover-leaf skull and shortening of the skull base with a variable secondary midfacial hypoplasia. Many of these FGFR3 mutations confer ligand dependent or ligand-independent 'gain of function' (Webster et al, 1996;Naski et al, 1996;Webster and Donoghue, 1996;D'Avis et al, 1998), and the phenotypic consequences of this have been investigated in vitro and in vivo. The achondroplasia mutant receptor has been demonstrated to promote growth retardation and failure of differentiation in a chondrocytic cell line (Henderson et al, 2000). Furthermore, activated – fgfr3 achondroplasia constructs in transgenic mice generate dwarfed phenotypes, with axial, appendicular and cranio-skeletal hypoplasia. Examination of the epiphysis demonstrates an age-dependent expansion (Iwata et al, 2000) or inhibition of the proliferating zone of chondrocytes (Naski et al, 1998;Chen et al, 1999;Wang et al, 1999;Segev et al, 2000), and a consistent inhibition of chondrogenic differentiation. Restriction of the hypertrophic zone is also seen in murine – fgfr3<sup>TD</sup> and human TD long bone articular cartilages (Brenner et al, 1996;Li et al, 1999), in concert with an expansion of FGFR3 expression (Delezoide et al, 1997;Li et al, 1999;Monsonego-Orman et al, 2000), suggesting that the FGFR3 protein is stabilised in activated form by the mutation, whereupon it acts to deregulate further chondrocyte differentiation (Brenner et al, 1996;Delezoide et al, 1997;Su et al, 1997;Legeai-Mallet et al, 1998;Henderson et al, 2000).

It is an apparent paradox that mutant FGFR3 functional gain causes an inhibition of chondrocyte differentiation, given that activating FGFR1 and 2 mutations are associated with accelerated membranous ossification, and that the activating fgfr3 – gly369Cys achondroplasia mouse model displays the upregulation of osseous differentiative markers in long-bone endochondral ossification (Chen et al, 1999). A chondrocyte-specific inhibitory pathway is
thereby suggested. FGF1, acting as activating ligand for FGFR3, causes inhibition of proliferation and DNA synthesis in a rat chondrosarcoma cell line which expresses only the FGFR3 homologue, yet causes a proliferation of the NIH3T3 fibroblast cell line expressing FGF1 and FGFR2; in the presence or absence of FGFR3. The effect in the rat chondrosarcoma cells correlates with the phosphorylation of the STAT1 transcription factor and its activation by nuclear translocation (Sahni et al, 1999). STAT1 activation is a shared consequence of constitutively activating FGFR3 mutations in human TD chondrocytes and the 293T cell line (Su et al, 1997;Legeai-Mallet et al, 1998); Gfr3 - Gly369Cys mice (Chen et al, 1999); and a mouse model for thanatophoric dysplasia (Li et al, 1999). In addition, the activation of STAT1 in human TD and various models of activating FGFR3 mutations correlates with the activation of cell cycle inhibitors (Su et al, 1997;Chen et al, 1999;Sahni et al, 1999), and this correlates with a potentially direct anti-proliferative and anti-differentiative role for activated FGFR3 signalling in chondrogenesis.

The induction of STAT1 - mediated cell - inhibitor pathways by FGFR3 in chondrogenesis appears to be cell - type and differentiation - stage specific (Sahni et al, 1999;Iwata et al, 2000). The activation of NIH3T3 fibroblasts by wt-FGFR3 or an achondroplasia mutant receptor was not found to correlate with STAT1 activation, but probably modulated by a MAP - kinase pathway (Sahni et al, 1999). The observation that FGF signalling does not inhibit chondrogenesis in the stat1 - null mouse (Sahni et al, 1999), suggests that stat1 activation is a limiting factor of the negative regulation of chondrogenesis by FGFR3 signalling. The lack of stat1 expression in the early mouse TDII model correlates with a chondro - proliferative response (Iwata et al, 2000), in contrast with the co - regulation of activated stat1 and restriction of proliferation in postnatal and adult gfr3 gain - of - function mutant mice (Chen et al, 1999;Li et al, 1999). The expression and activation of stat proteins (Stat1, Stat5a, Stat5b) may thus be the switch that regulates the proliferative and anti proliferative/differentiative effects of activation by FGFR3 (Legeai-Mallet et al, 1998;Li et al, 1999;Sahni et al, 1999). STAT1, activated by FGFR3 mutations in TD1 (Legeai-Mallet et al, 1998) and TDII (Su et al, 1997) is detected in human basicranial chondogenesis at 8 – 10 weeks. At 10 – weeks the STAT1 protein is detected in the hypertrophic chondrocyte nuclei of this human wild - type tissue, compared to detection in the cytosol of the perichondrial/resting chondrocyte cells. This suggests that STAT1 is activated in the absence of FGFR3 in the hypertrophic chondrocyte phase of human wild - type basicranial ossification at 10 – weeks; which correlates with the inhibition of chondrogenesis in preparation for endochondral ossification. Chondrocyte apoptosis is observed in hypertrophic chondrocytes prior to endochondral ossification in long - bones, and is upregulated by TD1 mutations in FGFR3 (Legeai-Mallet et al, 1998). Mutant FGFR3
functional gain in human basicranial ossification might therefore be predicted to stabilise FGFR3 protein dimers in the proliferating zone, and prematurely activate STAT transcription factors to curb chondrocytic proliferation in this zone beginning at 10-weeks. The hypertrophic chondrocyte zone would thus be restricted, and chondrocyte terminal osseous differentiation also be inhibited with a possible acceleration of apoptosis. The ossification of the skull base would thereupon follow on a dysplastic cartilage template. This is consistent with observations of the cranial base in the mouse model of the activating achondroplasia FGFR3 — Gly375Cys mutation. Premature ossification and closure of the basicranial synchondroses occurs, which results in an antero-posterior shortening of the skull base and secondary macrocephaly compared to age-matched wild-type (Chen et al, 1999). The predicted restriction of basicranial ossification would result in the skull base growth stenosis observed clinically in the chondrodysplasias, Crouzon- acanthosis nigricans, and the coronal synostoses. The variability of the basicranial presentation between these syndromes in turn reflects subtle differences between their specific causal FGFR3 mutations.

**Midface retrusion in FGFR — craniofacial dysostoses: genotype/phenotype correlations.**

FGFR expression in the midface becomes defined in a differential and tissue specific manner within the 8–13 week period of human craniofacial development. At eight weeks, maxillary osteogenesis has commenced as islands of osteoid surrounded by osteoblasts within mesenchymal condensations. The maxillary osteoblasts co-express osteonectin, which has been correlated with the early osteoblast phenotype (Jundt et al, 1987; Nakase et al, 1994), with the mRNAs of FGFR1 and the IgIIIa/c and IgIIIa/b isoforms of FGFR2. The equal expression of both the IgIIIa/c and the IgIIIa/b isoforms is unexpected in normal maxillary osteogenesis, as FGFR2 - IgIIIa/b is reportedly dominantly expressed in epithelia (Orr-Urtreger et al, 1993). Whereas the FGFR2 - IgIIIa/c isoform has high affinity for FGF2, co-expressed in human midfacial development, the IgIIIa/b isoform displays far higher affinity for FGF7 (Miki et al, 1992; Orr-Urtreger et al, 1993) and FGF4 (Orr-Urtreger et al, 1993). FGF4, which has been demonstrated to promote growth of facial mesenchyme (Richman et al, 1997), is co-expressed in human embryo—foetal midfacial osteogenesis at 8–13 weeks, but FGF7 cannot be demonstrated compared to positive control in foetal dermis. The diffusion of FGF7 from the epithelial domain to activate the IgIIIa/b protein in facial membranous bone might provide the molecular mechanism for the epithelio-mesenchymal induction of facial growth (Matovinovic and Richman, 1997). Furthermore, the ectopic expression of the IgIIIa/b isoform in the midface as a result of certain splicing
FGFR2 mutations (Oldridge et al, 1999) might generate the midfacial retrusion phenotype by inducing premature membranous ossification.

Differential expression of FGFR protein in human midfacial osteogenesis also reflects FGFR craniofacial phenotypes. FGFR1 and FGFR2 protein are dominantly expressed in midfacial membranous ossification compared to FGFR3. ‘Gain of function’ characterises many FGFR2 mutations causing craniofacial dysostosis, and may be ligand dependent (Anderson et al, 1998c), or independent (Wilkie, 1997; Webster and Donoghue, 1997a; Burke et al, 1998). FGFR2 functional gain in Apert calvarial osteoblasts has been shown to promote premature ossification (Lomri et al, 1998; Fragale et al, 1999) and this, in the presence of FGF2 in maxillary membranous ossification, would generate the severe Apert midface retrusion phenotype. Similarly, osteoblasts carrying the C342R Pfeiffer mutation show a low proliferation rate and high alkaline phosphatase expression tending to premature differentiation, but do not prematurely mineralise (Fragale et al, 1999). This behavioural difference in vitro may represent hetero-dimerisation between FGFR homologues (Nguyen et al, 1997) as a result of ligand – independent FGFR2 mutation, which would contribute to the phenotypic differences seen between the various craniofacial dysostoses. Phenotypic variability in these syndromes may also reflect variable FGF binding and the negative regulation of receptor expression (S-3.2). The addition of FGF2 to Apert and Pfeiffer osteoblast cultures stimulates proliferation and inhibits differentiation (Fragale et al, 1999), an effect which is maturity dependent in normal (wild – type) human osteoblasts (Debiais et al, 1998). A maturity - dependent switch in FGFR dominance during human craniofacial membranous ossification might mediate this effect (S-3.1). Mutant FGFR2 functional gain causes a negative regulation of the receptor domain (S-3.2; Lemonnier et al, 2000) and the variable effects of this specific to each mutation, the relative maturity of the osteoblast, and the influence of co – factors in the signalling cascade will all bear effect upon the clinical phenotype.

