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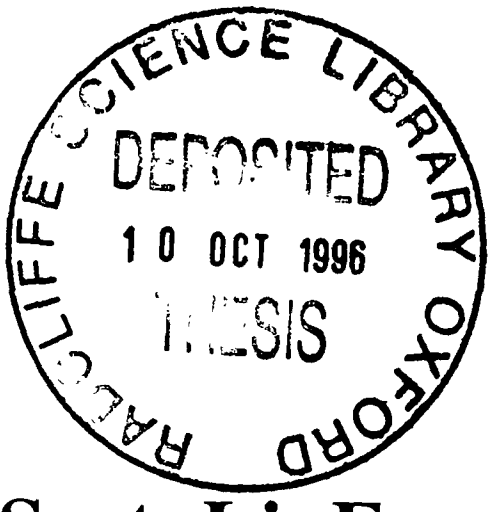
Characterization of the Biological Properties of FGF-9



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Abbreviations

AER	Apical ectodermal ridge
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFR	Cysteine-rich FGF receptor
CHAPS	3[(3-cholamidopropyl)dimethylammonio]-1 propanesulphonate
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
cpm	Counts per min
CSF-1	Colony-stimulating factor-1
d.p.c.	day post-coitum
DAG	Diacylglycerol
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Embryonal carcinoma
ECM	Extracellular matrix
EDC	N-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide hydrochloride
EDTA	Ethylenediamine tetra acetic acid
EGF	Epidermal growth factor
ELF	Enzyme-labelled fluorescence
ELISA	Enzyme-labelled immunosorbent assay
ERDF	Endothelium-derived releasing factor
ES	Embryonic stem
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	FGF receptor tyrosine kinase
GAF	Glia-activating factor

GAP	GTPase-activating protein
GDP	Guanosine diphosphate
GlcN	Glucosamine
GM-CSF	Granulocyte-macrophage colony stimulating factor
GST	Glutathione *S*-transferase
GTP	Guanosine triphosphate
HexA	Hexuronic acid
HPLC	High pressure liquid chromatography
HPRT	Hypoxanthine phosphoribosyl transferase
HS	Heparan sulphate
HSPG	Heparan sulphate proteoglycan
IdoA	Iduronic acid
Ig	Immunoglobulin
IP_3	Inositol 1,4,5-triphosphate
IPTG	Isopropyl-ß-D-thiogalactopyranoside
k_a	Association rate constant
kb	Kilobase
K_D	Equilibrium dissociation constant
k_d	Dissociation rate constant
kDa	Kilodalton
lacz	Bacterial ß-galactosidase gene
LIF	Leukaemia inhibitory factor
MALDI	Mass assisted, laser desorption/ionisation
MIF	Mesoderm-inducing factor
MMTV	Mouse mammary tumour virus
NBT	Nitro blue tetrazolium
NGF	Nerve growth factor
NHS	N-hydroxysuccinimide
nt	Nucleotide
O-2A	Oligodendrocyte-type-2 astrocyte
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma cell line
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
pgk	Phosphoglycerate kinase gene
PI3-kinase	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C

PLCγ	Phospholipase Cγ
PtdIns	Phosphatidylinositol
RA	Retinoic acid
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RU	Resonance units
SDS	Sodium dodecyl sulphate
SH2	Src-homologous domain 2
SPR	Surface plasmon resonance
SSC	Standard saline citrate buffer
SV40	Simian virus 40
UTR	Untranslated region

Abstract

Characterization of the Biological Properties of FGF-9

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The fibroblast growth factor family of polypeptides currently consists of nine structurally-related members. Cloning of the mouse homologue of the latest reported member of the family, FGF-9, is described in this study.

Mouse *Fgf9* exhibits a high level of sequence conservation with the human, rat and *Xenopus* counterparts. Of note is the lack of a hydrophobic signal peptide at the N-terminus of the coding sequence. The protein, however, appeared to be secreted by producer cells since a significant quantity of the protein could be purified from the culture supernatant of transfected cells.

Members of the FGF family are known to bind to cell surface tyrosine kinase receptors (FGFRs) to elicit a variety of physiological responses. These receptors themselves form a family of four structurally-related tyrosine kinases and each FGF member commonly has the ability to bind several members of the FGFR family. By using *in vitro* plate binding assays, FGF-9 is shown in this study to bind specifically to two FGFR members: FGFR2b and FGFR3c.

To further study the potential functional role of FGF-9, its expression pattern in the mouse embryo was examined by both RNase protection and RNA *in situ* hybridization analyses. The transcript was detected in a variety of embryonic tissues: the germinal epithelium of the central nervous system, the mesonephric cords, the somites, the gut primordia and the developing eye and ear, suggesting that the gene may have multiple roles during development. In addition, the potential involvement of FGF-9 in mediating adult brain functions was examined by double RNA *in situ* hybridization analysis of the distribution of both *Fgf9* and *Fgfr3* transcripts in the adult mouse brain. Most apparent areas of co-localization are the olfactory bulb and cerebral cortex. The two transcripts are also shown to have distinct distribution patterns in the cerebellum.

Chapter 1

Introduction

Many polypeptides which regulate the growth of animal cells have been identified. These molecules are termed growth factors and named according to the biological activities or assays by which they were originally isolated. A good example is the fibroblast growth factor (FGF) family of polypeptides. The term fibroblast growth factor was first used to describe the activity in brain and pituitary extracts which stimulated the proliferation of Balb/c 3T3 fibroblasts (Gospodarowicz, 1974). Since then, multiple related molecules have been isolated and a great deal of effort has been expended in the attempt to elucidate the biochemistry and biological roles of these molecules. An understanding of the functional role of each of these growth factors requires knowledge of not only its biological characteristics, but also the mechanisms by which its activity may be regulated. The aim of this review is to illustrate the unique features of each growth factor and the complexity and diversity by which the biological activity of this family of growth factors may be regulated. In greater detail in the following chapters, the characteristics and regulation of a specific member of the FGF family will be described, as an excellent example of a molecule which is a member of a family with many structurally related counterparts but which possesses a potentially unique functional role.

1.1 A Brief History

The existence of the FGFs were probably reported as early as the 1940s when crude brain homogenates were found to contain activities that stimulated primary fibroblasts to proliferate in culture (Trowell et al., 1939). Later, potent mitogenic activity for Balb/c 3T3 fibroblasts from pituitary extracts was described (Armelin, 1973). This discovery soon led to the partial purification of a 14 - 16 kDa polypeptide mitogen for the Balb/c 3T3 cell line from both pituitary and brain extracts (Gospodarowicz, 1975; Gospodarowicz et al., 1978) and was given the name FGF. Subsequently, the mitogenic activities in the brain and pituitary preparations were found to be mediated by two predominant but distinct molecules. One has an acidic isoelectric point (pI 5 - 6) and was called acidic FGF (or aFGF) (Maciag et al., 1979; Thomas et al., 1980) while the other has a basic isoelectric point (pI 9.5 - 10) and was named basic FGF (or bFGF) (Lemmon et al., 1982). By this time, a number of growth factors that modulate the growth and differentiation of neurotrophic and angiogenic responsive cells had also been independently identified and later found to be either aFGF or bFGF, reflecting the potentially diverse biological activities associated with the FGFs. As before, these molecules were named either after their tissue of origin or by the biological property by which they were isolated. They included eye-derived growth factor (EDGF), cartilage-derived growth factor (CDGF), astroglial growth factor (AGF), retinal-derived growth factor (RDGF), brain-derived growth factor, hypothalamus-derived growth factor, myogenic growth factor, pituitary growth factor, bone-derived growth factor, prostatic growth factor and endothelial cell growth factor (reviewed by Burgess and Maciag, 1989). This last property, the ability to stimulate endothelial cell proliferation and knowledge of the association of endothelial cells with mast cells which produce heparin, led subsequently to the discovery of the ability of heparin to enhance the angiogenic activity of the FGFs *in vitro* and *in vivo* (Taylor and Folkman, 1982). Both aFGF and bFGF were found to bind strongly to heparin affinity columns, with aFGF requiring 1 M NaCl and bFGF 1.5 M NaCl for elution (Gospodarowicz et al.,

1984; Maciag et al., 1984; Klagsbrun and Shing, 1985). Hence, they have also been known as heparin-binding growth factors (HBGF). This ability to bind avidly to heparin greatly facilitated purification and characterization of the FGFs (Shing et al., 1984). By 1985, the primary amino acid structures of aFGF and bFGF were available (Esch et al., 1985a, b; Giménez-Gallego et al., 1985) and the two molecules shown to be related polypeptides (Thomas and Giménez-Gallego, 1986). Both molecules were also found to be distantly related to interleukin-1ß (IL-1ß) (Gospodarowicz et al., 1978; Giménez-Gallego et al., 1985; Thomas et al., 1985) suggesting that all three molecules share a common ancestral origin. To date, complementary DNA clones encoding human and mouse aFGF (Jaye et al., 1986; Hébert et al., 1990) and cDNAs encoding for human, bovine, rat, murine, amphibian and chicken bFGF have been isolated and characterized (Abraham et al., 1986a, b; Kurokawa et al., 1987; Shimasaki et al., 1988; Kurokawa et al., 1988; Hébert et al., 1990; Kimelman et al., 1988; Mitrani et al., 1990; Borja et al., 1993).

1.2 A Family of Related Polypeptides

Following the discovery of aFGF and bFGF, more related genes including those encoding the INT-2, HST/K-FGF, FGF-5, FGF-6, FGF-7, AIGF and GAF proteins were reported (Moore et al., 1986; Sakamoto et al., 1986, Delli-Bovi and Basilico, 1987; Zhan et al., 1987; Tanaka et al., 1992; Naruo et al., 1993). The genes encoding for FGF-3, FGF-4 and FGF-5 were initially isolated as oncogenes. *Fgf3* was identified as a cellular oncogene at a site of frequent insertion of the mouse mammary tumour virus (MMTV) (Dickson et al., 1984). The gene encoding FGF-4, on the other hand, was identified as a transforming gene for NIH 3T3 cells from both Kaposi's sarcoma and human stomach tumours (Delli-Bovi and Basilico, 1987; Sakamoto et al., 1986). The *Fgf5* gene was similarly isolated by screening for genes present in tumours that were able to transform NIH 3T3 cells (Zhan et al., 1988). The gene for *Fgf6* was initially obtained by low stringency hybridization with the *Fgf4* gene as probe (Marics et al., 1989). The human and murine homologues of all three proto-oncogenes, *Fgf3, Fgf4* and *Fgf5*, have been isolated and the sequences

reported (Brookes et al., 1989a, b; Delli-Bovi et al., 1987; Taira et al., 1987; Zhan et al., 1988; Hébert et al., 1990). The *Xenopus*, chicken and zebrafish homologues of *Fgf3*, have also been isolated (Kiefer et al., 1993; Mahmood et al., 1995b; Kiefer et al., 1996) while PCR cloning using primers designed from the *Fgf4* sequence resulted in the identification of a FGF molecule closely related to FGF-4 and FGF-6 in *Xenopus*, called XeFGF (Isaacs et al., 1992).

Like the prototype FGFs, the remaining members known to date were identified mainly as mitogens for different cell lines. FGF-7 (or KGF) was originally purified as a mitogen for cultured murine keratinocytes from the conditioned medium of human embryonic fibroblasts (Rubin et al., 1989). The mouse homologue of this gene has also been reported (Mason et al., 1994). FGF-8 was identified as an androgen-induced growth factor (AIGF) that stimulated growth of the androgen-dependent SC-3 mouse mammary carcinoma cell line (Tanaka et al., 1992). The last member, FGF-9, was originally described as a glia-activating factor (GAF) as it was purified on the basis of its mitogenic activity on rat primary glial cells from the conditioned medium of a human glioma cell line (Naruo et al., 1993). The rat, murine and amphibian homologues of this gene have also been reported (Miyamoto et al., 1993; Seo and Noguchi, 1995; Hecht et al., 1995; Song, 1996).

The number of members in this family thus currently stand at nine. To avoid confusion, members of the FGF family will be named numerically according to the chronological order in which they were first identified (Baird and Klagsbrun, 1991), hence, aFGF is also known as FGF-1, bFGF as FGF-2, INT-2 as FGF-3, HST/K-FGF as FGF-4, AIGF as FGF-8 and GAF as FGF-9 (Table 1.1).

Classification into the FGF family is based on sequence homology at the amino acid level (Figure 1.1). Members of the family vary in length from 155 amino acids for the prototypic FGFs (FGF-1 and FGF-2) to 268 residues for FGF-5. They all encode polypeptides which share a conserved "core" of about 120 amino acids (Figure 1.2). Core homology between members range from 30% to 69% (reviewed by Goldfarb, 1990). This is in contrast to the dramatic sequence conservation between species for each FGF, with

Table 1.1. Current List of Members of the FGF Family and Interspecies Sequence Identity

Growth Factor	New Nomenclature	% Human-Murine FGF Sequence Homology
aFGF	FGF-1	95
bFGF	FGF-2	95
INT-2	FGF-3	89
K-FGF/HST	FGF-4	80
FGF-5	FGF-5	84
FGF-6	FGF-6	93
KGF	FGF-7	94
AIGF	FGF-8	N.D.
GAF	FGF-9	99.5

N.D., not determined.

Figure 1.1. Alignment of Amino Acid Sequences of Members of the FGF Family.
Sequences of nine members of the mouse FGF family are aligned based on the PileUp Program (GCG Wisconsin Package Version 8.0) and the output displayed by the Alscript Program (Geoff Barton, Molecular Biophysics, Univ. of Oxford). Colour legend: basic residues - red; acidic residues - green; non-polar residues - blue; most common residues - shaded grey.

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                1                   10                  20                  30                  40                  50                  60                  70
FGF-4   MSGPGTAAVALLPAVLLALLAPWAGRGGAAAPTAPNGTLEAELERRWESLVALSLARLPVAAQPKEAAVQ
FGF-6   ..........................................................................
FGF-5   ....MSLSFLLLLFFSHLILSAWAHGEKRLAPKGQPGPAATDRNPRGSSSRQSSSSSAMSSSSASSSPAAS
FGF-9   ..........................MAPLGEVGSYFGVQDAVPFGNVPVLPVDSPVLLSDHLGQSEAGG
FGF-3   ..........................................................MGLIWLLLLSLLEPGWPAAGPGAR
FGF-1   ..........................................................................MAEGEITT
FGF-2   ..........................................................................MAAGSITT
FGF-7   ......................MHKWILTWILPTLLYRSCFHIICLVG.TISLACNDMTPEQMATNVN
FGF-8   ..........................................MGSPRSALSCLLLHLLVLCLQAQVTVQSSPNF

                80                  90                  100                 110                 120                 130                 140
FGF-4   SGAGDYLLGIKRLRR.........LYCNVGIGFHLQALPDGRIGGAHADTRDSLLELSPVERGVVSIFG
FGF-6   ......GIKRQRR.........LYCNVGIGFHLQGLPDGRISGTHEENPYSLLEISTVERGVVSLFG
FGF-5   LGSQGSGLEQSSFQWSLGARTGSLYCRVGIGFHLQIYPDGKVNGSHEANMLSVLEIFAVSQGIVGIRG
FGF-9   LPRGPAVTDLDHLKGIL..RRRQLYCR..TGFHLEIFPNGTIQGTRKDHSRFGILEFISIAVGLVSIRG
FGF-3   LRRDAGGRGGVYEHLGGAPRRRKLYC..ATKYHLQLHPSGRVNGSLENSAYSILEITAVEVGIVAIRG
FGF-1   FTALTEKFN...LPPGNYKKPKLLYCSNG.GHFLRILPDGTVDGTRDRSDQHIQLQLSAESVGEVYIKS
FGF-2   LPALPEDGGSGAFPPGHFKDPKRLYCKNG.GFFLRIHPDGRVDGVREKSDPHIKLQLQAEERGVVSIKG
FGF-7   CSSPERHTRSYDYMEGGDIRVRRLFC.RT.QWYLRIDKRGKVKGTQEMKNNYNIMEIRTVAVGIVAIKG
FGF-8   TQHVREQSLVTDQLSRRLIRTYQLYSRTSGKHVQVLANKRINAMAEDGDPFAKLIVETDTFGSRVRVRGA

                150                 160                 170                 180                 190                 200                 210
FGF-4   VASRFFVAMSSKGKLYG.SPFFTDECTFKEILLPNNYNAYESYKYPGM.....................FIALSK
FGF-6   VRSALFVAMNSKGRLYA.TPSFQEECKFRETLLPNNYNAYESDLYQGT.....................YIALSK
FGF-5   VFSNKFLAMSKKGKLHA.SAKFTDDCKFRERFQENSYNTYASAIHRTE.....................KTGREWYVALNK
FGF-9   VDSGLYLGMNEKGELYG.SEKLTQECVFREQFEENWYNTYSSNLYKHV.....................DTGRRYYVALNK
FGF-3   LFSGRYLAMNKRGRLYA.SEHYSAECEFVERIHELGYNTYASRLYRTVSSTPGARRQPSAERLWYVSVNG
FGF-1   TETGQYLAMDTDGLLYG.SQTPNEECLFLERLEENHYNTYISKKHAE.....................KN..WFVGLKK
FGF-2   VCANRYLAMKEDGRLLA.SKCVTDECFFFERLESNNYNTYRSRKYT.....................S..WYVALKR
FGF-7   VESEFYLAMNKEGKLYA.KKECNEDCNFKELILENHYNTYASAKWTH.....................NGGEMFVALNQ
FGF-8   ETGLYICMNKKGKLIAKSNGKGKDCVFTEIVLENNYTALQNAKYEG.....................WYMAFTRKG

                220                 230                 240                 250                 260                 270                 280
FGF-4   NGKTKKG..NRVSPTMKVTHFLPRL..........................................................
FGF-6   YGRVKRG..SKVSPIMTVTHFLPRI..........................................................
FGF-5   RGKAKRGCSPRVKPQHISTHFLPRFKQSEQPELSFTVTVPEKKNPPSPIKSKIPLSAPRKNTNSVKYRLK
FGF-9   DGTPREG..TRTKRHQKFTHFLPRPVDPDKVPELYKDILSQS.....................................
FGF-3   KGRPRRGF..KTRRTQKSSLFLPRVLDHRDHEMVRQLQSGLPRPPGKGVQPRRRRQKQSPDNLEPSHVQA
FGF-1   NGSCKRG..PRTHYGQKAILFLPLPVSSD.....................................................
FGF-2   TGQYKLG..SKTGPGQKAILFLPMSAKS.....................................................
FGF-7   KGIPVRG..KKTKKEQKTAHFLPMAIT.....................................................
FGF-8   RPRKGSKTRQHQREVHFMKRLPRGHHTTEQSLRFEFLNYPPFTRSLRGSQRTWAPEPR.....................