TGFβ isoforms are powerful osteoblast mitogens (Bonewald and Dallas, 1994; Zellin et al, 1998). FGF2 has been shown to upregulate TGFβ1 and promote osseous differentiation in the rat osteoblast lineage (Nakamura et al, 1995). TGFβ isoforms also act in synergy with FGF2 in chondrogenesis, promoting DNA synthesis and articular chondrocyte proliferation in a rabbit model (Okazaki et al, 1996). Furthermore TGFβ1 selectively upregulates FGFR1 and FGFR2 in a time and dose dependent manner in human fibroblasts, resulting in potentiation of FGF/FGFR mitogenicity (Thannickal et al, 1998). An autocrine positive feedback system regulating Apert skeletogenesis is implied by the upregulation of TGFβ1 production by Apert osteoblasts (Locci et al, 1999). The co – ordinated upregulation of
TGFβ1 and FGF2 in calvarial ossification (Mehrara et al, 1999) further suggests synergism between the cytokine pathways, perhaps by direct autocrine stimulation (Kay et al, 1998), or the upregulation of essential matrix heparin sulphate proteoglycans (Nugent and Edelman, 1992) which modulate FGF/FGFR signalling. The co-expression of TGFβ1 and 3 is demonstrated in human basicranial chondrogenesis and membranous osteogenesis. That the TGFβ1 domain in the perichondrium/periosteum is particularly marked reflects its likely synergy with FGFR expression in cells of the early differentiative state in chondrogenic/osseous differentiation (Harris et al, 1994), and this will further regulate the genotype/phenotype relationship in FGFR - associated craniofacial dysostosis.

3.3.6 Conclusions

The craniofacial phenotypes of FGFR - associated craniofacial dysostosis correlate with the expression of FGF and FGFR in human craniofacial development. Midface retrusion, commonly a feature of Apert syndrome and the FGFR2 mutations characterising Crouzon, Pfeiffer and related syndromes, reflects the expression of both the FGFR2 Illa/c and Illa/b isoforms in midfacial osteogenesis. The unexpected Illa/b expression domain in maxillary ossification could provide the mechanism for the normal epithelio – mesenchymal induction of facial growth, and its pathogenic upregulation would induce mutant FGFR - associated midface retrusion.

Analogous mutations in FGFR1, FGFR2, and FGFR3 result in very different midfacial phenotypes. The P252R mutation in FGFR1 causes Pfeiffer syndrome, and the P253R FGFR2 mutation causes Apert syndrome, each of which displays significant midface retrusion reflecting the expression of FGFR1 and 2 in midfacial osseous development. The P250R mutation in FGFR3, however, causes coronal synostosis, which lacks a severe midface retrusion phenotype and reflects paucity of FGFR3 expression in midfacial ossification. The relative importance of FGFR1 and FGFR2 to human membranous ossification is emphasised; whereas FGFR3 appears to be primarily a modulator of endochondral, and therefore, basicranial, skeletogenesis. Midface retrusion in the FGFR3 syndromes is relatively benign, and possibly secondary to the skull base stenosis. The 'membranous – skeletal' phenotypic features of the FGFR3 syndromes, such as craniosynostosis, may be alternatively explained by receptor heterodimerisation and recruitment of FGFR1/2 domains. The expression of FGFR1, 2, and 3 and their ligands FGF2 and 4 in basicranial development emphasises the importance of growth dysplasia at this site in craniofacial dysostosis. Arrest of chondrocyte differentiation results in the
foreshortened skull base which characterises these syndromes. Asymmetrical inhibition of chondrocyte differentiation in the skull base by the FGFR3 - P250R mutation (perhaps sensitive to critical amounts of ligand, or local extracellular matrix conditions (Dinbergs et al, 1996)), would generate the facial scoliosis and orbital dystopia of unilateral coronal synostosis. These studies serve to illustrate that human basicranial and facial osteogenesis is characterised by the differential expression of FGFs/FGFRs, and that the subtle differences in their expression domains reflect clinical presentation of human FGFR — syndromes. The development of specific animal models, particularly the advent of transgenic mice with activating FGFR1/2 mutations, will further unravel the complexity of pathogenesis in this spectrum of conditions.
**Figure Legends**

**Figure 3.3-1**

Coronal sections through the midface of the 8-week human embryo; FGFR immunohistochemistry. FGFR1, FGFR2, and FGFR3 are similarly detected in the midfacial mesenchyme (mm), surrounding the orbit (pom — periocular muscles; on — optic nerve). All three proteins are detected in the maxillary osteoid (black arrows), and basicranial and paranasal cartilage (white arrows), where expression is marked in perichondrium. The nasal epithelium lining the nasal cavity and nasal septum (ns) labels strongly for all three proteins. Bar = 100 μm.

**Figure 3.3-2**

Coronal sections through the midface of the eight (panel i - iv) and ten week (panel v – vii) human embryo; FGFR and osteonectin in-situ hybridisation. Osteonectin is expressed in the osteoblasts of the maxillary osteoid (i, white arrows), but not the midfacial mesenchyme (mm) or palatal shelf (ps). Transcripts of FGFR1 (ii), FGFR2 – IgIIIa/c (iii), and IgIIIa/b (iv) are also expressed in the maxillary osteoid at eight weeks, becoming particularly strong in the periosteum by 10 weeks (v – vii, white arrows). FGFRs are expressed in vascular mesenchyme (vm). (ns = nasal space.) Bar = 30 μm.
Human 8 wk hemiface: negative control

FGFR1 protein

FGFR2 protein

FGFR3 protein
Osteonectin

FGFR1

FGFR2 - IgIIIa/c

FGFR2 - IgIIIa/b

FGFR1

FGFR2 - IgIIIa/c

FGFR2 - IgIIIa/b
Figure 3.3-3

Coronal sections through 10-week human midface; FGFR1, 2, and 3 immunohistochemistry, FGF2 expression, and control. FGF2 (II) is detected in the cartilagenous skeleton and perichondrium (ns – nasal septal cartilage, pnc – paranasal cartilage). It is detected in the maxillary osteoid (black arrow), midfacial mesenchyme (mm), and enamel epithelium (white arrow) of the tooth germ (tg). FGFR1, FGFR2 (composite image, III), and FGFR3 (IV), proteins are equally and maximally expressed in the enamel epithelium (white arrow) of the tooth germ (tg). Compared to the tooth domain, FGFR3 (IV) is poorly expressed in the maxillary osteoid (black arrow) and midfacial mesenchyme (mm); whereas FGFR1 and FGFR2 are strongly expressed in these areas (see also Fig4). Bar = 100 μm.

Figure 3.3-4

Coronal sections through maxillary osteoid of the human embryo at ten (panel I – III) and thirteen (panel IV & V) weeks; FGFR1, FGFR2, and FGFR3 immunohistochemistry. At ten weeks, FGFR1 protein (I) is expressed in cellular aggregates of the midfacial mesenchyme (mm), and consistently in the osteoblasts lining the maxillary osteoid (black arrows). FGFR2 (II) protein is similarly expressed in the condensing mesenchyme and osteoblast domains. By contrast, FGFR3 is only patchily expressed in a few cell aggregates (III, black arrows) and many cells of the mesenchyme and osteoid are unlabelled (white arrows). At 13 weeks of human facial development FGFR2 consistently labels maxillary osteoblasts (IV, black arrows) and condensing mesenchymal cells (mm). FGFR3, however, compared to the strong positive signal of the nasal epithelium (ne), labels a minority of the maxillary osteoblasts (V, black arrows). Many osteoblasts and mesenchymal cells remain unlabelled (white arrows). Bar = 30 μm.
Human 10 wk hemiface: Negative control

FGF2

FGFR1, FGFR2

FGFR3
Figure 3.3-5

Coronal sections through the midface at 8, 10, and 13 weeks; FGF2 and 4; immunohistochemistry. FGF4 (I) is expressed in the osteoblasts of the maxillary osteoid (black arrows) and is also expressed in the wider midfacial mesenchyme (mm, white arrows), compared to control (II). At 13—weeks, FGF4 (III; control, IV) is expressed in the maxillary osteoblasts and mesenchyme; and is expressed in nasal cartilage cells (nc, white arrows) more than the lining perichondrium (pc). FGF2 is expressed, at 10 weeks (V; and control, VI), in the nasal septal chondrocytes (nsc), and lining periosteum (pc, white arrows). FGF2 is strongly expressed in the osteoblasts lining maxillary osteoid (mo; black arrows), and condensing mesenchyme (mm) at 10 weeks (VII; and control, VIII). Bar = 30 μm.