                290
FGF-4   ..........
FGF-6   ..........
FGF-5   FRFG......
FGF-9   ..........
FGF-3   SRLGSQLEASAH
FGF-1   ..........
FGF-2   ..........
FGF-7   ..........
FGF-8   ..........X
```

typically greater than 90% overall homology over the predicted protein coding sequence. For instance, human, bovine, murine and *Xenopus* FGF-2 sequences share 84-98% identity between them. This remarkably low level of divergence suggests that the FGFs are not redundant *in vivo* and that all regions of each FGF are functionally important.

In general, the prototype FGFs, FGF-1 and FGF-2, are similar to the rest of the members in two ways: they have similar overall gene organization and the ability to bind heparin and heparan sulphate glycosaminoglycans. On the other hand, the prototypic FGFs differ from the other members in the lack of a classical hydrophobic secretory sequence and in their pattern of distribution. Moreover, while FGF-1 and FGF-2 appear to be relatively ubiquitous molecules, the other FGFs show restricted expression patterns and are mainly found in the developing embryo.

1.3 Structure of FGF

1.3.1 Gene Structure

Members of the FGF family appear to share a similar gene organization, consistent with the theory that they arise from a common ancestral gene. They typically consist of three coding exons separated by two introns which may be relatively long in length, as found for the prototypes (Gospodarowicz et al., 1987). The second exon is typically short while the third exon is known to contain a 3' untranslated region (UTR).

The 5' and 3' UTRs are variable and those of the prototypes have been well characterized. The 5' UTR of *Fgf1* was found to contain at least four upstream untranslated exons which are alternatively spliced to the protein-coding first exon (Philippe et al., 1992; Myers et al., 1993). These alternative transcripts appear to be differentially expressed in tissues and cell lines (Payson et al., 1993) and so, may be involved in the regulation of *Fgf1* expression. The 3' UTR typically contains multiple polyadenylation sites. For example, bovine retina *Fgf1* has a 3' UTR which gives rise to alternatively terminated *Fgf1* transcripts (Renaud et al., 1992).

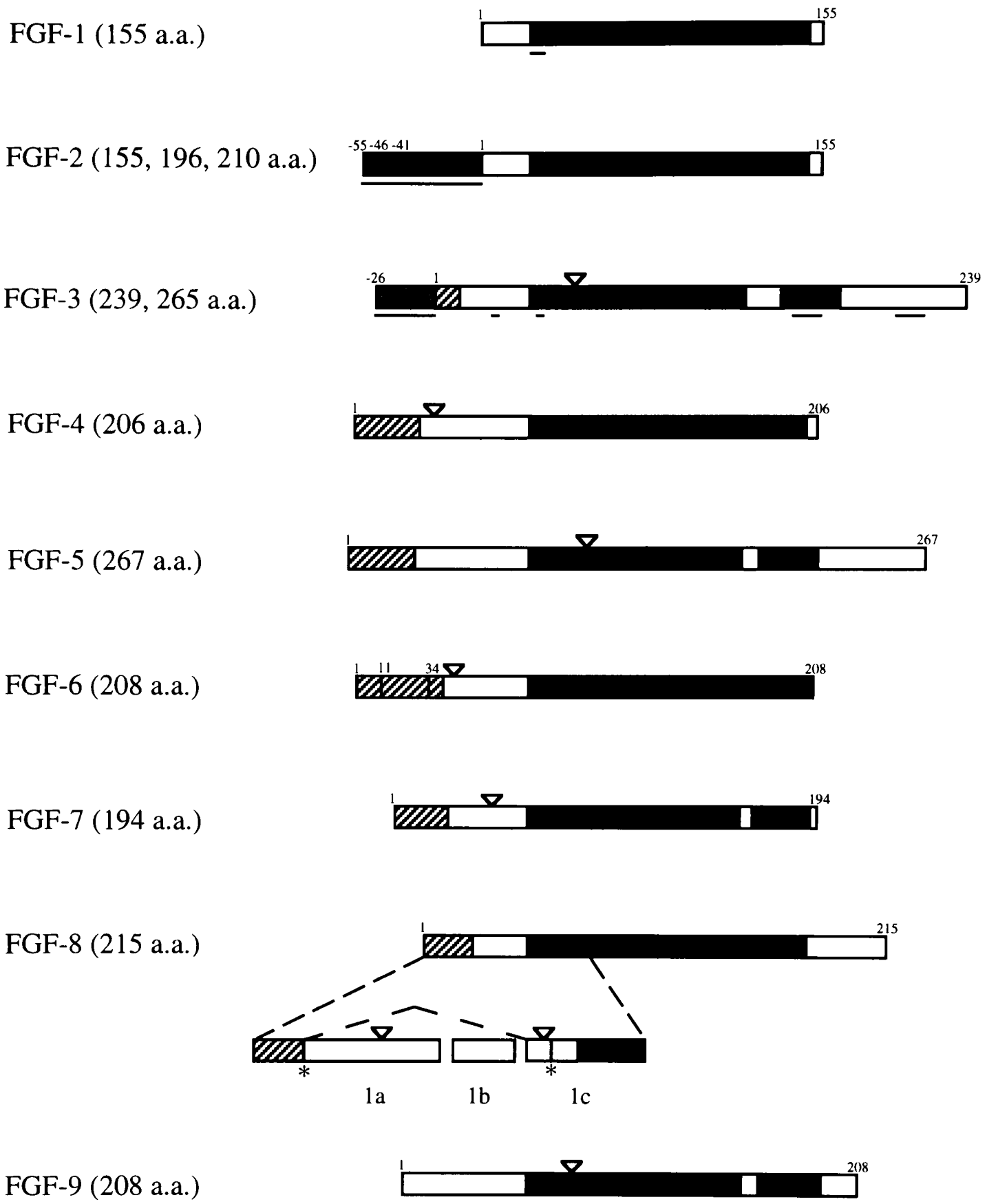


Figure 1.2. The Fibroblast Growth Factor Family of Related Polypeptides.
The nine human FGFs are aligned with respect to the conserved core of sequence homology (~120 a.a., black box). Areas of non-homology are indicated by open boxes. FGF-3 to FGF-8 contain amino-terminal hydrophobic secretory signals (hatched box). FGF-2 and FGF-3 may contain amino-terminal-extensions (shaded box) arising from translation initiation from upstream CUG codons, the positions of which are indicated relative to the AUG start site which is given the postion +1. There are three in-frame AUGs at positions 1, 11 and 34 in the coding region of FGF-6 which may serve as potential start sites for translation. FGF-8 contains at least three coding exons which correspond to the first exon of most of the other FGFs (shown below the 215 a.a. isoform identified by Tanaka et al. (1992) where exon 1a is spliced to exon 1c). Alternative splice donor and acceptor sites in exons 1a and 1c are indicated by *. Consensus sites for gylcosylation are indicated (open, inverted triangles). The lengths of the primary translation products initiated from the AUG or CUG codons are also shown. Putative nuclear localization signal is indicated (line beneath box).

Fgf2, isolated from human hepatoma cells or foreskin fibroblasts, also contains a long 3' UTR where at least eight polyadenylation sites can be found (Kurokawa et al., 1987; Prats et al., 1989). Indeed, multiple transcripts of FGF-2 have been reported from analysis of its expression pattern in different species (Abraham et al., 1986a, b; Kimelman et al., 1988; Mitrani et al., 1990). Hence, the typical detection of multiple FGF transcripts may be partially accounted for by multiple transcriptional termination. The human *Fgf2* gene promoter contains five GC boxes, which may represent Sp-1 binding sites, and one AP1 binding site instead of a canonical TATA box (Shibata et al., 1991). Its activity has been shown to be repressible by the tumour suppressor nuclear phosphoprotein p53 (Ueba et al., 1994).

The *Fgf3* gene contains three promoters and two termination polyadenylation sites, thus accounting for the presence of multiple *Fgf3* RNA transcripts (Grinberg et al., 1991). The *Fgf3* promoter region has been shown to have the ability to regulate expression of the gene in differentiated versus undifferentiated EC cells (Grinberg et al., 1991). The *Fgf3* and *Fgf5* genes have in common an out-of-frame open-reading-frame (ORF) upstream of the coding ORF (Dixon et al., 1989; Bates et al., 1991; Kiefer et al., 1993). These upstream ORFs may regulate gene expression by interfering with the correct translation initiation as removal of these upstream ORFs was shown to enhance the translation of both *Fgf3* and *Fgf5* genes.

The 5' flanking region of the mouse *Fgf4* gene contains a TATA box as well as two Sp-1, two AP2 consensus binding sequences and an alternating purine:pyrimidine motif (Tiesman and Rizzino, 1990). Analysis of the transcription regulation of murine *Fgf4* expression revealed the presence of at least three positive *cis*-regulatory elements: two in the enhancer element region located in the 3' UTR of the third exon and one in the 5' flanking region of the gene (Ma et al., 1992). The enhancer element contains a series of consensus binding sites for transcription factors including octamer-binding proteins and it has been shown to stimulate transcription from the weak promoter (Curatola and Basilico, 1990). A more recent study using reporter gene constructs indicated that the octamer binding motifs may be involved in the regulation of *Fgf4* expression in EC and ES cell lines (Rizzino and

Rosfjord, 1994). Thus, the enhancer element may determine the expression pattern of this gene as it promotes *Fgf4* transcription only in undifferentiated embryonal carcinoma cells and not in its differentiated counterparts or in fibroblast cells.

1.3.2 Multiple Peptide Forms

The coding sequence for FGF-2 predicts a primary translation product of 155 amino acids or 18 kDa (Abraham et al., 1986a, b). The 18 kDa form of FGF-2 was indeed purified from the human placenta and guinea pig brain (Moscatelli et al., 1987; Sommer et al., 1987). Smaller forms of FGF-2 have, however, been isolated. For instance, FGF-2 was isolated originally as a 146 amino acid protein product (Mr 16.5 kDa) from bovine pituitary, brain, retina, adrenal and human brain (Gospodarowicz et al., 1984; Esch et al., 1985a; Giménez-Gallego et al., 1986; Gospodarowicz et al., 1986). Amino terminal-truncated but biologically active forms of this molecule have also been purified from the corpus luteum, adrenal gland and the testis (Gospodarowicz et al., 1985; Ueno et al., 1987). Some of these truncated forms may be attributed to degradation by proteolysis during the extraction procedure (Klagsbrun et al., 1987). Indeed, when FGF-2 was purified in the presence of protease inhibitors, FGF-2 molecules of sizes greater than 16.5 kDa were obtained (Story et al., 1987; Ueno et al., 1986). Truncated forms of FGF-1 that are artefacts derived from extraction procedures were also observed (Burgess et al., 1986; McKeehan and Crabb, 1987).

Higher molecular weight forms of FGF-2 also exist, being present in the brain and most cell lines studied, including fibroblasts and endothelial cells (Presta et al., 1988; Ensoli et al., 1989; Florkiewicz and Sommer, 1989; Iberg et al., 1989; Rifkin and Moscatelli, 1989; Renko et al., 1990; Tsuboi et al., 1990). Subsequent analysis of the cDNA structure together with *in vitro* transcription and translation experiments and transient COS cell expression revealed that the multiple isoforms of FGF-2 were co-translated from a single mRNA transcript. Site-directed mutagenesis studies showed that the larger forms of FGF-2 arose from use of three upstream CUG codons as alternative initiation codons for

translation, resulting in three additional forms of FGF-2 of sizes 22, 23 and 24 kDa (Figure 1.2; Florkiewicz and Sommer, 1989; Prats et al., 1989). These larger isoforms are co-linear amino-terminal expansions of the well-characterized 18 kDa protein and may represent functionally distinct variants since the amino-terminal sequence upstream of the methionine AUG codon between the human and bovine cDNA is only 80% identical (Prats et al., 1989). Translation from any one of the four possible initiation codons may be regulated by five *cis*-acting mRNA elements located in the leader and 3' UTR of *Fgf2* which have been shown to exert differential effects on the efficiency of translation from one or more of the four initiation codons (Prats et al., 1992). The use of alternative CUG codons for initiation of translation was also exemplified by another member of the family, FGF-3 (Acland et al., 1990). In this case, only one CUG initiation codon is present upstream of the more commonly found AUG codon and is thought to be the major site of initiation of translation from *in vitro* mutagenesis and translation experiments (Kiefer et al., 1994) (Figure 1.2). Two other FGF members may be translated from multiple initiation codons. The *Fgf6* ORF may be initiated from three in-frame AUG codons (Coulier et al., 1991) (Figure 1.2) while *Fgf7* contains an additional in-frame AUG codon upstream from the normal initiation start codon in the genomic sequence but it is not known if it is present in the mature mRNA (Mason et al., 1994).

In addition, alternatively-spliced forms of several members of the FGF family have been reported. *Fgf1* transcripts lacking the second exon (Yu et al., 1992) and chick embryo *Fgf2* transcripts alternatively spliced with respect to the first exon (Borja et al., 1993) have been described. FGF-8 is by far the most complex of the FGFs to date. In the FGF-8 coding sequence, four exons that correspond to the first exon of the normal FGF gene are present (Crossley and Martin, 1995) (Figure 1.2). The consequence is that at least eight different FGF-8 isoforms can potentially be produced by alternative splicing. Indeed, seven cDNAs representing different FGF-8 isoforms have been isolated (Tanaka et al., 1992; Crossley and Martin, 1995; MacArthur et al., 1995b).

1.3.3 Tertiary Structure

The three-dimensional crystal structures of both prototype FGFs, human FGF-1 and bovine FGF-2, have been solved (Ago et al., 1991; Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991) and found to be highly similar. The three-dimensional structure of FGF-2 consists of twelve antiparallel ß-strands organized into a folding pattern analogous to a cylindrical barrel with an approximate three-fold internal symmetry (Figure 1.3). The structure is similar to the IL-1α and IL-1ß folding patterns (Fox et al., 1988; Priestle et al., 1988; Finzel et al., 1989; Graves et al., 1990). The amino terminal region of both FGF-1 and FGF-2 is apparently flexible and disordered. The carboxyl-terminal three residues of FGF-2 is also observed to be disordered. These amino- and carboxyl-terminal disordered configurations are thus not thought to contribute to the folding of the rest of the molecule. The ß-strands of the FGF-2 molecule are joined by twelve ß-turns, three of which are type II while the rest are type I.

The four cysteine residues in FGF-2, two of which are conserved between all family members except for FGF-8, are not likely to be involved in disulphide bridge formation due to their distant spatial positions in the tertiary structural fold. The non-conserved cysteine residues may, however, participate in intermolecular disulfide bridges, resulting in dimerization of the FGF-2 molecules under non-reducing conditions (Seno et al., 1988; Zhang et al., 1991). Indeed, site-directed mutagenesis of the cysteine residues in FGF-1, FGF-2 and FGF-7 have indicated that the formation of disulfide bonds is not necessary for biological activity (Fox et al., 1988; Arakawa et al., 1989; Linemeyer et al., 1990; Ortega et al., 1991; Bare et al., 1994). In fact, formation of a disulfide bond between conserved cysteine residues of FGF-1 resulted in virtually inactive products (Linemeyer et al., 1990). In FGF-7, the cysteine residues were found to contribute to its ability to bind heparin and its stability to heat and acid, suggesting that these residues may have a role in maintaining the structure of this protein (Bare et al., 1994).

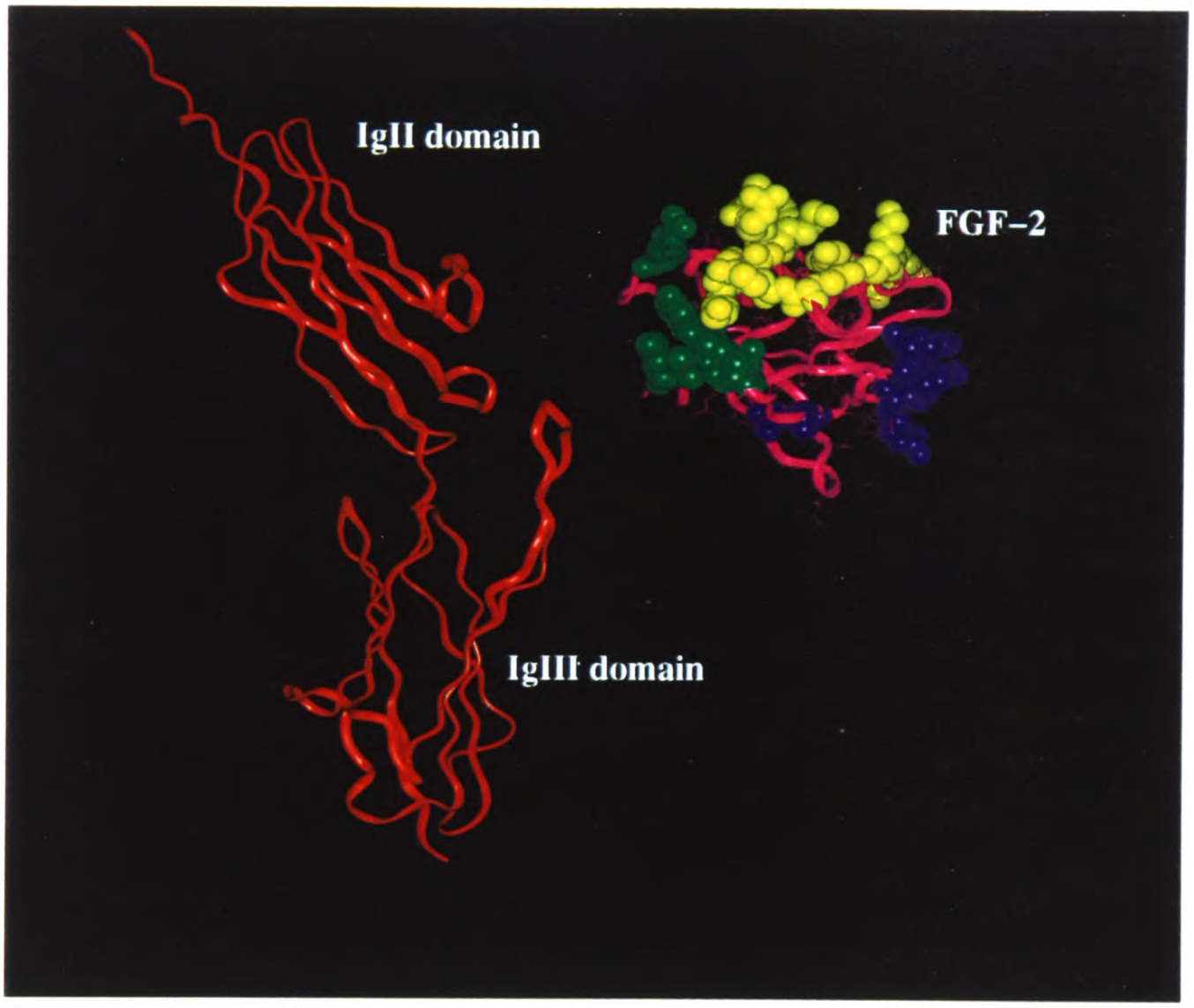


Figure 1.3. The Three-Dimensional Structure of FGF-2 and A Model of the Extracellular Domain of FGFR2.
The crystal structure of FGF-2 has been determined to consist of twelve anti-parallel ß-strands organized into a barrel-like structure with a three-fold internal symmetry. Colour legend: yellow - residues comprising the putative heparin-binding site (Thompson et al., 1994); green - residues forming the putative primary receptor-binding site (Pantoliano et al., 1994); purple - residues thought to form the secondary receptor-binding site (Seddon et al., 1995). It can be observed that the putative heparin-binding site occupies a position orthogonal to the two receptor-binding sites. A model of the tertiary structure of the extracellular domain of FGFR2 is also presented (RasMol software program), showing the immunoglobulin (Ig)-like loops II and III. FGF-2 is thought to interact with the receptor at a region comprising these two Ig-like loops.

1.3.4 Functional Domains

The FGF-2 molecule may be broken down into two major functional domains. As will be described in greater detail below, the FGFs bind to high affinity cell surface receptors and to heparin. Many attempts have been made to delineate the regions of the FGF-2 molecule that interact with each of these molecules.

The receptor-binding domain on FGF-2 was mapped to distinct sites on the molecule based on a variety of assays. Early studies using synthetic peptides representative of different regions of FGF-2 indicated that two regions, amino acids 32-76 and 114-123 have receptor-binding activities (Baird et al., 1987; Baird et al., 1988). Cross-linking assays on hippocampal neurons indicated that the sequence 111-154 participates in receptor interaction (Walicke et al., 1989). The receptor-binding domain was also shown to be distinct from the heparin-binding domain as antibodies that bind to FGF-2 and inhibits its binding to high-affinity receptors did not affect its affinity for heparin (Kurokawa et al., 1989). One study reported that phosphorylation of Thr-120 enhances the binding of FGF-2 to its receptor (Feige and Baird, 1989). The subsequent description of the three-dimensional crystal structure of FGF-2 enabled a more precise definition of the receptor-binding site (Eriksson et al., 1991; Zhang et al., 1991). The structure supports the peptide region between residues 114 and 123 as the receptor-binding site as it is a solvent-exposed loop located at the junction of the ninth and tenth ß-strands (Figures 1.3, 1.4). This domain is variant among the nine genetically distinct FGF ligands and thus, may provide the various members with distinct receptor binding characteristics. Cassette mutagenesis analysis confirmed this loop structure spanning from residue 117 to 121 as a receptor-binding site and that it functions to define the receptor binding specificity of FGF-2 (Seddon et al., 1995). It is thought that the specificity of receptor recognition may be dependent on the size, conformation and charge of this loop motif (Seddon et al., 1995). Another study based on site-directed mutagenesis and molecular modelling of FGF-2 reported the identification of a second site of higher affinity for receptor binding (Springer et al., 1994). This primary higher affinity site was found to consist of a group of

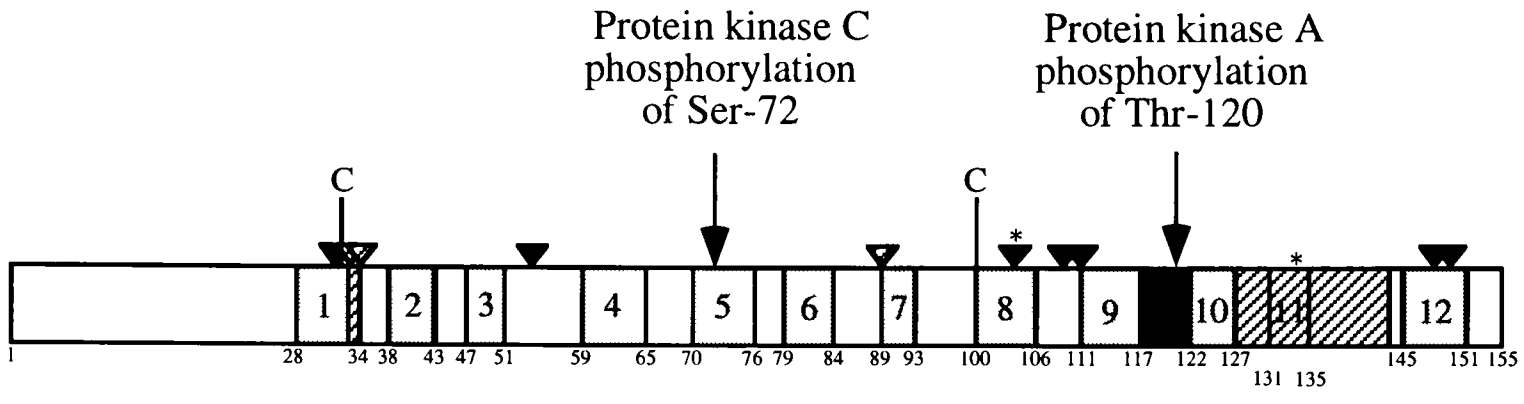


Figure 1.4. Functional Domains of FGF-2.
A schematic diagram representing the 155 amino acid form of FGF-2. Numbers on the bottom marks the location of the amino acid residues that form the limits of the ß-strands, relative to the initiation methionine designated as at position +1. The ß-strands are represented by spotted boxes and enumerated sequentially from the amino-terminus according to the numbering system described by Zhu et al., 1991. Residues forming the primary receptor-binding domain are typically found within the conserved ß-strands and are marked by filled inverted triangles, while residues forming the secondary lower-affinity receptor-binding domain are grouped in the variant solvent-exposed loop between the 9th and 10th ß-strands, as represented by the black box. Residues that constitute the heparin-binding domain are marked by hatched inverted triangles and represented by a hatched box where they are clustered together. Residues which are of special importance for binding to receptor or heparin are Glu-104 and Lys-133 respectively, the positions of which are marked *. Positions of conserved cysteine residues are marked as C.

hydrophobic amino acids (Tyr-32, Tyr-111, Leu-148 and Met-150) and a pair of polar residues (Arg-52 and Asn-109), all of which are solvent-exposed and typically highly conserved between members (Figures 1.3, 1.4). The existence of two receptor-binding sites on opposite faces of the FGF-2 molecule was supported by measurements from isothermal titrating calorimetry experiments (Pantoliano et al., 1994). In addition, the highly conserved residue Glu-104 was found to be essential for high affinity binding of FGFR1, probably by forging an electrostatic interaction (Zhu et al., 1995).

The heparin-binding domain of FGF-2 was initially thought to coincide with the receptor-binding sites, as determined by synthetic peptides corresponding to different parts of the molecule (Baird et al., 1987, 1988). The sequences shown to possess heparin-binding capacity were the following: amino acids 32-76, 101-128, 110-154 and 113-150. As mentioned above, neutralizing antibodies to FGF-2 subsequently demonstrated that the receptor- and heparin-binding domains on FGF-2 were actually distinct (Kurokawa et al., 1989). The crystal structure of FGF-2 suggests that a cluster of basic residues, including Lys-127, Arg-128, Lys-133 and Lys-143, located at the carboxyl terminus of the molecule may be involved in forming the heparin-binding site (Ago et al., 1991; Eriksson et al., 1991). This local concentration of positive charges may produce a significant amount of energy that could contribute to the latent instability of the molecule and binding to a polyanionic molecule such as heparin may counteract this instability (Ago et al., 1991). Analysis of the affinity of FGF-2 for heparin by deletion mutagenesis of the FGF-2 molecule supports the carboxyl-terminal amino acid residues 113-149 as the heparin-binding site (Seno et al., 1990). Further analysis by site-directed mutagenesis coupled with isothermal calorimetry and molecular modelling based on the crystal structure of FGF-2 indicated that key residues comprising the heparin-binding site are dispersed throughout the primary coding sequence and they include Lys-34, Asn-35, Arg-89, Lys-127, Arg-128, Thr-129, Gln-131, Lys-133, Lys-137, Gln-142 and Lys-143, all of which appear to be arranged at a site in the crystal structure that is oriented almost orthogonal to the receptor-binding sites (Figure 1.3; Thompson et al., 1994). These residues are not absolutely conserved between members. Conservation may not be essential since the binding

interaction was found to comprise of only 30% electrostatic interaction while hydrophobic effects and other noncovalent forces such as hydrogen bonding and van der Waals packing contribute the majority of the free energy for the binding reaction (Thompson et al., 1994). The location of these residues match the predictions derived from co-crystallization of sulphate anions with FGF-2 (Zhang et al., 1991; Eriksson et al., 1991). In particular, Lys-133 was shown to be the most important residue since mutation of this residue resulted in the most dramatic reduction in heparin-binding affinity relative to the wild-type (Thompson et al., 1994). The analogous Lys-133 residue in FGF-1 has also been shown to be crucial for heparin binding and not for interaction with high affinity receptor as observed from results of both chemical modification and site-directed mutagenesis procedures (Harper and Lobb, 1988; Burgess et al., 1991b). The heparin-binding site of FGF-1 may be predicted from the crystal structure of the 1:1 complex between FGF-1 and sucrose octasulphate (an analogue of heparin) which identified the binding site for sucrose octasulphate as comprising residues 112-127 (Zhu et al., 1993). Recently, the crystal structure of complexes between FGF-2 and synthetic trisaccharides were obtained, revealing the presence of two pairs of trisaccharide binding sites (1 and 1', and, 2 and 2') (Ornitz et al., 1995). Each pair of sites are symmetry related. Site 1 is similar to the region bound to sucrose octasulphate and sulphate anions determined previously (Zhu et al., 1993) while sites 2 and 2' were proposed to be involved in oligomerization of FGF-2 by oligosaccharides (Ornitz et al., 1995). These two pairs of trisaccharide binding sites are also reported to be FGF-2 self-association sites as observed in apo-FGF-2 crystal structures (Venkataraman et al., 1996).

1.4 FGF Post-translational Modifications

The amino-termini of full-length FGF-1 and FGF-2 are blocked by acetylation (Crabb et al., 1986; Ueno et al., 1986). Acetylation of FGF-1 is not thought to be biologically relevant since non-acetylated recombinant FGF-1 made in bacteria was found to have

equivalent mitogenic and angiogenic activities when compared to the wild-type molecule (Barr et al., 1988; Jaye et al., 1987).

The prototype FGFs are unlikely to be modified by glycosylation as the expression of these two proteins in transfected mammalian cell lines yield products of sizes equivalent to the predicted primary translation products. This may be accounted for by the lack of a signal peptide in these molecules which suggests that these molecules are not channelled through the normal subcellular processing compartments where glycosylation takes place. FGF-9, however, is a glycosylated protein even though it appears to lack a classical hydrophobic signal peptide (see Chapter 3; Naruo et al., 1993). The other secreted members of the FGF family share a common feature in possessing N-linked glycosylation sites (Delli-Bovi et al., 1988; Dixon et al., 1989; Finch et al., 1989; Bates et al., 1991; Coulier et al., 1991; Mason et al., 1994). FGF-5 may also undergo O-linked glycosylation (Bates et al., 1991). Hence, the remaining members of the FGF family are post-translationally processed by cleavage of an amino-terminal signal peptide and glycosylated.

As mentioned previously, truncated lower molecular weight forms of FGF-1 and FGF-2 have been purified (Burgess et al., 1985; Gospodarowicz et al., 1985; Burgess et al., 1986; Crabb et al., 1986; Ueno et al., 1987). This may imply protease-mediated processing of the full-length 18 kDa produce *in vivo*. This issue is, however, unresolved as some of these truncated products are artefacts arising from the extraction procedure which activated certain acid proteases (Klagsbrun et al., 1987). *Xenopus* FGF-3 is processed by proteolytic cleavage, not only of the signal peptide, but also of an additional short peptide sequence in the amino-terminus (Kiefer et al., 1993). FGF-9 may also be proteolytically-processed as the protein product has its amino-terminal three residues cleaved (Chapter 3).

The prototype FGFs have been shown to contain target sites for phosphorylation by the cAMP-dependent protein kinase A (PKA) and by the calcium- and phospholipid-dependent protein kinase C (PKC) (Feige and Baird, 1989). PKC was found to phosphorylate Ser-72 while the target amino acid for PKA was Thr-120 (or Ser-120 in bovine FGF-2). Thr-120 is located on the loop structure between the ninth and tenth ß-strands thought to be responsible for defining receptor-binding specificity of FGF-2

(Seddon et al., 1995) (Figure 1.4). Indeed, the PKA phosphorylated FGF-2 was found to have increased receptor-binding affinity (Feige and Baird, 1989). Phosphorylation on Ser-72 by PKC did not appear to alter the receptor- or heparin-binding capacity of FGF-2. FGF-1 has also been found to be a substrate for PKC but the effect of phosphorylation on the activity of this molecule has not been clearly addressed (Feige and Baird, 1989). The identity of the endogenous ecto-protein kinase is not known, but a protein kinase activity capable of phosphorylating FGF-2 has been discovered on the outer cell surface of hepatoma cells (Vilgrain and Baird, 1991). Hence, it is possible that phosphorylated FGF may be an important post-translational modification that contributes to the regulation of FGF activity.

1.5 FGF Receptors

The biological functions of FGF are thought to be mediated by interaction of these growth factors with cell surface receptors. To date, three classes of receptors have been identified for the FGFs. The first is represented by the high-affinity receptor tyrosine kinases which are members of the immunoglobulin superfamily of molecules (Williams and Barclay, 1988). A second class of receptor is distinct from the first in both structure and lower binding affinity for the FGFs (Burrus et al., 1992). The third group of biomolecules to which the FGFs bind are the heparan sulphate proteoglycans which are present as high capacity, low-affinity receptors.

1.5.1 FGF Receptor Tyrosine Kinases

The FGF receptors were initially identified based on binding and cross-linking studies using radiolabelled FGF-1 or FGF-2. These early studies revealed the presence of receptor species ranging from 125 to 165 kDa in a variety of cell lines including the BHK-21 baby hamster kidney cell line, Swiss 3T3 fibroblasts, MM14 murine myoblasts, murine lung capillary endothelial cells, bovine epithelial lens cells, HepG2 hepatoma cells, human glioma cells and fetal hippocampal neurons (Neufeld and Gospodarowicz, 1985; Olwin and

Hauschka, 1986; Friesel et al., 1986; Moenner et al., 1986; Libermann et al., 1987; Kan et al., 1988; Walicke et al., 1989). As these receptors appeared to bind specifically to both FGF-1 and FGF-2 with affinity values in the range 10 - 120 pM (Olwin and Hauschka, 1986; Kan et al., 1988; Walicke et al., 1989), it was hypothesized that a common receptor(s) exists for both ligands. This idea was, however, not supported by the observation that FGF-7 is uniquely not mitogenic for endothelial cells or fibroblasts which are responsive to both FGF-1 and FGF-2 (Rubin et al., 1989). Moreover, in other organisms, such as the fruit fly (*Drosophila melanogaster*), high affinity receptors specific for FGF-2 (140 kDa, $K_D \approx$ 100-200 pM) but not FGF-1 have been reported (Doctor et al., 1991). Other clues pertaining to the nature of the FGF receptors were derived from observations that FGFs induced the tyrosine phosphorylation of putative receptor molecules and other proteins, particularly one of 90 kDa (Huang and Huang, 1986; Coughlin et al., 1988; Friesel et al., 1989). Thus, the FGF receptors are associated with tyrosine kinase activities. N-linked glycosylation was also observed to be an important property of the FGF receptors as deglycosylated receptors were unable to bind FGF-2 (Feige and Baird, 1988).

Molecular cloning studies subsequently revealed that several related but distinct receptor tyrosine kinases exist for the FGF family of ligands. To date, four distinct genes encoding for a family of FGF receptor (FGFR) tyrosine kinases have been identified. The identity of the first FGF receptor was revealed in 1989 when a functional FGF receptor was purified from chicken embryos based on affinity chromatography on immobilized FGF-2 (Lee et al., 1989). This cDNA, also cloned by screening a chicken embryo cDNA expression library using anti-phosphotyrosine antibodies (*cek1*, for chicken embryo kinase 1; Pasquale and Singer, 1989) has a human counterpart, the tyrosine kinase encoded by the *fms*-like (*flg*) gene (Ruta et al., 1988). The full length sequences of the human, murine, *Xenopus* and *Pleurodeles* homologues were later obtained from screening a variety of cDNA libraries including human placental and endothelial cDNA libraries, a mouse brain library and a *Pleurodeles waltl* tail-bud cDNA library (Dionne et al., 1990; Isacchi et al., 1990; Itoh et al., 1990; Mansukhani et al., 1990; Musci et al., 1990; Reid et al., 1990; Safran et al., 1990; Friesel and Dawid, 1991; Shi et al., 1992). The chicken, murine,

Xenopus and newt homologues displayed 78 to 98% overall amino acid sequence identity to the human FLG (reviewed by Johnson and Williams, 1993). A second FGF receptor displaying high homology to FLG (84% sequence identity) was cloned initially from a mouse liver cDNA expression library based on its tyrosine kinase activity and named as BEK, for bacterially expressed kinase (Kornbluth et al., 1988). As for FLG, the human, mouse, chicken, *Xenopus*, newt and *Pleurodeles* homologues of BEK have been obtained (Dionne et al., 1990; Houssaint et al., 1990; Pasquale, 1990; Raz et al., 1991; Sato et al., 1991; Friesel and Brown, 1992; Poulin et al., 1993; Shi et al., 1994) and labelled variously, including TK14 for the human homologue (Houssaint et al., 1990) and CEK3, for the chicken homologue (Pasquale, 1990). A third receptor, differing from BEK only at a limited region, was initially identified as an amplified gene in the stomach cancer-derived KATO-III cell line and given the name K-*sam* (KATO-III cell-derived stomach cancer amplified gene) (Hattori et al., 1990). The coding sequence of this gene is identical to that of the so-called KGFR which was cloned from a human mammary epithelial cell cDNA library for its ability to transform NIH3T3 cells via interaction with FGF-7 (KGF) (Miki et al., 1992). The mouse homologue of K-SAM or KGFR was cloned using a similar strategy (Miki et al., 1991) and the chicken, newt and *Pleurodeles* counterparts have also been isolated (Sato et al., 1991; Poulin et al., 1993; Shi et al., 1993). The fourth receptor, called FGFR-3, was first cloned from the human chronic myelogenous leukaemia cell line K562 for its homology to the conserved tyrosine kinase sequence in the v-*sea* gene (Keegan et al., 1991). Incidentally, this gene was also identified in the process of screening for the candidate gene implicated in Huntington Disease (HD) (Thompson et al., 1991). The chicken homologue, called CEK2, was isolated from screening chicken embryo cDNA libraries (Pasquale, 1990; Sato et al., 1991) while the murine homologue was cloned from mouse brain (Ornitz and Leder, 1992). A *Pleurodeles* homologue of this receptor (PFR3) containing 75% sequence identity to human FGFR3 has also been isolated (Shi et al., 1993). The fifth receptor, FGFR4, encoded by a distinct gene, was cloned from the human K562 erythroleukaemia cell line as a novel tyrosine kinase by PCR and library screening (Partanen et al., 1991). The rat homologue of FGFR4 has also been cloned from rat lung

mRNA (Horlick et al., 1992). In addition, novel FGFRs structurally related to FGFR4 and showing homology of approximately 65% were recently isolated from *Xenopus* (XFGFR4 and XFGFR4B) and *Pleurodeles* (PFR4) (Shi et al., 1992; Shiozaki et al., 1995; Riou et al., 1996). Furthermore, two receptors that may represent new members of the FGFR family have also been cloned. One was obtained from quail embryonic RNA (FREK, for fibroblast growth factor receptor-like embryonic kinase) (Marcelle et al., 1994) and the other from *Caenorhabditis elegans* (EGL-15) (DeVore et al., 1995). A summary of the names given to identical or distinct FGFRs is listed in Table 1.2. To simplify discussion of the FGFRs described above, they will henceforth be referred to as FGFR1 (for FLG and related gene products), FGFR2 (for BEK and related gene products), FGFR3 and FGFR4, according to a previously proposed nomenclature (Keegan et al., 1991). The four human FGFR gene product shows overall amino acid identities in the range of 55 to 72%, with FGFR1 and FGFR2 being the most closely related while FGFR1 and FGFR4 showed the greatest divergence from each other (Figure 1.5; Johnson and Williams, 1993). The FGFRs are well conserved between species (Figure 1.5).

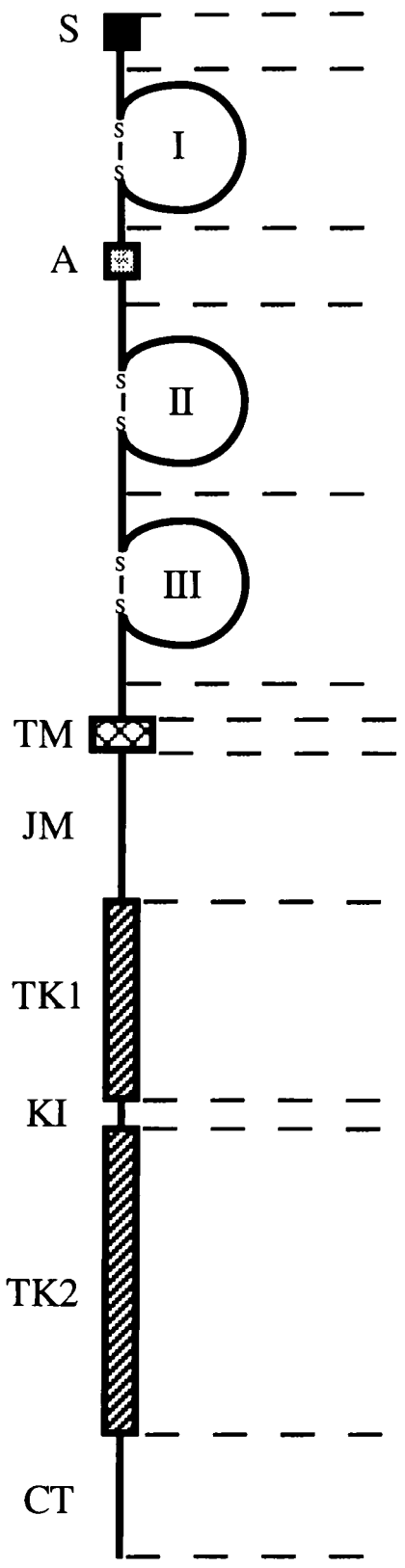
1.5.1.1 General Structure of FGFR

Members of the vertebrate FGFR family share a common basic structure. The general structure of a FGFR tyrosine kinase consists of an amino-terminal hydrophobic leader sequence, an extracellular domain, a single membrane-spanning region, an intracellular tyrosine kinase domain and a carboxy-terminal tail (Figure 1.5). The extracellular domain comprises of multiple immunoglobulin (Ig)-like repeats while the cytoplasmic kinase region is interrupted by a kinase insert, two features analogous to those found on the PDGF and CSF-1 receptor tyrosine kinases (Ullrich and Schlessinger, 1990). This structural make-up of the FGFR placed it under subclass IV of the family of receptor tyrosine kinases or RTKs (Ullrich and Schlessinger, 1990). Unique features which distinguished the FGFRs from other RTKs include the exoplasmic region containing three Ig-like domains (instead of five, as for the PDGF and CSF-1 receptors), an acidic region consisting of 4 to 8 consecutive

Table 1.2. Nomenclature and Sizes of Members of the FGF Tyrosine Kinase Receptor Family.

FGFR	FGFR1	FGFR2		FGFR3	FGFR4	Others
Splice Form	FGFR1c	FGFR2b	FGFR2c	FGFR3c	FGFR4	
Human	FLG, h2, h3, h4, h5	K-SAM, K-SAM', KGFR	BEK, K-SAM-I, TK14, TK25	FGFR3	JTK2	
Mouse	FLG, N-bFGFR	KGFR	BEK	FLG-2		
Rat					FGFR4	
Chicken	CEK1, bFGFR		CEK3	CEK2		
Quail						FREK
Newt	FGFR1	KGFR	BEK			
Xenopus	XFGFR1, XFGFRA1		XFGFR2			XFGFR4, XFGFR4B
Pleurodele	PFR1	PFR2-IIIb, PKGFR	PFR2-IIIc	PFR3	PFR4	
Drosophila						DFGF-R1, DFGF-R2
C. elegans						EGL-15
M. W. (kDa)	150	135		135	110	

The numerical nomenclature of the FGFRs (top row) is based on the chronological order in which they were first identified. The suffix"b" or "c" refers to the Ig-like domain III splice forms of the receptors. The table lists some of the alternative names given to homologues of members of the FGFR family isolated from different animal sources. The column "Others" refer to FGFRs which may represent new members of the FGFR family since it has not been clearly established if they are homologues of any of the known receptors. The apparent molecular weights shown are given for the three Ig-like domain forms of the receptors.



	% Identity	
	Between Homologues	Between FGFR Members
S	50 - 93	17 - 43
I	54 - 93	19 - 40
A	73 - 97	27 - 43
II	79 - 100	63 - 81
III	79 - 97	74 - 82
	44 - 100	13 - 38
TM	57 - 90	24 - 62
JM	81 - 100	31 - 66
TK1	92 - 100	75 - 88
KI	50 - 93	14 - 50
TK2	95 - 99	84 - 92
CT	75 - 95	38 - 60
% Overall Identity	78 - 98	56 - 71

Figure 1.5. Structure of the FGF Tyrosine Kinase Receptor and Protein Sequence Identity Between Different Homologues and Between Members of the FGFR Family.
A schematic diagram of the three Ig-like domain form of the FGFR is shown. Loops I, II and III represent the three extracellular Ig-like domains. The figure shows the range of amino acid identity between the human, mouse, chicken and *Xenopus* homologues of FGFR1 for the various domains of the receptor. The homology range between the corresponding domains of the four human FGFRs, FGFR1, FGFR2, FGFR3 and FGFR4 are also shown. S, signal peptide; A, acidic region; TM, transmembrane region: JM, juxtamembrane region; TK, tyrosine kinase domains: KI, kinase insert; CT, carboxy-terminal tail.

acidic residues between the amino-terminal Ig-like loops I and II, an unusually long (about 80 amino acids) juxtamembrane region between the transmembrane domain and the first kinase domain (kinase 1) relative to that of other RTKs (typically 49 - 51 amino acids in PDGF, CSF-1, EGF and insulin receptors) and a short kinase insert (about 13 amino acids) separating the two parts of the catalytic domain which is considerably longer in other RTKs (e.g. 78 amino acids in the PDGF receptor). The second and third Ig-like loops as well as the tyrosine kinase domain are highly conserved between the different FGF receptors (greater than 60% amino acid identity). In contrast, the signal peptide, Ig-like loop I, the membrane-proximal region, the transmembrane domain and the kinase insert are the least conserved between the different receptors (as low as 7% in the kinase insert domain between FGFR1 and FGFR4) (Figure 1.5). The receptor also contains several potential N-linked glycosylation sites while the carboxy-terminal tail includes tyrosine residues which may be involved in modulating the interaction of the activated receptor with cellular substrate molecules and other proteins (reviewed by Givol and Yayon, 1992).

The invertebrate FGF receptor, DFGF-R1, identified in *Drosophila*, differs from the basic structure of the vertebrate FGFRs only in the presence of five Ig-like domains in the extracellular region (Klämbt et al., 1992), but otherwise, it is similar in structure and overall organization to its vertebrate counterparts (Glazer and Shilo, 1991). A second *Drosophila* FGFR homologue (DFGF-R2) containing only 30% amino acid sequence identity to DFGF-R1 in the extracellular domain has also been isolated, suggesting a different ligand-binding profile for this receptor (Klämbt et al., 1992).

1.5.1.2 Multiple FGFR Structural Variants

A unique feature of the vertebrate FGFR family is the presence of multiple structural variants. The possible mechanisms by which these variants arose can be deduced from analysis of the genomic organization of *Fgfr1* which defined the complex exon-intron structure of the receptor gene (Johnson et al., 1991). The FGFR1 gene is made up of up to nineteen exons showing distinct exon-domain relationships in several domains (Figure

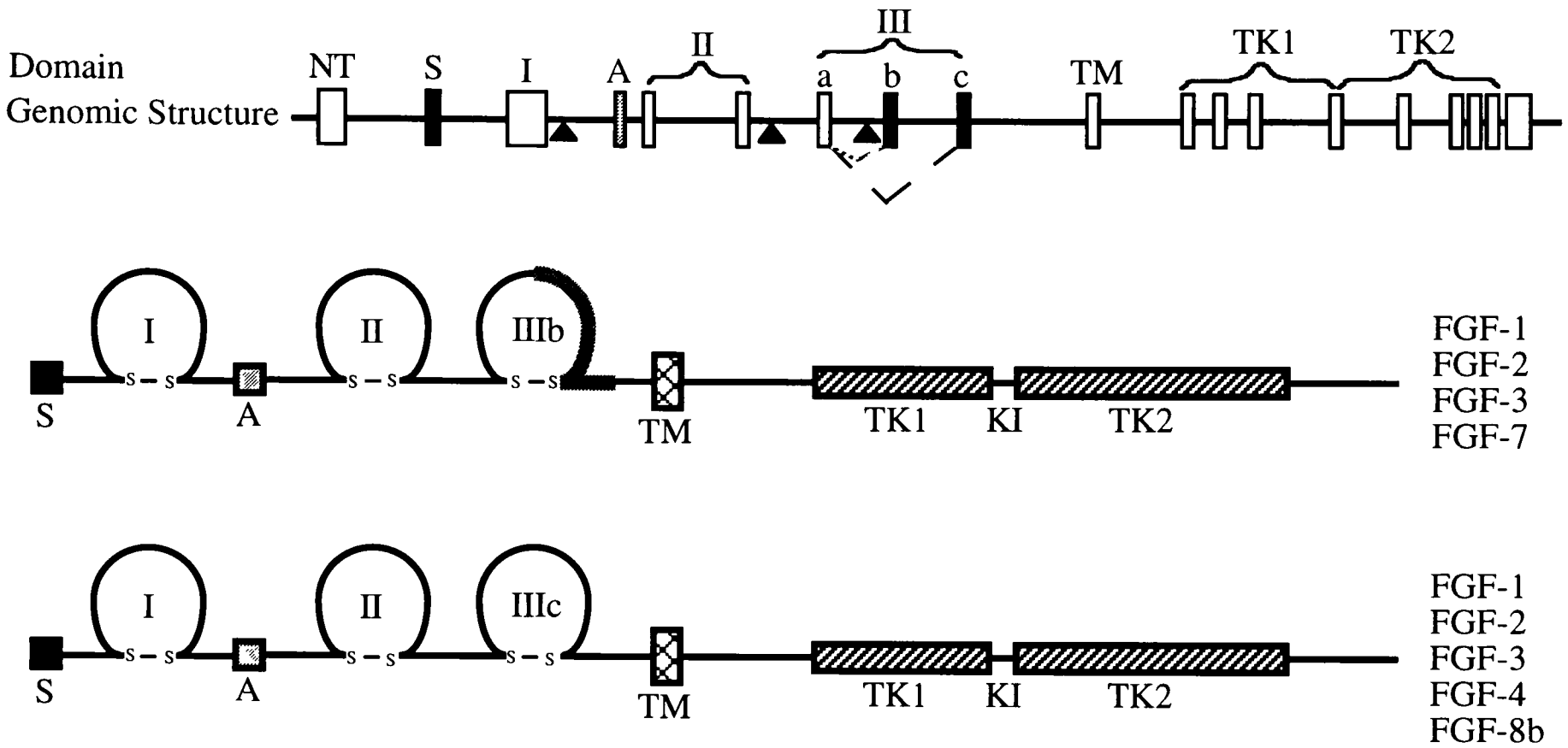


Figure 1.6. Structure of the FGFR1 gene.
The schematic diagram on top shows the exon-intron arrangement of the FGFR1 gene described by Johnson et al. (1991), with additional information from Saito et al. (1992). The gene consists of 19 exons (boxes) which shows some correlation with discrete domains in the receptor structure. Note that the genomic structure of the region encoding the third Ig-like domain half consists of three exons: IIIa, IIIb and IIIc. Exon IIIa encodes the "constant" amino-terminal half of Ig-like domain III while the carboxy-terminal half is encoded by either exon IIIb or IIIc which are alternatively spliced in a mutually exclusive fashion to exon IIIa. A similar genomic arrangement is present in the FGFR2 and FGFR3 genes. With respect to FGFR2, for which the ligand binding profile is well-studied, differential splicing in this region generates receptor variants that exhibit distinct ligand binding specificities, the ligand profile being shown on the right of either variant. Internal polyadenylation sites are indicated by filled triangles. NT, non-translated exon; S, signal peptide; A, acidic region; I, II, III, Ig-like domains I, II and III respectively; TM, transmembrane region; TK1, TK2, tyrosine kinase domains; KI, kinase insert.

1.6). In addition, putative polyadenylation sites were identified in three introns within the extracellular domain coding region, suggesting possible multiple alternatively spliced transcripts as well as prematurely terminated transcripts without the intracellular coding sequence. Hence, structural variants may be generated by either alternative RNA splicing or differential polyadenylation, as typified by the existence of multiple isoforms of FGFR1 and FGFR2. These isoforms would account for the multiple protein species typically cross-linked to iodinated FGF-1 or FGF-2 in early studies. More importantly, analyses of the biological properties of these receptor isoforms provided invaluable information about the functional domains of the FGFR.

The full-length FGFRs consisting of three Ig-like loops in the extracellular ligand binding domain and the complete tyrosine kinase domain in the intracellular catalytic domain have been isolated (Figure 1.7A; Lee et al., 1989; Dionne et al., 1990; Houssaint et al., 1990; Isacchi et al., 1990; Pasquale, 1990; Reid et al., 1990; Safran et al., 1990; Bernard et al., 1991; Champion-Arnaud et al., 1991; Hou et al., 1991; Raz et al., 1991; Miki et al., 1992). The three Ig-like domain forms of FGFR1 and FGFR2 bound FGF-1 and FGF-2 with high affinities in the range of 20-100 pM and 50-150 pM respectively (Dionne et al., 1990).

The amino-terminal Ig-like loop I in the extracellular domain may be spliced out from the transcript leaving isoforms of FGFR1 and FGFR2 lacking Ig-like loop I (Figure 1.7B, E). The two Ig-like loop forms of FGFR1 or FGFR2 have indeed been isolated and found to retain the ability to be chemically cross-linked to radiolabelled FGF-1 and/or FGF-2 (Johnson et al., 1990; Hou et al., 1991; Kouhara et al., 1991; Friesel and Dawid, 1991; Bernard et al., 1991) as well as to mediate biological responses to FGF-1, FGF-2 and FGF-4 (Mansukhani et al., 1990; Bernard et al., 1991). Similarly, iodinated FGF-7 could also be covalently cross-linked to the two Ig-like domain form of KGFR (Miki et al., 1991; Miki et al., 1992). These results suggest that Ig-like domain I is dispensable for high affinity receptor interaction with FGF. Ig-like loop I may, however, be interactive with the adjacent Ig-like loops II and III to lower FGFR1 affinity for both FGF-1 and heparin, an

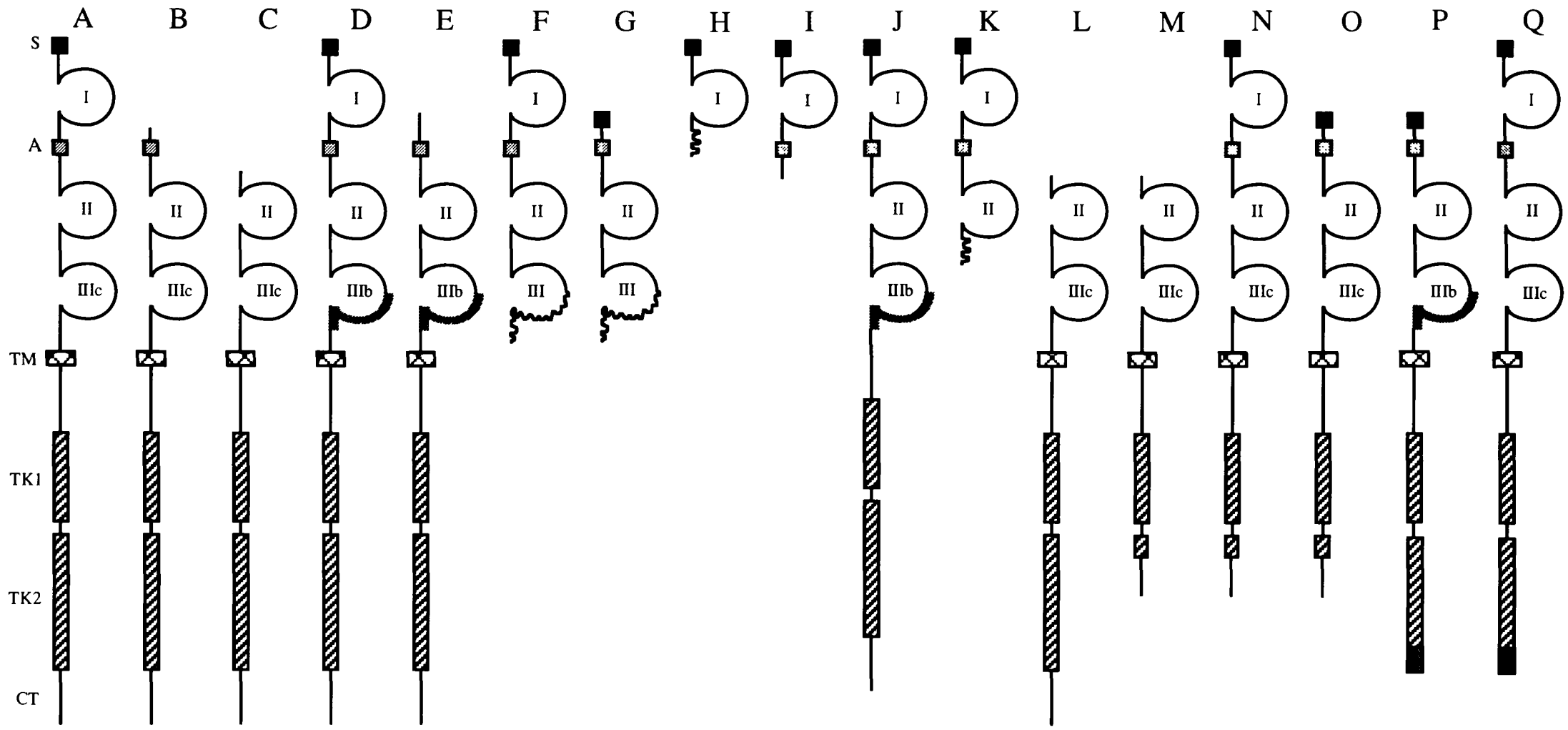


Figure 1.7. Schematic Representations of FGFR Variants.
The isoforms represented by the reported cDNA sequences of FGFR1 (A, B, F, G, H, L, M, N, O), FGFR2 (A, B, C, D, E, I, J, K, P, Q), FGFR3 (A, D) and FGFR4 (A) are shown. Both FGFR1 and FGFR2 may be produced with or without Ig-like loop I and with alternatively spliced Ig loop III (A, B, D, E). FGFR1 and FGFR2 may also be secreted (F, G, H, I, J, K) or produced as intracellular molecules (L, M, N, O). Kinase-defective FGFR1 (M, N, O) and variants of FGFR2 containing mutated carboxy-terminal tails (shaded box, P, Q) may additionally be generated. S, signal peptide; A, acid box; TM, transmembrane region; TK1, TK2, tyrosine kinase domain; CT, carboxy-terminal tail.

interaction that is most likely to be facilitated by the interloop sequence between Ig-like loops I and II (Shi et al., 1993; Wang et al., 1995b).

A variant of FGFR2 lacking both Ig-like loop I and the acidic motif has also been described (Figure 1.7C; Crumley et al., 1991). This receptor isoform also retained the property of binding FGF-1 and FGF-2 with high affinities and to undergo phosphorylation upon ligand binding (Crumley et al., 1991). A two Ig-like domain form of the KGFR lacking the acidic domain was also able to bind FGF-1 and FGF-7 with high affinity (Miki et al., 1992). This implies that the acidic domain is also not essential for ligand binding or receptor activation.

The amino terminus of Ig-like domain II is potentially important for receptor function (Figure 1.8). This region includes sequences containing the conserved tripeptide His-Ala-Val (commonly termed HAV) which are homologous to those found in cadherin and haemagglutinins where they are implicated in mediating protein-protein interactions (Byers et al., 1992). Specific deletion of the triplet sequence from *Xenopus* FGFR abolished the ability of the receptor to respond to FGF (Byers et al., 1992). This region also contains the 18-residue peptide (K18K) identified as a component of the heparin-binding site of FGFR1 (Kan et al., 1993).

The KGFR gene (Miki et al., 1992) encodes a receptor that is completely identical to FGFR2 (Dionne et al., 1990) except for a stretch of divergent 49 amino acids spanning the carboxy-terminal half of the third Ig-like loop (Figure 1.7D; Miki et al., 1992). Examination of the genomic structure at the region of Ig-like loop III indicated that this Ig-like loop is encoded by three co-linear exons: a "constant" exon IIIa encoding the common amino-terminal half of Ig-like loop III present in both FGFR2 and KGFR and two "variable" exons, exon IIIb or exon IIIc which encodes the second half of Ig-like loop III (Figure 1.6; Champion-Arnaud et al., 1991). Exon IIIa may be spliced to IIIb or IIIc in a mutually exclusive fashion. Splicing of exon IIIa to IIIb results in K-SAM or KGFR while splicing of exon IIIa with IIIc yields BEK or FGFR2. To differentiate between these gene products, FGFR2 expressing exon IIIb (i.e. K-SAM, KGFR) will be known as FGFR2b and that containing the IIIc exon as FGFR2c (i.e. BEK, Table 1.2). FGFR2b binds FGF-1

and FGF-7 with high affinity but interacts with FGF-2 with at least 20-fold lower affinity (Bottaro et al., 1990; Miki et al., 1991; Dell and Williams, 1992; Miki et al., 1992). In contrast, FGFR2c binds both FGF-1 and FGF-2 with high affinity but not FGF-7 (Dionne et al., 1990; Mansukhani et al., 1992; Miki et al., 1992). This differential ligand binding specificity determined by the third Ig-like loop was further confirmed by the observation that a FGFR2c chimera where the variable region of Ig-like loop III was replaced by that from FGFR2b gained the ligand binding profile of FGFR2b (Yayon et al., 1992). In a separate but similar study where Ig-like loops of FGFR1 were replaced with those from FGFR2b, it was shown that full capacity of the FGFR1 chimera to interact with FGF-7 required the presence of both Ig-like loops II and III from FGFR2b (Zimmer et al., 1993). A recent study using receptor chimeras consisting of individual Ig-like loops of FGFR2b fused to the HFc domain of IgG showed that the Ig II-HFc chimera was able to bind FGF-1 with high affinity but not FGF-7; in contrast, the Ig III-HFc chimera bound FGF-7 with high affinity but not FGF-1; finally, the presence of both Ig-like loops II and III in the chimera bound both FGF-1 and FGF-7 with high affinity (Cheon et al., 1994). It therefore appears that both Ig-like loops II and III are important for contributing to high affinity ligand binding and specificity (Figure 1.8). More recent evidence indicates that a heparin-protected FGFR1 peptide fragment containing Ig II, the inter-loop sequence between Ig II and III, and the amino-terminus of Ig III is sufficient for interaction with FGF-1, FGF-2 and FGF-7, while an extension to this peptide of five residues that are invariant throughout the four receptor genes and receptor isoforms, restricted binding of only FGF-7 (Wang et al., 1995a). This study suggests that the structural domain encoded by exon IIIc is not actively involved in ligand binding while that encoded by exon IIIb plays an active role in abrogating the restriction on FGF-7 binding.

The genomic organization of FGFR1 and FGFR3 in the region of the third Ig-like loop shows a conservation of structure similar to that of FGFR2, suggesting that these receptors may also be generated as alternatively spliced forms with respect to Ig-like domain III (Johnson et al., 1991; Werner et al., 1992a; Avivi et al., 1993; Chellaiah et al., 1994). The IIIc exon is expressed in all of the FGFR1 isoforms isolated so far, which are

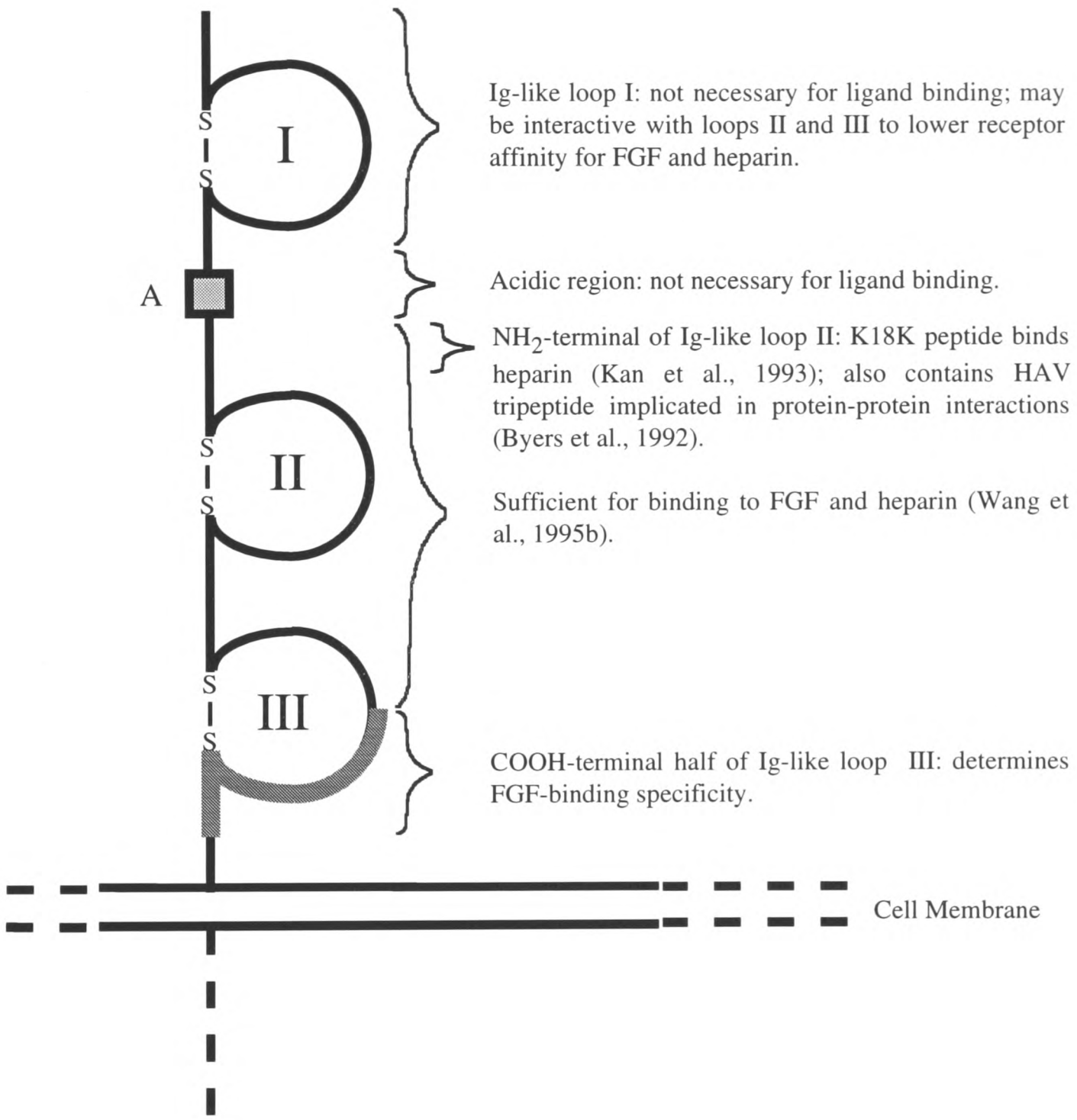


Figure 1.8. FGFR Motifs Involved in Intermolecular Ligand Interactions.
The extracellular region of the three Ig-like domain form of FGFR may be separated into essential domains for binding to FGF and heparin, or for regulation of ligand binding. A, acidic region.

accordingly labelled as FGFR1c. The complete cDNA sequence encoding the FGFR1b isoform has not yet been isolated but it is expected to display about 50-fold lower affinity for FGF-2 compared to FGFR1c, as shown by chimeric receptor binding analysis (Werner et al., 1992a). Similarly, all of the FGFR3 cDNA clones isolated so far are of the IIIc type. The FGFR3b isoform is, however, predicted to be activated only by FGF-1 to which it binds with high affinity (Chellaiah et al., 1994). Replacement of the variable region of Ig-like domain III of FGFR3b with that from FGFR2b has been shown to confer upon the chimeric receptor the ability to bind FGF-1, FGF-2 and FGF-7 where previously only FGF-1 was the only interactive ligand (Chellaiah et al., 1994), indicating yet again, that the alternatively spliced domain of Ig-like loop III contains the necessary information to define the ligand binding specificity of the FGFR. The genomic structure of FGFR4 appears to deviate from this typical gene organization as only one exon (exon IIIc') encodes the carboxy-terminal half of the third Ig-like domain (Vainikka et al., 1992). Thus, FGFR4 is not thought to be expressed as variants containing alternatively spliced Ig-like III domains.

Soluble FGFR variants that may be secreted have also been isolated in the course of cloning for the cDNAs of FGFR1 and FGFR2. FGFR1 mRNAs encoding the two or three Ig-like domain forms of the receptor containing extracellular domain sequences up till the amino-terminal half of Ig-like loop III followed by short unique sequences but not transmembrane or cytoplasmic coding sequences have been reported (Figure 1.7F, G; Johnson et al., 1990; Werner et al., 1992a). Other putative secreted forms include FGFR1 containing only the signal peptide, Ig-like loop I and 31 unique residues (Figure 1.7H; Eisemann et al., 1991). Potentially secreted FGFR2 variants have also been identified. These include an isoform containing only Ig-like loop I and the acidic motif (Figure 1.7I; Crumley et al., 1991), a variant containing all sequences except the transmembrane coding sequence (Figure 1.7J; K-SAM-III; Katoh et al., 1992), and a form containing all the extracellular domain up till Ig-like domain II and including 5 unique amino acids but did not include the transmembrane or tyrosine kinase domains (Figure 1.7K; K-SAM-IV; Katoh et al., 1992). These isoforms may be expressed as a result of splice site skipping due to the presence of polyadenylation signals located at the intronic sequences between the following

domain-coding exons: Ig-like domain I and the acidic region exons, Ig-like domain II exon and exon IIIa, and between exons IIIa and IIIb (Figure 1.6; Johnson et al., 1991). Unique sequences are thus derived from parts of the intronic sequences. A study of the putatively secreted two Ig-like FGFR1 isoform (Figure 1.7G) indicated that it possessed FGF-1 and FGF-2 binding activity (Werner et al., 1992a). The generation of the secreted variants involving possibly competition between the alternative splicing and polyadenylation events may lead to potentially differential biological consequences. Proposed functions for the secreted forms of FGFRs include: (1) as a carrier for extracellular secretion of FGFs, particularly for members which lack typical secretory signals; (2) as an extracellular reservoir of FGFs, analogous to the proposed role of matrix heparan sulphate proteoglycans; (3) as non-competitive inhibitor of FGF binding by forming heterodimers between the active and inactive forms of the receptor which thereby modulates the action of FGF by competing with the high affinity cell surface receptors; and (4) as direct inhibitors/antagonists to circulating forms of FGFs.

A putative intracellular form of FGFR1 has also been isolated (Hou et al., 1991). In this instance, an alternative splicing event in the 5' region of the receptor transcript generates stop codons upstream from Ig-like loop I and re-initiation of translation at an internal methionine start codon yields a receptor containing Ig-like domains II and III and the cytoplasmic catalytic domain without a signal peptide (Figure 1.7L). A kinase-defective intracellular isoform has also been identified (Figure 1.7M). Intracellular FGFR1 forms possessing kinase or potentially defective kinase function may serve unique functions, particularly since FGF-1 and FGF-2 have been localized intracellularly (see section 1.7.1.1).

Many other isoforms of the FGFRs have been documented. These include FGFR1 isoforms where two amino acids (Arg-59, Met-60; Johnson et al., 1990) are either deleted or included between the acidic box domain and Ig-like loop II (Johnson et al., 1990; Eisemann et al., 1991; Kiefer et al., 1991). This variant is thought to be the result of alternative use of two possible donor splice sites. Similarly, FGFR1 and FGFR2 (K-SAM, TK14, K-SAM-II) variants differing in the presence or absence of two amino acids (Thr-

Val) at the 3' end of the juxtamembrane domain have been reported (Hou et al., 1991; Champion-Arnaud et al., 1991; Houssaint et al., 1990; Hattori et al., 1990). This dipeptide in the juxtamembrane region may be involved in phosphorylation by serine-threonine protein kinase which has been implicated in a variety of regulatory functions such as alteration of ligand affinity, kinase activity as well as receptor internalization (Ullrich and Schlessinger, 1990). In addition, FGFR1 and FGFR2 isoforms truncated or mutated with respect to the carboxy-terminal region have been found. Variants of FGFR1 where most of the second kinase domain is deleted have been reported (Figure 1.7M, N, O; Hou et al., 1991). These isoforms are potentially defective in kinase activity and may modulate the function of intact receptors via the formation of heterodimers (Shi et al., 1993). FGFR2 variants containing truncated and mutated carboxy-terminal tails (Figure 1.7P, Q; TK 25, K-SAM; Champion-Arnaud et al., 1991) may alter receptor function as this domain has been implicated in negative control of receptor activity, possibly by interacting with the substrate binding sites of the tyrosine kinase domain and modulating its capacity to interact with cellular substrate molecules (Ullrich and Schlessinger, 1990). Isolation of an mRNA encoding a form of FGFR2 lacking the potential tyrosine residue required for phospholipase Cγ interaction suggests yet another mechanism by which different signal transduction pathways may be activated by one ligand (Yan et al., 1993b).

In contrast to the multiplicity of variant forms discovered for FGFR1 and FGFR2, only the three Ig-like domain forms of FGFR3 (Keegan et al., 1991; Ornitz and Leder, 1992) and human FGFR4 (Partanen et al., 1991) have been isolated to date. A variant of FGFR4 lacking the amino-terminal Ig-like loop I and the acidic region has, however, been reported to be expressed in the rat lung (Horlick et al., 1992). Secreted forms of FGFR4 containing the extracellular ligand binding domain up to and including the amino-terminal of Ig-like loop III are not predicted to be produced since there are no stop codons or polyadenylation sites in the intron between exon IIIa and IIIc'.

1.5.1.3 Ligand Binding Specificity of FGFR

A second important feature of the FGFR family of tyrosine kinases is the high level of cross-reactivity between the receptors and their ligands (Table 1.3). For instance, FGFR1c binds with high affinity to both FGF-1 ($K_D \approx$ 20-80 pM) and FGF-2 ($K_D \approx$ 50-150 pM) (Dionne et al., 1990; Mansukhani et al., 1990; Ornitz and Leder, 1992; Vainikka et al., 1992) but with significantly lower affinity to FGF-4 ($K_D \approx$ 1.6 nM; Vainikka et al., 1992; Mansukhani et al., 1990; Ornitz and Leder, 1992) and it is partially activated by FGF-5 and FGF-6 (Ornitz and Leder, 1992; Vainikka et al., 1992). FGFR2c exhibits an almost identical ligand binding profile as FGFR1c, except for its apparently higher affinity for FGF-4 ($K_D \approx$ 70 pM; Johnson and Williams, 1993; Dionne et al., 1990; Mansukhani et al., 1992) and its ability to bind mouse FGF-3 (Mathieu et al., 1995a). FGF-8b has also been shown to activate FGFR2c with moderate potency (MacArthur et al., 1995a). FGFR3c binds with high affinity to FGF-1 ($K_D \approx$ 223 pM) followed by FGF-4 and FGF-2 in order of decreasing affinity (Ornitz and Leder, 1992; Chellaiah et al., 1994). Both FGF-5 and FGF-6 induced a relatively weak but detectable mitogenic response in cells expressing FGFR3c (Ornitz and Leder, 1992; Chellaiah et al., 1994). FGFR4 binds with high affinity to FGF-1 ($K_D \approx$ 0.2 nM) while FGF-2 and FGF-4 interact with the receptor with 10-fold lower affinity ($K_D \approx$ 2 nM) (Vainikka et al., 1992). The FGF-8 isoforms, FGF-8b and FGF-8c appeared to activate FGFR4 with moderate to high efficiency (MacArthur et al., 1995a). In addition, FGFR4 has the unique property of being a receptor for heparin which has the ability to activate classic receptor-mediated biological responses including FGFR4 autophosphorylation and downstream substrate activation (Gao and Goldfarb, 1995).

In marked contrast to the "c" splice forms, receptors expressing the exon IIIb in the third Ig-like loop exhibit more restricted ligand binding profiles. FGFR1b is predicted to have a high affinity for FGF-1 while its affinity for FGF-2 was about 50-fold lower relative to FGF-1 (Werner et al., 1992a). Similarly, FGFR2b binds with high affinity to FGF-1 and 15- to 30-fold lower affinity to FGF-2 (Miki et al., 1991; Miki et al., 1992; Chellaiah et al., 1994). The Ig-like IIIb splice forms of both FGFR1 and FGFR2 also bind mouse

Table 1.3. Ligand Binding Specificity of the FGFR Members.

FGFR	Ig III Splice Form	Ligand Binding Profile
FGFR1	IIIa†	FGF-2, FGF-1
	IIIb	FGF-1, FGF-2, FGF-3*
	IIIc	FGF-1, FGF-2, FGF-4, FGF-5, FGF-6
FGFR2	IIIb	FGF-1, FGF-3*, FGF-2, FGF-7
	IIIc	FGF-1, FGF-2, FGF-3§, FGF-4, FGF-8b*
FGFR3	IIIb¶	FGF-1
	IIIc	FGF-1, FGF-2, FGF-4, FGF-5, FGF-6, FGF-8b, FGF-8c
FGFR4	IIIc'	FGF-1, FGF-2, FGF-4, FGF-5, FGF-8b, FGF-8c

†, FGFR1-IIIa refers to an isoform of FGFR1 containing coding sequences up to the invariant amino-terminal half of Ig-like domain III that is predicted to be secreted (see Figure 1.5 F, G). ¶, FGFR3b does not appear to bind FGF-2. *, *Xenopus* FGF-3 homologue has also been reported to bind with high affinity to both FGFR2b and FGFR2c, and with lower affinity to FGFR1 and FGFR3 relative to FGF-1 (Mathieu et al., 1995b).

FGF-3 with high affinity (Mathieu et al., 1995a). In addition, FGFR2b binds FGF-7 with high affinity ($K_D \approx 200$ pM), a property that is conserved in all the homologues, from human to newt (Miki et al., 1992; Johnson et al., 1991; Miki et al., 1991; Shi et al., 1994; Patrie et al., 1995). The last receptor isoform on the list, FGFR3b, appears to be the most discriminative member of the receptor family, favouring binding to only FGF-1 (Chellaiah et al., 1994).

Several immediate conclusions may be drawn from the overall ligand binding profiles of members of the FGFR family described above: (1) FGF-1 appears to be a pan-acting ligand for all of the receptors and isoforms identified to date, suggesting that it interacts with epitopes common to all the receptors; (2) each receptor isoform has the potential capacity to bind multiple members of the FGF family, often with differing affinities; (3) different receptor isoforms may bind to the same ligand. These observations and the presence of at least nine different FGFs raise the question as to the nature of the regulatory mechanisms that define the specificity of the biological response induced by individual members of the FGF family. There are examples where the induction of a biological response is selective for a specific FGF. FGF-2 but not FGF-1 has been reported to stimulate mitogenesis in human melanoctyes (Halaban et al., 1987). FGF-1 and FGF-2 were also found to exert differential effects on the same cell type, as found for cardiac myocytes where FGF-1 inhibits skeletal α-actin transcription while FGF-2 has a stimulatory effect (Parker et al., 1990). Furthermore, FGF-7 is known to stimulate growth of keratinocytes but not fibroblasts or endothelial cells (Rubin et al., 1989). In a similarly discriminative manner, FGF-9 is mitogenic towards fibroblasts but not endothelial cells (Naruo et al., 1993). It may hence be predicted that endothelial cells do not express the necessary receptor(s) that recognize FGF-7 or FGF-9 as ligands. Hence, the establishment and maintenance of selective cellular responsiveness to individual members of the FGF family is expected to involve regulatory controls of the FGFRs by specifying : (1) ligand specificity and affinity of different receptor isoforms; (2) tissue-specific FGFR gene expression; (3) tissue-specific alternative splicing of the expressed FGFR; and (4)

differential signalling potential transduced by the different FGFRs. Evidence supporting the relevance of these suppositions will be discussed below and in later sections.

1.5.1.4 Signal Transduction By FGFRs

Signal transduction by FGFRs conforms to the universal model proposed for growth factor receptor tyrosine kinases. The model suggests that ligand binding to its cognate receptor induces oligomerization of receptors, resulting in conformational changes in the receptor extracellular domains which enhance their ligand binding affinity as well as stabilize the interaction between the adjacent cytoplasmic domains such that the receptor kinase function is activated (Ullrich and Schlessinger, 1990; Heldin, 1995). One event following activation of kinase activity is tyrosine phosphorylation of the receptors themselves (autophosphorylation) which occurs via an intermolecular transphosphorylation reaction. Phosphorylation of tyrosine residues in the receptor intracellular domain may maintain the tyrosine kinase in an activated state and more importantly, modulate the interaction between the activated receptor and cellular proteins. Interaction between the activated receptor and cellular substrates results in phosphorylation of the latter molecules on tyrosine residues. Examples of intracellular molecules known to physically associate with activated receptors and thought to be important in signal transduction include phospholipase Cγ (PLCγ), *ras* GTPase activating protein (GAP) and phosphatidylinositol 3-kinase (PI3-kinase) (Ullrich and Schlessinger, 1990). In general, these molecules have two features in common, they contain: (1) specific domains, termed *src*-homologous domain 2 (SH2) which binds to specific phosphotyrosine residues on autophosphorylated RTKs, as exemplified by the interaction of PDGFR with PI3-kinase and GAP (Fantl et al., 1992); and (2) catalytic activities that are regulated by tyrosine phosphorylation. These molecules are thought to mediate distinct signalling pathways leading to different biological responses.

The existence of at least four FGFRs, combined with a large combinatorial repertoire of splice isoforms with unique as well as overlapping FGF binding specificity and affinity, presents a situation whereby a plethora of distinct responses may be generated.

Given that the nature of the biochemical and functional responses mediated by each receptor is determined by sequences in the intracytoplasmic domain, the diversity of the signalling potential of FGFRs may be achieved by two mechanisms. Firstly, potential receptor oligomerization between different members of the FGFR family or between different splice variants (heterodimerization) would result in pairing of different receptor cytoplasmic domains with different complements of autophosphorylation sites. As a result, different combinations of SH2-domain-containing signalling molecules may be recruited leading to altered signalling responses. Heterodimerization may also increase the repertoire of ligands that can bind to the receptors such that the cellular response may be fine-tuned according to the FGF environment. Secondly, subtle differences in the cytoplasmic domain sequence of each receptor member may trigger different signalling pathways.

There is evidence to indicate that FGFR activation induced by FGF binding involves FGFR oligomerization. For instance, kinase-defective FGFR2 may be phosphorylated in the presence of intact FGFR2, an outcome that presumably involved dimerization of the defective receptor with its wild-type counterpart (Bellot et al., 1991). Dimerization between different FGFR members (heterodimerization) have also been shown to occur. FGFR1 may transphosphorylate kinase-defective FGFR2 and vice versa (Bellot et al., 1991). Moreover, non-functional FGFR1 containing no cytoplasmic sequences was able to abolish the signalling functions of not only its wild-type counterpart but also that of FGFR2 and FGFR3 (Ueno et al., 1992), a phenomenon better known as the "dominant-negative" effect. Similarly, FGFR1, truncated with respect to the cytoplasmic domain was shown to block the signalling function and biological effects of the wild-type receptor(s) in *Xenopus* (Amaya et al., 1991). In addition, different splice variants of a FGFR may also heterodimerize. Indeed, multiple isoforms of FGFR1, including variants which are kinase-defective, have the potential to form heterodimers in the differentiated HepG2 liver cell line (Shi et al., 1993). Taken together, these observations support the notion that both homodimers and heterodimers, composed of different FGFRs or different isoforms of the same receptor, may occur *in vivo*. These receptor dimers, which may be active or inactive

with respect to kinase activity, would then be responsible for eliciting and/or modifying FGF-dependent biological effects.

Different members of the FGFR family may possess different signalling and mitogenic potentials. This difference is particularly apparent between FGFR1 and FGFR4, the latter being the most divergent amongst the family members (Partanen et al., 1991). Of interest is the lack of two conserved tyrosine residues in FGFR4 that are present in FGFR1 and FGFR2, reflecting perhaps a divergence in function for FGFR4 (Partanen et al., 1991). A study comparing the abilities of FGFR1 and FGFR4 to phosphorylate PLCγ, a major substrate for FGFR1 (Burgess et al., 1990), indicated that FGFR4 was much less efficient in this activity (Vainikka et al., 1992). A subsequent report showed more differences between the two receptors: firstly, FGFR4 expressed in BaF3 cells was unable to transduce a mitogenic response in the presence of FGF-1 while FGFR1 activation induced cell proliferation; secondly, FGFR1 was more potent than FGFR4 at inducing the phosphorylation of various signalling substrates including SHC, ERKs (extracellular signal-related kinases) and an uncharacterized 80 kDa protein; and thirdly, FGFR4 failed to induce *fos* and *tis11* RNA expression in response to FGF-1 while activation of FGFR1 dramatically induced this response (Wang et al., 1994). Hence, different FGFRs do elicit different signalling and biological responses.

On the other hand, there are reports indicating similarities in FGFR-mediated responses. For example, activation of FGFR1 or FGFR3 ectopically-expressed in *Xenopus* oocytes is linked to a rapid calcium release from internal stores (Johnson et al., 1990; Musci et al., 1990; Keegan et al., 1991) while activation of FGFR1, FGFR2 or FGFR3 over-expressed in murine myeloid or CHO cells induces a similar response: cell proliferation (Mansukhani et al., 1992; Ornitz and Leder, 1992). Likewise, ligand activation of both FGFR1 and FGFR2b expressed in MM14 myoblasts prevented terminal differentiation of these cells, suggesting that both receptors transduce similar signals (Patrie et al., 1995). The calcium influx response generated upon ligand binding may be linked to activation of PLCγ, an enzyme that catalyses the hydrolysis of phosphatidylinositol (PtdIns) to generate diacylglycerol (DAG) and free inositol 1,4,5-triphosphate (IP_3). DAG

in turn activates protein kinase C (PKC) while IP_3 mediates calcium release from intracellular stores. PLCγ has been shown to bind to the carboxy-terminal tail of FGFR1 at the phosphotyrosine-766 autophosphorylation site via its SH2 domains (Figure 1.9; Mohammadi et al., 1991; Mohammadi et al., 1992; Peters et al., 1992a). The activation of PLCγ in response to FGF-1 or FGF-2 has been demonstrated by assays based on the measurement of increased cellular DAG, cytoplasmic calcium and activation of PKC (Kaibuchi et al., 1986; Brown et al., 1989; Presta et al., 1989). PLCγ activation is, however, not linked to the mitogenic response induced by FGF (Mohammadi et al., 1992; Peters et al., 1992a). It may, however, be associated with the ability of FGF to modulate cellular differentiation. For instance, cellular differentiation during early embryonic pattern formation may be mediated by PtdIns hydrolysis induced by FGF (Maslanski et al., 1992). PLCγ activation may also be responsible for the inhibitory effect of FGF on myogenesis. PKC, activated by PLCγ function, was found to mimic the effect of FGF-2 in inhibiting muscle-specific gene expression and in phosphorylating a conserved site in the DNA-binding domain of myogenin, thereby suppressing terminal myoblast differentiation (Li et al., 1992). In addition, the PKC activity associated with the differentiation of *Xenopus* ectoderm to mesoderm induced by FGF may function as a negative feedback mechanism for regulation of FGFR signalling (Gillespie et al., 1992). It is likely that the mitogenic response induced by FGF-mediated activation of FGFR may involve autophosphorylation of other sites in the intracellular domain of the receptor. A recent report described the discovery of six other autophosphorylation sites, two of which (Tyr-653 and Tyr-654) are conserved in all known members of the FGFR family and essential for the kinase activity of FGFR1 as well as for its ability to induce mitogenesis in L-6 myoblasts and neuronal differentiation in PC12 cells (Figure 1.9; Mohammadi et al., 1996).

Besides the activation of phospholipid signalling, FGFR1 signalling has also been associated with the Ras pathway. Members of the Ras pathway, including Ras, Raf-1, SH-PTP2, MEK and MAP kinase have been implicated in FGFR signalling in *Xenopus, Drosophila, C. elegans* and in PC12 cells (Altin et al., 1991; Kremer et al., 1991; Whitman and Melton, 1992; MacNicol et al., 1993; Tang et al., 1995; Umbhauer et al., 1995).

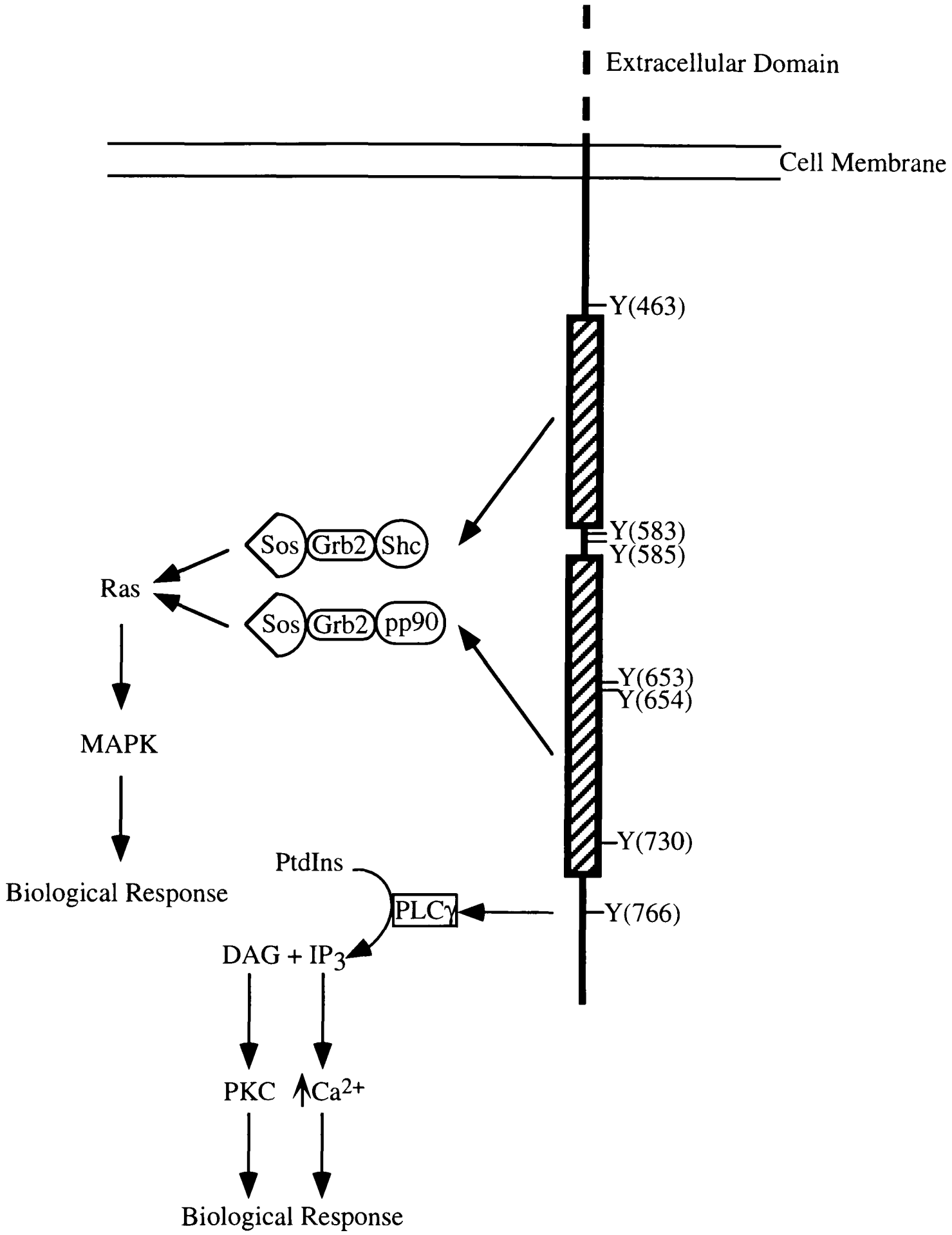


Figure 1.9. Schematic Representation of the Known Tyrosine Autophosphorylation Sites on FGFR1 and Signal Transduction.
The seven tyrosine autophosphorylation sites (Y) identified on the intracellular domain of FGFR1 are shown. Autophosphorylation on tyrosines 653 and 654 activates the receptor kinase activity. Activated receptor is found to result in tyrosine phosphorylation of Shc and/or an identified 90 kDa protein (pp90) which has the potential to interact with the Grb2/Sos complex thereby linking the receptor to the Ras/MAPK signalling cascade. Autophosphorylation of tyrosine 766 couples the receptor to the phospholipase Cγ (PLCγ) pathway. Activated PLCγ catalyses phosphatidylinositol (PtdIns) hydrolysis to diacylglycerol (DAG) and free inositol 1,4,5-triphosphate (IP_3) which in turn leads to the activation of protein kinase C (PKC) and influx of Ca^{2+} respectively. This latter pathway is thought to mediate biological responses other than mitogenesis.

FGFR1 activation may trigger the Ras signalling cascade by tyrosine phosphorylation of the adaptor Shc or an unidentified 90 kDa protein (pp90) (Figure 1.9; Mohammadi et al., 1996). Phosphorylated Shc can interact with another SH2-, SH3-containing adaptor molecule, Grb2, which forms a complex with Sos, a protein that facilitates GDP/GTP exchange on Ras. Activated Ras in turn triggers downstream Raf and MAP kinase signalling pathways. Indeed, Raf was shown to be an important component of the FGFR signalling pathway in mesoderm induction in the *Xenopus* and in tracheal cell migration in *C. elegans* (MacNicol et al., 1993; DeVore et al., 1995). A recent study of the significance of the MAP kinase pathway in FGFR1 signalling showed that it is insufficient to promote differentiation of the rat adrenal tumour-derived PC12 cell line (Renaud et al., 1996). The signalling molecule involved in these cells may instead be Src, member of a family of protein tyrosine kinases (Kremer et al., 1991). Indeed, FGFR1 has been reported to interact with c-Src (Zhan et al., 1994). Activated Src is also thought to be responsible for the FGF-1-dependent tyrosine phosphorylation of cortactin, a molecule known to associate with the cytoskeleton and so may have a role in cell proliferation or migration (Zhan et al., 1993; Zhan et al., 1994).

1.5.1.5 Receptor Downregulation

Cell responsiveness to FGF is additionally regulated by ligand-induced receptor internalization. Exposure of a variety of cell lines, including murine lung capillary endothelial cells, bovine capillary endothelial cells, baby hamster kidney cells and NIH 3T3 fibroblasts, to FGF-1 or FGF-2 was found to result in FGF receptor downregulation on these cells (Friesel et al., 1986; Moscatelli, 1988; Moscatelli and Devesly, 1990; Prudovsky et al., 1994). In addition, NIH 3T3 cells transformed by the over-expression of FGF-2 or FGF-4 also displayed a decreased number of cell surface receptors which can be restored to normal levels by blocking FGF-receptor interaction (Moscatelli and Quarto, 1989). Hence, downregulation occurs only upon ligand binding. Internalized receptors do not appear to be recycled to the cell surface. In fact, repopulation of the cell surface receptors was observed

to require protein synthesis, suggesting that FGFRs in the plasma membrane is not replenished by internal stores (Moscatelli and Devesly, 1990).

Studies where exogenous FGF-2 was allowed to interact with cell surface FGFR demonstrated that receptor downregulation is associated with the rapid internalization of FGF-2 (18 kDa) into the cytoplasmic compartment where it is processed to a stable 16 kDa polypeptide and eventually to 10 kDa and 8 kDa polypeptides (Moscatelli, 1988; Bikfalvi et al., 1991; Briozzo et al., 1991). In several cases, internalized FGF-2 following receptor interaction has been observed to be translocated to the nucleus (Cattini et al., 1991) where it is localized in the nucleolus (Bouche et al., 1987; Baldin et al., 1990; Shiurba et al., 1991; Walicke and Baird, 1992). There is evidence that FGF-2 translocation to the cytosol requires the added participation of heparan sulphate proteoglycans since in its absence, FGF-2 failed to enter the cytosol even though receptor-mediated endocytosis has occurred (Reiland and Rapraeger, 1993). Nuclear translocation following receptor interaction was also found for FGF-1 (Zhan et al., 1993). It now appears that FGFR1 may also be translocated near the nucleus upon binding to FGF-1 as a functional tyrosine kinase (Prudovsky et al., 1994). In HepG2 hepatoma cells, treatment with FGF-1 under conditions that induce internalization of the transmembrane receptor-ligand complex results in the appearance of a nuclear- and perinuclear-associated 40 kDa complex consisting of FGF-1 and fragments of the extracellular domains of FGFR1 and FGFR4 (Feng et al., 1996). These observations implicate a potential regulatory role of the internalized FGF-FGFR complex in the cytoplasm, perinuclear region or nucleus.

1.5.2 Cysteine-Rich FGF Receptor

A second receptor type that is distinct from the tyrosine kinase receptors described in the above sections but binds to FGF has been reported. This FGF receptor candidate was originally purified from embryonic chick tissue by FGF affinity chromatography as a 150 kDa glycosylated polypeptide whose binding to FGFs can be disrupted by heparin (Burrus and Olwin, 1989). cDNA cloning of the receptor indicates that it contains a signal peptide, a

large extracellular domain characterized by 16 cysteine-rich repeats, a membrane-spanning domain and a short intracellular domain comprising of 13 amino acids and two threonine residues that may represent consensus residues for phosphorylation by protein kinase C and cyclic AMP/cyclic GMP-dependent protein kinase (Figure 1.10; Burrus et al., 1992). This receptor, known as cysteine-rich FGF receptor (CFR), binds FGF-1, FGF-2 and FGF-4 but its affinity for FGF-2 was low compared to the FGFRs, being approximately 1 nM (Burrus et al., 1992). In addition, its interaction with FGFs does not seem to require regulation by heparin, a property that distinguishes it from the RTK family of FGF receptors (Burrus et al., 1992). CFR appears to be highly conserved between species, with 90% sequence identity between the chicken and mouse homologues (Burrus et al., 1992). The presence of an additional class of polypeptide receptor for the FGF family of ligands suggests that the manifestation of FGF biological activity is likely to involve multiple distinct signalling pathways.

1.5.3 Heparan Sulphate Proteoglycans

A third class of biomolecules that feature as low affinity ($K_D \approx 1$ nM), large capacity binding sites for FGF has been identified as the glycosaminoglycan heparan sulphate (HS) on cell surfaces and in the extracellular matrix (ECM) (Moscatelli, 1987; Vlodavsky et al., 1987). HS is a complex mammalian glycosaminoglycan (GAG) consisting of a basic disaccharide repeat unit (iduronic acid-N-sulphated glucosamine) found in N- and O-sulphate-rich domains which are separated by regions of low sulphation containing predominantly N-acetylated disaccharides (Gallagher et al., 1992). The strong affinity of FGFs for *in vivo* HS was highlighted first by the ability of these growth factors to bind tightly to heparin, a heterogeneously sulphated GAG that is highly similar in structure to HS (Shing et al., 1984; Maciag et al., 1984; Klagsbrun and Shing, 1985). Heparin is, however, different in saccharide composition and is also more highly sulphated (Gallagher and Walker, 1985). In addition, as heparin is produced exclusively by mast cells associated

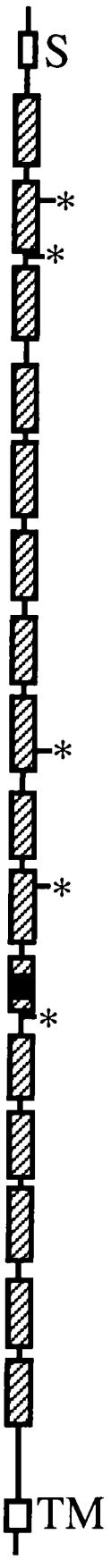


Figure 1.10. Structure of the Cysteine-Rich FGF Receptor (CFR).
A schematic representation of the CFR which consists of 16 conserved cysteine-rich repeats (hatched box). Open boxes indicate the signal peptide (S) and membrane-spanning (TM) domains. The putative heparin-binding domain with sequence similarity to the K18K peptide in FGFR1 (Kan et al., 1993) is represented by a black box. Consensus sites for attachment of N-linked oligosaccharide are marked *.

with connective tissues, its distribution is limited and therefore unlikely to represent the principal cell-associated HS-like molecules involved in interaction with the FGFs.

Heparan sulphates are commonly present in proteoglycans as a diverse group of macromolecules associated with the cell surface and extracellular matrix of a wide range of cells. A role for these macromolecules in regulation of FGF activity was first indicated by the observation that FGF-2 activity can frequently be extracted from the ECM deposited by cultured vascular, capillary and corneal endothelial cells (Baird and Ling, 1987; Vlodavsky et al., 1987; Rogelj et al., 1989; Vlodavsky et al., 1991). The binding of FGF-2 to the ECM appears to be specific for HS or heparin-like GAGs as FGF binding is inhibited in the presence of heparin or treatment of the ECM with heparinase or heparitinase but not in the presence of chondroitin sulphate, dermatan sulphate or hyaluronic acid or treatment of the ECM with chondroitinase, chondro-4-sulphatase, chondro-6-sulphatase or hyaluronidase (Baird and Ling, 1987; Bashkin et al., 1989; Olwin and Rapraeger, 1992). This specificity for HS is supported by the finding that FGF-2 bound to the ECM can be released by heparinase, heparatinase, soluble heparin or high salt which disrupts the FGF-heparin interaction and not by chondroitin sulphate, keratan sulphate, hyaluronic acid or chondroitinase (Folkman et al., 1988; Bashkin et al., 1989).

1.5.3.1 Requirement for Heparan Sulphate

Substantial evidence has been obtained from a variety of studies to show that the binding of FGFs to heparan sulphate or heparin is potentially biologically important. First of all, binding to heparin or HS appears to potentiate the biological activity of FGF-1 (Thornton et al., 1983; Schreiber et al., 1985; Lobb et al., 1986; Mueller et al., 1989; Kunou and Hatanaka, 1995). Hyaluronic acid, chondroitin sulphate and dextran sulphate were inactive in this respect (Mueller et al., 1989). This ability of heparin to enhance FGF activity may be correlated with the observation that heparin binding induced the apparent structural stabilization of FGF-1 and restored the biological activity of inactive FGF-1 (Schreiber et al., 1985; Jaye et al., 1987; Ortega et al., 1991). Secondly, heparin or HS is known to

protect FGF from thermal denaturation, extremes of pH as well as proteolytic degradation by trypsin or plasmin (Gospodarowicz and Cheng, 1986; Rosengart et al., 1988; Saksela et al., 1988; Sommer and Rifkin, 1989; Copeland et al., 1991). Thirdly, heparin and HS binding to FGF appears to be necessary for FGF-induced signal transduction. It has been well demonstrated that the activity of FGF-2 is abolished if its binding to cellular HS is prevented, as in the case where cells were treated with heparitinase or chlorate (a reversible inhibitor of GAG sulphation), or in suspension culture cells which lack cell surface heparan sulphate proteoglycans (HSPGs) but expressing transfected FGFR cDNA (Bernard et al., 1991; Rapraeger et al., 1991; Li and Bernard, 1992; Mansukhani et al., 1992; Olwin and Rapraeger, 1992). This property is shared by FGF-1, FGF-4 and FGF-9, suggesting that the requirement of HS for signal transduction is a general characteristic of this family of growth factors (Bernard et al., 1991; Li and Bernard, 1992; Mansukhani et al., 1992; Ornitz and Leder, 1992; Hecht et al., 1995; Santos-Ocampo et al., 1996).

Heparin or HS participation in FGF function is thought to be due to the ability of these macromolecules to facilitate FGF-FGFR binding interactions. Heparin has been shown to increase the binding affinity of FGF-1 for FGFR1 and FGFR2 by two- to three-fold (Kaplow et al., 1990). Furthermore, studies utilizing mutant Chinese Hamster Ovary (CHO) cells or myeloid cells defective in the metabolism of HSPG have shown that high affinity binding of transfected FGFRs to FGF-2 failed to occur in the absence of concomitant binding to HS or heparin, a condition that can be restored upon the addition of exogenous heparin or HS (Bernard et al., 1991; Rapraeger et al., 1991; Yayon et al., 1991). Assays based on a cell-free system that employ soluble ectodomains of FGFRs also demonstrated the requirement of heparin for FGF-1 or FGF-2 binding to FGFR1 (Ornitz and Leder, 1992; Ornitz et al., 1992). This is, however, in contrast with other studies, which indicate that the presence of heparin is not obligatory for FGF-FGFR interaction (Moscatelli, 1988; Kiefer et al., 1991; Pantoliano et al., 1994; Roghani et al., 1994). A consensus amongst these latter studies is that heparin, though not absolutely necessary for FGF binding to FGFR1 or FGFR2, serves to increase the affinity of the binding interaction, possibly by reducing the rate of dissociation of the ligand from the receptor

(Moscatelli, 1992). Heparin or HS may also mediate FGF signal transduction by interacting with FGFR. It has been shown that heparin interacts with FGFR1 and FGFR4 independent of FGF ligand (Hou et al., 1991; Kan et al., 1993; Pantoliano et al., 1994; Gao and Goldfarb, 1995). Again, any conclusions drawn from these findings may be disputed as other workers reported the inability to detect significant heparin-FGFR interactions (Ornitz et al., 1992; Spivak-Kroizman et al., 1994). Despite the lack of a general consensus, it is nevertheless clear that heparin and heparan sulphates are potentially important components of the FGF-FGFR binding interaction that results in a biological response.

Many proposals regarding the mechanism by which HS-like molecules enhances the biological activity of FGFs have been advanced. A proposed "induced-fit" mechanism suggests that FGF binding to HS induces a conformational change in the ligand so that its affinity for FGFR is enhanced (Yayon et al., 1991). This dual receptor model is supported by observations that heparin binding to FGF-1 has the effect of stabilizing its activity and modifying its antibody recognition epitopes (Schreiber et al., 1985). Moreover, spectroscopic studies indicated that HS and heparin can induce a reproducible conformational change in FGF-2 (Prestrelski et al., 1992). Further support comes from recent evidence suggesting that heparin binding invokes a secondary receptor-binding site on FGF-2 (Pantoliano et al., 1994). The structure of FGF-1, however, does not appear to be altered by heparin binding though it is stabilized against thermal denaturation (Copeland et al., 1991). A second hypothesis is that HSPG on the cell surface serves to concentrate or accumulate the FGF ligand and mediate its transfer to high affinity receptors optimally (Klagsbrun and Baird, 1991). Cell-associated HSPGs may also aid in increasing the probability of FGF and FGFR encounters by reducing the dimensionality of ligand diffusion from three to two dimensions and at the same time, imparting rapid lateral mobility to the bound FGF molecules. An alternative view is that HS is a component of a ternary complex involving FGF and its receptor which is stabilized by strong cooperative binding via the simultaneous interaction of FGF with both HS and FGFR (Nugent and Edelman, 1992). The most tested hypothesis, however, is the role of heparin in facilitating oligomerization of FGF molecules such that multivalent binding to the receptor is achieved,

resulting in receptor dimerization and consequent activation (Spivak-Kroizman et al., 1994). Cross-linking experiments have provided evidence that heparin and heparin fragments containing 6 monosaccharide units and above can promote the formation of FGF-2 dimers and trimers (Ornitz et al., 1992). Other physical studies involving light scattering experiments, sedimentation analysis, surface plasmon resonance analysis and isothermal titration calorimetry also indicated that FGF-1 binds to heparin with high stoichiometries, with one FGF-1 molecule binding to 4 to 6 monosaccharide units of heparin or heparan sulphate (Mach et al., 1993; Spivak-Kroizman et al., 1994). Oligomerization of FGF was shown to be important for receptor activation as the heparin analogue, sucrose octasulphate, which binds to FGF-1 in a 1:1 complex (Spivak-Kroizman et al., 1994), is unable to induce FGFR2 dimerization in the presence of FGF-1 (Spivak-Kroizman et al., 1994) and have little or no effect in promoting FGF-1- or FGF-2-induced mitogenic activity in BaF3 cells expressing FGFR1 or FGFR2 (Spivak-Kroizman et al., 1994; Ornitz et al., 1995). New molecular modelling and crystallographic data suggests that FGF-2 preferentially undergoes self-association and that heparin serves to stabilize the oligomerized FGF-2 molecules without the induction of any conformational changes (Venkataraman et al., 1996).

1.5.3.2 Structural Specificity of Heparin-FGF Interaction

The binding of heparin or HS to FGF appears to be highly specific, as in the example of the interaction between anticoagulant heparin and thrombin requiring a specific pentasaccharide sequence in heparin (Kjellén and Lindahl, 1991). Attempts in defining the structural characteristics of heparin or HS required for binding to FGF highlighted the existence of a general structural pattern. There is a general correlation between the degree of sulphation of the heparins and their relative potencies in promoting FGF-2-FGFR binding interactions (Bashkin et al., 1989; Yayon et al., 1991). A minimal size of 8 sugar residues in a heparin fragment is required for FGF-2 binding to FGFR1 expressed in mutant CHO cells deficient in cell surface HSPG (Ornitz et al., 1992). Another group reported that heparin fragments

containing 4 sugar units or more can displace FGF-2 from immobilized heparin (Bashkin et al., 1989; Aviezer et al., 1994b) although only heparin fragments comprising 12 or more monosaccharide units are as effective as native heparin in binding to immobilized FGF-2 or in restoring high affinity binding of FGF-2 to FGFR1 (Aviezer et al., 1994b; Walker et al., 1994). The minimal structure of heparin required for FGF-1 binding has been determined to consist of three consecutive disaccharide repeats: [IdoA($2SO_4$)-GlcNSO_3($6SO_4$)]$_3$ where IdoA and GlcN refer to iduronic acid and glucosamine respectively (Bârzu et al., 1989). A heparan sulphate sequence derived from human skin fibroblasts that binds to FGF-2 with an affinity as high as the parent molecule is found to be a tetradecasaccharide motif (Oligo H) composed essentially of five consecutive [IdoA($2SO_4$)-GlcNSO_3] disaccharide units (Turnbull et al., 1992). Another report described a similar structure in the FGF-2-binding domain of HS involving three such disaccharide units (Habuchi et al., 1992). The minimal binding structure, however, is described as a pentasaccharide sequence, [HexA-GlcNSO_3-HexA-GlcNSO_3-IdoA($2SO_4$)] where HexA refers to hexuronic acid which may be either glucuronic acid or iduronic acid (Maccarana et al., 1993). This minimum sequence has a much lower affinity than Oligo H, indicating that additional sequences are required for optimal binding interaction between HS and FGF-2.

Recent evidence indicates that a minimal HS sequence capable of binding FGF-2 may not be sufficient for promoting the biological activity of the growth factor. Indeed, a heparin or heparan sulphate sequence containing at least 10 monosaccharides is required to elicit a biological response induced by FGF-2 (Ornitz et al., 1992; Ishihara et al., 1993; Guimond et al., 1993; Walker et al., 1994). A dodecasaccharide sequence that possesses the same activity as native heparin in promoting the biological activity of FGF-2 on chlorate-treated 3T3 fibroblasts is found to be enriched in the disaccharide repeats similar to those described above by Turnbull et al. (1992) and Habuchi et al. (1992), confirming that the binding interaction abides by a strict structural requirement (Walker et al., 1992). It has been proposed that a longer saccharide sequence than the minimum pentasaccharide structure for FGF-2 binding is required for promoting the mitogenic activity of FGF-2 because binding of the heparin fragment to both FGF-2 and receptor may be essential

(Guimond et al., 1993). This, however, may not be the case as it was recently reported that non-sulphated trisaccharides could stimulate the mitogenic activities of FGF-1 and FGF-2 (Ornitz et al., 1995). As cross-linking studies showed that disaccharides and trisaccharides can induce FGF oligomers, ligand oligomerization may be sufficient for receptor activation.

1.5.3.3 Regulation of FGF Activity by Endogenous Heparan Sulphates

As HS are commonly present on cell surfaces as HSPGs, attempts have been made to isolate these molecules that may act as endogenous binding receptors for FGF. Several HSPGs are now known to occupy the cell surface of various cell types including syndecan, fibroglycan, glypican, ryudocan, betaglycan and amphiglycan (Gould et al., 1992; Yanagishita and Hascall, 1992; David, 1993). One of these, syndecan, a developmentally-regulated transmembrane HSPG, was isolated as a FGF-2 binding protein from the hamster kidney cell line using an expression cloning strategy (Kiefer et al., 1990). Another possible candidate is a cellular HSPG containing a putative phosphatidylinositol plasma membrane anchor identified as the major FGF-2-binding protein in primary human bone marrow culture (Brunner et al., 1991). An uncharacterized 150 kDa HSPG has also been isolated from a rat parathyroid cell line which has a high affinity for FGF-2 (Sakaguchi et al., 1991). In addition, perlecan, the major HSPG species residing in the pericellular matrix and basal lamina, has recently been shown to possess many characteristics that define it as a low affinity, accessory receptor for FGF-2. These features include its ability to promote FGF-2-FGFR1 binding, stimulate mitogenic response of HSPG-deficient cells to FGF-2 and induce neovascularization *in vivo* (Aviezer et al., 1994a). A more recent study suggests that an increase in perlecan expression may be correlated with increased FGF-2 mitogenic activity in sub-cultures of bovine retinal pigmented epithelial cells (Guillonneau et al., 1996).

The activity of FGFs *in vivo* may be regulated by the nature of the HSPGs in the surrounding microenvironment. Current data suggests that the FGF-binding HSPGs may be classified into two major categories: inhibiting or activating. Syndecan appears to be of

the inhibiting type as enhanced expression of this proteoglycan in 3T3 cells specifically repressed FGF-1 or FGF-2-induced cell proliferation (Mali et al., 1993). Additional evidence indicates that syndecan not only lacks the capacity to promote high affinity receptor binding of FGF-2, it also inhibits heparin-induced binding of FGF-2 to FGFR1 (Aviezer et al., 1994b). This is in marked contrast to the activation function displayed by perlecan (Aviezer et al., 1994a). Hence, the bioavailability and activity of the FGFs may be determined by a balance between the inhibiting and activating species of HS present in a particular locale. The degree and extent of FGF-induced cellular responses may thus be fine-tuned by regulation of the expression patterns of the different HSPGs on the cell surface, basement membrane, or in the extracellular matrix.

Cellular responsiveness to a specific FGF may also be regulated by alteration of the GAG side chains of interacting species of HSPGs. A good example is the discovery that neuroepithelium HSPGs at two different stages of murine embryonic development displayed differential binding affinities for FGF-1 and FGF-2 and correspondingly different abilities to stimulate the mitogenic activity of these two growth factors (Nurcombe et al., 1993). The FGF-binding HSPG species from the two stages of development seem to differ only in the pattern of glycosylation, suggesting that a developmental switch in post-transcriptional and/or post-translational modifications of one HSPG species can alter the binding specificity of the proteoglycan for different FGFs. In addition, it was found that the pattern of sulphation of the sugar residues in heparin has differential effects on FGF-1 and FGF-4 mitogenic activities on 3T3 cells (Guimond et al., 1993). These observations thus lend further support to the idea that the FGF-binding specificity of HSPG is localized in the GAG side chains.

The prototype FGFs are present in many adult tissues at concentrations often above that which is necessary for supporting cell growth. A prime example is the high level of the potent endothelial cell mitogen, FGF-2, associated with endothelial cell basement membranes and ECM (Baird and Ling, 1987; Vlodavsky et al., 1987; Rogelj et al., 1989). Endothelial cell proliferation in normal tissues is usually very low, with turnover time measured in years (Hobson and Denekamp, 1984). Hence, the activity of this growth factor

is expected to be tightly regulated under normal conditions to ensure the maintenance of the resting state in the adult vasculature. It is suggested that this may be attained by sequestering the FGF with inhibiting HSPGs such as syndecan-1 (Aviezer et al., 1994a). There is evidence to suggest that syndecan expression in NIH 3T3 fibroblasts is enhanced in the presence of FGF-2 (Elenius et al., 1992). Furthermore, FGF-2 exposure is known to have an effect in the rate of synthesis and structural features of membrane-associated HS in corneal endothelial and arterial smooth muscle cells (Schmidt et al., 1995a, b) which may then regulate its activity.

1.5.3.4 Physiological Implications of FGF-Heparan Sulphate Proteoglycan Interaction

FGF bound to cell- or tissue-associated HSPGs may have important physiological relevance. In addition to participation as putative co-receptors with FGFRs in FGF-induced signalling, the physiological function of cell surface or ECM-resident HSPG may be to serve as an efficient, large capacity reservoir of FGFs thereby, providing a short- or long-term access of the growth factor for interaction with the receptors. This property may be particularly important under conditions where an acute cellular responsiveness to FGF is required, as at sites of wound healing, tissue remodelling and growth. FGF bound to the matrix may be rapidly mobilized by heparanases (Vlodavsky et al., 1987; Ishai-Michaeli et al., 1990), plasmin (Saksela and Rifkin, 1990) which cleaves the core protein of HSPGs thereby releasing FGF as a noncovalent complex with HSPG or GAG, or by phospholipase C when the HSPG is anchored to the plasma membrane by an inositol phospholipid constituent (Brunner et al., 1991). Plasmin production is known to be modulated by FGF activity on endothelial cells (Saksela and Rifkin, 1990). FGF-2 complexed to heparin or HSPG appears to interact with its receptor in a similar manner to free FGF-2 (Moscatelli, 1987; Saksela et al., 1988; Bashkin et al., 1989; Rogelj et al., 1989). The release of FGF as a complex with either HSPG or GAG provides several potential advantages, including stabilization of the FGF against denaturation and proteolytic

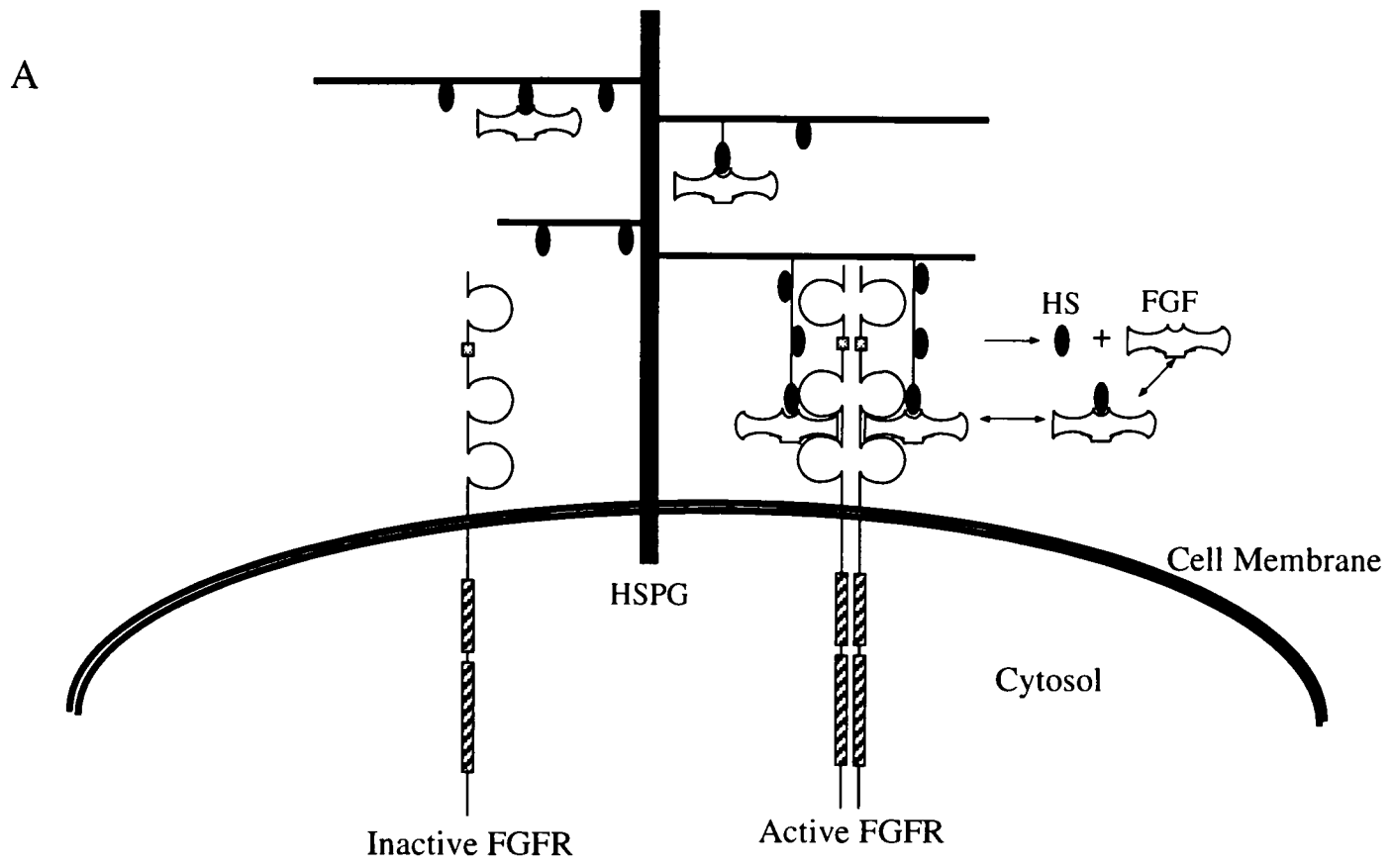
degradation, increased mobility of diffusion since the complexes do not bind to the ECM and the presentation of an active complex of FGF oligomers for binding to FGFRs. Hence, FGF may reach its target cells at sites distant from its site of release more rapidly to achieve paracrine activation of cellular response.

FGF may also be released from its storage sites in the ECM by the action of heparin. Intravenous administration of heparin has been shown to result in a rapid release of FGF-like activity into the circulation of normal rabbits (Thompson et al., 1990). *In vitro* studies also demonstrated that soluble heparin can act as carriers for FGF-2, increasing the radius of its diffusion, thereby potentially increasing the availability of this growth factor to cells distant form its site of release (Flaumenhaft et al., 1990). This function of heparin may be important during repair processes and capillary growth as mast cells, a source of heparin, is present at the leading edge of developing blood vessels (Azikhan et al., 1980). In addition, heparin may form a ternary complex with FGF and FGFR, and so enhances FGF-receptor interaction and subsequent signalling.

All in all, it is becoming increasingly clear that the activity of members of the FGF family is intimately regulated by interactions with HSPGs. This has important implications as the ECM may play an active role in controlling the bioactivity and bioavailability of these growth factors and so increasing a level of complexity and intricacy to the control of FGF action.

1.6 Models for the Role of Heparan Sulphate in FGF-FGFR Interaction

The reported obligatory requirement for heparin or heparan sulphates in promoting FGFR activation may be summarized by two models proposed previously (Figure 1.11). The first is based on the function of heparan sulphate or heparin to oligomerize multiple FGF molecules so that multivalent binding of FGFRs is effected, resulting in receptor dimerization and activation (Schlessinger et al., 1995) (Figure 1.11A). The second model is derived from the finding that two receptor-binding sites exist on a single FGF-2 molecule



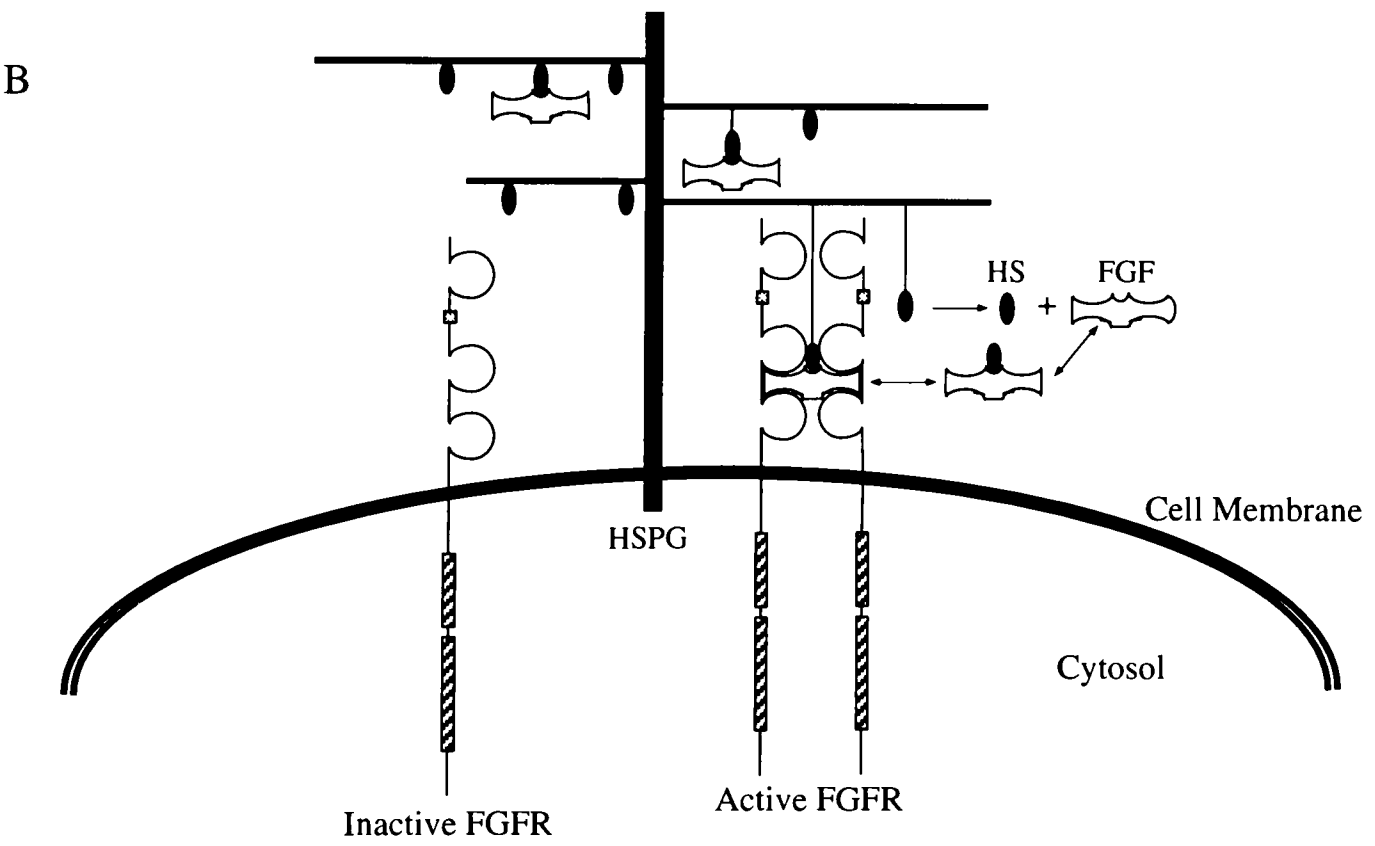


Figure 1.11. Interactions Between Heparan Sulphate Proteoglycan, FGF and FGFR in Effecting Receptor Dimerization and Activation.
Two models proposed for the obligatory requirement of heparan sulphate proteoglycans (HSPGs) in effecting FGFR activation are shown. (A) A cell surface proteoglycan binds to multiple FGF molecules which increases the avidity of binding of the ligands to the FGFR. The multivalent binding of the multimeric FGF-heparan sulphate complex stimulates receptor dimerization and activation. (B) Alternatively, binding to heparan sulphate (HS) results in a conformational change of FGF which exposes a hitherto concealed second receptor binding site such that one FGF ligand can interact with two receptor molecules in a quarternary complex to accomplish receptor dimerization.

(Springer et al., 1994) and that interaction with heparin may induce the binding of a second receptor molecule to a secondary, lower affinity site on a FGF-2 molecule that is already complexed to a receptor molecule via a primary higher affinity site (Pantoliano et al., 1994) (Figure 1.11B). The second model thus describes a mechanism whereby monomeric FGF-2 facilitates receptor dimerization by interacting with two FGFR molecules via its two receptor-binding epitopes localized on opposite faces of the molecule, in a manner analogous to the growth hormone model of receptor dimerization with the exception that the latter system does not require the recruitment of co-factors (Lemmon and Schlessinger, 1994).

1.7 Biological Functions

The establishment of a functional role for a growth factor requires that the molecule is expressed at the correct spatial and temporal position coincidental with its ability to induce a biological effect on the cell/tissue in question.

1.7.1 Ligand Distribution

1.7.1.1 Extracellular, Cellular and Subcellular Localization

One major difference between the prototype FGFs and most other members of the family is the lack of a classical consensus secretory signal peptide in FGF-1 and FGF-2 (Jaye et al., 1986; Abraham et al., 1986a, b). FGF-9 is similar in lacking a signal peptide (Miyamoto et al., 1993). How these potentially cell-associated FGFs modulate the autocrine stimulation of cell growth is thus of interest. The other members of the FGF family, including XeFGF, possess consensus hydrophobic signal peptides for secretion (Delli-Bovi et al., 1987; Taira et al., 1987; Finch et al., 1989; Bates et al., 1991; Coulier et al., 1991; Isaacs et al., 1992), with the exception of FGF-3 which uses a short stretch of hydrophobic amino acid at the amino terminus as an atypical secretory signal sequence (Dickson and Peters, 1987). These latter group of growth factors have, indeed, been shown to be secreted from cells (Kiefer et

al., 1991; Kiefer et al., 1993; Delli-Bovi et al., 1988; Bates et al., 1991; Tanaka et al., 1992).

The supposition that the prototypic FGFs are cell-associated molecules as opposed to secreted factors is corroborated by various observations. For instance, FGF-2 secretion was not detectable in bovine aortic endothelial cells or human hepatoma cells that normally synthesize the protein (Klagsbrun et al., 1986; Moscatelli et al., 1986; Presta et al., 1986; Vlodavsky et al., 1987). FGF-2 was also not found to be secreted in transformed or tumour-derived cell lines such as *ras*-transformed 3T3 cells (Iberg et al., 1989), rhabdomyosarcoma (Schweigerer et al., 1987) and melanoma (Lobb et al., 1986; Scott et al., 1991). Consistent with these data is the difficulty in detecting bioactive FGF-2 in circulating plasma or cell culture fluid (Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989; Zimering et al., 1990). DNA transfection studies also failed to find significant levels of FGF-2 released from the cells even when they are induced to express high levels of the protein (Quarto et al., 1989; Rogelj et al., 1988). Indeed, FGF-2 expressed by transfection of NIH 3T3 cells was not found to be associated with cytoplasmic structures characteristic of a secretory protein (Renko et al., 1990). Immunolocalization and subcellular fractionation experiments have localized FGF-2 in the cytosol of normal producer and DNA transfected cells (Gonzalez et al., 1990; Renko et al., 1990; Florkiewicz et al., 1991; Dell'Era et al., 1991; Bugler et al., 1991). FGF-1 is also inefficiently released by cells that produce it and is cell associated in transfected cell lines (Jaye et al., 1988; Blam et al., 1988; Cao and Pettersson, 1990).

There are, however, contradictory reports that FGF-2 is somehow released into the extracellular medium where it exerts its effect via high affinity cell surface receptors. Early reports identified the presence of significant amounts of FGF-2 in the basement membranes and extracellular matrix produced by cultured endothelial cells, such as bovine corneal and aorta endothelial cells (Klagsbrun et al., 1986; Baird and Ling, 1987; Vlodavsky et al., 1987; Folkman et al., 1988; DiMario et al., 1989; Weiner and Swain, 1989). Its mitogenic or differentiation modulating activity in this locale can be inhibited by protamine sulphate, a protein that binds avidly to heparin (Neufeld and Gospodarowicz, 1987) and by exogenous

anti-FGF-2 antibodies (Rogelj et al., 1989). Similarly, the ability of cultured bovine aortic endothelial cells to grow, migrate and produce basal levels of the protease plasminogen activator, can be inhibited by the addition of exogenous antibodies that bind to FGF-2 (Sakaguchi et al., 1988; Sato and Rifkin, 1988; Matsuzaki et al., 1989). Another study also indicated that these FGF-2 neutralizing antibodies can reverse the transformed phenotype of Balb/c 3T3 cells made to express high levels of FGF-2 (Sasada et al., 1988). More recently, FGF-2 has been detected in cell culture medium and extracellular matrix of fetal chondrocytes by radioimmunoassays (Hill and Logan, 1992). The case for FGF-9 is mainly based on DNA transfection studies where a substantial quantity of the protein may be purified from the culture medium, as described in Chapter 3 and elsewhere (Miyamoto et al., 1993). Western-blot of cell lysates from transfected COS cells could not detect the presence of FGF-9, suggesting that most of the protein was secreted into the culture supernatant. Furthermore, FGF-9 can be purified, albeit in relatively low abundance, from the culture medium of a human glioma cell line (Naruo et al., 1993), substantiating the idea that FGF-9 is indeed a secreted protein.

The release of FGF-2 and FGF-9 into the extracellular medium may be accounted for by cell damage or lysis. Studies of the chemotactic effect of FGF-2 on single NIH 3T3 cells, however, suggests that the protein is released by viable cells to mediate cell functions via an autocrine mechanism (Mignatti et al., 1991). The mechanism(s) by which secretion can occur is unclear. One proposal was the specialized transport of FGFs as an intracellular complex with heparan sulphate which is then inserted into the cell surface and/or liberated into the extracellular matrix (Baird and Ling, 1987; Bashkin et al., 1989). Other possible pathways hypothesized are based on examples of proteins which lack signal peptides but are yet secreted from cells. The export of the yeast mating pheromone a-factor (McGrath and Varshavsky, 1989) and hemolysine (Mackman et al., 1987) is dependent on ATP-driven membrane transport proteins related to the multidrug resistance P-glycoprotein. The

secretion of muscle lectin L-14 is associated with evaginated segments of the plasma membrane of differentiating myoblasts (Cooper and Barondes, 1990). Another example is IL-1ß which appears to be released from activated human monocytes by a novel pathway that requires the presence of intact intracellular vesicles (Rubartelli et al., 1990).

The non-secreted forms of FGF-1 and FGF-2 may have roles in the nucleus as suggested by more recent evidence presenting a case for the nuclear localization of these two molecules. Initial findings indicated that exogenous FGF-2 added to aortic endothelial cells was translocated into the nucleus in a growth cycle-dependent manner (Bouche et al., 1987; Baldin et al., 1990). DNA transfection experiments subsequently revealed the preferential translocation of higher molecular weight forms of FGF-2 initiated from alternative CUG codons to the nucleus (Renko et al., 1990; Bugler et al., 1991; Florkiewicz et al., 1991). The putative nuclear localization signal required for active translocation of the protein through the nuclear pore complex is thought to reside in the residues between the third CUG codon and the initiating AUG codon which include several stretches of alternating glycine and arginine residues (Sommer et al., 1989; Bugler et al., 1991; Burgess et al., 1991a). Indeed, fusion of the amino-terminal extension upstream from the initiating AUG codon to the coding sequence of a reporter gene which is normally cytoplasmic, resulted in translocation of the chimeric protein to the nucleus (Bugler et al., 1991). Immunolocalization analyses confirmed that FGF-2 is indeed localized to the nucleus of both adult and fetal bovine aortic endothelial cells, bovine cardiac muscle cells, human hepatoma cells, ovine fetal growth plate chondrocytes and in *Xenopus* during gastrulation (Kardami and Fandrich, 1989; Renko et al., 1990; Dell'Era et al., 1991; Hill and Logan, 1992; Shiurba et al., 1991). Nuclear localization *in vivo* was shown to be not limited to the higher molecular weight forms of FGF-2 but included the AUG-initiated 18 kDa form as well (Dell'Era et al., 1991; Pasumarthi et al., 1994). Moreover, injection of *Xenopus* FGF-2 RNA that does not encode amino-terminally extended sequences into fertilized *Xenopus* embryos resulted in translocation of the over-expressed protein to the cell nucleus (Thompson and Slack, 1992). The *in vivo* translocation of FGF-2 isoforms to the nucleus may be regulated in specific cell types at defined developmental stages. For

example, in the developing chicken kidneys, FGF-2 in all isoforms, is distributed initially in the cytoplasm in most epithelial cells of the pro- and mesonephros but is subsequently located predominantly in the nucleus of differentiating podocytes of the metanephros where it forms the glomerular filtration unit of the functional kidney (Dono and Zeller, 1994).

Likewise, FGF-1 has been detected in the nucleus by immunohistochemical studies on mesenchymal cells (Sano et al., 1990; Speir et al., 1990). Immunofluorescence and immunoblot studies indicated the translocation of exogenous FGF-1 into the cytosol soluble factions and nuclei of fibroblasts and endothelial cells, with nuclear localization occurring preferably at the late G1 phase of the cell cycle (Imamura et al., 1994). Other experiments utilizing FGF-1 tagged to a farnesylation signal as a marker for intracellular translocation also showed that exogenous FGF-1 was translocated into the cytosol and nucleus (Wiedlocha et al., 1995). Residues 21-27 of FGF-1 appear to resemble the nuclear localization signal of other nuclear proteins (Imamura et al., 1990). Indeed, deletion of the first twenty-seven amino acid residues of FGF-1 inhibited nuclear translocation of the protein (Imamura et al., 1992) and abolished its ability to induce DNA synthesis and proliferation of responsive cells, a property which can be rescued by grafting a nuclear localization signal from yeast histone 2B (H2B) to the amino terminus (Imamura et al., 1990). Recently, FGF-1 is reported to be associated with fragments of the extracellular domains of FGFR4 and FGFR1 in a 40 kDa complex that is selectively localized in the nucleus and perinuclear cytoskeletal elements (Feng et al., 1996).

The CUG-initiated form of FGF-3 was also reported to be localized in the nucleus (Acland et al., 1990). A combination of deletion and replacement mutants coupled to chimeric analyses demonstrated that the AUG-initiated FGF-3 was typically secreted while the CUG-initiated form was found in both the nucleus and the juxtanuclear region that is associated with the secretory pathway. Nuclear localization of this protein is shown to be mediated by a relatively weak bipartite nuclear localization signal and by amino-terminal sequences upstream of the signal peptide (Kiefer et al., 1994). Recent data indicated that additional basic domains located in the carboxy-terminal region of FGF-3 participate in its nuclear uptake and accumulation in the nucleolus (Figure 1.2; Kiefer and Dickson, 1995).

The differential localization of FGF-1 and FGF-2 to the nucleus or cytosol may be correlated with specific biological effects elicited by these molecules. For instance, constitutive expression of the AUG-initiated form of FGF-2 resulted in cell transformation while expression of the CUG-initiated forms led to cell immortalization (Couderc et al., 1991). Other studies showed that mutant FGF-2 lacking the putative nuclear localization signal sequence have reduced ability to induce plasminogen activator synthesis in endothelial cells (Isacchi et al., 1991) and that intracellular FGF-2 was required for the trans-differentiation of cultured Schwann cells into melanocytes (Sherman et al., 1993). In addition, a recent report suggests that the different molecular weight forms of FGF-2 may activate distinct but convergent signalling pathways to effect cell migration and growth in low serum (Bikfalvi et al., 1995). As for FGF-1, both nuclear localization and receptor binding appear to be essential components of its proliferative effect on cells (Imamura et al., 1990; Wiedlocha et al., 1994; Wiedlocha et al., 1996). In contrast, nuclear FGF-3 is reported to inhibit growth of mouse mammary epithelial cells (Kiefer and Dickson, 1995).

1.7.1.2 Tissue Localization

The second major difference between the prototypic FGFs and the other members is in their expression pattern. The prototype FGFs are widely distributed in normal adult tissues (reviewed by Basilico and Moscatelli, 1992). Since their initial purification from bovine pituitary and brain, they have also been purified from diverse tissues including the adrenal gland, bone, cartilage, corpus luteum, hypothalamus, kidney, liver, placenta, prostate, retina, testis and thymus (Baird et al., 1986; Gospodarowicz et al., 1987). Indeed, immunolocalization analyses of FGF-2 expression in normal adult tissues indicated its expression by a wide variety of cell types, including skeletal muscle cells, cardiac and smooth muscle cells, epithelial cells, endothelial cells, neuronal and glial cells (reviewed by Basilico and Moscatelli, 1992; Pettmann et al., 1986; Finkelstein et al., 1988; Emoto et al., 1989). FGF-1 expression may be more limited, being primarily found in neural tissue (Wagner, 1991).

The distribution of other members of the FGF family is markedly different from that of FGF-1 and FGF-2. They are rarely found in normal adult tissues but are prevalent during embryogenesis and in specific tumours. For instance, *Fgf4* expression appears to be highly restricted in normal cells and its transcript has not been detected in the tissues of the adult animal (Yoshida et al., 1988b). The other members appear to be expressed in the adult mouse but their distribution is extremely limited, as summarized in Table 1.4. Of interest is that FGF-7 is a specific product of stromal fibroblasts in tissues with distinct epithelial and stromal cell compartments (Yan et al., 1992; Yan et al., 1993). This, and the observation that its receptor FGFR2b is expressed by epithelial cells, implicate FGF-7 as a candidate mediator of directional stromal-epithelial cell interactions which are important in processes such as kidney and tooth development, generation of the male and female reproductive tracts and liver regeneration (Ekblom, 1989; Yan et al., 1992a; Thesleff et al., 1995).

The expression patterns of the FGFs during embryonic development are discussed in Chapter 5.

1.7.2 FGF Receptor Distribution

1.7.2.1 Expression Pattern of FGFR Isoforms