Figure 3.3-6

Coronal sections through the basicranium of the 13-week embryo; FGF2, FGFR1, FGFR2, and FGFR3 immunohistochemistry. At 13 weeks the basicranium has a cartilagenous template (I-IV, black arrows), consisting medially of a layered perichondrium and adjacent hypertrophic cartilage matrix (hc). Laterally, the perichondrial chondrocytes ‘fan’ into a proliferating zone (pz), and interface more laterally with membrane cranial bone with a well-defined periosteum (white arrows). The eye (e) is inferolaterally placed and surrounded by periocular mesenchyme (pom). FGF2 protein is ubiquitously expressed in membranous and endochondral craniofacial skeletogenesis (II, white and black arrows respectively), and in the periocular facial mesenchyme (pom) surrounding the extraocular muscles (om) and optic nerve (n). FGFR1 and FGFR2 (III, composite image) are strongly expressed in the proliferating zone and hypertrophic cartilage (black arrows). Both receptors are also expressed in cranial membrane bone (white arrows) and periocular mesenchyme (pom). FGFR3 is strongly expressed in perichondrium and proliferating zone (IV, pz), but weakly in the hypertrophic cartilage matrix (hc), and facial mesenchyme (see also Fig 4, Fig 7). Bar = 100 μm.
Human skull base, 13 wk: Negative control

FGF2

FGFR1/FGFR2

FGFR3
Coronal sections through the 10 and 13 week human basicranium; FGFR in-situ hybridisation and immunohistochemistry. FGFR1 protein (i) is detected in the proliferating zone (pz), the layered perichondrium (white arrows) and the hypertrophic zone (hc, black arrow). FGFR2 is expressed as both IgIIIa/α and IgIIIa/β transcripts in the 10-week basicranial perichondrium (ii, & iii; white arrows). By 13 weeks, FGFR2 protein (iv) is detected in the proliferating zone (pz), hypertrophic cartilage (hc; black arrow), and layered perichondrium (white arrows). FGFR3 protein (v) is strongly detected in the layered perichondrium and proliferating zone; but is not detected in the hypertrophic cartilage. Bar = 100 µm.

Expression of Stat1 in the human skull base cartilage, 8 weeks and 10 weeks; immunohistochemistry. The Stat1 transcription factor is detected in the nuclei of the 8-week basicranial chondrocytes (i, sbc; white arrows) compared to age-matched control (iii). There is no expression in the adjacent cranial mesenchyme (i, cm), and there is a weak signal in the perichondrium (p; black arrows). In contrast, by 10-weeks (ii) Stat1 is strongly detected in the nuclei and throughout the cytoplasm of the perichondrial cells (p, black arrows) and adjacent loose stroma. Stat1 is detected in the nuclei, but not the cytosol, of the hypertrophic chondrocytes (hc; white arrows) of the skull base cartilage (sbc). Nuclear localisation of Stat1 is associated with activation of the transcription factor (see text). Bar = 30 µm.
Coronal sections through the basicranium of the thirteen week embryo; TGFβ1 and TGFβ3 immunohistochemistry (panel I – II); FGF2 and FGF4 immunohistochemistry, with control (panel III – V). TGFβ1 (I) is expressed in the membranous pericranium (white arrows), periocular mesenchyme (pom) and most strongly in the perichondrium (black arrows). By contrast, TGFβ3 (II) is expressed equally in both cartilage matrix and perichondrium (black arrows), as well as the wider range of skeletogenic tissue. FGF2 (IV) is strongly expressed in perichondrium and cartilage (sbc, black arrows) as well as cranial membrane osteoid (cno, white arrows), whereas FGF4 (V), whilst weakly expressed in the perichondrium during endochondral ossification (black arrows), is preferentially expressed in the cranial membrane osteoid (white arrows). (eom = extraocular muscle.) Bar = 100 μm, panel I-II, and 30 μm, panel III-V.
3.4 Towards pathogenesis of Apert cleft palate — FGF, FGFR, and TGFβ genes are differentially expressed in sequential stages of human palatal shelf fusion.

3.4.1 Abstract

Critical cellular events at the palatal medial edge epithelium (MEE) occur in unperturbed mammalian palatogenesis, the molecular control of which involves a number of growth factors including TGFβ3. Apert syndrome is a monogenic human disorder in which cleft palate has been significantly correlated to the FGFR2 - Ser252Trp mutation. The relative expression of these genes in human palatogenesis is currently reported.

The expression of the IgIIIa/b and IgIIIa/c transcript isoforms of FGFR2 and the proteins FGFR1, FGFR2, and FGFR3 was studied in situ throughout the temporospatial sequence of human palatal shelf fusion and correlated with the expression of TGFβ3. In addition, the immunolocalisation of the ligand FGFs, 2, 4, and 7, was undertaken together with the intracellular transcription factor STAT1, which is selectively activated by FGFR signalling. FGFRs are differentially expressed in the mesenchyme and epithelia of fusing palatal shelves, in domains overlapping those of their ligands FGF4 and FGF2 but not FGF7. Co-expression is seen with TGFβ3, which is implicated in MEE dynamics and FGF and FGFR upregulation; and STAT1, an intracellular transcription factor that mediates apoptosis.

The co-regulation of molecules of the FGFR signalling pathway with TGFβ3 throughout the stages of human palatal fusion suggests their controlling influence upon apoptosis and epithelio-mesenchymal transdifferentiation at the MEE. Potential mechanisms in the pathogenesis of Apert cleft palate are discussed.
3.4.2 Introduction

The sensitivity of mammalian palatogenesis to disruption is illustrated by the great number of animal models that display cleft palate as a consequence of embryonic or molecular experimentation designed to investigate completely independent systems. Perhaps because of this sensitivity, no single unifying aetiology can be described for human cleft palate, which is considered to be multifactorial. Clinical observation suggests that mechanical obstruction will perturb human palatogenesis, for example by basal encephalocele (Shimizu et al, 1999) or intraoral space occupying lesions (Britto et al, 2000b). These cases are, however, rare and sporadic, and give little clue of the prevailing molecular controls; the best models for which are clinical cleft palate syndromes with known molecular etiology.

Human palatogenesis occurs from the 8 - 13 week of development. The primary palate forms from a posterior protrusion of the fused maxillary prominences, whereas the secondary palate forms by the elevation of the initially vertically orientated palatal shelves of the maxilla. The horizontal palatal shelves appose, then fuse in the midline to each other and the nasal septum, thereby separating nasal and oral cavities. This process is dependent upon critical cellular events at the medial edge epithelium (MEE) which promote epithelial fusion and subsequent dissolution, to allow palatal shelf continuity across the midline. The β3 isoform of transforming growth factor β (TGFβ3) has been specifically implicated in this process. Negation of endogenous TGFβ3 activity in embryonic mouse palatal cultures by antisense oligonucleotides or blocking antibodies causes a failure of murine palatogenesis (Brunet et al, 1995). TGFβ3 - null mice (TGFβ3 -/-) display isolated cleft palate in the absence of wider craniofacial anomaly (Kaartinen et al, 1995;Proetzel et al, 1995); and in organ culture, the transgenic TGFβ3 (-/-) mouse cleft palate can be 'rescued' to fuse under the influence of exogenous TGFβ3 (Kaartinen et al, 1997;Taya et al, 1999). Furthermore, exogenous TGFβ3 will cause the normally cleft avian palate to fuse in culture (Sun et al, 1998). There is, however, no human clinical correlate of a TGFβ3 cleft palate syndrome.

By contrast, the Apert syndrome is a monogenic human craniofacial dysmorphism in which posterior palatal clefts occur in 75% of cases (Kreiborg and Cohen, 1992). Whilst the craniofacial features of Apert syndrome are extensive, the cleft palate phenotype has, in particular, been significantly correlated with the Ser252Trp mutation of the fibroblast growth factor receptor (FGFR) 2 gene (Slaney et al, 1996). The isoform - specific expression of FGFR2, together with the homologous genes FGFR1 and FGFR3 has therefore been studied in sequential stages of human palatal fusion. In addition, the expression of the FGF ligands
2, 4, and 7, and the downstream transcription factor \textit{STAT 1} has been investigated; and related to the expression of \textit{TGFβ3} in human palatal fusion at the MEE. The potential relevance of the differential expression of these genes to mechanisms of human palatogenesis is discussed.

3.4.3 Materials \& Methods

\textit{Preparation of embryonic \& early foetal material}

Human embryo – foetal tissue aged 8 to 14 weeks (n=6) was provided by the Human Tissue Resource maintained at the Institute of Child Health and University College Hospital, London. Craniofacial tissue was collected and prepared as previously described (S-2.1). Coronal sections of the palatal elements of these samples are used to illustrate the differential expression of \textit{FGF/FGFR, STAT1} and \textit{TGFβ3} genes throughout progressive states of palatal fusion in these studies.

\textit{Studies of FGFR and allied gene expression}

\textit{FGFR1}, and the \textit{IgIIIa/c (BEK)} and \textit{IgIIIa/b (KGFR)} isoforms of \textit{FGFR2} mRNA transcripts were detected by \textit{in - situ} hybridisation using isoform - specific riboprobes. Probe synthesis and \textit{in – situ} hybridisation steps are described in S-2.2.

The protein receptors \textit{FGFR1}, \textit{FGFR2}, and \textit{FGFR3}; the ligand FGFs 2, 4, and 7; and the \textit{TGFβ3} and \textit{STAT 1} proteins were detected by immunohistochemistry using commercially available specific antibodies (S-2.2.3). The anti – receptor antibodies have epitopes corresponding to the carboxy – terminal sequences of the receptor protein, and therefore label both \textit{IgIIIc} and \textit{IgIIIb} isoforms in immunohistochemistry studies.
3.4.3 Results

The histomorphology of human palatal shelf apposition and fusion from 8–13 weeks is illustrated in Figures 3.4-1 – 3.4-6. At eight weeks of human craniofacial development, the anterior part of the secondary palate in coronal section displays two horizontal palatal shelves in apposition with each other at the midline medial epithelial edge (MEE), and with the nasal septum to form an inverted 'T' (Figs 3.4-2 & 3.4-3). The 'junctions' of palatal and naso-septal mesenchyme are separated by intact epithelia. These intact epithelia form 'seams' of contact, which are expanded, in accordance with previous observations (Smiley and Dixon, 1968), into triangular areas at the oral aspect of the MEE. In the mid-part of the secondary palate, the closely apposed epithelia form a tight seam, the integrity of which is reduced in part by 'tongues' of interposed mesenchymal continuity (Fig 3.4-4). The oral 'epithelial triangle' remains intact. More posteriorly in coronal section, the palatal shelves are observed in various phases of separated elevation to meet the descending septum (Fig 3.4-1). It therefore appears that the mid-part of the human secondary palate meets and fuses first, closely followed by the anterior secondary palate, with the posterior secondary palate lagging behind. By ten weeks, the elevated, horizontal palatal shelves are completely apposed with each other and the nasal septum, and dissolution of the intervening epithelia, apparently beginning at the naso-septal aspect, is in process (Fig 3.4-6). Confluent mesenchyme separates 'islands' of epithelia. By thirteen weeks, only a residual medial epithelial edge remains, there is palatal mesenchymal continuity across the midline, with complete loss of the nasal – palatal epithelial interface (Fig 3.4-6).

Palatal shelf elevation

Coronal section of the post-elevation, pre-appositional, human palate at eight weeks (Fig 3.4-1) reveals a simple palatal mesenchyme with a surface epithelium in continuity from the nasal to the oral surface via the medial epithelial edge (MEE). The IgIIIa/c isoform of FGFR2 is expressed as transcript, and protein FGFR2 is detected in all three epithelial domains; protein labels particularly strongly at the oral aspect of the MEE. FGFR1 is co-expressed as protein these epithelia. Both FGFR1 and FGFR2 are sparsely expressed in the palatal mesenchyme. FGF2, a preferential ligand for the FGFR2 – IgIIIa/c isoform (Dionne et al, 1990; Miki et al, 1992; Orr-Urtreger et al, 1993) is particularly co-expressed with it at the oral aspect of the MEE, in company with FGF4, which is expressed throughout all three epithelial domains. FGF4, which also activates FGFR2 - IgIIIa/c, is a preferential ligand for the IgIIIa/b isoform of FGFR2 (Orr-Urtreger et al, 1993). STAT1, a downstream
modulator FGFR signalling (Su et al, 1997; Hart et al, 2000) is also expressed in all three palatal epithelial domains in the pre-appositional human palate. TGFβ3 expression characterises the palatal mesenchyme and the overlying epithelial domains.

**Palatal shelf apposition and dissolution of the MEE**

As the palatal shelves and nasal septum come into apposition (Figs 3.4-2 & 3.4-3), TGFβ3 is dominantly expressed in the MEE and nasal septal perichondrium, compared to the epithelia separating the palate and nasal septum. STAT1, strongly expressed in the nasal septal perichondrium, is poorly expressed in the appositional MEE (Fig 3.4-2). FGF2 is strongly expressed in all appositional epithelia (Fig 3.4-2), as are the FGFRs 1, 2, and 3 proteins (Fig 3.4-3). Both isoforms of FGFR2, IgIIα/ε and IgIIα/β are expressed as mRNA transcript in the nasal junctional and medial edge epithelia, and in the nasal and oral epithelia (Fig 3.4-3). Where the elevated palatal shelves appose the nasal septum, the integrity of the MEE begins to break down, commencing at the nasal side of the MEE (see Fig 3.4-3; i, iv) with loss of continuity of epithelial cells. Dissolution of the MEE progresses with ‘tongues’ of mesenchymal continuity forming between ‘islands’ of epithelial cells, and an increase in mesenchymal cellular density subjacent to the remaining MEE (Fig 3.4-4; i, iv - vi). The epithelial islands continue to express FGFR proteins and both IgIIα/ε and IgIIα/β isoform mRNAs of FGFR2 (Fig 3.4-4); whereas the associated subjacent dense ‘halo’ of mesenchymal cells express FGF2 (Fig 3.4-5) but not the receptors. The ligands FGF2 and FGF4 are co-expressed with their receptors in the epithelial islands of the remaining MEE, whereas FGF7 is not expressed in human palatal epithelia (not detected at 8 or 13 weeks; see Fig 3.4-7). TGFβ3 and STAT1 proteins are strongly expressed in the residual MEE of the fusing palate but not the mesenchymal cells (Fig 3.4-5).

Progression of the palatal mesenchyme to confluence across the midline appears to commence at the nasal aspect of the MEE at points where the elevated palatal shelves come into apposition with the nasal septum, and progresses to the oral aspect. At points posterior to the nasal septum, corresponding to the midline fusion of the soft palate, there is no obvious vector to the dissolution of the MEE (Fig 3.4-5; v and vi). Strong expression of FGF/FGFR at the nasal aspect persists (Fig 3.4-5; v), with the formation of an epithelial triangle, as the oral aspect of the MEE becomes attenuated. At complete fusion of the soft palate, there is no residual label for FGFRs in the mid-palatal domain (Fig 3.4-5, vi).
Completion of palatal confluence across the midline

By ten weeks of human palatal development, there is residual MEE as circumscribed islands of epithelial cells expressing FGFRs and FGFs2, and 4 (Fig 3.4-6, i – ii). The surrounding mesenchyme is undergoing membranous ossification as the palatal shelves of the maxilla, and labels strongly for FGF2 and 4 and TGFβ which are powerful osteoblast mitogens. Mesenchymal confluence across the palatal – septal margin is complete. By thirteen weeks, the MEE remains as a few epithelial islands at the most oral aspect of the palatal midline which strongly express FGFR and STAT1. Expression of STAT1 is tightly regulated to the remaining epithelial cells, FGFR1 and 2 are expressed in the mesenchyme undergoing osseous differentiation.

3.4.4 Discussion

Clinical observation (Shimizu et al, 1999; Britto et al, 2000b) and animal models (Griffith and Hay, 1992; Sun et al, 1998) indicate that successful apposition of the medial epithelial edge (MEE) of the palate is essential to normal palatal shelf fusion. Under normal circumstances, MEE subsequently undergoes adherence and then dissolution to allow confluence of the palatal shelf mesenchyme across the midline and with the nasal septum. Despite the evidence linking TGFβ3 signalling to MEE dynamics in palatal fusion, and the implied role of the FGFR signalling pathway (Slaney et al, 1996) the molecular events that control this process in human palatogenesis are incompletely understood. Controversial evidence supports three potential mechanisms for the dissolution of the MEE; the epithelial – mesenchymal transdifferentiation of MEE to mesenchyme, the migration of the MEE to populate the nasal and oral epithelia, and the dissolution of the MEE by programmed cell death (apoptosis).