The function of each FGF member may be regulated by the temporal and spatial regulation of expression of its receptors. Expression of the alternatively spliced forms of members of the FGFR family appear to be regulated in a tissue-specific manner. For instance, expression of the three or two Ig-like domain forms may be differentially regulated. The three Ig-like domain form of FGFR1 is exclusively expressed during development, particularly in the embryonic brain and on birth, in the brain and kidney (Bernard et al., 1991; reviewed by Johnson and Williams, 1993). In comparison, the two Ig-like form of FGFR1 is apparently expressed only after birth (Johnson and Williams, 1993). There may also be a correlation between expression of the longer (three Ig) or shorter (two Ig) form of FGFR1 and the transition from benign to malignant phenotype in cells and tissues. For

Table 1.4. Summary of the Expression Patterns of Members of the FGF and FGFR Family in Adult Tissues.

Gene	Expression Pattern	References
Ligand		
FGF-1	Wide-spread; predominantly in neural tissues	Burgess and Maciag, 1989
FGF-2	Wide-spread	Burgess and Maciag, 1989
FGF-3	Brain, testis†	Dickson et al., 1984, 1989; Jacobovits et al., 1986;
FGF-4	Not detected	Yoshida et al., 1988
FGF-5	Brain	Haub et al., 1990
FGF-6	Heart, skeletal muscle, testis	de Lapeyrière et al., 1990
FGF-7	Gastrointestinal tract, kidney, skin (dermis)	Finch et al., 1989
FGF-8	Ovary, Testis	Lorenzi et al., 1995; MacArthur et al., 1995a
FGF-9	Brain, kidney	Miyamoto et al., 1993
Receptor		
FGFR1*	Brain*, bone, heart, kidney, lung, muscle, skin	Wanaka et al., 1990; Peters et al., 1992b; Werner et al., 1992a
FGFR2*	Brain*, kidney, liver, lung, skin, temporal lope	Kornbluth et al., 1988; Peters et al., 1992b
FGFR3	Bone, kidney, intestine, lung, skin	Johnson and Williams, 1993
FGFR4	Kidney, liver, lung	Korhonen et al., 1992

†, *Fgf3* is reported to be not detectably expressed in the adult mouse, with the possible exception of the brain and testicular tissues. The receptor distribution pattern is not distinguished for splice variants. *, *Fgfr1* transcripts are predominantly expressed in neuronal populations in the brain while *Fgfr2* transcripts are mainly expressed in glial cells.

instance, the three Ig-like domain form is the predominant form in normal human adult and fetal brain, and normal pancreas while the two Ig-like domain form is more prevalent in glioblastomas, astrocytomas, pancreatic adenocarcinomas and other tumours including leiomyosarcomas, mammary and bladder carcinoma (Eisemann et al., 1991; Kobrin et al., 1993; Morrison et al., 1994b; Yamaguchi et al., 1994; Luqmani et al., 1995; Penault-Llorca et al., 1995). Thus, the two Ig-like form of FGFR1 may contribute to aberrant autocrine and paracrine pathways in a variety of cancers. There are, however, cell types that show no apparent discrimination between the three and two Ig-like domain forms of FGFR1. These include hepatoma cells (Hou et al., 1991), human umbilical vein endothelial cells (Johnson et al., 1990), human embryonic lung fibroblasts (Eisemann et al., 1991), neuroepithelial and fibroblast cell lines (Reid et al., 1990; Bernard et al., 1991), human ovarian carcinoma cells (Eisemann et al., 1991) and in normal tissues, the heart, lung, breast and muscle (Bernard et al., 1991; Luqmani et al., 1995).

Secreted forms of FGFR1 have been detected in the brain, skeletal muscle and skin (Werner et al., 1992a), human umbilical vein endothelial cells (Johnson et al., 1990) and human embryonic lung fibroblasts (Eisemann et al., 1991). Secreted FGFR1 may play an important role in the transporting, sequestering or delivery of FGFs throughout the body as three FGF-binding proteins (FGF-BD) with immunological similarity, FGF-binding characteristics and sequence identity to the two Ig-like form of FGFR1 have been identified in blood (Hanneken et al., 1994). A potentially intracellular form of FGFR1 was found to have increased expression in rat prostate tumours (Yan et al., 1992b). Other FGFR1 splice forms have been reported in tumour cells like hepatoma (Hou et al., 1991). Multiple splice forms of FGFR2 is also produced in human stomach cancer-derived cells (KATO-III cells) (Katoh et al., 1992) and secreted FGFR2 have also been isolated in human brain stem (Crumley et al., 1991). In contrast, only the three Ig-like form of human FGFR4 is reported to be the predominant receptor expressed in hepatoma cells and hepatocytes (Feng et al., 1996). In the rat, a potentially intracellular form of FGFR4 is expressed in the lung (Horlick et al., 1992).

Alternative splicing in the third Ig-like loop which generates receptors exhibiting different ligand binding properties also appears to be tissue-specific. FGFR1b is expressed predominantly in the skin, with lower levels in the brain, kidney, muscle and placenta (Werner et al., 1992a). In contrast, the "c" splice form is expressed in most tissues, except the liver (Werner et al., 1992a) and it is the only form detected in foreskin fibroblasts (Johnson et al., 1991). In general, where both splice forms are expressed, FGFR1c was found to be more abundant (Johnson et al., 1991; Werner et al., 1992a). Similarly, FGFR2c is detected in most cell types while FGFR2b is found predominantly in cell types such as keratinocytes (Bottaro et al., 1990), epithelial cells (Miki et al., 1992), chicken embryonic fibroblasts (Sato et al., 1991), stomach carcinoma cells (Hattori et al., 1990) and teratoma cells (Katoh et al., 1992). In addition, the progression of epithelial cells derived from a non-malignant prostate tumour to malignancy was found to be accompanied by a switch in the exclusive expression of exon IIIb to exon IIIc in the FGFR2 gene (Yan et al., 1993a). As for FGFR3, PCR analysis have identified the "b" splice form in the mouse embryo and in the adult mouse skin and epidermal keratinocytes (Avivi et al., 1993). Highest levels of expression of this isoform was detected in the skin while lower levels were found in the kidney, liver and lung (Chellaiah et al., 1994). FGFR3c, in contrast, is expressed predominantly in the brain. The expression patterns of the four FGFR genes in the adult animal are summarized in Table 1.4.

The distribution of the FGFRs in the developing mouse embryo is discussed in Chapter 5.

1.7.3 Biological Activities *In Vitro*

The FGFs are a family of molecules implicated in functions such as cell growth and survival, differentiation, chemotaxis, angiogenesis as well as developmental processes. These roles are deduced from the biological effects they elicit in various target cell types *in vitro*.

The prototype FGF molecules, FGF-1 and FGF-2, were originally purified from a variety of tissues as polypeptide mitogens (Burgess and Maciag, 1989) and they are mitogenic towards a broad spectrum of cells of epithelial, mesenchymal and neuroectodermal origin. In addition to fibroblasts from which their names were derived, they induce the *in vitro* proliferation of many other cell types including vascular endothelial cells, smooth muscle cells, adrenal cortex cells, prostate epithelial cells, retina epithelial cells, chondrocytes, skeletal myoblasts, osteoblasts, hepatocytes, and glial cells (reviewed by Burgess and Maciag, 1989). Other members may exhibit unique mitogenic characteristics. While FGF-3, FGF-4, FGF-5, FGF-6 and FGF-9 have all been shown to stimulate fibroblast growth, FGF-7 is exceptional in being a potent mitogen for keratinocytes but not fibroblasts (Rubin et al., 1989). The prototype FGFs also promotes the survival of neuronal cells, including those derived from the hippocampus, cerebral cortex, thalamus, striatum, cerebellum, spinal cord and parasympathetic ganglia, suggesting a neuronal role for these growth factors (Walicke et al., 1986; Morrison et al., 1986; Schubert et al., 1987; Unsicker et al., 1987; Hatten et al., 1988; Walicke, 1988).

The differentiation of several cell types *in vitro* appear to be modulated by the actions of FGF. The prototype FGFs were shown to promote the differentiation of adipocytes (Serrero and Khoo, 1982) and post-mitotic neurons, commonly observed as induction of neurite outgrowth (Togari et al., 1983; Togari et al., 1985; Morrison et al., 1986; Wagner and D'Amore, 1986; Walicke et al., 1986; Neufeld et al., 1987; Hatten et al., 1988; Lipton et al., 1988; Walicke, 1988). In addition, members of the FGF family, including FGF-1, FGF-2, FGF-4 and FGF-5 have the ability to induce differentiation of the *Xenopus* embryo to form mesoderm (Kimelman and Kirschner, 1987; Slack et al., 1987; Paterno et al., 1989; Slack, 1991). On the other hand, the effect of FGFs on other cells may be one of inhibition of differentiation. For instance, FGF-2 has been shown to suppress the terminal differentiation of skeletal muscle myoblast precursors (Lathrop et al., 1985; Clegg et al., 1987; Seed and Hauschka, 1988; Fox et al., 1994), growth plate chondrocytes (Kato and Iwamoto, 1990) and granulosa cells in culture (Gospodarowicz and Bialecki, 1978). Therefore, the effects of the FGFs on the differentiation status of

various tissues appears to be cell type-specific and may be dependent on different regulatory and signal transduction pathways.

The prototype FGFs may function as chemoattractants for cells in culture. They have been shown to be chemotactic for endothelial cells, fibroblasts, astroglial cells and teratocarcinoma cells (Terranova et al., 1985; Moscatelli et al., 1986; Presta et al., 1986; Senior et al., 1986; Sato and Rifkin, 1988; Mignatti et al., 1991; Alanko et al., 1994). FGF-4 also appears to possess chemotactic properties as it has been shown to contribute to the cell motility and invasion characteristics of NIH 3T3 cells (Taylor et al., 1993). In endothelial cells, the mitogenic and chemotactic effects of FGFs may be coupled to the abilities of these growth factors to induce production of proteolytic enzymes such as plasminogen activator and collagenase (Moscatelli et al., 1986; Presta et al., 1986; Gospodarowicz et al., 1987; Delli-Bovi et al., 1988; Sato and Rifkin, 1988; Mignatti et al., 1989; Tsuboi et al., 1990) to induce a true angiogenic response, a process describing the formation of new blood vessels (Folkman and Klagsbrun, 1987). Indeed, FGF-2 was reported to cause capillary-derived endothelial cells to invade a three-dimensional collagen matrix and promote their organization into tubular structures akin to vascular capillaries (Montesano et al., 1986). The angiogenic process involves increased endothelial cell proliferation and concomitant enzymatic degradation of microvascular basement membranes which facilitates endothelial cell migration and invasion. Hence, the FGFs are putative angiogenic factors since they could stimulate most of these steps *in vitro* .

In vitro biological data implicating a role for the FGFs in embryonic development are presented in Chapter 5.

1.7.4 Pathophysiological Implications

Some of the activities of FGF have also been ascertained in *in vivo* assay systems, which together with the *in vitro* biological data, implicate the involvement of FGFs in various physiological and pathological processes, including angiogenesis, wound healing, tissue regeneration and oncogenesis.

The role of FGFs as angiogenic factors *in vivo* has been well-studied. In fact, the FGFs are the first angiogenic polypeptides reported (Folkman and Klagsbrun, 1987). The ability of the prototype FGFs to induce blood vessel capillary growth *in vivo* was shown in experiments using chick embryo chorioallantoic membrane, rat or rabbit cornea, and hamster cheek pouch (Lobb et al., 1985; Shing et al., 1985; Thomas et al., 1985; Gospodarowicz et al., 1987; Hayek et al., 1987). The FGFs have also been demonstrated to induce rapid neovascularization in the kidney, skin and fibroplasia in the dermis as well as the formation of highly vascularized granulation tissue in sponges implanted subcutaneously in the rat (Davidson et al., 1985; Hayek et al., 1987). A strategy employing retrovirus-mediated gene transfer into neural and non-neuroectodermal cells of fetal brain transplants also showed that FGF-4 has angiogenic activity *in vivo* (Brüstle et al., 1992). Angiogenesis is an important process that occurs physiologically in the development of the vascular system during embryonic, fetal and adolescent growth, and in the growth of the uterine lining during the menstrual cycle.

In addition to angiogenesis, endothelial cells are important in other pathophysiological processes. As they constitute the nonthrombogenic interface between blood and tissue, they may perform mechanistic roles in other physiological processes including wound repair, regulation of arterial blood pressure, and tissue regeneration. They may also contribute directly or indirectly to pathological states such as tumour angiogenesis, thrombosis, diabetic retinopathy, rheumatoid arthritis, and atherosclerosis. As FGFs have significant impact on the biology of endothelial cells, they may be involved in these other pathophysiological events (reviewed by Gospodarowicz, 1990).

Wound healing involves the formation of granulation tissue accompanied by neovascularization. The prototype FGFs have been shown to be mitogenic for many of the cell types potentially involved in the wound healing process *in vivo* and *in vitro*. These cell types include capillary endothelial cells, vascular smooth muscle cells, fibroblasts, chondrocytes, myoblasts, and keratinocytes (reviewed by Gospodarowicz, 1990). Many of these same cells including vascular endothelial cells (Moscatelli et al., 1986; Schweigerer et al., 1987b; Vlodavsky et al., 1987), vascular smooth muscle cells (Gospodarowicz, 1988;

Gospodarowicz, 1990), and macrophages (Baird et al., 1985) are known to produce FGF-2. FGF-1 and FGF-2 are thought to be released through dead or dying cells so that the release of FGF following cell damage could play an important role in tissue healing. *In vivo* experimental models of injury repair have established the potential of FGF in tissue remodelling. FGF-2 was shown to increase granulation tissue formation (Buntrock et al., 1982a, b; Buntrock et al., 1984; Davidson et al., 1985; Davidson et al., 1988), accelerate normal rat wound healing (McGee et al., 1988), promote re-epithelialization rate when epidermis was detached from the dermis (Fourtanier et al., 1986), promote cartilage repair *in vivo* (Cuevas et al., 1988), improve normally impaired healing of dermal wounds in diabetic mice (Tsuboi and Rifkin, 1990), and mediate more rapid healing of tympanic membrane perforations (Fina et al., 1991). Similarly, FGF-1 has the ability to accelerate corneal wound healing (Fredj-Reygrobelet et al., 1987) and to promote endothelial regeneration in injured rat carotid arteries with parallel inhibition of intimal thickening (Bjornsson et al., 1991). FGF-7 has also been implicated in wound healing as its mRNA expression has been shown to be dramatically induced by more than 160-fold after skin injury (Werner et al., 1992b).

The endothelium may regulate arterial blood pressure by releasing factors such as endothelium-derived releasing factor (ERDF) which act on vascular smooth muscle cells to effect vasodilation. Both FGF-1 and FGF-2 have been reported to produce a hypotensive effect on rats or rabbits injected systemically with the proteins, an effect that is partly attributed to ERDF (Cuevas et al., 1991). Hence, FGFs may also modulate the arterial blood pressure regulatory function of endothelial cells.

The regeneration of damaged tissues may involve FGF action. It is known that certain amphibian species such as the newt have the capacity to regenerate lost appendages, a process that requires the initial generation of blastema cells which are thought to originate from either de-differentiating myoblasts or chondrocytes (Gospodarowicz, 1990). FGF-1, FGFR1 and FGFR2 are present in the newt limb blastema (Boilly et al., 1991; Poulin et al., 1993) and FGF infusion was reported to promote the resumption of blastema mitotic activity in the denervated newt forelimb (Mescher and Gospodarowicz, 1979;

Gospodarowicz and Mescher, 1980). As for the chick limb bud, normally unable to mount a regenerative response following amputation at any stage of development, the local application of FGF-2 onto an amputated chick limb bud resulted in the formation of limbs featuring a regeneration of digit-like structures (Taylor et al., 1994).

FGFs may also participate in liver regeneration. Upon partial hepatectomy, hepatocytes initiate DNA synthesis to restore liver mass. Under this condition, FGF-1 expression was found to be induced and total FGF-like mitogenic activity from extracts of regenerating liver tissue was a thousand-fold greater than that from normal liver tissue, suggesting a role for FGF-1 in the autocrine stimulation of hepatocyte proliferation (Kan et al., 1989). A recent report describing the mitogenic activity of FGF-7 on rat hepatocytes in culture indicates this growth factor as another potential candidate for promoting rat liver regeneration (Strain et al., 1994).

The ability of FGF-2 to promote tissue regeneration may account for the benign phenotype of the dystrophic mdx mouse, an animal model of the X-chromosome gene mutation responsible for Duchenne muscular dystrophy in humans (DiMario et al., 1989). Elevated FGF-2 expression in the mdx mouse muscle is thought to be responsible for the persistent regeneration of new muscle fibres such that the lethal phenotype seen in Duchenne patients is negated.

On the other hand, FGF may contribute adversely to a disease state. For instance, FGF-2 localized in the senile plaques of Alzheimer's disease may facilitate abnormal neurite sprouting into plaques and promoting their growth (Cotman and Gómez-Pinilla, 1991). In the case of transgenic mice aberrantly expressing the granulocyte-macrophage colony stimulating factor (GM-CSF) gene, high expression of FGF-2 may be partly responsible for the syndrome characterized by blindness, tissue damage and wasting (Lang et al., 1992).

1.8 Oncogenic Potential and Tumorigenesis

Cancer is the result of multiple progressive genetic changes affecting a number of specific genes which may be classified as oncogenes and tumour suppressor genes. Aberrant expression of oncogenes is frequently associated with a transformed phenotype of cells cultured *in vitro*. Over-expression of several members of the FGF family, from FGF-1 to FGF-6, have been shown to result in transformation of fibroblast cells (Delli-Bovi et al., 1987; Zhan et al., 1987; Jaye et al., 1988; Neufeld et al., 1988; Sasada et al., 1988; Marics et al., 1989; Moscatelli and Quarto, 1989; Goldfarb et al., 1991; Kiefer et al., 1991), suggesting a potential involvement of these polypeptides in tumorigenesis. These observations are not surprising as *Fgf3*, *Fgf4* and *Fgf5* were originally isolated as proto-oncogenes associated with certain tumours. FGF-3, in particular, is implicated in mouse mammary tumorigenesis as the *Fgf3* gene is a main target for mouse mammary tumour virus (MMTV) insertional activation (Dickson et al., 1984). FGF-3 can stimulate the growth of mouse mammary epithelial cells in transgenic mice (Muller et al., 1990) and secreted FGF-3 expressed in mammary cells can initiate tumorigenesis of the mammary gland in a hormone-independent manner (Hajitou and Calberg-Bacq, 1995). In addition, transgenic mice expressing *Fgf3* under the regulation of a binary system of GAL4/UAS control elements exhibited mammary hyperplasia (Ornitz et al., 1991). As *Fgf4* is located in a region 17 to 35 kb downstream from *int-2* in the same transcription orientation in both the mouse and human chromosomes (Huebner et al., 1988; Wada et al., 1988; Yoshida et al., 1988a; Peters et al., 1989), it is also transcriptionally activated by the insertion of MMTV (Peters et al., 1989). In addition, another member of the family, *Fgf8*, is recently reported to be transcriptionally activated in mammary tumours resulting from MMTV insertions in *Wnt-1* transgenic mice (MacArthur et al., 1995b).

Evidence for the role of the prototypic FGFs in tumour growth is not supported by observations that over-expression of FGF-1 or FGF-2 in 3T3 fibroblasts is insufficient to result in a fully transformed or tumorigenic phenotype. These reports indicated that over-expression of native FGF-2 in NIH 3T3 cells led to no evident transformation effects but

that transfection of *Fgf2* cDNA modified by conjugation to a secretory signal derived from either the immunoglobulin G gene or growth hormone gene was required for the cells to become transformed and cause tumour formation when injected into nude mice (Rogelj et al., 1988; Blam et al., 1988). Hence, secretion of the prototypic FGFs is a necessary event for tumorigenesis and that the transforming potential of these growth factors is dependent on the activation of a mechanism for extracellular release. FGF-2 is known to be expressed by a variety of tumour cell lines (Klagsbrun et al., 1986; Schweigerer et al., 1987; Penault-Llorca et al., 1995) and exogenous anti-FGF-2 neutralizing antibody can suppress proliferation of some of these cell lines, including glioma cells (Morrison et al., 1993) and transformed human breast epithelial cells (Souttou et al., 1994), suggesting that FGF-2 is indeed released by transformed cells. A particularly intriguing example is the apparent release of FGF-2 into the extracellular medium during the advanced stages of dermal fibrosarcomas while in normal dermal fibroblasts and during initial stages of the pathological condition, FGF-2 remains cell-associated (Kandel et al., 1991).

The chemotactic and angiogenic properties of FGF-2 may contribute towards tumour metastasis and invasion. A recent report showed that FGF-2 has the potential to induce migration of human embryonic carcinoma cells in a three-dimensional matrix (Alanko et al., 1994) while another study using *Fgf2*- and *Fgf4*-transfected NIH 3T3 cell lines reported a significant correlation between cell locomotion and malignant potential (Taylor et al., 1993). FGF released from the extracellular matrix of endothelial cells as a result of alterations in basement membrane structure and turnover could also facilitate tumour angiogenesis, a complex process involving endothelial cell migration towards and into the tumour tissue to form capillary blood vessels. Tumour angiogenesis is necessary for tumour progression as the increased vascular supply is needed for delivery of oxygen and nutrients as wells as removal of waste products from the actively growing tumours. FGF-2, however, may not always affect tumour angiogenesis as it has been observed that high-level expression of anti-FGF-2 antibodies in mice did not lead to a reduction in angiogenesis of the tumours in these animals (Matsuzaki et al., 1989).

1.9 Animal Models of FGF Function

The expression patterns of the FGFs and its receptors as well as their many *in vitro* and *in vivo* biological activities suggest various potential biological roles for the FGFs. In order to elucidate the *in vivo* biological functions of the individual FGFs and their receptors in the whole animal, efforts have been made towards developing animal models featuring disruption or activation of FGF-mediated signal transduction. Disruption of FGF function may be accomplished by gene targeting techniques which generate null alleles of the gene for the ligand or receptor via the mechanism of homologous recombination. Inactivation of FGF-dependent signalling may also be achieved through the application of dominant-negative FGFR mutants which lack the cytoplasmic catalytic domains but which are otherwise similar to the wild-type receptors. These mutated receptors inhibit signalling by wild-type receptors via the formation of inactive heterodimers upon ligand binding (Ueno et al., 1992). On the other hand, activation of FGF function may be attained by over-expression of the gene in transgenic mice or by ectopic application of the ligand.

1.9.1 Inactivation of FGF Function via Homologous Recombination

The biological consequences of deleting the functions of several members of the FGF family have established an important point: individual members have discrete biological functions. For example, mice carrying homozygous null mutations for *Fgf3* are viable with defects in the development of the tail and inner ear (Mansour et al., 1993), while mice inactivated for FGF-5 function are also viable but apparently defective only in hair development (Hébert et al., 1994). The activities of some members of the FGF family may be critical for early embryonic development. For instance, mouse embryos homozygous for the null allele of *Fgf4* failed to develop after uterine implantation as development of the inner cell mass is arrested, indicating an embryonic lethal phenotype (Feldman et al., 1995). Similarly, the absence of *Fgfr1* signalling in homozygous mutant embryos led to early lethality with an aberrant mesodermal patterning phenotype (Deng et al., 1994;

Yamaguchi et al., 1994). Hence, while useful in delineating the requirement of some FGFs in early development, the method of generating homozygous null mutations may not reveal the full range of developmental functions of a gene if loss of the gene product is lethal at an early stage of development, as in the case of FGF-4. Moreover, loss of one member may be compensated by another due to their overlapping expression patterns, hence, it is important that the inactivation effects be analysed for the complete set of ligand, receptors and cofactors, alone and in combination, to obtain a more accurate picture of endogenous FGF function.

1.9.2 Inactivation of FGFR Signalling via Dominant-Negative Receptors

Inhibition of FGFR function by injecting mRNA encoding a cytoplasmically-truncated form of FGFR1 into *Xenopus* embryos reflected the phenotype observed in mice homozygous for null alleles of *Fgfr1* described above (Amaya et al., 1991). Specifically, frog embryos injected with the dominant-negative FGFR1 construct showed defects in gastrulation and posterior development as grossly abnormal trunks were formed. The mutant embryos also displayed abnormal morphogenesis of the digestive tract which may be attributed to delayed differentiation of mesenchyme-derived smooth muscle cells (Saint-Jeannet et al., 1994). A similar experiment was carried out on zebrafish embryos and deficiencies in trunk and tail development were also observed in injected embryos (Griffin et al., 1995). There is a disadvantage to this approach of studying gene function as the mutant receptor may dominantly inhibit signalling from all FGFR members by combining with endogenous wild-type FGFRs to form inactive heterodimers (Ueno et al., 1992), and so obscuring the specific function of the particular FGFR in question.

The dominant-negative FGFR approach has also been used to study the effect of inactivation of FGFR signalling on the development of specific organs. This has been carried out by targeted expression of the mutant receptor using tissue-specific promoters in transgenic mice or by infecting specific cell types with retroviruses engineered to constitutively express the mutant receptor. Targeted expression of the dominant-negative

Fgfr2b gene in the lung-bud epithelium of transgenic mice by use of the human surfactant protein C promoter resulted in perinatal death due to a defect in airway branching and epithelial differentiation of the developing lung (Peters et al., 1994). The same mutant receptor, when designed to be expressed specifically in basal keratinocytes by fusing the cDNA downstream of the human keratin 10 promoter, resulted in transgenic mice exhibiting abnormalities in the morphogenesis of the epidermis and hair follicles, dermal thickening and impaired wound re-epithelialization (Werner et al., 1994). Disruption of FGFR signalling in the lens of the eye by using the lens fibre cell-specific αA-crystalline promoter to express a dominant-negative form of FGFR1 resulted in transgenic mice with eye defects that may be attributed to reduced differentiation and apoptosis of fibre cells, and diminished number of lens epithelial cells (Chow et al., 1995; Robinson et al., 1995). In a similar manner, the consequence of disrupting FGFR signalling in the retina of the eye was examined by expressing mutant FGFR1 and FGFR2 genes deleted with respect to the tyrosine kinase domain, under the control of the bovine rhodopsin promoter (Campochiaro et al., 1996). Transgenic mice expressing the mutant receptors were found to exhibit progressive photoreceptor degeneration. The role of FGFR1 in embryonic limb muscle development has also been examined by an alternative approach where retroviruses expressing a dominant-negative construct of FGFR1 are injected into newly formed somites of avian embryos (Itoh et al., 1996). Cells expressing the mutant receptor failed to migrate to the somatopleure and terminal differentiation within the myotome was induced. These latter approaches are informative as regards FGFR signalling in specific tissues at later developmental stages.

1.9.3 Inactivation of FGFR Signalling via Mutagenesis of FGFR

The *Drosophila* genome is particularly amenable to mutational and deletional studies. For example, deletional mutagenesis by the use of enhancer trap P elements for DNA excision has been successfully applied for examining gene function. The *Drosophila* FGFR homologue, DFGF-R1, has been examined by this method (Klämbt et al., 1992). Mutants

deleted with respect to the DFGF-R1 locus exhibited defects in the migration of tracheal cells and midline glial cells, the two cell types where it is known to be expressed (Glazer and Shilo, 1991). The role of DFGF-R1 in the process of tracheal cell migration appears to involve providing migration capacity to the cells rather than guiding the directionality of tracheal cell migration (Reichman-Fried et al., 1994).

In the *C. elegans* model, mutation of a FGFR homologue, EGL-15, is associated with abnormal migration of sex myoblasts in the animals (DeVore et al., 1995). In this instance, the *C. elegans* homologue of FGFR appears to be involved in the normal guidance of sex myoblast migration rather than for providing motility to these cells.

1.9.4 Activation of FGF Signalling by Ectopic Over-Expression of FGF

Activated FGF signalling may result in biological responses that are important clues to the potential functions played by the FGFs *in vivo*. For example, over-expression of FGF-4 in chimeric mice resulted in the appearance of ectopic structures resembling early phases of limb development (Abud, 1995; Abud et al., 1996). The ability of FGF-4 to induce additional ectopic limbs was subsequently verified in the chick by local application of FGF-soaked beads (Cohn et al., 1995), or by implanting FGF-4-expressing cells (Ohuchi et al., 1995). Ectopic expression of FGF-2 fused to an artificial signal peptide in the chick limb bud was also observed to cause skeletal duplications (Olwin et al., 1994). In other organs, a secreted form of FGF-1 expressed under the control of the lens-specific αA-crystalline promoter was recently shown to result in lens abnormalities including lens epithelial cell differentiation in transgenic mice (Robinson et al., 1995). These findings indicate yet again the important roles played by FGFs, particularly in limb outgrowth and patterning and in the normal biology of the eye. Hence, the value of assays exploiting activated FGF signalling is immense, providing biological data for a growth factor at stages of development where gene-ablation experiments may be non-revealing, as in the case of FGF-4.

1.10 Involvement in Human Disease

FGF-1 and FGF-2 expressions have been detected in multiple tumour cell lines (reviewed by Burgess and Maciag, 1989) and tumour samples, such as human breast tumours (Penault-Llorca et al., 1995). As for FGF-3 and FGF-4, their genes have been reported to be amplified in various cancers, including invasive bladder cancer and oesophageal cancer (Tsuda et al., 1988; Tsutsumi et al., 1988). FGF-4 may also be involved in other tumours as it was identified as a transforming gene in Kaposi's sarcoma and in stomach cancers (Sakamoto et al., 1986; Delli-Bovi and Basilico, 1987) (Table 1.5). FGFR expressions, particularly that of FGFR1, FGFR2 and FGFR4, are also elevated in a large number of breast tumours (Adnane et al., 1991; Penault-Llorca et al., 1995) and elevated expression of FGFR1 is associated with human glioblastomas and astrocytic tumours (Morrison et al., 1994a; Yamaguchi et al., 1994). In addition, FGFR2b was isolated as a product of an amplified and over-expressed gene in a stomach carcinoma cell line (Hattori et al., 1990) (Table 1.6).

The most important discovery, however, is the establishment of a link between a spectrum of autosomal-dominant human genetic disorders relating to bone morphogenesis and mutations in the genes encoding the FGFRs (reviewed by Wilkie et al., 1995a). Seven known skeletal disorders, namely, Pfeiffer syndrome, Apert syndrome, Crouzon syndrome, Jackson-Weiss syndrome, achondroplasia, hypochondroplasia and thanatophoric dysplasia are associated with specific mutations in FGFR1, FGFR2 and FGFR3 (Table 1.6). Several of these syndromes are typically characterized by craniosynostosis, a clinical description for the premature fusion of the skull which is presented as a tower-shaped head, widely-spaced down-slanting eyes, and a receding midface. The various syndromes may be distinguished by the severity of the hand and feet anomalies. Patients with Pfeiffer syndrome often carry a point mutation in exon IIIa of FGFR1 (Muenke et al., 1994; Schell et al., 1995) and/or three point mutations with the splice acceptor site of exon IIIc of FGFR2 (Lajeunie et al., 1995; Rutland et al., 1995; Schell et al., 1995). Skeletal defects found in Jackson-Weiss syndrome and Crouzon

Table 1.5. Chromosomal Localizations of the Human FGF genes, and Involvement in Human Disease.

FGF	Gene Mapping	Disease or Syndrome	Mutation
FGF-1	5q31-33	-	-
FGF-2	4q25	-	-
FGF-3†	11q13	Invasive bladder cancer; oesophageal cancer	Amplification
FGF-4†	11q13	Kaposi's sarcoma; stomach cancer; invasive bladder cancer; oesophageal squamous cell carcinoma	Amplification
FGF-5	4q21	-	-
FGF-6	12p13	-	-
FGF-7	N.D.	-	-
FGF-8	10	-	-
FGF-9	N.D.	-	-

†, the close chromosomal localization of *Fgf3* and *Fgf4* may account for the amplification of the two genes as one amplication unit in human cancers. N.D., not determined.

Table 1.6. Chromosomal Localizations of the Human FGFR genes, and Involvement in Human Disease.

Receptor	Gene Mapping	Disease or Syndrome	Affected Domain: Mutation
FGFR1	8p12	Pfeiffer	Ig IIIa: Pro252Arg
FGFR2	10q26	Apert	Ig IIIa: Ser252Trp Pro253Arg
		Crouzon	Ig IIIa: Ser267Pro Cys278Phe Gln289Pro Trp290Gly Ig IIIc: Tyr328Cys Tyr340His Cys342Tyr or Arg or Ser or Phe or Trp Ala344Ala* Ser347Cys Ser354Cys
		Pfeiffer	Ig IIIc: Asp321Ala Thr341Pro Cys342Arg or Tyr
		Jackson-Weiss	Ig IIIc: Ala344Gly Cys342Arg
		Stomach cancer	FGFR2b: amplification
FGFR3	4p16	Achondroplasia	Transmembrane domain: Gly375Cys
		Hypochondroplasia	Gly380Arg
		Thanatophoric dysplasia: type I	Tyrosine kinase domain: Asn540Lys
		Thanatophoric dysplasia: type II	Extracellular domain: Arg248Cys Ser371Cys Base substitution of stop codon
			Tyrosine kinase domain: Lys650Glu
FGFR4	5q35	Breast and ovarian tumours	Amplification

*, mutation at this position may alter splicing.

syndrome are linked to mutations in FGFR2 (Jabs et al., 1994; Reardon et al., 1994; Park et al., 1995; Steinberger et al., 1995; Wilkie et al., 1995a). In Apert syndrome, the most severe form of the mentioned skeletal disorders, two adjacent mutations on exon IIIa of FGFR2 can be found (Wilkie et al., 1995b). As for the other four known skeletal dysplasias, dominant mutations in the *Fgfr3* locus are thought to be responsible for the disorders. For instance, two mutations localized to the transmembrane domain of FGFR3 are consistently found in individuals with achondroplasia, the most common genetic form of dwarfism (Rousseau et al., 1994; Shiang et al., 1994; Bellus et al., 1995; Ikegawa et al., 1995; Stoilov et al., 1995; Superti-Furga et al., 1995). Likewise, hypochondroplasia, a condition with milder phenotypes than those seen in achondroplasia, is associated with a recurrent mutation in the proximal tyrosine kinase domain of FGFR3 (Bellus et al., 1995). More recently, extracellular domain and stop codon mutations in FGFR3 have been correlated with thanatophoric dwarfism type I (TDI), a condition featuring a cloverleaf skull, bowed long bones, platyspondyly and short ribs (Rousseau et al., 1995). A second form of thanatophoric dysplasia (TDII), characterized by the lack of a cloverleaf skull and straight femurs, is also associated with a FGFR3 mutation located in the distal tyrosine kinase domain of the receptor (Tavormina et al., 1995).

These clinical disorders are not ascribed to loss of FGFR function as individuals deleted with respect to the short arm of chromosome 4, leaving presumably one copy of FGFR3, do not have achondroplasia. Similarly, individuals with deleted chromosome 8 or partially deleted chromosome 10q which removes FGFR1 and FGFR2, do not have craniosynostosis (reviewed by Muenke and Schell, 1995). Hence, the disorders may be interpreted as gain-of-function mutations involving activation of FGFR signalling, possibly by the induction of ligand-independent receptor activation. This hypothesis is supported by a recent finding that mice deficient for FGFR3 function exhibit enhanced and prolonged bone growth, suggesting that FGFR3 signalling is involved in negatively regulating chondrocyte proliferation (Deng et al., 1996). The overlapping phenotypes observed in the syndromes may be accounted for by disturbance of possible heterodimerization between the different members of the FGFR family.

1.11 This Thesis

It is apparent from the current literature that the biological activities of the FGFs are under immensely complex and intricate regulatory controls. Regulatory control of FGF function may be effected at multiple levels by restricting (1) the expression pattern of each FGF; (2) its receptor binding specificity; (3) distribution of the interacting receptor(s) as exemplified by the FGF receptor tyrosine kinases (FGFR) or cysteine-rich FGF receptor (CFR); (4) alternative splicing profile of the expressed FGFR; and (5) expression pattern of co-factors as represented by matrix- and cell-associated heparan sulphate proteoglycans which may be either activating or inhibitory. In addition, an appropriate cocktail of secondary downstream signal transduction molecules must be present to elicit the desired biological response. Thus, the ability of FGF to induce a response is dependent on the presence of all the essential players: ligand, receptor and activating co-factor, at the right place and time.

Perhaps one of the main goals of recent times is the establishment of the role of each FGF *in vivo*, particularly as regards its participation in development. The developmental importance of several FGF genes has been determined through the use of homologous recombination to generate null alleles, dominant-negative receptors and ligand over-expression. These studies also showed that the FGFs are functionally non-redundant. The connection of FGF signalling with normal embryonic development is further strengthened by the recent finding of FGFR mutations in several human autosomal genetic diseases affecting skeletal development. Hence, there is a compelling need to elucidate the precise role and biochemistry of each member of the growing family of FGFs in embryogenesis.

As a step forward towards a greater understanding of the biology of the fibroblast growth factor family of polypeptides, this thesis attempts to unravel the biological properties of the most recently described member of the family, FGF-9. The regulation of this growth factor in terms of its receptor binding specificity and its expression patterns in the embryo and adult mouse were examined. The potential role of this growth factor as a neurotrophic factor was also analysed with respect to its distribution in the brain. The

method of gene targeting as a means for solving the role of FGF-4 in murine development was also explored and described.

Chapter 2

Materials and Methods

All basic molecular biology protocols are derived from the manual "Molecular cloning, a laboratory manual", 2nd edition by Sambrook et al. (1989). All chemicals were obtained from BDH unless otherwise specified.

2.1 Cloning and Expression of FGF-9

2.1.1 Cell Culture

Rat glioma C6 and COS cells (provided by the William Dunn School of Pathology Cell Bank) were maintained in growth medium comprising Dulbecco's MEM/Nut Mix F-12 (1:1, Gibco BRL) supplemented with 10% foetal calf serum (Globepharm), 0.12% $NaHCO_3$, 2mM glutamine and 1 unit/100 ml of penicillin (Sigma) and 0.1 mg/100 ml streptomycin (Sigma).

2.1.2 Isolation of mRNA

mRNA from C6 cells was isolated with the aid of the QuickPrep mRNA Purification Kit (Pharmacia) according to the manufacturer's instructions.

2.1.3 First Strand cDNA Synthesis

Approximately 2 µg of C6 mRNA was added to 16.5 µl of DEPC-treated water and 2 µl (1 µg) of oligo(dT) primers (Promega). The mixture was heated to 70 °C for 10 minutes, quenched on ice and added to 10 µl 5x buffer (Gibco BRL), 5 µl 0.1 M DTT (BRL), 5 µl 10 mM dNTP mix, 0.5 µl (20 units) of 40 U/µl RNasin (Promega) and 1 µl (200 units) Superscript reverse transcriptase (Gibco BRL). The reaction was carried out at 37 °C for 1 h, after which, 1 µl (1.5 units) RNase H (Promega) was added and incubated at 37 °C for 15 minutes. The reaction was terminated by heating at 95 °C for 5 minutes. The resulting cDNA mixture was extracted once with phenol/chloroform before further use.

2.1.4 Polymerase Chain Reaction and Cloning of Rat *Fgf9* cDNA

A third of the C6 cDNA preparation was amplified in a 100 µl reaction volume containing 1x buffer (Promega), 2.5 mM each dNTP, 500 ng of each primer, 2 mM $MgCl_2$ and 5 units Taq polymerase (Promega). The first round of PCR was carried out with the external primers S152-5'-CTTGTGATTAAAAGCCGAGTCCTC-3' and A888-5'-GGCACTGCT AATCAATAAGAACCC-3' under the following conditions: annealing at 40 °C for 3 min, extension at 72 °C for 3 min, and denaturation at 94 °C for 2 min, for a total of 30 cycles followed by a final extension step of 72 °C for 7 min. The second round of PCR was carried out with the internal primers S178-5'-**AAAGGATCC**ATGGCTCCCTTAGGTG AAGTTG-3' (*Bam*HI restriction site in bold) and A812-5'-**AAGAATTC**AAGGGCTC AAGTGAAGACACTGT-3' (*Eco*RI restriction site in bold) under the following conditions: annealing at 60 °C for 3 min, extension at 72 °C for 3 min, and denaturation at 94 °C for 2 min, for a total of 30 cycles followed by a final extension step of 72 °C for 7 min. A *Bam*HI and an *Eco*RI restriction sites were incorporated into the S178 and A812 primers respectively (bold). Following amplification, 5 µl 10% SDS and 1 µl proteinase K (1 mg/ml) was added to the reaction and heated at 55 °C for 30 min. The amplified material was extracted with phenol/chloroform and precipitated with ethanol followed by digestion

with *Bam*HI and *Eco*RI. The 635 bp amplified and digested band was excised from a 2% TAE gel and purified by means of the Geneclean II kit (Bio 101 inc.). The purified insert was cloned into *Bam*HI/*Eco*RI digested pBluescript KS(+) (Stratagene) and named pBSrFGF-9. Sequence analysis of the insert was carried out with T3-5'-ATTAACCCTCACTAAAG-3' and T7-5'-AATACGACTCACTATAG-3' primers, both of which are present in the pBluescript vector. The primers S178 and A812 were also used for sequencing.

2.1.5 Sequencing

Sequencing reactions were carried out using the Sequenase Version 2.0 DNA sequencing kit (USB). The protocol was as described by the supplier.

2.1.6 Screening for the Murine *Fgf9* Genomic Homologue

The mouse D3 embryonic stem cell genomic library was screened for the mouse homologue to the *Fgf9* gene with the 635 bp rat cDNA as probe. The library was plated at high density on 150-mm diameter plates and transferred onto nylon filters in duplicate. The probe was labelled using the Random Priming Labelling kit as described by the supplier (Boehringer Mannheim). Generally, about 100 ng of DNA probe was labelled with (α^{32}P)dATP (3000 Ci/mmol, Amersham). The probe was denatured by heating at 100 °C for 2 to 5 min prior to addition to the hybridization mix. Hybridization with (α^{32}P)dATP-radiolabelled probe in Church Buffer (7% SDS, 1% BSA, 0.5 M Na_2HPO_4 [pH 7.2] and 1 mM EDTA) was carried out at low stringency at a temperature of about 57 °C for 12 to 14 h. After hybridization, the filters were washed two times with 2x SSC, 0.1% SDS at the same temperature as the hybridization procedure, before being exposed to X-ray film (Kodak).

Positive clones were picked with large Gilson tips cut at the end and resuspended in 300 µl SM buffer (0.1 M NaCl, 14.5 mM $MgSO_4.H_2O$, 50 mM Tris-HCl, pH 7.5 and 0.01% (w/v) gelatin) containing 5 µl chloroform and allowed to elute for 4 h at room temperature or overnight at 4 °C. 1 µl of each eluate (the appropriate volume used being

dependent on the titre) was then added to 250 µl of *E. coli* (strain LE392) and incubated at 37 °C for 30 min before being added to 13 ml of 0.7% top agarose containing 10 mM $MgSO_4$ at 45 °C. The agarose mix was then quickly plated onto 2% base agar previously warmed to 37 °C and allowed to set before being incubated at 37 °C overnight.

Four positive clones were obtained and confirmed by Southern analyses with two probes: (1) the full-length 635 bp rat cDNA probe, and (2) a 170 bp probe spanning the non-conserved 5' sequence of the rat *Fgf9* coding sequence generated by digesting the plasmid pBSrFGF-9 (section 2.1.4) with *Bam*HI and *Dra*I. One of the clones (lambda clone 4) was subsequently cloned into pBluescript KS (Stratagene) and was labelled pBSλD3/4. A 4 kb fragment of lambda clone 4 was also obtained by digestion with *Xba*I and cloned into pBluescript KS, resulting in the plasmid pBSXbaI-4kb. This latter plasmid was subjected to digestion with *Hae*III and ligated with *Sma*I-digested pBluescript KS. Subsequent sequence analysis of the resultant plasmid, pBSλD3/4/HaeIII confirms the identity of the insert as *Fgf9* (for sequence, see Chapter 3). Primers used for the sequencing reactions include BSX4, BSXH260, BSXI2, C20/1, BSXH280, A888, T3 and T7 (for nucleotide sequences, see Appendix I).

2.1.7 Screening for the Murine *Fgf9* cDNA Homologue

The mouse kidney cDNA library in the Uni-ZAP™XR vector (Stratagene) was screened for the mouse homologue to the *Fgf9* gene with the 635 bp rat cDNA probe as in section 2.1.6. The screening procedure was as described in the instruction manual provided by Stratagene. Briefly, approximately 50 000 plaque-forming units of the phage library was mixed with 600 µl of OD_{600} = 0.5 XLI-Blue host cells, incubated at 37 °C for 15 min and then added to 10 ml of top agarose at 48 °C. The mixture was plated on 150-mm NZY plates and incubated at 37 °C for about 8 h. The plates were refrigerated at 4 °C for 2 h to chill. Duplicate nylon hybridization filters (Amersham) were then placed on each plate for 2 to 4 min. The filters were subsequently denatured with 1.5 M NaCl and 0.5 M NaOH for 2 min and neutralized with 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0). After blotting briefly

on Whatman 3MM paper, the filters were baked at 80 °C for 2 h followed by hybridization with the 635 bp rat *Fgf9* cDNA probe as described in section 2.1.6.

Putative clones were picked as described in section 2.1.6, and allowed to elute at room temperature for 6 to 8 h in 1 ml of SM buffer containing 20 µl chloroform. Secondary screening to obtain a single positive plaque was then carried out by plating about 1000 plaques per 100-mm NZY plate. Plaque lifts and hybridization were done as before.

The pBluescript phagemid can be excised from the Uni-ZAP vector by an *in vivo* excision protocol using the ExAssist/SOLR system. A single positive plaque was cored from the agar plate and added to 500 µl of SM buffer containing 20 µl of chloroform. The phage particles were eluted into the SM buffer by vortexing the mixture and incubating at room temperature for 1 to 2 h. The excision process was then carried out by first mixing 200 µl of OD_{600} = 1.0 XL1-Blue MRF' cells, 250 µl of phage stock (containing more than 1 x 10^5 phage particles) and 1 µl of ExAssist helper phage (more than 1 x 10^6 pfu/ml), followed by incubation at 37 °C for 15 min. 3 ml of LB broth was then added and incubation continued at 37 °C for 2 to 2.5 h with shaking. The cells were pelleted by centrifuging at 2000x g for 15 min. The supernatant so obtained was transferred to a fresh tube, heated at 70 °C for 15 min and centrifuged again at 4000x g for 15 min. The supernatant from this step contained the excised phagemid pBluescript packaged as filamentous phage particles and can be stored at 4 °C for 1 to 2 months. The rescued phagemids were plated by adding 100 µl of the phage stock obtained from the last step to 200 µl of freshly grown SOLR cells (OD_{600} = 1.0) and incubating at 37 °C for 15 min. 50 µl of the mixture was then plated on LB-ampicillin (100 µg/ml) agar and incubated overnight at 37 °C. Colonies that appeared on the plate contained the pBluescript double-stranded phagemid including the *Fgf9* cDNA insert. Of 5 x 10^5 plaques screened, four putative clones (no. 18, 19a, 19b and 20) were obtained. The clones were hybridized with the 170 bp *Bam*HI-*Dra*I probe (section 2.1.6) for confirmation. Sequencing reactions with a series of primers (T3, T7, C20/1, C20/2, C20/3, C20/4, C20/5, C20/6, Ex2/3', BSXH280 and A558; for nucleotide sequence, see Appendix I) revealed that clone 18 did

not contain the *Fgf9* sequence while clones 19a, 19b and 20 were identical, carrying an insert with 95% homology to rat *Fgf9* cDNA according to the BLAST homology search program (GCG Wisconsin Package Version 8.0; Altschul et al., 1990). All future work, in particular, protein expression of FGF-9, was based on clone 20, named pBSmFGF-9.

2.1.8 Construction of FGF-9 Expression Vector for Mammalian Cells

The expression plasmid pEE14 (Celltech Ltd) contains a strong cytomegalovirus promoter enhancer, a cloning site for the insertion of the mouse *Fgf9* cDNA, transcriptional signals for the processing of the cDNA transcript, and a replication origin from SV40 (Bebbington, 1991). The complete coding sequence of mouse *Fgf9*, spanning from the ATG codon to the stop codon, was generated by PCR. The 5' primer, X178-5'-**GCTCTAGA**ATGGCTCC CTTAGGTGAAGTTG-3' was designed to introduce an *Xba*I restriction site (bold) adjacent to the ATG initiation codon (underlined). The 3' primer used was A812 (see section 2.1.4) which incorporates an *Eco*RI restriction site into the PCR product. The complete coding sequence was amplified from 10 ng of the pBSmFGF-9 plasmid template in a 50 µl reaction volume containing 1x buffer (Promega), 2.5 mM each of dNTP, 500 ng of each primer, 2 mM $MgCl_2$ and 5 units AmpliTaq polymerase (Promega). The PCR conditions were: annealing at 61 °C for 1 min, extension at 72 °C for 1 min, and denaturation at 94 °C for 1 min, for a total of 25 cycles. The PCR product was subsequently digested with *Xba*I and *Eco*RI and ligated between the same sites in the pEE14 expression plasmid, yielding the plasmid pEE14mFGF-9. The insert was checked for DNA sequence errors before being used for protein expression experiments.

2.1.9 Expression of Murine FGF-9 in COS Cells

COS cells are CV-1 cells (an established line of simian cells permissive for lytic growth of SV40), transformed with SV40 defective for the origin of replication (Gluzman et al., 1980). COS cells therefore express the functional large T antigen together with all the factors required for the replication of any plasmid DNA containing the SV40 origin of

replication. These cells are transfected with the expression vector by electroporation (Chu et al., 1987). Approximately 4 x 10^7 COS cells were transfected with 40 µg of the pEE14mFGF-9 plasmid by electroporation using a Biorad gene pulser at 330 V and 500 µF. The transfected cells were allowed to stand at room temperature for 10 min before being plated onto 162 cm^2 tissue-culture flasks (Costar) containing 30 ml DME/F12 (Gibco BRL) with 10% fetal calf serum (Globepharm). The medium was replaced with 100 ml DME/F12 supplemented with 10 µg/ml transferrin (Sigma) the following day. The cells were cultured for a further 48 h before the culture medium was collected, centrifuged, and passed through a 0.22 µm filter (Costar) prior to processing for purification. The purification procedure was essentially similar to that described previously for the rMK protein (Nurcombe et al., 1992). The COS cell-conditioned medium (200 ml) was pumped onto a 1 ml Hitrap heparin affinity column (Pharmacia Biotech) which was equilibrated with about 5 volumes of 0.01 M sodium phosphate buffer pH 7. The process was carried out at 4 °C and the flow rate maintained at about 200 µl/min. The column was then washed with 10 ml of 50 mM phosphate buffer (pH 7) containing 0.5M NaCl. FGF-9 was eluted by washing the column with 5 x 1 ml of phosphate buffer (pH 7) containing 1 M NaCl. Protein products were analysed by SDS-polyacrylamide gel electrophoresis followed by silver staining. The protein concentration in the eluted fractions were assayed using the Coomassie plus protein assay reagent kit (Pierce).

A sample of FGF-9 was further purified by reverse phase high-pressure liquid chromatography (HPLC) using a poros R6/8 column. Fractions from the heparin affinity column that contained FGF-9 were applied directly onto the HPLC column at a flow rate of 2 ml/min. The protein was eluted with an acetonitrile gradient (10 to 60%) in a solvent containing 0.1% trifluoroacetic acetic acid in acetonitrile. The protein profile was determined by absorbance at 280 nm. 1 ml fractions were collected and the acetonitrile removed by Speed Vac concentrator (Savant Instruments). Samples were analysed by electrophoresis on a 12.5% SDS-polyacrylamide gel followed by staining by the silver-staining method.

2.1.10 Expression of Murine FGF-9 in 293T Cells

293T cells are 293 cells (an established line of human epithelial cells derived from the kidney) that express the functional SV40 large T antigen. 293T cells were grown in DMEM medium (Gibco BRL) supplemented with 10% fetal calf serum (Globepharm), 0.12% $NaHCO_3$, 2 mM L-glutamine (Sigma), 1 unit penicillin, 1 µg/ml streptomycin (Sigma) and 1 mM sodium pyruvate (Sigma). The cells were plated at a density of about 2 x 10^6 per 100-mm diameter tissue culture plate (Costar) the day before transfection. Calcium phosphate-mediated DNA transfection (Wigler et al., 1979) was carried out by mixing 30 µg of the pEE14mFGF-9 plasmid at a concentration of 1 mg/ml with 62 µl of 2 M $CaCl_2$ and 408 µl of water (pH 7). The mixture was then added, drop by drop, to 0.5 ml of 2x HBS buffer (1.64% NaCl (w/v), 1.19% Hepes (w/v) and 0.04% Na_2HPO_4 (w/v)) and allowed to stand for 5 min. The precipitate was then added to the previously plated 293T cells and the plates returned to the 37 °C incubator. At the end of that period, the medium was removed and replaced with 10 ml/100-mm plate of UltraCHO medium (Biowhittaker) after one rinse with PBS. The plates were then left in the incubator for the next 72 to 96 h for expression of the protein to occur. Supernatants containing the protein were subsequently collected and centrifuged at 12 000 rpm for 15 min in a Beckman J2-HS centrifuge. The clarified supernatant was treated with phenylmethylsulfonyl fluoride (PMSF) and EDTA to 1 mM final concentration and the concentration determined by Coomassie plus protein assay reagent kit (Pierce).

2.1.11 Construction of FGF-9 Expression Vector for *E. coli*

The expression vector for synthesis of FGF-9 in *E. coli* was constructed based on the pGEX-2T vector (Pharmacia). The expression vector contains the strong *tac* promoter inducible by the presence of the nonmetabolizable gratuitous inducer isopropylthiogalactoside (IPTG), the complete coding sequence of glutathione *S*-transferase, cleavage-recognition sequences for the site-specific protease thrombin, an

origin of replication obtained from the multicopy plasmid pBR322, an ampicillin selectable marker and a restriction cloning site for insertion of the *Fgf9* cDNA. The murine *Fgf9* coding sequence, deleted with respect to the N-terminal 48 amino acids, was generated by PCR using the 5' primer, L-42-5'-**AAAGGATCC**CCCGCAGTCACGGACTT-3' and the 3' primer, A812 (see section 2.1.4). The L-42 primer contains a *Bam*HI restriction site (bold) adjacent to the codon for the 49th amino acid, proline (underlined). The PCR conditions were as described in section 2.1.8. The PCR product was treated as described in section 2.1.4 and double-digested with *Bam*HI and *Eco*RI before being ligated into the pGex-2T vector. The resulting plasmid obtained was known as L-42 clone 20 and the integrity of the coding sequence was confirmed by sequencing.

2.1.12 Expression of Murine FGF-9 in *E. coli*