Fate mapping studies have been undertaken in both palatal culture and in – vivo models of mammalian palatogenesis. DiI is a fluorescent, lipid soluble carbocyanine dye that irreversibly incorporates into cell membranes, and remains present for a number of subsequent cell doublings. Sequential time - course analysis by confocal microscopy of DiI fluorescence in separated sheets of intact palatal epithelia, and palatal cultures undergoing fusion, have been interpreted to indicate the migration of palatal MEE cells orally and nasally to populate these epithelial domains (Carette and Ferguson, 1992). In these studies, in mouse posterior palate cultures, the DiI positive domains were not directly correlated with the cellular histology of the fusing palate and their significance is therefore open to question.
Serial sections of the fusing human palate do not easily support the ‘MEE migration theory’ for the entirety of antero-posterior human palate fusion. In the anterior and mid-palate, where fusion with the nasal septum also occurs, a line of epithelial apposition and adherence forms at the naso-palatal junction by eight weeks. By ten and thirteen weeks this has become a confluent mesenchyme, and there is no evidence of migratory palatal epithelial domains or a heaped ‘recipient’ nasal epithelium in any of the sections examined. The appearance of epithelial islands at the oral aspect of the MEE might represent static images of a migrating epithelium, however, persistence of such islands in the central MEE as late as thirteen weeks suggests an alternative process is affecting the whole length of the MEE concurrently. In the human post-septal soft palate, heaping of the epithelium and the formation of a nasal epithelial triangle does occur, and it is possible that MEE migration occurs in this posterior part of the palate consistent with the model used by Carette and Ferguson (1992).

The interpretation of these DiI labelling studies, however, remains controversial. Similar studies in sequential static sections of post-septal mouse palatal cultures have been interpreted, together with immunolabelling of cell cytoskeletal elements, to support transdifferentiation of the MEE to confluent mesenchyme (Shuler et al, 1991), an interpretation that has been corroborated by in-utero observations (Shuler et al, 1992). Studies in the rat palate indicate that the MEE breaks up into epithelial islands, the cells of which form filopodia, lose their epithelial polarity, and shift their cytoskeletal phenotype from a cytokeratin (epithelial – type) to a vimentin (mesenchymal – type) filament structure (Fitchett and Hay, 1989). Fate-mapping the MEE with CCFSE label, a more robust and long-lived alternative to DiI, supports the contention that the MEE transdifferentiates to mesenchyme with a fibroblast cell type in both in vivo and in vitro models (Griffith and Hay, 1992). The fibroblast – like mesenchymal cells continue to populate the midline and nasal–septal junction, but also migrate laterally. Furthermore, CCFSE labelled fibroblast–like cells resulting from epithelio-mesenchymal transdifferentiation characterise the avian palatal fusion model under the influence of exogenous TGFβ3 (Sun et al, 1998); suggesting that the differentiation of MEE to palatal mesenchyme is highly conserved amongst vertebrates and is triggered by palatal adherence in midline seam formation.

This study demonstrates that human palatal fusion is characterised by the formation of epithelial islands separated by tongues of confluent mesenchyme, which gradually become the dominant cell type as the epithelium attenuates. This series of static images in human palatal fusion, is of course, open to ambiguous interpretation, but may be usefully correlated with experimental work in animal models. Electon micrographic examination of rat palatal fusion
indicates that the dissolution of the MEE progresses from the nasal to the oral aspect, and that islands of epithelial cells are each surrounded by a 'halo' of mesenchymal cells; an observation consistent with this human data. Ultrastructural analysis of the 'halo' mesenchymal cells in rat palatal fusion suggests that they contain phagocytosed apoptotic debris (Gibbins et al, 2000). The expression of alpha smooth muscle actin in the MEE during seam development supports a role for epithelio – mesenchymal transdifferentiation (Gibbins et al, 2000), however; and both transdifferentiation and apoptosis may contribute to the dissolution of the MEE in – vivo (Martinez-Alvarez et al, 2000). Murine MEE cells have also been demonstrated to undergo apoptosis, with the resulting cell debris scavenged by macrophages (Martinez-Alvarez et al, 2000). Apoptotic DNA – fragmentation markers are localised to the cells of the epithelial triangles, the central MEE, and also those of the nasal septo – palatal line of fusion; but do not characterise these epithelia during palatal shelf elevation or midline adhesion (Mori et al, 1994).

The molecular control of transdifferentiation and/or apoptosis in the human MEE may involve a combination of FGF/FGFR and TGFβ mediated pathways. FGF/FGFR signalling been implicated in neurite (Chao, 1992) and osteoblast differentiation (Lomri et al, 1998;Fragale et al, 1999), and may play a role in epithelial trans - differentiation also. The FGFR2- IgIIIa/b (KGFR) homozygous null mouse has a cleft palate phenotype (De Moerlooze et al, 2000), whereas the heterozygote is normal. This implies a dose – dependent functional role for KGFR signalling in normal murine palatogenesis. In Apert syndrome, cleft palate occurs in 75% cases (Kreiborg and Cohen, 1992), and the incomplete expressivity is consistent with a dose dependent effect of Apert – FGFR2 mutations upon human palatal shelf fusion via the IgIIIb/KGFR isoform. The Apert Ser252Trp and Pro253Arg mutations confer 'gain – of – function' upon the mutant IgIIIc/BEK isoform by the selective facilitation of ligand binding. This results in prolonged homodimerisation and signal transduction (Anderson et al, 1998c), which is phenotypically demonstrated by accelerated differentiation in osteoblasts (Lomri et al, 1998;Fragale et al, 1999). Apert mutant activation of IgIIIb/KGFR, possibly resulting from an increased affinity for ligand FGF7 in the skin (Finch et al, 1995;Danilenko et al, 1995), results in the acneiform Apert cutaneous phenotype, which is similar to that associated with a somatic FGFR2-Ser252Trp mutation (Munro and Wilkie, 1998). To remain consistent with the cleft palate phenotype of the KGFR – null mouse, the Apert mutations might be predicted to confer a dominant – negative functional effect upon KGFR in human palatal epithelia, resulting in failure of fusion at the MEE.

Such a mechanism would appear to be exquisitely associated with the Apert activating mutations of FGFR2. The 'Crouzon – Pfeiffer' group of craniofacial dysostoses, in which
severe midfacial retrusion may occur in combination with similar palatal (Peterson and Pruzansky, 1974) and nasopharyngeal (Peterson-Falzone et al, 1981) morphology to Apert syndrome, do not display cleft palate (Kreiborg and Cohen, 1992) despite their association with activating FGFR2 mutations. Furthermore, Apert cleft palate significantly co-segregates with the Ser252Trp mutation, whereas the Pro253Arg mutation significantly co-segregates with the Apert syndactyly phenotype (Slaney et al, 1996). It may be therefore, that the Ser252Trp mutation confers a specific functional effect upon KGFR signalling, perhaps via changes in the normally high-affinity FGF4/KGFR binding association (Orr-Urtreger et al, 1993), to result in a disruption of human palatal shelf fusion by dose-dependent dominant-negative means. Both the Ser252Trp and Pro253Arg mutations in Apert syndrome cause changes in ligand specificity for the IgIIIb/KGFR and IgIIIc/BEK receptor proteins (Yu et al, 2000); and this might reduce the functional efficiency of IgIIIb/KGFR expressed in the human palatal MEE. Further support for this theory comes from the observation of a cleft palate phenotype in E18.5 transgenic mice in which a soluble, dominant-negative, kinase-deficient kgfr protein is expressed as a chimera with mouse immunoglobulin (Celli et al, 1998). Specific KGFR/FGFR2—IgIIIb loss-of-function co-segregates with murine cleft palate in a dose dependent manner.

The contributory evidence for a dominant-negative role for KGFR signalling in the pathogenesis of Apert cleft palate is compelling and might be tested in a variety of models. Possible downstream consequences include disruption of epithelial–mesenchymal transdifferentiation at the MEE or a disruption of apoptosis, or both. STAT1 is an intracellular DNA binding protein which is activated by FGFR signalling (Su et al, 1997; Chen et al, 1999), and is particularly strongly expressed in the epithelial islands of the fusing palate. STAT1 is specifically implicated in promoting apoptosis in a range of cell types (Chin et al, 1997) and in response to activated FGFR (Legesai-Mallet et al, 1998); and therefore provides a candidate downstream target for KGFR signalling in the normal apoptotic dissolution of the MEE. FGFR and TGFβ signalling pathways synergistically promote apoptosis in rat palatal fibroblasts (Funato et al, 1997), in which FGFR is upregulated by the TGFβ1 isoform (which is also expressed in the human MEE). The TGFβ3 (−/−) cleft palate mouse has a reduced apoptotic rate in the MEE, thus TGFβ3 may be an upstream mediator of FGF upregulation (Kay et al, 1998) in the maintenance of normal palatal apoptosis. The co-regulation of FGF/FGFR and TGFβ3 may also have a role in processes as diverse as proteoglycan regulation in the palatal mesenchymal matrix (Sharpe et al, 1993), and the maintenance of the MEE cell surface filopodia, which are critical to the adhesion of the palatal midline seam (Martinez-Alvarez et al, 1996; Taya et al, 1999; Martinez-Alvarez et al, 2000).
3.4.5 Conclusions

The studies presented in this report, taken together with that from animal models, suggest that human palatal fusion in the midline and at the nasal septal junction is achieved by a combination of mechanisms under the influence of TGFβ3 and specific FGF/KGFR signalling pathways. There is a gradual dissolution of the human MEE, which breaks up along its length into 'epithelial islands' surrounded by a dense mesenchymal 'halo'. Evidence from animal models implicates both apoptosis and epithelio – mesenchymal transdifferentiation in this process, in which the mesenchymal cells play a both a scavenging and supportive role. It is hypothesised that apoptosis in human MEE is driven by the co-regulation of TGFβ3 and FGF/KGFR signalling causing the upregulation of STAT1. Furthermore, KGFR/TGFβ co-regulation may influence both palatal midline adhesion (by inducing epithelial filopodia) and the epithelio – mesenchymal transdifferentiation of the MEE to a palatal fibroblast phenotype.