The murine FGF-9 protein, truncated with respect to the first 48 amino acids was produced using the pGEX-2T bacterial expression system. The protein was produced by transfecting the Ca^{2+}-competent *E. coli* strain JM109 with the plasmid L-42 clone 20 (section 2.1.11). A single colony was then inoculated into 100 ml LB medium supplemented with 100 µg/ml ampicillin and grown overnight at 37 °C. The following day, 500 ml of LB supplemented with 100 µg/ml ampicillin was seeded with the 100 ml overnight culture and shaked at 37 °C for about 3 hours until the OD_{600} reading is between 0.6 and 1.0. The culture was then cooled down to 25 °C - 30 °C by shaking in a 25 °C incubator for half an hour before induction was carried out by adding 0.1 mM IPTG (final concentration) to the culture. The culture was grown for a further 4 h at 25 °C until the OD_{600} reading is about 1.2. The cells were then spun down at 5000 rpm for 5 min at 4 °C before being resuspended in 5 ml MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3). At this point, the cell suspension may be flash-frozen in liquid nitrogen and stored at -70 °C or further processed. The protease inhibitors EDTA and PMSF (Sigma) were each added to 1 mM final concentration and the cell suspension sonicated 4 times for 15 s each time with a Soniprep 150 sonicator at an amplitude of about 12 microns. Triton X-100 (Boehringer

Mannheim) was added to 1% final concentration and vortexed. The suspension was then centrifuged at 12 000 rpm for 15 min at 4 °C. The resulting supernatant was allowed to flow through a column of 0.3 ml glutathione-sepharose beads (Pharmacia) previously equilibrated with 10 bed-volumes of MTPBS. The column was then washed once with 3 ml MTPBS, twice with 3 ml wash buffer (50 mM Tris-Cl (pH 8.5) and 150 mM NaCl) and twice with 3 ml of elution buffer (50 mM Tris-Cl (pH 8.5), 150 mM NaCl and 2.5 mM $CaCl_2$). 0.5 ml of elution buffer containing 10 µl of thrombin (1.5 mg/ml, Sigma) was then added to the beads, mixed and allowed to stand at room temperature for 5 h, with mixing every other hour. The cleaved protein was eluted by forcing the eluate from the column with a 10 ml syringe and the elution was repeated two more times with 0.5 ml of 1.5 M KCl.

2.1.13 N-terminal Sequencing and Amino Acid Analysis

The FGF-9 protein product synthesized in the mammalian cells was analysed with respect to the N-terminal sequence and amino acid composition. These analyses were performed by T. Willis (Molecular Biophysics Unit, Department of Biochemistry, University of Oxford). N-terminal sequencing was done on the Applied Biosystems 470A protein sequencer (Applied Biosystems, Warrington, UK), while amino acid analysis was carried out on the ABI 420A derivatiser/analyser (Applied Biosystems, Warrington, UK).

2.1.14 Molecular Mass Analysis

The mass of FGF-9 produced by the pGEX-2T bacterial expression system was determined by mass spectrometry using the mass assisted, laser desorption/ionisation (MALDI) technique. The experiment was carried out by the Joint Mass Spectrometry Facility (AltaBioscience, University of Birmingham).

2.1.15 Cell Proliferation Assay

The biological activities of the FGFs were monitored based on DNA synthesis in Balb/c 3T3 clone A319 cells as described previously (Sullivan and Klagsbrun, 1986). Mouse Balb/c 3T3 cells were grown in DMEM (Gibco BRL) containing 10% fetal calf serum (Globepharm) till about 50% confluence. The cells were passaged and plated onto 96-well microtiter plates (Costar) at about 5000 cells/200-µl well. The cells were left in the incubator at 37 °C for 7 to 14 days for quiescence to be reached before growth factors were added. In the bioassay, 20 µl of the growth factor sample and 1 µl of tritiated methyl-thymidine (1 µCi/µl, Amersham) were added to the medium atop the cells. After an incubation period of 36 to 48 h, the cells were treated as follows: two washes each with 0.15 M NaCl and methanol, at 5 min per wash; four rinses four with water before treatment with 5% cold trichloroacetic acid (twice, 10 min each time at 4°C); and finally after another 4 rinses in water, the cells were lysed by incubation with 200 µl per well of 0.3 N NaOH for more than 1 min. The lysates were then transferred to scintillation vials (L.I.P. Ltd) and 2 ml of OptiPhase 'HiSafe' scintillation fluid (Wallac scintillation products) added to each vial. Counting was done in a Beckman LS 1701 scintillation counter at 3 min per vial. Controls were done with recombinant FGF-2 (17 kDa) obtained from PeproTech EC Ltd.

2.2 Receptor Binding Profile of FGF-9

2.2.1 Cell Culture and Transfection

293T cells were used for the expression of the FGF receptors and the procedure was as described in section 2.1.10.

2.2.2 Construction of FGFR Expression Vector

The expression vector for soluble FGFR-Fc fusion protein was constructed based on the pIG vector (Simmons, 1993). The vector consists of the powerful cytomegalovirus (CMV) enhancer and promoter which drives expression of the foreign gene inserted at a polylinker

cloning site. Downstream from this site is the coding sequence for human IgG1 Fc (520 - 2000 nucleotide, GENBANK HUMIGCC4). A splice acceptor site exists at its 5' end while an SV40 intron and polyadenylation site exist at the 3' end of this gene fragment. When the foreign gene is designed with a splice donor site at its 3' end, it can be spliced in-frame onto the IgG1 Fc coding sequence. The foreign gene or FGFR coding sequence in this instance, is then expressed as a secreted dimeric fusion protein with the human IgG1 Fc peptide. In addition, the pIG vector contains both an SV40 and a polyoma origin of replication such that vector replication can occur in both primate cell lines (such as COS-7 and 293T cells) and murine polyoma transformed cell lines. The vector also contains elements for replication in *E. coli*. The presence of a suppressor tRNA (supF) suppresses amber stop codons in ampicillin and tetracycline resistance genes carried on a stable episome in the *E. coli* strain MC1061. This allows drug selection of bacterial cells carrying the plasmid so that the latter can be propagated in *E. coli*. Other features of the vector include an M13 origin of replication for production of single stranded plasmid templates when appropriate *E. coli* strains carrying the plasmid are superinfected with filamentous f1 phage such as M13.

The extracellular ligand binding domain of each FGF receptor, up to but not including the transmembrane domain, was isolated by PCR. The 5' primer included a *Hind*III cloning site and the Kozak sequence GCCACC (Kozak, 1991) placed immediately adjacent to the ATG start codon of each receptor, while the 3' primer included a splice donor site (ACTTACCTGT) as well as a *Bam*HI cloning site. The splice donor site allows the FGFR ligand-binding domain to be fused in-frame with the Fc fragment which contains a splice acceptor site. The plasmids containing the FGFR1c and FGFR2c cDNAs were gifts from Y. Yarden. The murine FGFR1c or FLG (Safran et al., 1990) sequence encoding the extracellular ligand-binding domain was obtained with the following set of primers: 5'FLG-5'-CCCAAGCTTGCCACCATGTGGGGCTGGAAGTGCCTCC-3'; 3'FLG-5'-ACGGA TCCACTTACCTGTCACAGCTGGTCTCTCTTCCAGG-3'. The plasmid containing the human FGFR1b-encoding sequence was a gift from S. Werner (Werner et al., 1992). The ligand binding domain of FGFR1b was generated with the following set of primers:

hFGFR1IIIbIgI-5'-CCCAAGCTTGCCACCATGTGGAGCTGGAAGTGCCTCC-3'; 3'hFGFR1IIIb-5'-ACGGATCCACTTACCTGTCATCCTGCCGGCCTCTCTTCC-3'. The murine FGFR2c or BEK (Raz et al., 1991) fragment was obtained with the following set of primers: 5'BEK-5'-CCCAAGCTTGCCACCATGGTCAGCTGGGGGCGCTTCA-3'; 3'BEK-5'-ACGGATCCACTTACCTGTATCTGGGGAAGCCGT-3'. The plasmid containing the murine FGFR2b cDNA sequence was provided by C. Dickson (Miki et al., 1992). The ligand-binding fragment of FGFR2b was obtained with the following set of primers: 5'KGFR-5'-CCCAAGCTTGCCACCATGGTCAGCTGGGGGCGCTTCAT-3'; 3'KGFR-5'-ACGGATCCACTTACCTGTATAATCTGGGGAAGCCGTGATCT-3'. The plasmid containing the coding sequence for murine FGFR3c was a gift from D. Ornitz (Ornitz and Leder, 1992). The ligand binding domain for FGFR3c was generated with the following set of primers: 5'FGFR3-5'-CCCAAGCTTGCCACCATGGTAGTCCCGGCC TGCGTGCTA-3'; 3'FGFR3-5'-ACGGATCCACTTACCTGTCTCATCAGTTTCCATCA GCTCCT-3'. The plasmid containing the murine FGFR4-encoding sequence was provided by J. MacMahon (Stark et al., 1991). The ligand-binding domain for FGFR4 was obtained with the following set of primers: 5'FGFR4-5'-CCCAAGCTTGCCACCATGGGCTGCT CTTGGCCCTGT-3'; 3'FGFR4-5'-ACGGATCCACTTACCTGTTCTGGCCTCAGGGG TTGCT-3'. The PCR fragments, after digestion with *Hind*III and *Bam*HI, were gel-purified and ligated with the pIG vector. The *E. coli* strain MC1061 was used for transformation of the ligation mix.

2.2.3 Concentration Determination of FGFR-Fc Fusion Product

Nunc Maxisorp™ plates were first coated with 100 µl per well of 1µg/ml mouse anti-human IgG1 Fc monoclonal antibody (Sigma) for 2 to 16 h at room temperature. The plates were then blocked with 150 µl/well of a 1% BSA/PBS solution for 1 h. On rinsing 2 times with PBS containing 0.05% Tween-20, 100 µl samples in triplicate were added to the wells. For the standard curve, triplicates of human IgG1 (Sigma) at concentrations of 500, 375, 250, 125, 62, 31, 16 and 8 ng/ml were used. The covered plates were incubated with

the samples and standards at room temperature for 1 h. On discarding the plate contents and washing 2 times with PBS containing 0.05% Tween-20, 100 µl/well of a horseradish peroxidase-conjugated polyclonal goat anti-human IgG Fc (1/5000 dilution in 1% BSA/PBS, Pierce) was added and the plate incubated covered at room temperature for 1 h before discarding. After washing two times each with PBS/0.05% Tween-20 and PBS, substrate for the peroxidase label was added. Colour development was effected by the addition of 100 µl/well of a solution of 1,2-phenylenediamine (OPD) [4 x 2 mg tablets of OPD (Dako Ltd) in 12 ml of 0.1 M citric acid-phosphate buffer pH 5.0 containing 35 mM citric acid and 67 mM $Na_2HPO_4.12H_2O$] containing 0.0125% final concentration of hydrogen peroxide. The colour development was allowed to occur in the dark and should be satisfactory within 10 to 15 min. The reaction was stopped by the addition of 75 µl/well of 1 M H_2SO_4. The plate was read at 492 nm on an ELISA plate reader.

2.2.4 Labelling of FGF with Biotin

A solution of Biotin-BMCC (Pierce) was prepared immediately before use by dissolving 2.5 mg Biotin-BMCC in 0.55 ml DMSO (Sigma) to yield a final concentration of approximately 8.5 mM. FGF was biotinylated by mixing with a 50-fold molar excess of Biotin-BMCC in a 200 µl reaction volume made up with PBS so that the final biotinylated-FGF concentration is approximately 100 µg/ml. The mixture was gently rocked at 4 °C for about 5 h before being dialysed against 1 l of PBS/1 mM EDTA with a 6000 - 8000 Da exclusion limit dialysis membrane (Gibco BRL) to remove any unreacted biotin. Dialysis was carried out overnight with the aid of a peristaltic pump (Pharmacia) at 4 °C. The FGF-9 protein used was prepared as in section 2.1.12 while recombinant FGF-1 (15.5 kDa) and FGF-2 (17 kDa) were obtained from PeproTech EC Ltd.

2.2.5 Receptor Binding Assay

Protein A (2 μg/ml, Sigma) was coated onto each well of a 96-well Maxisorp plate (Nunc) and incubated overnight at room temperature. To immobilize FGFR-Fc fusion proteins onto the plate, the protein A-coated wells were blocked with PBS containing 1% BSA (150 μl per well) for 2 h at room temperature, washed two times with PBS containing 0.05% Tween-20 (Sigma), incubated with FGFR-Fc fusion proteins (50μl per well) for 1 h at room temperature followed by washing two times with phosphate-buffered saline containing 0.05% Tween-20. For the direct binding assay, the FGFR-Fc-coated plate was next incubated with 100 μl per well of binding buffer (DMEM medium containing 0.5% crystalline BSA) containing varied concentrations of biotinylated FGF and 10 - 25 μg/ml heparin (Sigma). For the competition assay, the FGFR-Fc-coated plate was incubated with binding buffer containing a fixed concentration of biotinylated FGF and 10 - 25 μg/ml heparin in the presence of varied concentrations of unlabelled FGF. After incubation at 4 °C overnight, the plates were washed twice with PBS containing 0.05% Tween-20 followed by the addition of 100 μl of streptavidin-horseradish peroxidase conjugate (Amersham, diluted one thousand times in PBS) per well. After incubation at room temperature for 1 h, the plate was washed two times with PBS and colour development achieved as described in section 2.2.3. The amount of biotinylated FGF bound was plotted against the concentration of the competitor and the dose that inhibits maximum binding by 50% (ID_{50}) was calculated.

2.2.6 Purification of FGFR-Fc Fusion Proteins

Protein A-Sepharose was equilibrated by washing the column sequentially with 5 ml of 0.1 M Tris HCl, pH 8.0, 5 ml of 0.1 M citrate buffer, pH 3.0 and 5 ml of PBS. The 293T culture supernatant containing the FGFR-Fc fusion protein (50 ml) was centrifuged at 12 000 rpm for 15 min at 4 °C before being loaded onto 300 μl of protein A-Sepharose® 4 Fast Flow (Pharmacia Biotech) column two times. The Fc-fusion protein was eluted in 8 fractions. 300 μl of 0.1 M citrate buffer, pH 3.0. 30 μl of 1 M Tris base, pH 10 was added

immediately to each eluted fraction to neutralize the pH of the protein solution. The appropriate fractions containing the highest protein concentrations were dialysed against PBS using the micro-dialysis apparatus (Pharmacia) at 4 °C. Sodium azide was added to each fraction to 0.02% and the protein concentration assayed by both Coomassie assay and ELISA as described in section 2.2.3.

2.2.7 Surface Plasmon Resonance Analysis

All experiments were performed on a BIAcore™ 2000 biosensor (Pharmacia Biosensor AB, Uppsala, Sweden) at 25 °C.

2.2.7.1 Kinetic Determination of FGF-9-FGFR3c Interaction

FGFR3-Fc dimers were immobilized to the sensorchip CM5 (Pharmacia Biosensor AB). The hydrogel matrix was first activated by injecting N-hydroxysuccinimide (NHS) and N-ethyl-N'-[3-(diethylamino)propyl]carbodiimide (EDC) (Pharmacia Biosensor AB) over the chip surface. 35 µl of FGFR3-Fc (100 µg/ml) in 10 mM sodium acetate (pH 5) was then injected at a flow rate of 5 µl/min. Unreacted sites on the matrix was subsequently blocked with 1 M ethanolamine hydrochloride (pH 8.5) (Pharmacia Biosensor AB). In this way, the amount of receptor covalently immobilized to the chip surface was measured as approximately 9000 RU.

Binding reactions were performed at a constant flow rate of 5 µl/min with HEPES buffer (HBS: 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA and 0.005% surfactant P20). Samples of FGF-9 (see section 2.2.12) at varying concentrations (12.5, 25, 50, 100 and 200 nM) and diluted accordingly in HBS, were injected sequentially over the sensorchip surface containing immobilized FGFR3. Each injection volume was 30 µl and contained 25 µg/ml heparin (Sigma). In addition, each injection step was linked to a regeneration step consisting of the injection of 10 µl of 10 mM HCl once a stable HBS reading was obtained at the end of each binding interaction. This latter step brought the signal back to almost the baseline level. Each binding interaction (including the association

and dissociation processes) was measured continuously as data points and evaluated as described in section 4.3.7.1.

2.2.7.2 Receptor Specificity of FGF-9

Immobilization of biotinylated FGF-9 to sensorchip SA5 (contains streptavidin immobilized on the surface; Pharmacia Biosensor AB) was performed by injecting 35 µl of biotinylated FGF-9 (100 µg/ml, in PBS, section 2.2.4) over the sensor chip surface at a flow rate of 5 µl/min. The amount of immobilized material was determined to be about 50 RU.

Each binding cycle was carried out with a constant flow of HBS buffer at a flow rate of 5 µl/min. Purified samples of FGFRs (FGFR3c-, FGFR1c-, FGFR2c-, and FGFR1b-Fc), each diluted to 100 µg/ml with HBS buffer, were injected across the surface of the chip sequentially via an automated sampling port. The injection volume of each FGFR was 10 µl at a constant flow rate of 5 µl/min. No regeneration of the surface layer was required as the signal returned to baseline level at the end of each injection cycle. Positive binding was indicated by the presence of the association phase in the sensorgram obtained.

2.2.7.3 Heparin Dependence of FGF-2 Interaction with FGFR

Biotinylated FGF-2 (100 µg/ml, 35 µl) was immobilized to sensorchip SA5 and the binding conditions were as described before (section 2.2.7.2). The amount of immobilized material was determined to be 2100 RU. Samples of FGFR1b-Fc, FGFR1c-Fc, FGFR2c-Fc and FGFR3c-Fc (30 µl-volume each, all at 200 µg/ml except for FGFR1b-Fc which was applied at 100 µg/ml) in the absence or presence (10 µg/ml) of heparin were injected over the sensorchip surface coated with FGF-2. Regeneration of the surface after each injection cycle was achieved by injecting 5 µl of 1 mM HCl.

2.3 Expression Pattern of *Fgf9*

2.3.1 Cell Cultures

E14 embryonic stem cells were maintained as described by Rathjen et al. (1990). ES cell medium consisted of DMEM/Nut Mix F-12 (1:1, Gibco BRL) supplemented with 15% foetal calf serum (ICN Flow), 1% each of glutamine and penicillin/streptomycin (Sigma), 0.12% $NaHCO_3$, 100 μM betamercaptoethanol (Sigma, tissue-culture grade) and 20 ng/ml recombinant leukaemia inhibitory factor (Smith et al., 1988). 1009 EC cells were maintained in growth medium comprising DMEM/Nut Mix F-12 (1:1, Gibco BRL) supplemented with 10% foetal calf serum, 1% glutamine, 1% penicillin/streptomycin and 0.12% $NaHCO_3$.

E14 ES cell differentiation was achieved either by treatment with 5 μM retinoic acid (Sigma) for 5 days (in the presence or absence of leukaemia inhibitory factor) or by culture in the absence of leukaemia inhibitory factor (Smith et al., 1988). Differentiation of 1009 EC cells was carried out by plating the cells at a density of 8 x 10^5 cells per 75 cm^2 flask and adding 5 μM of retinoic acid (final concentration) to the medium the following day.

mRNAs from the ES and EC cell lines (differentiated and undifferentiated inclusive) were prepared using the QuickPrep mRNA Purification Kit (Pharmacia) as mentioned in section 2.1.2.

2.3.2 Isolation of Total RNA from Tissues

C57BL/6 mice were sacrificed by cervical dislocation at various stages of pregnancy and the embryos were dissected from the uterus. The age of the embryo or foetus was counted from the day the copulation plug was observed. This was taken to be 0.5 days post coitum (0.5 d.p.c.). Newborn mice (20.5 d.p.c.) were killed by cervical dislocation. Various adult mouse organs including the brain, heart, lungs, kidneys and ovaries were also collected. The tissues were immediately frozen in dry ice and stored at -70 °C until used for RNA isolation.

Total cellular RNA was isolated as described previously (Chomczynski and Sacchi, 1987). About 0.5 g of tissue was homogenised on ice in 5 ml of a guanidinium solution containing 4 M guanidinium isocyanate and 25 mM sodium citrate with 0.72% of ß-mercaptoethanol added just prior to use. The following solutions were then added in order, mixing well between additions: a solution of sarcosyl to 0.5% final concentration, 1/10 volume of 2M sodium acetate at pH 4.0, an equal volume of water-saturated phenol and 1/5 volume of chloroform/isoamyl-alcohol (49:1 ratio). The mixture was agitated vigorously for 10 s and placed on ice for 15 min before being centrifuged at 8000 rpm for 20 min at 4 °C. The upper aqueous layer containing the RNA was transferred to a fresh tube and an equal volume of isopropanol added. The precipitation was carried out at -20 °C for more than 1 h. After centrifugation at 8000 rpm for 20 min at 4 °C, the pellet was dissolved in 1.5 ml of the guanidinium solution and re-precipitated with an equal volume of isopropanol at -20 °C for more than 1 h. The pellet was washed with 70% ethanol, air-dried and finally resuspended in an appropriate volume of DEPC-treated water, typically 100 to 200 µl per sample.

2.3.3 RNase Protection Analysis

The RNase protection analysis procedure was based on the RPA II kit supplied by AMS Biotechnology. The *Fgf9* antisense probe was derived from the genomic clone pBSλD3/4HaeIII (see section 2.1.6). The portion of the insert containing part of intron II and all of exon III stretching from base 441 to 812 was amplified by PCR with the primers I441-5'-**TCCCCCGGGGG**AATGGAGAATATTGCTTTTTAA-3' and A812 (section 2.1.4). I441 included a *SmaI* site (bold) while A812 contained an *EcoRI* site. On double-digestion with *Sma*I and *Eco*RI, the PCR fragment was cloned into pBluescript KS(+) and the sequence confirmed by sequencing. On linearising with *Sma*I, the *Fgf9* antisense riboprobe was synthesized by mixing the following reagents in order: 2 µl of 5x transcription buffer (Boehringer Mannheim), 1 µl of 100 mM DTT (Boehringer Mannheim), 0.5 µl of 40 U/µl RNAsin, 2 µl of nucleotide mix containing 2.5 mM each of

rATP, rUTP and rGTP, 1.5 µl of 0.75 µg/µl DNA template (described above), 5 µl of 40 µCi/µl (α-^{32}P)CTP (Amersham) and 2.5 µl T3 RNA polymerase (Promega). The mixture was incubated at 30 °C for 1 h. A control probe for detection of ß-actin mRNA was also synthesized as above. After the 1 h incubation, 1 µl of RNase-free DNase (Boehringer Mannheim) was added and the mixture incubated at 37 °C for 15 min. 5 µl of gel loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue and 2 mM EDTA, AMS Biotech) was then added, heated to 80 °C for 5 min and the whole mixture loaded onto a 6% polyacrylamide gel. The gel was run till the leading dye band (bromophenol blue) was approximately 60% to the end of the gel. The gel was then covered with a plastic wrap and exposed to an X-ray film (Fuji) for 30 s. The location of the probe on the gel was marked, excised with a razor bade and transferred to a fresh eppendorf tube. 350 µl of elution buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS, AMS Biotech) was added to the gel slice and elution was carried out by incubating at 37 °C overnight. The amount of radioactive label in 1 µl of the eluted probe was determined in a Beckman LS 1707 scintillation counter. Approximately 2 x 10^4 cpm and 2 x 10^5 cpm of the ß-actin and FGF-9 probes were used respectively for each hybridization reaction.

For the hybridization reaction, 50 µg of total RNA sample was mixed with 1 µl of probe in screw-cap eppendorfs. For each probe used, two control tubes were set up containing 10 µg of yeast RNA and 1 µl of probe. NH_4OAc was added to a final concentration of 0.5 M followed by 2.5 volumes of ethanol. On mixing thoroughly, the tubes were placed in a -20 °C freezer for 15 min. The RNAs were pelleted by centrifuging at 15 000 rpm for 15 min at 4 °C. After the ethanol supernatant was removed carefully with a drawn-out pasteur pipette, the pellets were dissolved in 20 µl of hybridization buffer (80% deionized formamide, 100 mM sodium citrate pH 6.4, 300 mM sodium acetate pH 6.4 and 1 mM EDTA, AMS Biotech) and vortexed briefly. The tubes were heated to 90 ± 5 °C for 4 min to denature the RNAs before incubation at 45 °C for 18 h for hybridization to occur.

For RNase digestion of the hybridized probe and sample RNA, a solution containing 250 units/ml RNase A and 10 000 units/ml RNase T1 (solution R, AMS

Biotech) was diluted 100 times in solution Bx (AMS Biotech). 200 µl of this diluted RNase mixture was added to each hybridization reaction and one of the yeast RNA control tubes while 200 µl of solution Bx was added to the other yeast RNA control tube. The tubes were incubated at 37 °C for 30 min to digest the unprotected single-stranded RNA. 300 µl of solution Dx was then added to stop the RNase reaction and to precipitate the protected RNA fragments. The tubes were transferred to a -20 °C freezer for at least 15 min followed by centrifuging at 15 000 rpm for 15 min at 4 °C. All supernatant was removed with a drawn-out pasteur pipette and the protected RNA fragments resuspended in 8 µl of gel loading buffer (AMS Biotech). The solution was heated to 90 ± 5 °C for 4 min before being loaded onto a 5% polyacrylamide gel. For the control reaction not subjected to RNase treatment, only 1 µl of the sample was loaded on the gel. About 10 cps of each probe was also loaded to check the integrity of the probe.

2.3.4 *In Situ* Hybridization Analysis

2.3.4.1 Embryo Preparation

C57BL/6 mice at various stages of pregnancy were sacrificed by cervical dislocation and the embryos retrieved by dissecting into PBS. Conception was defined as midnight prior to plug detection. The embryos were fixed in 4% paraformaldehyde/PBS at 4 °C overnight. After fixation, the embryos were taken through several sequential washes: (1) 2 times in PBT (0.1% Triton X-100/PBS) for 10 min each; (2) 25% methanol/PBT for 20 min; (3) 50% methanol/PBT for 20 min; (4) 75% methanol/PBT for 20 min; and (5) two times in 100% methanol for 20 min each. The dehydrated embryos were then stored in 100% methanol at -20 °C. On the day of use, the embryos were rehydrated by taking them through the above washing steps in reverse order followed by washing three times with PBT for 10 min each.

2.3.4.2 Whole Mounts

Whole-mount *in situ* hybridizations were carried out based on a modified form of the protocol described previously (Wilkinson, 1992). The template for generating the *Fgf9* sense and antisense riboprobes is the plasmid pBSmFGF-9. The plasmid pBSmFGF-9 (see section 2.1.7) contains the complete coding sequence of murine FGF-9 between the cloning sites *EcoR*I and *Xho*I in the pBluescript SK(-) vector. The antisense probe was generated by linearising the plasmid with *EcoR*I and using T7 RNA polymerase in the transcription reaction. Conversely, the same plasmid digested with *Xho*I or *Bgl*II was used to transcribe the control sense probe with T3 RNA polymerase. Both sense and antisense probes were labelled with digoxygenin (DIG) by transcription from the template. The transcription reaction was carried out by mixing the following reagents in order: 9.5 µl DEPC-treated water, 4 µl of 5x transcription buffer (Promega), 2 µl of 0.1 M DTT (Promega), 2 µl of 10x DIG RNA labelling nucleotide mix (Boehringer Mannheim), 1 µl of 1µg/µl linearised plasmid, 0.5 µl of 40 U/µl placental RNase inhibitor (Boehringer Mannheim) and finally 1 µl of 20 U/µl RNA polymerase (Promega). The reaction was carried out at 37 °C for 2 h, after which 2 µl of 10 U/µl DNase I (Boehringer Mannheim) was added and incubated at 37 °C for 15 min. The transcripts were then precipitated by addition of 100 µl TE, 10 µl of 4 M LiCl and 300 µl of ethanol and left at -20 °C for 30 min, before being centrifuged at 14 000 rpm for 10 min. The pellet was washed once with 70% ethanol, air-dried, resuspended in 100 µl ice-cold TE and taken through the LiCl/ethanol precipitation step again. Finally, the pellet was resuspended in 50 µl ice-cold TE to yield a concentration of approximately 0.1 µg/µl and stored at -20°C. 2 to 10 µl of this transcription solution was used per ml of hybridization mix.

Before probe hybridization, the rehydrated embryos (see section 2.3.4.1) were treated with 10 µg/ml proteinase K in PBT. The length of time of the latter treatment was dependent on the age of the embryos. For examples, 6.5 and 7.5 d.p.c. embryos were exposed to proteinase K for up to 2 min while 11.5 d.p.c. embryos were treated with the enzyme for 20 to 30 min. The enzyme reaction was stopped by washing the embryos twice

with PBT for 5 min each, followed by refixation with 0.2% glutaraldehyde/4% paraformaldehyde/PBT (Sigma) for 20 min. After washing two times with PBT for 10 min each, the embryos were prehybridized with 1 ml of prehybridization mix (50% formamide, 5x SSC, 2% Boehringer Blocking powder, 0.1% Triton X-100, 0.5% CHAPS (Sigma), 1 mg/ml yeast RNA (Boehringer Mannheim), 5 mM EDTA (Sigma), and 50 µg/ml heparin (Sigma)), allowed to equilibrate, and then replaced with 1 ml of fresh prehybridization mix for incubation at 65 °C overnight. For hybridization, whole-mount embryos were hybridized to sense or antisense DIG-labelled *Fgf9* riboprobes in 0.5 ml fresh hybridization mix at 65 °C overnight. The post-hybridization washes consisted of the following steps: (1) 100% solution 1 (50% formamide, 5x SSC, 0.1% Triton X-100, 0.5% CHAPS) for 5 min; (2) 70% solution 1/30% 2x SSC for 5 min; (3) 30% solution 1/70% 2x SSC for 5 min; (4) two times with 2x SSC/0.1% CHAPS for 30 min; (5) two times with 0.2x SSC/0.1% CHAPS for 30 min each, and finally (6) two times with TBT (50 mM TrisHCl, 150 mM NaCl, 0.1% Triton X-100, pH 7.5) for 10 min each. Steps (1) to (5) were carried out at 65 °C while the last step (6) was done at room temperature. The embryos were preblocked with 10% sheep serum (Sigma) in TBT containing 2% BSA for 2 to 3 h at room temperature. This was replaced with anti-DIG antibody (Boehringer Mannheim) (previously preabsorbed with embryo powder in preblocking solution) and incubated at 4 °C overnight, with rocking. The antibody was removed by five washings with 0.1% BSA/TBT at room temperature for 1 h each, and followed by an overnight wash at 4 °C. On the next day, the embryos were washed two times in TBT for 30 min each and equilibrated with NTM (100 mM NaCl, 100 mM TrisHCl, 50 mM $MgCl_2$, pH 9.5) by washing the embryos three times in it, 10 min each. Colour development was achieved by incubating the embryos with NTM containing 0.45% (v/v) nitro blue tetrazolium (NBT) and 0.35% (v/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (X-phosphate; Boehringer Mannheim) in the dark, rocking for the first 20 min and allowing to stand thereafter until the colour has developed to the desired extent. The embryos were then washed with PBT and in some instances, washed overnight with 1% Triton X-100/PBS at 4 °C to reduce

background. The embryos were then fixed with 4% paraformaldehyde/PBT at 4 °C overnight, washed with PBT and photographed in 30% glycerol/PBT.

2.3.4.3 Frozen Embryo Sections

In situ hybridizations on frozen sections were performed using the single stranded DIG-labelled RNA probes as described in section 2.3.4.2. Embryos pretreated as in section 2.3.4.1 were incubated with 30% glycerol/PBS at 4 °C for at least 24 h before being embedded in O.C.T. and cryosectioned to a thickness of 15 µm.

The sections were prehybridized in hybridization buffer containing 50% formamide, 1.3x SSC, 10% dextran sulphate, 1 mg/ml yeast RNA (Boehringer Mannheim) and 1x Denhardt's solution for more than an hour at 65 °C. Hybridization was performed with 150 µl of hybridization buffer containing the appropriate probe, covered with a coverslip and incubated in a humidified chamber at 65 °C overnight. Following this step, the slides were subjected to several washing steps: (1) three times in a washing solution containing 1x SSC, 50% formamide and 0.1% Tween-20 at 65 °C for 30 min each; and (2) two times in MABT (100 mM maleic acid (Sigma), 150 mM NaCl, 0.1% Tween-20, pH 7.5) at room temperature for 30 min each, with agitation. The slides were then preblocked with 700 µl blocking solution (20% sheep serum and 2% Boehringer Blocking powder in MABT) per slide for at least two hours at room temperature. Following this step, 300 µl of anti-DIG antibody (diluted 1000x in blocking solution, Boehringer Mannheim) was added per slide and incubated in a humidified chamber at room temperature. On the next day, the slides were washed 5 times with MABT for 1 h each, followed with two more washings in NTMT for 10 min each. The colour reaction was performed as before (section 2.3.4.2) and stopped by immersing the slides in PBW containing 1 mM EDTA. For mounting in DPX, the sections were first dehydrated by washing the slides sequentially in 25% methanol/PBT, 50% methanol/PBT and two times in 100% methanol for 2 min each followed by a 4 minute treatment with xylene.

Anatomical positions of the mouse embryo were identified based on the description by Kaufman (1992).

2.3.4.4 Double In situ Hybridization on Adult Mouse Brain Sections

The probe for *Fgfr3* was generated as described in a previous report (Peters et al., 1993). The cDNA fragment encoding for the transmembrane and juxtamembrane parts of FGFR3 was generated by PCR with the following set of primers: mR3TJ5'-5'-GGAATTCCCTGCAGGCGCTAACACCACCGA-3'; mR3TJ3'-5'-TCCCCCGGGGGA CATTGGCCAGAACAGGACCTTCTCC-3'. The mR3TJ5' primer has incorporated an *Eco*RI site while the mR3TJ3' primer a *Sma*I site to the FGFR3 cDNA fragment which was then cloned into the pBluescript KS vector. To obtain labelled antisense and sense transcripts, the plasmid was linearised with *Eco*RI or *Sma*I and transcribed using T7 or T3 RNA polymerase (Promega) respectively. Each of the linearised plasmid was first purified from low melting-point agarose by Wizard DNA Clean-Up System (Promega) yielding a DNA solution of concentration 1 µg/µl. The transcription reaction was as described above (section 2.3.4.2) and the riboprobes for *Fgf9* and *Fgfr3* were labelled with either DIG or fluorescein. To obtain fluorescein-labelled probes, the fluorescein RNA labelling nucleotide mix (Boehringer Mannheim) was used instead of the DIG mix.

Mouse adult brains were fixed overnight in 4% paraformaldehyde/PBS, cryoprotected by immersion in 15% sucrose/PBS overnight at 4 °C and embedded in O.C.T. for cryosectioning. Prior to hybridization, the sections were treated with 1 µg/ml proteinase K for 10 min at 37 °C. This enzyme reaction was stopped by immersing sections in 0.2% glycine/PBS for 30 min at room temperature. On washing twice with PBS, the sections were fixed in 4% paraformaldehyde/PBS for 15 min at room temperature followed by two other washings with PBS. The *in situ* hybridization method used here is similar to that described above for frozen embryo sections (section 2.3.4.3) and modified from the protocols of D. Henrique, P. Ingham and D. Ish-Horowictz (ICRF Developmental Biology Unit, University of Oxford), R. Conlon (Mt. Sinai, Toronto) and D. Wilkinson (NIMR,

London). Double *in situ* hybridization was carried out by adding hybridization buffer (50% formamide, 1.3x SSC, 5 mM EDTA (Sigma), 1 mg/ml yeast RNA (Boehringer Mannheim), 10% Tween-20 (Sigma), 10% CHAPS, 100 µg/ml heparin (Sigma)) containing a combination of DIG-labelled *Fgf9* RNA probe and fluorescein-labelled *Fgfr3* RNA probe or the same probes labelled in the reverse manner. Post-hybridization washes were done in the following order: three rinses in pre-warmed hybridization mix; two washes with the same hybridization mix at 60 °C for 30 min each time; one wash with pre-warmed hybridization mix/MABT (1:1) for 20 min with rocking; three rinses with MABT; and finally two washes with MABT for 30 min each time, with rocking. After incubation with MABT containing 2% Boehringer Blocking Reagent (BBR) for 1 h, the sections were pre-blocked with MABT containing 2% BBR and 20% serum for 2 h. For detection of the first probe, either anti-DIG (diluted from 2000x to 8000x, Boehringer Mannheim) or anti-fluorescein (diluted from 1000x to 8000x, Boehringer Mannheim) antibody was incubated with the sections. The application of the anti-fluorescein antibody required the use of 20% heat-inactivated horse serum (Sigma) instead of the goat serum in the blocking solution. The first antibody was always detected by the NBT-BCIP reaction. When the colour development has reached the desired level, the staining reaction was stopped by two washes in PBT containing 10 mM EDTA. The alkaline phosphatase activity of the first antibody was then inactivated by incubation in 100 mM glycine/HCl pH 2.2 for 1 h. After washing in MABT and preblocking, the slides were incubated with the second antibody for the other probe. The alkaline phosphatase tag of this second antibody was then detected by fluorescence histochemistry. The sections were washed 4 to 6 times in MABT for 20 min each at room temperature and followed by three times in ELF washing buffer (Molecular Probes Europe) for 5 min each. The ELF substrate working solution was prepared fresh by mixing the substrate reagent and additives in developing buffer, all provided in the kit (Molecular Probes Europe). 100 to 200 µl of the staining solution was then added to the slides and the reaction allowed to develop in the dark overnight. When the fluorescence has developed to the desired extent, the slides were washed in the dark with PBT containing 10 mM EDTA for three times, 5 min each. The sections were then fixed in post-fix solution

(2% formaldehyde in PBS, 20 mg/ml BSA) for 30 min, washed quickly with PBS and mounted in ELF Mounting Medium provided by the kit.

Photography was performed using Leitz microscopes with Fuji RTP or Kodak Ektachrome 64T professional slide film.

2.4 Targeted Disruption of the *Fgf4* Gene

2.4.1 ES Cell Culture

BL-6/III ES cells derived from C57BL6 mice were maintained in the undifferentiated state by growth on *neo* resistant STOOB500 fibroblasts (irradiated at 195 rad/min for 1 hr) in DMEM/F-12 medium (Gibco BRL) supplemented with 15% foetal calf serum (Boehringer Mannheim), 10^{-4} M betamercaptoethanol (Sigma) and 20 ng/ml of recombinant leukaemia inhibitory factor (LIF). The cells were fed with fresh medium containing LIF everyday and grown in a humidified atmosphere with 5% CO_2 at 37 ^{o}C.

2.4.2 Plating Efficiency Assay

500 and 1000 ES cells were separately plated in duplicate on 10-mm tissue culture plates and cultured as described above. The cells were allowed to grow for 5 days before the colonies were counted.

2.4.3 Transfection Efficiency Assay

Both E14GT2a and BL-6/III cell lines were tested for transfection efficiency. 1.34 x 10^7 E14GT2a cells (passage 21) were used per electroporation, while 1.41 x 10^7 BL-6/III cells (passage 30) were used per electroporation. The electroporation conditions tested were: 200 V, 960 μF; 240 V, 250 μF; 240 V, 500 μF; 240 V, 960 μF; and 800 V, 3 μF. The plasmid (uncut) used for the transfections contained the *lacz* gene driven by a *pgk* promoter. Controls for each cell line were done using water in place of the plasmid, with electroporation conditions of 240 V, 500 μF to ensure that there was no nonspecific

staining. The cells were suspended in 1 ml of medium for each electroporation. After electroporation, another 1 ml of medium was added to each and all of the contents plated equally on two plates, i.e. 1 ml per plate (or about 0.7 x 10^7 cells per plate in duplicate). The cells were allowed to grow for 2 nights before being fixed and stained with X-Gal (section 2.4.4). The plates were stained overnight at 37 °C and blue colonies counted the next day. As it was observed that only one or two cells per colony were stained, each staining cell was considered as the result of a successful transformation event. So, each staining "colony" as such actually refers to one or a group of staining ES cells within a colony. The transfection efficiency represents the number of staining "colonies" per number of cells electroporated.

2.4.4 X-Gal Staining

The presence of the *lacz* gene product was detected by staining with the substrate X-Gal (Boehringer Mannheim). The cells transfected with the *lacz* gene was first rinsed two times with PBS followed by treatment with a fixative (0.2% glutaraldehyde, 0.1 M phosphate buffer, 2 mM $MgCl_2$, 5 mM EGTA, pH 7.3). The cells were then washed three times in wash solution (0.1 M phosphate buffer, 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.02% NP40, 0.05% BSA, pH 7.3) for 20 min each at room temperature. The staining reaction was carried out in the dark, at 37 °C overnight with the staining solution (50 mg/ml X-Gal/dimethylformamide in wash solution containing 0.24% spermidine, 5 mM $K_3Fe(CN)_6$, 5.5 mM $K_4Fe(CN)_6$, and 0.0013% NaCl).

2.4.5 Determination of the Lower Limit of G418 Selection

1.4 x 10^5 BL-6/III cells were plated onto each well of a 6-well plate (each previously seeded with irradiated STO fibroblasts). In practice, 6 x 10^4 cells/well will suffice for the experiment. The cells were allowed to recover for a day, after which, each well was treated with medium containing 350, 300, 250, 200, 150, 100 and 50 µg/ml respectively of G418

(Sigma). Each concentration test was carried out in duplicate. A duplicate control was done without G418 treatment. The selection was stopped on the 10th day of culture. The cells were fixed first with 2 to 3 drops of acetic acid for 2 to 3 min and then again with 1 ml of acetic acid for 10 min followed by staining with Giemsa for 15 min. After carefully washing off the stain with water and drying, the colonies can be observed as darker staining spots against a background of faintly staining feeder layer.

2.4.6 Electroporation and Selection of ES Cells

3 x 10^7 BL-6/III cells (passage 21) were transfected with 50 µg of the insert from the plasmid PS1 digested with *Kpn*I and *Not*I by electroporation with the Bio-Rad Gene Pulser set at 250 V and 500 µF. The time constant of the electroporation was 7.9. On allowing the cells to recover for 5 to 10 minutes, the cells were diluted 10 times so that about 10^6 cells were seeded onto each of a 100-mm tissue culture plate (each previously seeded with 1/2 of a 3/4-confluent flask of irradiated feeders). One control plate was seeded with cells electroporated with 20 µg of the plasmid pPGKneoß (time constant 8.8). 24 h after electroporation, the cells were fed with medium containing 150 µg/ml G418. The G418 concentration was reduced to 100 µg/ml on the 13th day of selection. Selection was continued for up to 16 days.

On the last day of the selection process, the plates were washed twice with PBS. On covering each plate with 5 ml of PBS, each colony was picked with a Gilson tip into a well of a 96-well plate containing 50 µl of 1/10 diluted TVP (13.7 mM NaCl, 2.7 mM KCl, 8 mM KH_2PO_4, 25% (w/v) trypsin, 37% (w/v) EDTA, 1% (v/v) chick serum, pH 7.4). After waiting for about 15 mins, 50 µl of fetal calf serum (Boehringer Mannheim) was added to each well and the mixture pipetted up and down 20 times to disperse the colonies. The content of each well was then transferred to a well on a 48-well plate that had previously been seeded with 3 x 10^4 of irradiated *neo*-resistant STO fibroblasts and containing 500 µl of LIF-containing medium. The cells in each well were allowed to grow to reasonable confluence before being transferred to a 24-well plate (similarly plated with

feeder cells) and allowed to grow. Half the cells from each clone in the 24-well plate was then frozen down and the rest transferred to a well of a 6-well plate to expand the clone so that sufficient cells are obtained for genomic DNA extraction. For the latter purpose, the cells were grown in LIF without feeders.

2.4.7 Southern Blot Analyses

Genomic DNA was prepared from each clone by the dimethylformamide method. ES cell pellets were washed two times with phosphate-buffered saline (PBS) and lysed in 200 µl of 500 µg/ml proteinase K (Boehringer Mannheim), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8), 0.1 M NaCl and 1% SDS at 37 °C overnight. The DNA was then precipitated by the addition of 1 ml of 5% dimethylformamide in 70% acetone, washed two times with 70% ethanol and finally dissolved in an appropriate volume of TE.

Genomic DNA (10 µg) was digested with *Bam*HI or *Sac*I (Boehringer Mannheim) for 12 h and subjected to agarose gel (0.8%) electrophoresis. Following gel denaturation in 1.5 M NaCl and 0.5 M NaOH and neutralization in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2) and 1 µM EDTA, the DNA was transferred onto nylon filters (Amersham) in 20x SSC and baked at 80 °C for 2 h. Filters was hybridized with (α^{32}P)dATP-radiolabelled probes in Church buffer (7% SDS, 1% BSA 0.5 M Na_2HPO_4 [pH 7.2] and 1 mM EDTA) for 12 - 14 h. Filters were washed two times with 2x SSC, 0.1% SDS at 65 °C and two times with 0.5x SSC, 0.1% SDS at 65 °C at 15 min per wash, before being exposed to film (Kodak).

2.4.8 Preparation of Screening Probe

A plasmid p18FNPCR was made to prepare the screening probes. It contained a 1.9 kb *Xba*I-*Sac*I fragment of genomic *Fgf4* DNA that included only the third exon (Brookes et al., 1989b). This fragment was preceded by a 273 bp *Sph*I-*Hinc*II fragment of *neo* derived from the plasmid pMC1Neo (Stratagene), inserted in the same orientation. Both fragments were cloned into PUC18.

2.4.9 Mapping the 3' End of *Fgf4*

BL-6/III wild-type genomic DNA was digested with a series of enzymes: *Bam*HI, *Eco*RI, *Hind*III, *Sac*I, *Sal*I, *Bam*HI + *Eco*RI, *Bam*HI + *Hind*III, *Bam*HI + *Sac*I, *Bam*HI + *Sal*I, *Eco*RI + *Sac*I, *Hind*III + *Sac*I and *Hind*III + *Sal*I and subjected to agarose gel electrophoresis. DNA was transferred onto nylon filters as described above (section 2.4.8) and hybridized with the 327 bp *Dra*I-*Sac*I probe.

2.4.10 Karyotype Analysis

It is important that the ES cells are in a state of active growth to obtain sufficient mitotic spreads. Good results were obtained using cultures sub-cultured 1 to 2 days before harvest. Colcemid (Sigma) was added to a final concentration of 0.05 µg/ml to each cell sample grown on a well in a 6-well plate. The plates were incubated at 37 °C for one hour. The cells were then trypsinized and resuspended in 5 ml of 0.56% (w/v) KCl solution. The treated cells were left at room temperature for 12 min. At the end of the incubation, the cells were pelleted by gentle centrifugation at 500 rpm for 5 min. After most of the KCl was aspirated, 5 ml of freshly prepared ice-cold Carnoy's fixative (volume of absolute methanol: glacial acetic acid is 3:1) was rapidly added to avoid cell clumping. The cells were then pelleted and the fixative was changed. This last step was repeated one more time before resuspending the cells finally in 0.5 - 1 ml of fixative. Single drops of the cell suspension were then applied onto pre-cleaned slides, ensuring that the drop spread rapidly and evenly. The slides were air-dried and observed under phase contrast (200x magnification) to check the spreads. For counting the chromosomes, the slides were stained with 3% Giemsa in PBS for 13 to 15 min and then washed with distilled water. On air-drying the slides, each slide was mounted with a coverslip with 1 drop of DPX solution and left to set overnight. The chromosome spreads were counted using a magnification of 1000x under oil immersion.

Chapter 3

Cloning & Expression of Mouse FGF-9

Summary

Fibroblast growth factor-9 (FGF-9) was first identified as a glia-activating factor purified from the culture supernatant of a human glioma cell line. To study the functions of FGF-9 in the mouse as a model system, both the genomic and cDNA sequences encoding the FGF-9 homologue in the mouse were isolated. The mouse homologue differs from the human counterpart by only one residue of the 208 amino acids encoded by the open reading frame identified. COS and 293T cells transfected with the mouse *Fgf9* cDNA expressed two products of 30 and 27 kDa, the latter predicted to be a non-glycosylated version of the first. Both products were secreted into the culture supernatant despite the lack of a typical secretory signal at the N-terminus. They bind quantitatively to heparin-Sepharose with high affinity and so can be purified by heparin affinity chromatography. This method of protein synthesis and purification, however, provided low yields and the product may be contaminated with heparin. Hence, an N-terminal-truncated form of the protein was also synthesized in *E. coli* and purified in the absence of heparin. Both the mammalian- and bacteria-expressed proteins are not only mitogenically active on quiescent Balb/c 3T3 cells but are also capable of inducing mesoderm formation in *Xenopus* animal cap explants. This suggests a possible role for FGF-9 in embryonic development.

3.1 Introduction

The mammalian fibroblast growth factor (FGF) family comprises of at least nine related genes that are classified as members based on conserved coding sequence (reviewed by Basilico and Moscatelli, 1992). FGF-9 is the most recently described member of the FGF family of growth factors. The protein was originally purified as a glia-activating factor (GAF) from the culture supernatant of a human glioma-derived cell line (Naruo et al., 1993). This protein displays properties characteristic of the FGF family of proteins. These diagnostic features include its ability to bind to heparin and stimulate growth of a number of cell types including oligodendrocyte type 2 astrocyte progenitor cells, Balb/c 3T3 fibroblasts, and PC12 cells. It, however, has no mitogenic activity towards human umbilical vein endothelial cells, a feature distinct from the prototype FGFs, FGF-1 and FGF-2. Both the human and rat *Fgf9* cDNAs were subsequently cloned and found to encode a polypeptide of 208 amino acids with an estimated 30% homology to the other members of the FGF family (Miyamoto et al., 1993). The protein shares a similar structural organization with the other family members including the conservation of two cysteine residues and other consensus sequences. FGF-9 appears to resemble the prototype FGFs in lacking a typical signal sequence in its N-terminus even though it is secreted into the culture medium of cDNA-transfected COS cells (Miyamoto et al., 1993).

In this report, the cloning of the mouse homologue of *Fgf9* is described. In view of the restricted expression pattern of *Fgf9* in the adult rat brain and kidney (Miyamoto et al., 1993), a kidney cDNA library was used to isolate the mouse cDNA homologue with the rat *Fgf9* cDNA as probe. The latter was derived by PCR on cDNA obtained from the rat C6 glioma cell line. The mouse genomic homologue was also obtained by high stringency hybridization of the rat *Fgf9* cDNA probe on a D3 ES cell genomic library. The genomic homologue was only partially analysed while the cDNA clone was fully sequenced. The mouse, human and rat *Fgf9* coding sequences show structural conservation.

FGFs display a myriad of biological activities *in vitro*, eliciting such responses as cell proliferation, angiogenesis, mesoderm induction and cellular differentiation (reviewed by Basilico and Moscatelli, 1992). To study the biological properties of FGF-9, mouse FGF-9 was synthesized in two mammalian cell lines: COS and 293T cells. As the recombinant DNAs transfected into both COS and 293T cells are maintained as autonomous replicating episomes, expressions of foreign genes in both COS and 293T cells are transient. Other disadvantages of using the mammalian expression system include greater difficulty in regulating transcription, lower yield of products per volume of culture medium and higher cost of production since large amounts of media, sera as well as tissue culture plastics must be expended. On the other hand, the major advantage of using mammalian cells to express *Fgf9* is the ability to obtain proteins that have undergone the correct post-translational modifications such as glycosylation, phosphorylation, amidation, and proteolytic cleavage. In other words, this method of protein expression allows sufficient quantities of the protein similar to the native molecule to be synthesized for biochemical analyses.

The results obtained in this study suggest that FGF-9 expressed from the above mammalian cell lines are subjected to post-translational modifications. As for the human form of the protein (Miyamoto et al., 1993), mouse FGF-9 is efficiently secreted from both COS and 293T cells and it can be purified by heparin affinity chromatography. Its affinity for heparin is similar to that for most other members of the FGF family.

While proteins derived from the mammalian system allow molecules similar to the native counterparts to be studied, the product yield is relatively low and the protein may be contaminated with heparin due to the nature of the purification procedure. Hence, the feasibility of expressing FGF-9 in a bacterial system using *E. coli* was explored. There are, however, disadvantages in using this system. For example, the *E. coli* system is unable to perform post-translational modifications such as glycosylation and phosphorylation that are common modifications of proteins in eukaryotic cells; in addition, proteolytic processing is lacking and there is a bias of codon usage associated with the prokaryotic system of protein expression. On the other hand, there are many advantages in using *E. coli* for protein

expression, including ease of manipulation, short generation time, low cost, ease of culture, and most important of all, relatively large quantities of proteins can be obtained. The work here shows that FGF-9 truncated with respect to the N-terminal 48 amino acids can be overexpressed and synthesized in the *E. coli* expression system. The expression level obtainable was much higher than that achieved with the mammalian system.

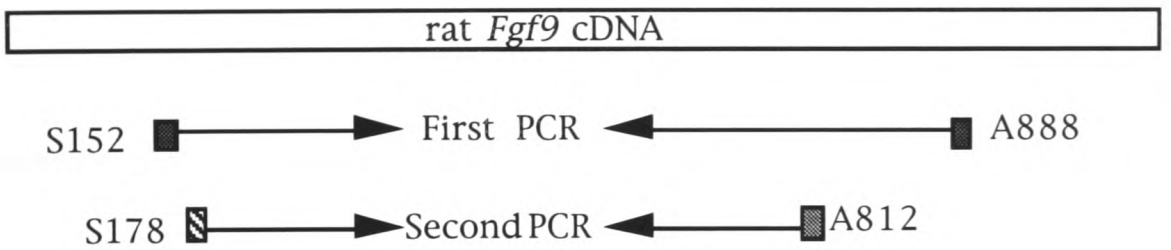
Analysis of the mitogenic activity of mouse FGF-9 on Balb/c 3T3 fibroblasts is also described. It was observed that FGF-9 produced from both the eukaryotic and prokaryotic systems were able to induce DNA synthesis in quiescent Balb/c3T3 cells. This activity was found to be comparable to that of FGF-2, in agreement with a previous observation for the native molecule isolated from the human glioma cell line (Naruo et al., 1993). The potential of mouse FGF-9 to act as a mesoderm inducer in *Xenopus* animal explants was also examined, in collaboration with J. Song (ICRF, Developmental Biology Unit, Department of Zoology, University of Oxford). FGF-9 produced from both mammalian and prokaryotic cell lines was found to possess potent mesoderm inducing activity. FGF-9, therefore, shows many features and properties similar to other members of the FGF family.

3.2 Results

3.2.1 Isolation of Rat *Fgf9* cDNA

The rat *Fgf9* cDNA was isolated by nested polymerase chain reactions on cDNA derived from the C6 glioma cell line. For this purpose, two sets of oligonucleotides were designed based on the rat cDNA sequence reported by Miyamoto et al. (1993). The first PCR was carried out with the external primers S152 and A888 (see section 2.1.4) at a low annealing temperature of 40 ^{o}C so that as much material as possible was amplified. The size of the amplified fragment was observed to be about 737 bp as predicted from the published sequence (Figure 3.1). The second PCR was carried out on the product of the first PCR with primers that corresponded to sequences internal to the fragment amplified by the first PCR. The resulting amplified fragment was shown to have a smaller size (635 bp) as predicted from the sequence (Figure 3.1). This amplified fragment encompassed the coding

A



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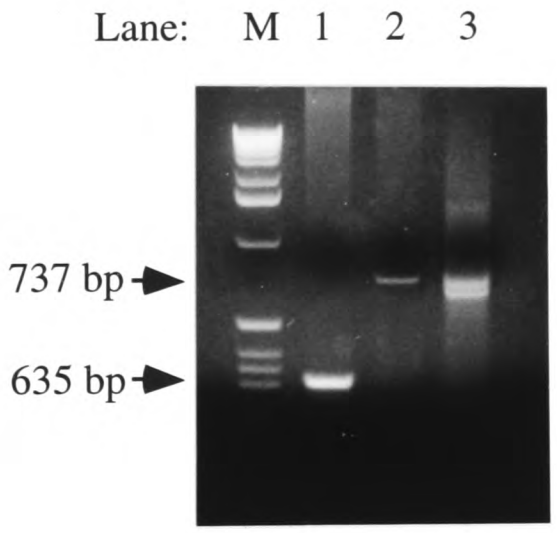


Figure 3.1. Strategy for PCR Cloning of the Rat *Fgf9* cDNA.
(A) The external primers S152 and A888 were designed to amplify a fragment of size 737 bp while the nested, internal primers S178 and A812 incorporating a *Bam*HI site and an *Eco*RI site respectively were designed to amplify a fragment of size 635 bp. (B) Agarose gel analysis of PCR products. PCR was carried out in 100 µl volume containing 250 µM of each of the dNTP and 5 units of Taq polymerase (Promega) with 500 ng of each of the primers. Lane 2: the first PCR was carried out using the external primers and the following program was used: 2 min at 94 °C, 3 min at 40 °C and 3 min at 72 °C for 30 cycles with a final primer extension step of 72 °C for 7 min. Lane 3: the second PCR was carried out using the internal primers and the following program was used: 2 min at 94 °C, 3 min at 60 oC and 3 min at 72 °C for 30 cycles with a final extension step of 72 °C for 7 min. Lane 1: A control PCR was carried out using the *hprt* A and G primers under the same cycle conditions as the first PCR. Five µl out of 100 µl was applied to a 1.8% agarose gel and the products were sized based on the 1 kb ladder DNA marker (M).

sequence of FGF-9, including both the initiation codon ATG and the termination codon TGA. The subcloning of this final PCR product into the pBlueScript KS(+) vector was facilitated by the incorporation of a *Bam*HI and an *Eco*RI restriction sequence into the internal primers. The authencity of the PCR product was confirmed by sequencing.

3.2.2 Isolation of Mouse *Fgf9* Genomic DNA

The mouse D3 embryonic stem cell genomic library (Stratagene) was screened for the mouse homologue to the *Fgf9* gene originally identified in a human cell line. The library was constructed in the lambda FIX™ II vector with *Xho*I as the cloning site. Of six clones isolated with the full-length rat *Fgf9* cDNA probe, two seemed to be identical and further hybridized with a 170 bp probe derived from the 5' end of the rat *Fgf9* cDNA (see section 2.1.6). As this latter probe covered non-conserved sequences amongst the FGF members, this suggested that the above-mentioned pair of clones may contain the mouse sequences for *Fgf9*. The genomic insert of one of these clones, which was larger than 10 kb, was isolated by restriction digest with *Not*I and cloned into pBluescript KS(+). The resulting clone was sequenced partially and the deduced amino acid sequence shown (Figure 3.2). The clone was found to contain exon III of *Fgf9* and part of intron II where sequences diverged from the reported cDNA sequences (Miyamoto et al., 1993). The start of the putative exon III sequence is consistent with the conservation of the exon II - exon III boundary characteristic of the FGF family of molecules (Dickson et al., 1989). Sequencing was achieved using multiple primers (see section 2.1.6; for nucleotide sequence of primers, see Appendix I).

3.2.3 Isolation of Mouse *Fgf9* cDNA

As rat *Fgf9* was reported to be expressed in the adult rat kidney (Miyamoto et al., 1993), the strategy for obtaining the cDNA clone of mouse *Fgf9* was to screen a mouse kidney cDNA library (Stratagene). Three apparently identical positive clones were obtained from 1 x 10^6 plaques screened. On sequencing, they were indeed confirmed to contain sequences