The Apert linker – sequence mutations in FGFR2 may potentially cause a change in ligand specificity, thereby reducing the functional efficiency of KGFR in palatal shelf fusion, to cause cleft palate. These studies, linking TGFβ3 and FGF/KGFR co-regulation in human palatogenesis, followed the correlation of clinical observation in Apert syndrome with animal KGFR and TGFβ3 'loss – of – function' models. The molecular models of the control of palatal fusion that are proposed here may now be investigated in experimental systems.
S-3.4 Figure Legends

Figure 3.4-1

Palatal shelves of the human posterior palate; post elevation, prior to apposition; coronal section at 8 weeks (x4 mag). FGFR 1 (ii) is expressed in the palatal epithelium in nasal (n), oral (o), and medial epithelial edge (MEE) (white arrows) domains compared to control (i). Weaker expression characterises the palatal mesenchymal cells (pm). FGFR2 protein (iii), is expressed predominantly on the oral side of the MEE, despite an even expression of transcript mRNA (iv) in all epithelial domains. FGF2 protein (v) is also dominantly expressed at the oral aspect of the MEE, whereas FGF4 (vi) is expressed evenly from nasal to oral epithelium. Stat1 protein (vii) is expressed in the nasal, oral and MEE domains. TGFβ3 (viii) is expressed in the palatal mesenchyme and has strongest epithelial expression in the nasal domain in the pre-appositional elevated palate. Bar = 30 μm, uniform scale.

Figure 3.4-2

Human anterior hard palate, palatal shelves in apposition, and with nasal septum (black arrows); coronal section at 8 weeks. TGFβ3 (ii) is dominantly expressed in the medial epithelial edge (MEE) compared to negative control (i), and positive control in the perichondrium of the nasal septal cartilage (nsc). It is not expressed in the palatal mesenchyme (pm), the dense peri-epithelial mesenchyme (white arrows), or vomeronasal body (vnb). STAT1 protein (iii) is weakly expressed in the MEE compared to nasal perichondrium. FGF2 is expressed in all apposed palatal epithelia (black arrows) and in the oral (o) and nasal (ne) epithelia. Note the increase in palatal mesenchymal cell density around the apposed MEE (white arrows; particularly ii, and iv). Bar = 30 μm.
8wk negative control
FGFR1 protein
FGFR2 protein
FGFR2 - IgIIIa/c mRNA
FGF2 protein
FGF4 protein
STAT 1 protein
TGFβ3 protein
Control: Antr palate 8 wks

STAT1 protein

TGFS3 protein

FGF2 protein
Figure 3.4-3

Human mid-hard palate, shelves in apposition with the nasal septum; coronal section at 8 weeks (x4 mag). FGFR2 protein (i) is expressed in the apposed MEE, and nasal septal epithelia (black arrows). Both the IgIIIa/b and IgIIIa/c isoforms (ii and iii) are expressed as mRNA in these epithelial domains. FGFR1 and FGFR3 (composite image, iv) are co-expressed in the apposed epithelium (ne - nasal epithelium; o - oral space, vnb - vomeronasal body). The midline epithelial ‘seam’ is broadened at the oral aspect of the MEE into an ‘epithelial triangle’ (see also Figs 4, 5), not seen at the nasal septal aspect. Bar = 30 μm.

Figure 3.4-4

Human mid-hard palate; epithelial – mesenchymal differentiation; coronal section at 8 weeks (x4 mag). The MEE breaks up into islands of cells (white arrows), expressing FGFR2 (i – iii), FGFR1 (iv) and FGFR3 (v) compared to negative control (vi). Epithelial – mesenchymal differentiation progresses in a nasal - septal to oral (o) direction. Mesenchymal cells (black arrows), accumulate around and between the epithelial islands, and do not express FGFRs (see also Fig5). Each epithelial island is surrounded by a dense ‘halo’ of mesenchymal cells. Note the ‘epithelial triangle’ formed by a widening of the MEE at its oral aspect (see also Fig5). Bar = 30 μm.
FGFR2 protein

FGFR2 - Igllla/b mRNA

FGFR2 - Igllla/c mRNA

FGFR1/FGFR3 protein
**Figure 3.4-5**

Human mid-hard palate, showing dissolution of the MEE; coronal section at 8 weeks (x4 mag). MEE transdifferentiation (white arrows), commencing at the nasal septal aspect, is accompanied by the expression of FGF2, FGF4, TGFβ3 and STAT1 in the epithelial islands (i – iv). FGF2 is expressed in the immediate peri-epithelial condensations of mesenchymal cells (black arrows, see also Fig4), but FGF4, STAT1, and TGFβ3 are not. In the posterior palate, without influence of the nasal septum (plate v, 156 microns posterior to iv), MEE dissolution does not progress in a vectoral manner. The nasal aspect of the MEE is maintained, with the formation of an ‘epithelial triangle’, as the oral aspect attenuates (v). At complete soft palate fusion (vi), FGFRs are not expressed across the midline compared to nasal (n) or oral (o) epithelia. Bar = 30 μm.

**Figure 3.4-6**

Human palatal fusion at 10 weeks (i and ii) and 13 weeks (iii, iv); coronal section (x4). At 10 weeks, the MEE is entirely separated into epithelial islands (black arrows), expressing TGFβ3 (i) and FGF2, 4, and the FGFRs (example FGF4; ii). Dense cellular condensations, immuno-negative for these proteins, persist immediately around these islands. Surrounding mesenchyme is undergoing membranous ossification to palatal bone, with the expression of TGFβ3, FGF2, 4 and FGFRs. The line of epithelial fusion with the nasal septum (white arrows) is replaced by mesenchyme. At 13 weeks, the MEE has nearly completely disappeared (iii, iv), surrounded and replaced by mesenchyme, condensations of which (mc) are forming membranous palatal bone. STAT1 expression is closely regulated to the remaining epithelial domain (iv), whereas the FGFRs are expressed in condensing mesenchyme. Bar = 30 μm.
FGF2 protein  
FGF4 protein  
TGFβ3 protein  

STAT1 protein  
FGFR3 protein: post soft palate  
FGFR1 protein: fused soft palate
TGF3 protein: 10 wks, MEE fusion

FGFR2 protein: 13 wks, remaining MEE

FGF4 protein: 10 wks, MEE fusion

STAT1 protein: 13 wks, remaining MEE
**Figure 3.4-7**

Coronal section of fusing palate at 8 and 13 weeks, FGF7 immunohistochemistry. FGF7 is not detected as protein in palatal fusion at 8 or 13 weeks in the MEE (i, ii), compared to positive control in the 10-week human cranial dermis (iii, iv; negative control). *Bar* = 30 µm.
FGF7 negative in fusing palate

FGF7, 10 wk cranial dermis

FGF7, 10 wk cranial dermis, control
4. Conclusions

Expression of FGFRs in human craniofacial development and craniosynostosis: Discussion, Conclusions, and Future Work

The studies in this thesis were undertaken to

1. Investigate the expression of FGFR1, 2, and 3 genes in human craniofacial development in a longitudinal series of tissues encompassing basicranial (endochondral) and membranous (calvarial/midfacial) modes of ossification.

2. Describe the expression of the FGFR and FGF genes in human infant sagittal synostosis.

3. Investigate whether the activation of FGFR2 by representative mutations causing Apert and Pfeiffer syndromes has an effect upon the differential expression of FGFR genes in human calvarial ossification in situ.

4. Investigate whether the expression of FGF, FGFR and TGFβ genes co-regulates with sequential stages of human palatal shelf fusion.

Differential FGFR expression is observed in wild-type human craniofacial development, and provides a correlate for the human phenotypes of activating FGFR mutations. FGFR3, in displaying differentiation stage-specific expression in basicranial chondrogenesis, is proposed as a specific controlling gene in the transition from 'proliferating' to 'hypertrophic' chondrocyte. The evidence from both 'gain-of-function' and 'loss-of-function' mouse models supports this contention for human endochondral ossification (S-3.3). Furthermore, FGFR3 expression in human basicranial chondrogenesis may be site-specific. Independent reports, published while these studies were ongoing, have characterised the expression of FGFR3 in wild-type human long-bone growth plates. The growth-plate in the human appendicular skeleton in a highly organised structure characterised by different stages of
chondrogenic differentiation ('resting', 'proliferative', 'pre-hypertrophic', 'hypertrophic' chondrocytes). In work by the same research group, the expression of FGFR3 has been reported to characterise the hypertrophic chondrocytes in long-bone growth plates (Delezoide et al, 1997; Delezoide et al, 1998). This was not the case in the human basicranium (S-3.3), suggesting that the regulation of chondrogenesis may be subtly site-specific. Further work is required to resolve this issue, but differences in the human FGFR3-phenotypes provide a potential illustration that this may be the case. The Pro250Arg and Ala391Glu mutations in FGFR3 cause 'basicranial' phenotypes but not dwarfism, whereas the range of activating chondrodysplasia mutations causes dysmorphogenesis at selected growth plates of long bones (S-1.3). Such differences may reflect the differential modes of activation of these mutations, however, developmental site-specificity in the regulation of endochondral ossification by FGFR3 may contribute.