```
  1  ccaatgaatcataaagttttggttattgttaatttccatgtgtgacttttttgcttgt
 61  aacttttaaaaaaataataaagtttaaagtaaaaaaaaaaaaagattttattaggtgtg
121  tatggctcaaacatccttgatttggaattctgaactgtgaagtgtccccatatttaaact
181  ttttttttcagcagtaacatgatgtcacaagtggaaaactccatatatgggcattttaat
241  gaaaacatatatgcaagattaccttcgggctatttgtagatgatatatttgaagcataag
301  tgaattttgtgtttagatatgggtcccatatcctacatatcttactatgcaaatgcaaat
361  gttccaaaatgtgaaaaaagtagttgaagactatctggttcaaagatttttgaataatgg
421  ggattcaacttgtgtgtaataatggagaatattgctttttaatgaggcagctacattttg
481  caatgtatttatttgctttccgtttatttttttacagGAAAAACTAACACAGGAATGTGT
                                       E  K  L  T  Q  E  C  V
541  GTTCAGAGAACAGTTTGAAGAGAACTGGTACAACACCTACTCTTCCAACCTCTATAAACA
      F  R  E  Q  F  E  E  N  W  Y  N  T  Y  S  S  N  L  Y  K  H
601  TGTGGACACCGGAAGGAGATACTATGTTGCATTAAATAAGGACGGGACTCCAAGAGAAGG
      V  D  T  G  R  R  Y  Y  V  A  L  N  K  D  G  T  P  R  E  G
661  GACCAGGACTAAACGGCACCAGAAATTTACACATTTTTTACCTAGACCAGTGGACCCTGA
      T  R  T  K  R  H  Q  K  F  T  H  F  L  P  R  P  V  D  P  D
721  CAAAGTACCTGAACTATATAAGGATATTCTAAGCCAAAGCTGAcaaagacagtgtcttca
      K  V  P  E  L  Y  K  D  I  L  S  Q  S  *
781  cttgagcccttaaaacataaccactataaatgctttcatgcggtgggttcttattgatta
841  gcagtgccgtcacctcagctccactgttgccaaactttgtcgcatgcatatgaatgatgg
901  aagcttggatgaggacttgccaatttgctctgcacttactggctggtcctcctggagggc
961  tgcctag
```

intron II

exon III

Figure 3.2. Partial Genomic Structure of Murine *Fgf9*.
Genomic nucleotide sequence of the C-terminal region of *Fgf9*. Exons are in uppercase and introns or non-coding sequences in lower case. The predicted amino acid sequence encoded by exon III is shown below the nucleotide sequence. The exon III sequence boundary is conserved as for other members of the FGF family (Dickson et al., 1989).

identical to that obtained from the genomic clone (Figure 3.2). One of these clones was excised from the phagemid vector as a pBluescript plasmid and found to contain the full-length murine *Fgf9* cDNA sequence (named pBSmFGF-9). The nucleotide sequence of mouse *Fgf9* cDNA and the deduced amino acid sequence are shown (Figure 3.3).

The cDNA sequence of mouse *Fgf9* shows 97% homology to the published rat sequence and 92% homology to that of human, according to the BLAST homology search program (Altschul et al., 1990). The sequence obtained here is identical to three other recent reports of the mouse *Fgf9* cDNA sequence (Hecht et al., 1995; Santos-Ocampo et al., 1995; Seo and Noguchi, 1995). There is a TAA stop codon 18 bases upstream from the first ATG codon at nucleotide position 484 in the open reading frame identified. Hence, the murine *Fgf9* cDNA is deduced to encode a polypeptide of 208 amino acids similar to that for the rat homologue. The deduced amino acid sequence is identical to that of the rat homologue. As for the human homologue, the mouse FGF-9 differs in only one amino acid at the 9th position from the N-terminus where a serine residue is replaced by an asparagine residue (Figure 3.4). Comparison of the amino acid sequence with other members of the FGF family shows about 30% homology (Figure 3.5). Two cysteine and other consensus residues were conserved as for other members of the FGF family. Like FGF-1 and FGF-2, no canonical secretory signal sequence was found in the N-terminus. A glycosylation site is deduced by the presence of the residues NGT at the 80 - 83th position from the N-terminus (Figure 3.3).

The *Xenopus* homologue of *Fgf9* has also been cloned in collaboration with our laboratory (Song, 1996). The *Xenopus* cDNA was found to be 80% homologous to the mouse homologue while the deduced amino acid sequence was observed to be 93% homologous to the human homologue (Figure 3.4). This high level of conservation of the amino acid sequence between species suggests a critical conservation of function. Thus, FGF-9 may possess essential functions in the mammalian system.

Hydropathy plot analysis (Figure 3.6) indicated that hydrophobicity of the N-terminal end of FGF-9 is low relative to that of other known secreted proteins (von Heijne, 1985). This analysis also highlighted a stretch of hydrophobic residues in the central region

```
  1   ggcacgagaaaaacagcgcatgcctttctggagtcaggatccgtaaattctgacgtagcc
 61   cgagcatcttaaaaatccctgtaatatcgcccaggcacttacgttgctatggtcattctg
121   atctctaagcaaatggagaaactacggatttttttcccttattacggtcggatgggatga
181   agaccttctgcctgctgagagtcgaggctccatatgtagcgatgcatagctgtgttgatc
241   aatgtcagtgtgtgagtataaagtggtggcttcttagactatcagtggtttgaccttgaa
301   cctgtgccagagaaacagccgattacttttatttatgcatcggatggattgaagaaaaga
361   acccttttttccctctctgtctgcaactgcggcaagggaggggagttggatatacctcgcc
421   tagtgtctcctggttgataccatcattattgtttattcttgtgcttaaaagccgagtcct
481   ctgATGGCTCCCTTAGGTGAAGTTGGGAGCTATTTCGGTGTGCAGGACGCGGTACCGTTC
         M  A  P  L  G  E  V  G  S  Y  F  G  V  Q  D  A  V  P  F    19

541   GGGAACGTACCGGTGTTGCCGGTGGACAGTCCGGTGTTGCTAAGTGACCACCTGGGTCAG
      G  N  V  P  V  L  P  V  D  S  P  V  L  L  S  D  H  L  G  Q    39