FGFR3 - chondrodysplasia mutations have been reported to stabilise the FGFR3 protein and arrest chondrocytic differentiation (S-3.3). The preliminary evidence presented here suggests that activating FGFR2 mutations in Apert syndrome and Pfeiffer syndrome cause negative regulation of the expression of FGFR2 - IgIIIc in membranous ossification in situ (S-3.2). These data provide a developmental mechanism for the generation of phenotypic diversity within the FGFR - skeletal dysplasias. One might speculate that differential activation of FGFR2 protein by various FGFR2 mutations would cause differential negative auto-regulation of the IgIIIc/BEK isoform. This in turn would have a functional effect in situ, against the background environment of matrix co-factors including HSPG (S-1.2.3) and the TGFβ isoforms (S-3.3; S-3.4). The characterisation of the expression of HSPGs such as perlecan and syndecan in basicranial development and the fusing suture may yield useful data to explain the phenotype diversity. The complexity of the matrix might, for example, explain how the same Pro250Arg mutation in FGFR3 generates both the uni-corneal and bi-corneal synostosis phenotypes. This and other examples of genotype-phenotype diversity (S-1.4) may also reflect the influence of mechanisms as diverse as the bioavailability of ligand, the specific action of modifier genes, downstream events post-FGFR activation, and epigenetic, environmental, constraints.

The human infant sagittal suture provides a useful model of accelerated calvarial osteogenesis independent of activating FGFR mutations. The expression of FGFR1-3 in human membranous ossification is differentiation-stage specific in a manner that differs from the mouse (S-3.1). Whereas the expression of human FGFR1 characterises the proliferative and
early differentiative stages of human membranous ossification, FGFR2 - IgIIIc characterises the latter stages. The IgIIIb isoform is transiently expressed in the osteoblast stage, and the preliminary evidence indicates that it is upregulated by activating FGFR2 mutations (S-3.2) and in suture fusion (S-3.1). Murine fgfr2- IgIIIb (kgfr) characterises an early stage of wild-type osteoblast differentiation (S-3.1), and thus models the wild-type human phenotype. It remains to be demonstrated whether murine kgfr 'gain - of - function' models will provide a craniosynostosis phenocopy. Whereas fgfr1/fgfr2 characterise different stages of murine calvarial ossification than human FGFR1/FGFR2 (S-3.1), murine fgfr3 characterises the same stages of chondrogenesis ('resting' and 'proliferating', but not 'hypertrophic' domains; S-1.2.2), as human FGFR3 in the basicranium. The collective data confirms that whilst the mouse models will undoubtedly provide a useful guide to activated fgfr - signalling in murine skeletogenesis in vitro, the parallels may be limited. The chick model has provided useful data on vertebrate craniofacial morphogenesis, as a relatively fast - growing and dispensible model. The expression of cek-3 (FGFR2) in chick cranial membranous ossification is a useful correlate for modelling human cranial FGFR2 mediated dysmorphogenesis, however, the lack of cek-3 (FGFR2) expression in the chicken maxilla reduces its value in midfacial modelling (S-1.2.2). Factors such as these will be of importance as various animal models are characterised and potentially therapeutic interventions are considered.

The degree to which the mouse provides a faithful model of the human phenotype may reflect the variable induction of cell - type specific downstream pathways, as demonstrated by STAT1 activation in chondrogenesis and the MAPK pathway in fibro - osseous differentiation (S-3.3). The variable cutaneous phenotype of human FGFR2 mutations, for example, suggests that there is a variable downstream effect of FGFR 'gain - of - function' in epithelia, and this has yet to be modelled in the mouse. There are no specific reports that the murine palatal medial edge epithelium (MEE) expresses FGF or FGFR genes. Independent reports, however, observe a dose - dependent failure of murine palatal shelf fusion when kgfr is deleted or expressed as a functionally dominant - negative protein (S-3.4). This suggests that KGFR signalling may be functionally limiting in human palatogenesis also. The Apert syndrome is the only FGFR2 'gain - of - function' phenotype with a consistent, but not complete, expressivity for cleft palate. It remains to be seen how, or whether, the inherently activating Apert mutations cause cleft palate via the KGFR - isoform in a dose - dependent, loss - of - function, manner in the palatal MEE, when functional gain characterises Apert osseous differentiation. The effects of the Apert mutations are ligand - dependent, and exquisitely perturb the IgII - IgIII linker - region to change ligand specificity (S-1.4; S-3.4). It
may be that the availability of ligand in the palatal MEE is functionally limiting in the presence of the Apert mutations. Functional limitation by ligand in the Apert mutations may also define the reason why some FGFR - syndromes have cutaneous manifestations, and others do not. The Apert, Beare - Stevenson, and Crouzon acanthosis - nigricans mutant receptors retain ligand - binding affinity, whereas many of the FGFR2 - craniosynostosis syndromes, which lack skin anomaly, abrogate ligand - binding. Ligand binding in the skin by activating FGFR mutants may thus correlate with cutaneous pathology. These issues have yet to be resolved in a controlled manner. However, exciting possibilities are raised for the role of mutant IgIIIb isoform - signalling in FGFR - cutaneous pathology; and also specifically for KGFR in wild – type palatogenesis and the pathogenesis of cleft palate. Palatal culture systems are well – described, and both murine 'knock - out' and dominant negative models may be exploited to resolve these issues by experimental means. Phenotypic correlations with the 'level of function gain' of the activating mutant receptor are common in the FGFR3 - chondrodysplasias (S-1.3.3.2) and their various published murine models, and so provide a parallel for a 'functional dose - dependent 'role for KGFR - signalling in palatogenesis.

The differentiation stage – specific expression of human FGFR1-3 genes in membranous ossification correlates with the clinical presentation of the mutant FGFR – phenotypes, in which mutant 'gain - of – function' in FGFR2 causes accelerated midfacial and calvarial prematurity. Similarly the co - expression of these genes in the basicranial cartilage provides evidence of the importance of the skull base as a site of growth control craniofacial development. The phenotypes of the craniofacial dysostoses have been described in some detail (S-1.3), and include a variety of extracranial manifestations, which have yet to be examined in the murine models with the same rigour as the skeletogenic phenotype. There is no Kfr 'gain - of – function' mouse model as yet, and it will be interesting to note whether this faithfully phenocopies human dysplasias, given the differentiation – stage specific differences in their expression patterns. In particular the generation of a mouse model for syndactyly, as an Apert limb phenocopy, will provide the opportunity to further delineate how common molecular mechanisms control craniofacial and limb development. There is preliminary evidence that kgfr – upregulation causes differential severity of syndactyly (Oldridge et al, 1999), in concert with a specific expression of KGFR in the human digital metaphyseal shaft periosteum (Britto et al, 2000a), but the specific molecular pathways that generate the phenotype have yet to be delineated.

The intracranial hypertension that characterises the 'Crouzon – Pfeiffer' group, in particular, is a severe clinical problem. The neuro – developmental consequences of activating FGFR mutations have yet to be investigated, and may yield useful information to clinical benefit.
Pathologies such as anomalous dural – sinus anatomy and intracranial venous drainage co–segregate with the 'Crouzon – Pfeiffer' phenotypes in particular (Taylor et al, 2000). The molecular pathogenesis of these specific aspects of the phenotype is unknown, yet result in considerable clinical sequelae. FGFR expression in vertebrates is widespread, and 'gain – of – function' mutations would appear to have exquisitely cell – type specific effect. The future is open for the development and investigation of in – vitro and in – vivo models to further explore the pathogenesis neuro – developmental delay, anomalous venous drainage, skeletal dysplasia and cleft – palate in these patient groups.
Publications

Britto JA, Evans RD, Hayward RD, Jones BM
From Genotype to Phenotype: the differential expression of FGF, FGFR, and TGFβ genes characterises human cranioskeletal development.
Plast Reconstr Surg 2001  in press

Britto JA, Evans RD, Hayward RD, Jones BM
Towards pathogenesis of Apert cleft palate – FGF, FGFR, and TGFβ genes are differentially expressed in sequential stages of human palatal shelf fusion.
Cleft Palate Craniofac J 2001  in press

Britto JA, Moore R, Evans RD, Hayward RD, Jones BM
Negative autoregulation of FGFR2-IgIIIa/c expression characterises cranial development in Apert (P253R) & Pfeiffer (C278F) syndromes, and suggests a basis for differences in their cranial phenotypes.
J Neurosurg  in press

Britto JA, Chan C-t J, Evans RD, Hayward RD, Jones BM
Differential expression of FGFRs in human digital development suggests common pathogenesis in complex acrosyndactyly and craniosynostosis.
Plast Reconstr Surg 2001; 107(6); 1331 - 1338

Britto JA, Chan C-t J, Evans RD, Hayward RD, Thorogood P, Jones BM
Fibroblast Growth Factor Receptors are expressed in Craniosynostotic Sutures.
6. Appendices

Appendix A: Embedding fixed tissue.

Embedding Human embryo and 14 week foetal samples in wax block

The human embryonic tissues were fixed and dehydrated prior to embedding according to the following protocol:

Samples were progressively dehydrated in ethanol (85%, 95%, 100%, 100%).

Samples were treated by sequential immersion in Histoclear (Fissons) (2 fresh solutions, 30 minutes each, 70°C), then Histoclear/wax (50/50 solution, 30 minutes, 70°C).

Samples were then immersed in wax at 70°C, which was freshly changed at 30 minutes, in which they were embedded by pouring into a block mold, orientating, and allowing to set at room temperature prior to storage at 4°C.

Embedding Human post-natal suture material and 27 week foetal samples in wax block

The human late foetal and postnatal tissues were fixed, dehydrated, and decalcified prior to embedding according to the following protocol:

Samples were progressively dehydrated in ethanol (85%, 95%, 100%, 100%).

Samples were treated by sequential immersion in Histoclear (Fissons) (2 fresh solutions, 45 minutes each, 70°C), then Histoclear/wax (50/50 solution, 45 minutes, 70°C).

Samples were then immersed in wax at 70°C, which was freshly changed at 45 minutes, in which they were embedded by pouring into a block mold, orientating, and allowing to set at room temperature prior to storage at 4°C.
Appendix B: Sectioning of fixed tissue

Pretreatment (subbing) of glass slides

Single frosted end glass slides were used throughout. Slides were immersed by the rack in 70% ethanol, 10% hydrochloric acid, then thoroughly rinsed in water. Slides were then dried overnight in a 60°C oven.

Slides were immersed by the rack in 350 mls 98% 3-amino propyltriethoxy-silane (TESPA 2% v/v in acetone; Sigma Co) and lavaged for 2 minutes under a fume hood.

Slides were then thoroughly lavaged twice by the rack in 350 mls acetone with (in a fume hood).

Slides were lavaged in distilled water, dried and stored in tin foil.

Sectioning of en-bloc wax samples

Wax blocks containing samples were orientated and secured on the microtome and serial sections of 6 – 8 μm were cut.

Sections were mounted on the subbed slides in DEPC water, excess fluid was carefully drained, and the slides allowed to dry on a 37°C hot plate.

Prepared slides were wrapped in tissue paper, and kept at 4°C until use.
Appendix C: Stock Solutions, Chemicals & Buffers

Tissue fixation & Preparation

PFA. Paraformaldehyde, 4%, used as fixative throughout. Made as 4% by volume with PBS, dissolved at 70°C and cooled to 0°C prior to use. Stock solution kept at −20°C

PBS. Phosphate buffered saline. PBS (x10): 1.37M NaCl, 27mM KCl, 80mM Na₂HPO₄, 15mM KH₂PO₄. Treated with 0.05% DEPC and then autoclaved. For 1L: 80g NaCl, 2g KCl, 11.5g anhydrous Na₂HPO₄, 2g KH₂PO₄.

Ethanol series. These were made freshly each week and used for dehydrating/rehydrating slides, and for removing paraffin wax from cut sections. In routine histology, laboratory grade IMS was used and diluted with water for 80%, 70%, 50%, 30% dilutions as necessary. For preparation for in situ hybridisation, separate stocks were made up in autoclaved bottles from 100% absolute ethanol and DEPC milliQ water, and used for this purpose only.

DNA Minipreps, Linearisation step

All bacterial preparations used were stock attenuated E Coli

LB Broth. Made from broth base supplied by GibCo BRL, made up as 20.0 gm/l in distilled water and autoclaved at 121C for 15 mins.

TE 10mM Tris.Cl (appropriate pH), 1mM EDTA. Autoclaved.
For RNA work, Tris was made up in DEPC water and filter sterilised.
2M Tris HCl  pH adjusted to 7.6 and 8.0.
For RNA work this solution was made up with DEPC treated water.

RNA'se.  RNA'se A was made up as a stock solution at 10mg/ml in 0.01M sodium acetate at pH 5.2, and brought to final pH7 with 1.0 M Tris – HCl.

Agarose gel  For electrophoresis. All gels for DNA estimation were made up as 0.8% agarose (GibCo BRL, Ultrapure. Electrophoresis grade); 0.8gm/100mls, dissolved in TAE for 2 mins in a laboratory microwave oven.

TAE (x50)  0.8M Tris, 40 mM EDTA. For 1L of 20x, 242g Tris, 100ml 0.5M EDTA, set pH to 8.2 with about 57ml of glacial acetic acid.

0.5M EDTA  pH adjusted to 8.0 with NaOH pellets. Autoclaved with DEPC.

Ethidium bromide. 10 mg/ml in TE.

3M Sodium acetate  pH adjusted to 5.2 with glacial acetic acid. Autoclaved.
For RNA, treated with 0.05% (v/v) DEPC then autoclaved.

**Riboprobe synthesis, In – situ hybridisation & washings**

DEPC-treated water  0.05% (v/v) DEPC (Diethyl pyrocarbonate) was added to milliQ water and then autoclaved.

DTT, 1Molar  1M DTT made up in DEPC water and stored at -20°C.

GAC mix  2.5mM (each) GTP, CTP and ATP.

Sephadex G-50  Preswollen in 10mM Tris pH8.0, 1mM EDTA, 0.1%SDS.

Elution buffer  10mM Tris.Cl (pH 7.6), 1mM EDTA (pH 8.0), 0.1% SDS, 10mM DTT

Alkaline SDS  0.2M NaOH, 1% SDS (lauryl sulphate).
Synthesised riboprobes were maintained in hybridisation mix as stock at $-20^\circ C$:

<table>
<thead>
<tr>
<th>Hybridisation mix</th>
<th>for 20 mls</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% formamide</td>
<td>10 mls formamide</td>
</tr>
<tr>
<td>0.3 M NaCl</td>
<td>1.2 mls 5M NaCl</td>
</tr>
<tr>
<td>20mM TRIS-HCL pH 8.0</td>
<td>0.2 mls TRIS pH 8.0</td>
</tr>
<tr>
<td>5mM EDTA pH 8.0</td>
<td>0.2 mls 5mM EDTA</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>0.31g DTT or 2mls 1M DTT</td>
</tr>
<tr>
<td>10% dextran sulphate</td>
<td>2g dextran sulphate</td>
</tr>
<tr>
<td>Denhardts solution</td>
<td>0.2 mls Denhardts solution</td>
</tr>
<tr>
<td>0.5 mg/ml baker’s Yeast RNA</td>
<td>0.5 mls, 0.5 mg/ml baker’s Yeast RNA</td>
</tr>
</tbody>
</table>

Denhardts solution (100x) 2% (w/v) BSA fraction V, 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrolidone. Filter sterilised and stored at $-20^\circ C$.

Proteinase K. Made up in 50mM Tris pH8.0, 1mmCaCl2, as a 10ml stock, kept at $-20^\circ C$ and used as 10μg/ml Proteinase K in PBS

SSC (x20). 3M NaCl, 0.3M Na citrate treated with 0.05% DEPC and autoclaved to provide an SSC stock, from which aliquots were used in post hybridisation washings. (Low stringency – 5xSSC, 10mM DTT, to high stringency 2xSSC, and 0.1xSSC)

Formamide wash solution.
50% formamide, 2xSSC, 10mM DTT (for 800mls, 400mls formamide, 80mls 20xSSC, 8mls 1M DTT, 320 mls dH2O)

TEN Buffer. 0.5M NaCl, 10mM Tris HCl, pH7.5, 5mM EDTA (for 1 litre, 29.22g NaCl, 5ml Tris pH 7.5, 10ml 0.5mM EDTA, dH2O to 1 litre)
Reagents used in Plasmid Minipreparation

<table>
<thead>
<tr>
<th>Solution</th>
<th>Final concentration</th>
<th>Composition (in water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>25mM TRIS pH8</td>
<td>12.5 ml 2M TRIS pH8</td>
</tr>
<tr>
<td>(1000mls)</td>
<td>10mM EDTA pH8</td>
<td>20 ml 0.5M EDTA pH8</td>
</tr>
<tr>
<td>Solution II</td>
<td>1% SDS 0.2M NaOH</td>
<td>170 ml dH2O</td>
</tr>
<tr>
<td>200mls</td>
<td></td>
<td>20 mls 2% SDS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 ml 4M NaOH</td>
</tr>
<tr>
<td>Solution III</td>
<td>3M KoAc pH4.8</td>
<td>100 ml 6M KoAc</td>
</tr>
<tr>
<td>200mls</td>
<td></td>
<td>23 ml acetic acid</td>
</tr>
</tbody>
</table>
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