601   TCCGAAGCAGGGGGGCTGCCCCGGGGCCCCGCAGTCACGGACTTGGATCATTTAAAGGGG
      S  E  A  G  G  L  P  R  G  P  A  V  T  D  L  D  H  L  K  G    59

661   ATTCTCAGGCGGAGGCAGCTGTACTGCAGGACTGGATTTCATTTAGAGATCTTCCCCAAC
      I  L  R  R  R  Q  L  Y  C  R  T  G  F  H  L  E  I  F  P  N    79

721   GGTACTATCCAGGGAACCAGGAAAGACCACAGCCGCTTCGGCATTCTGGAATTTATCAGT
      G  T  I  Q  G  T  R  K  D  H  S  R  F  G  I  L  E  F  I  S    99

781   ATAGCAGTGGGCCTGGTCAGCATTCGCGGTGTGGACAGTGGACTCTACCTCGGCATGAAC
      I  A  V  G  L  V  S  I  R  G  V  D  S  G  L  Y  L  G  M  N   119

841   GAGAAGGGGGAGCTGTATGGATCAGAAAAACTAACACAGGAATGTGTGTTCAGAGAACAG
      E  K  G  E  L  Y  G  S  E  K  L  T  Q  E  C  V  F  R  E  Q   139

901   TTTGAAGAGAACTGGTACAACACCTACTCTTCCAACCTCTATAAACATGTGGACACCGGA
      F  E  E  N  W  Y  N  T  Y  S  S  N  L  Y  K  H  V  D  T  G   159

961   AGGAGATACTATGTTGCATTAAATAAGGACGGGACTCCAAGAGAAGGGACCAGGACTAAA
      R  R  Y  Y  V  A  L  N  K  D  G  T  P  R  E  G  T  R  T  K   179

1021  CGGCACCAGAAATTTACACATTTTTTACCTAGACCAGTGGACCCTGACAAAGTACCTGAA
      R  H  Q  K  F  T  H  F  L  P  R  P  V  D  P  D  K  V  P  E   199

1081  CTATATAAGGATATTCTAAGCCAAAGCTGAcaaagacagtgtcttcacttgagcccttaa
      L  Y  K  D  I  L  S  Q  S  *

1141  aacataaccactataaaaaaaggggg
```

Figure 3.3. cDNA Structure of Murine FGF-9.
Nucleotide and predicted amino acid sequence of *Fgf9* cDNA. The cDNA encodes a protein of 208 amino aicds, the sequence of which is shown below the nucleotide sequence, as represented by one-letter codes. A potential N-glycosylation site is underlined.

```
          1       *                                                  50
Mouse     MAPLGEVGSY FGVQDAVPFG NVPVLPVDSP VLLNDHLG.Q SEAGGLPRGP
  Rat     MAPLGEVGSY FGVQDAVPFG NVPVLPVDSP VLLSDHLG.Q SEAGGLPRGP
Human     MAPLGEVGNY FGVQDAVPFG NVPVLPVDSP VLLSDHLG.Q SEAGGLPRGP
Xenopus   MAPLGEVGNY FGVQDAVSFG NVPVLQVDTP VLLSDHMSHH SEAGGLPRGS

          51                                                 100
Mouse     AVTDLDHLKG ILRRRQLYCR TGFHLEIFPN GTIQGTRKDH SRFGILEFIS
  Rat     AVTDLDHLKG ILRRRQLYCR TGFHLEIFPN GTIQGTRKDH SRFGILEFIS
Human     AVTDLDHLKG ILRRRQLYCR TGFHLEIFPN GTIQGTRKDH SRFGILEFIS
Xenopus   AVTDLEHLKG ILRRRQLYCR TGFHLEIFPN GTIQGTRQDH NRFGILEFIS

          101                                                150
Mouse     IAVGLVSIRG VDSGLYLGMN EKGELYGSEK LTQECVFREQ FEENWYNTYS
  Rat     IAVGLVSIRG VDSGLYLGMN EKGELYGSEK LTQECVFREQ FEENWYNTYS
Human     IAVGLVSIRG VDSGLYLGMN EKGELYGSEK LTQECVFREQ FEENWYNTYS
Xenopus   IAVGLVSIRG VDSGLYLGMN EKGELYGSEK LTQECVFREQ FEENWYNTYS

          151                                                200
Mouse     SNLYKHVDTG RRYYVALNKD GTPREGTRTK RHQKFTHFLP RPVDPDKVPE
  Rat     SNLYKHVDTG RRYYVALNKD GTPREGTRTK RHQKFTHFLP RPVDPDKVPE
Human     SNLYKHVDTG RRYYVALNKD GTPREGTRTK RHQKFTHFLP RPVDPDKVPE
Xenopus   SNIYKHADTG RRYYVALNKD GTSRDGTRTK RHQKFTHFLP RPVDPEKVPE

          201
Mouse     LYKDILSQS
  Rat     LYKDILSQS
Human     LYKDILSQS
Xenopus   LYKDILSQS
```

Figure 3.4. Comparison of Human, Rat, Mouse and *Xenopus* FGF-9 Amino Acid Sequences.
The predicted sequence is 100% conserved between the rat and mouse homologues. The human homologue differs from the rat and mouse homologues in only one amino acid at the ninth position where the serine residue is replaced by an asparagine residue, as indicated by a *. The *Xenopus* sequence is 93% identical to the human counterpart (Song, 1996). Sequence alignment was performed based on the PileUp Program (GCG Wisconsin Package Version 8.0).

Figure 3.5. Alignment of the Amino Acid Sequence of FGF-9 with that of Other Members of the FGF Family.
Amino acid sequences of the murine FGF family are aligned. The two conserved cysteine residues are in bold while the signature sequence structure which defines the family is boxed. Members are also identified by a conserved core of about 120 amino acids, the limits of which are indicated by arrows. Sequences are aligned based on the PileUp Program (GCG Wisconsin Package Version 8.0).

```
          1                                                            50
FGF-4     ..MAKR.... .....GPTTG TLLPRVLLAL VVALADRG.. ..........
FGF-6     MALGQRLFIT MSRGAGRVQG TLQALVFLGV LVGMVVPS.. ..........
FGF-5     .......... .......... .MSLSLLFLI FCSHLIHSAW AHGEKRLTPE
FGF-1     .......... .......... .......... .......... ..........
FGF-2     .......... .......... .......... .......... ..........
FGF-9     .......... .......... .......... ......MAPL GEVGSYFGVQ
FGF-7     .......... .......... .......... ...MRKWILT RILPTLLYRS
FGF-3     .......... .......... .......... .......... ..........
FGF-8     .......... .......... .......... .......... ..........

          51                                                          100
FGF-4     ..TAAPNGTR HAELGHGW.D GLVARSLARL PVAAQPPQAA VRSGAGDYLL
FGF-6     ..PAGARANG TLLDSRGW.G TLLSRSRAGL AGEI....SG VNWESG.YLV
FGF-5     GQPAPPRNPG DSSGSRGRSS ATFSSSSASS PVAASPGSQG SGSEHSSFQW
FGF-1     .......... .......... ..MAEGEITT FAALTERFN. .LPLG...NY
FGF-2     .......... .......... ..MAASGITS LPALPEDGGA AFPPG...HF
FGF-9     DAVPFGNVPV LPVDSPVLLS DHLGQSEAGG LP....RGPA VTDLD...HL
FGF-7     CFHLVCLVGT ISLACNDMSP EQTATSVNCS SPERHTRSYD YMEGG...DI
FGF-3     .......... .MGLIWLLLL SLLEPSWPTT GPGTRLRRDA GGRGGVYEHL
FGF-8     ..MGSPRSAL SCLLLHLLVL CLQAQVTVQS SPNFTQHVRE QSLVTD..QL

          101                                                         150
FGF-4     GL..KRLRRL YCNVGIGFHL QVLPDGRIGG VHAD.TRDSL LELSPVQRGV
FGF-6     GI..KRQRRL YCNVGIGFHL QVPPDGRISG THEE.NPYSL LEISTVERGV
FGF-5     SPSGRRTGSL YCRVGIGFHL QIYPDGKVNG SHEA.SVLSI LEIFAVSQGI
FGF-1     K...KP.KLL YCSNG.GHFL RILPDGTVDG TRDRSDQHIQ LQLSAESAGE
FGF-2     K...DP.KRL YCKNG.GFFL RIHPDGRVDG VREKSDPHVK LQLQAEERGV
FGF-9     KGILRR.RQL YCR.T.GFHL EIFPNGTIQG TRKDHSRFGI LEFISIAVGL
FGF-7     RV.....RRL FCR.T.QWYL RIDKRGKVKG TQEMKNSYNI MEIRTVAVGI
FGF-3     GGAPRR.RKL YC.AT.KYHL QLHPSGRVNG SLENSA.YSI LEITAVEVGV
FGF-8     SRRLIRTYQL YSR.TSGKHV QVLANKRINA MAEDGDPFAK LIVETDTFGS

          151                                                         200
FGF-4     .VSIFGVASR FFVAMSSRGK LFG.VPFFTD ECKFKEILLP NNYNAYESYA
FGF-6     .VSLFGVKSA LFIAMNSKGR LYT.TPSFHD ECKFRETLLP NNYNAYESDL
FGF-5     .VGIRGVFSN KFLAMSKKGK LHA.SAKFTD DCKFRERFQE NSYNTYASAI
FGF-1     .VYIKGTETG QYLAMDTEGL LYG.SQTPNE ECLFLERLEE NHYNTYTSKK
FGF-2     .VSIKGVCAN RYLAMKEDGR LLA.SKCVTE ECFFFERLES NNYNTYRSRK
FGF-9     .VSIRGVDSG LYLGMNEKGE LYG.SEKLTQ ECVFREQFEE NWYNTYSSNL
FGF-7     .VAIKGVESE YYLAMNKEGK LYA.KKECNE DCNFKELILE NHYNTYASAK
FGF-3     .VAIKGLFSG RYLAMNKRGR LYA.SDHYNA ECEFVERIHE LGYNTYASRL
FGF-8     RVRVRGAETG LYICMNKKGK LIAKSNGKGK DCVFTEIVLE NNYTALQNAK

          201                                                         250
FGF-4     YPG.......  ...M......  FMALSKNGRT KKG..NRVSP TMKVTHFLPR
FGF-6     YRG.......  ...T......  YIALSKYGRV KRG..SKVSP IMTVTHFLPR
FGF-5     HRT.......  ...EKTGREW  YVALNKRGKA KRGCSPRVKP QHVSTHFLPR
FGF-1     H.........  .....AEKNW  FVGLKKNGSC KRG..PRTHY GQKAILFLPL
FGF-2     Y.........  .....S..SW  YVALKRTGQY KLG..SKTGP GQKAILFLPM
FGF-9     Y.........  .KHVDTGRRY  YVALNKDGTP REG..TRTKR HQKFTHFLPR
FGF-7     W.........  .THSGGEM..  FVALNQKGIP VKG..KKTKK EQKTAHFLPM
FGF-3     YRTGSSGPGA QRQPGAQRPW  YVSVNGKGRP RRG..FKTRR TQKSSLFLPR
FGF-8     YEG.......  .........W  YMAFTRKGRP RKG..SKTRQ HQREVHFMKR

          251                                                         300
FGF-4     L.........  ..........  ..........  ..........  ..........
FGF-6     I.........  ..........  ..........  ..........  ..........
FGF-5     FKQSEQPELS  FTVTVPEKKK  PPVKPKVPLS  QPRRSPSPVK  YRLKFRFG..
FGF-1     PVSSD.....  ..........  ..........  ..........  ..........
FGF-2     SAKS......  ..........  ..........  ..........  ..........
FGF-9     PVDPDKVPEL  YKDILSQS..  ..........  ..........  ..........
FGF-7     AIT.......  ..........  ..........  ..........  ..........
FGF-3     VLGHKDHEMVRLLQSSQPRAPGEGSQPRQRRQKKQSPGDHGKMETLSTRATPST
FGF-8     LPRGHHTTEQSLRFEFLNYPPFTRSLRGSQRTWAPEPR..............

          301
FGF-4     ..........
FGF-6     ..........
FGF-5     ..........
FGF-1     ..........
FGF-2     ..........
FGF-9     ..........
FGF-7     ..........
FGF-3     QLHTGGLAVA
FGF-8     ..........
```

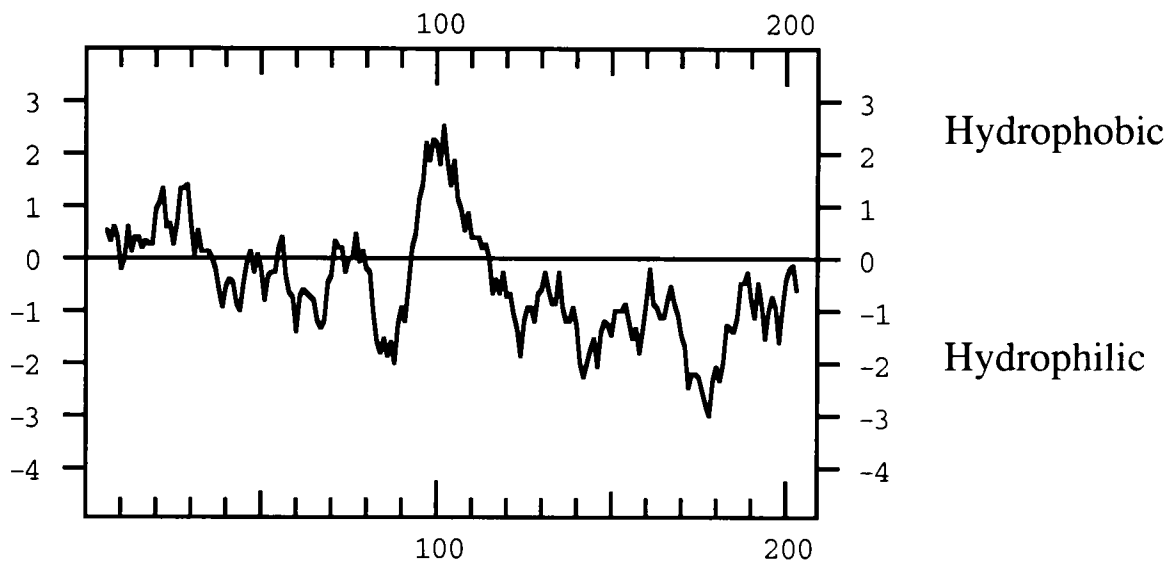


Figure 3.6. Hydropathy Plot Analysis of FGF-9.
The method of Kyte and Doolittle (1982) (DNA Strider Program) was used to calculate the hydrophobicity index of the protein. The degree of hydrophobicity or hydrophilicity is indicated by the y-axis while the position of the amino acid residue in the sequence is indicated by the x-axis. A secretory signal is not thought to be included in the N-terminus due to its low hydrophobicity.

of the protein sequence. The isoelectric point of the protein was calculated to be 7.6 by the Isoelectric Program (GCG Wisconsin Package Version 8.0).

The three-dimensional structure of FGF-9 can be inferred to be similar to that of FGF-2 for which the crystal structure is known (Ago et al., 1991; Eriksson et al., 1991; Zhang et al., 1991; Zhu et al., 1991). An alignment of the deduced amino acid sequence of FGF-9 with that of FGF-2 shows conservation of the residues that form the ß-strands in FGF-2 (Figure 3.7).

3.2.4 Expression of Mouse FGF-9 in Mammalian Cells

The mouse *Fgf9* cDNA was cloned into the vector pEE14 designed to allow expression of the insert in mammalian cells under the control of the cytomegalovirus promoter. The resultant pEE14mFGF-9 plasmid (Figure 3.8A) was transiently transfected into the monkey kidney fibroblast-derived COS cells by electroporation. FGF-9 protein was purified from the culture supernatant of the transfected COS cells by heparin affinity chromatography. The protein products recovered were analysed by SDS-polyacrylamide gel electrophoresis and two protein species of sizes approximately 30 and 27 kDa were observed (Figure 3.8B). Elution was achieved with 1.0 - 2.0 M NaCl, but not at a lower concentration of 0.5 M. The 30 kDa protein product can be resolved further by reverse-phase HPLC into a single peak (Figure 3.9). N-terminal amino acid sequence analysis of the 30 kDa protein revealed the first few residues to be Leu-Gly-Glu-Val-Gly-Ser-Tyr-Phe-Gly-Val-. This confirmed that the 30 kDa protein was indeed FGF-9 as predicted from the cDNA sequence. In addition, the purified protein appeared to start from the fourth amino acid leucine, consistent with that reported for the native product purified from the conditioned medium of a human glioma-derived cell line (Naruo et al., 1993). Expression level in this cell line was low, typically less than 10 µg per 100 ml of culture supernatant.

The plasmid pEE14mFGF-9 was also used for the transient transfection of 293T cells. 293T cells are human kidney epithelial-derived 293 cells previously transformed with the SV40 large T antigen and transfection was based on the calcium phosphate DNA

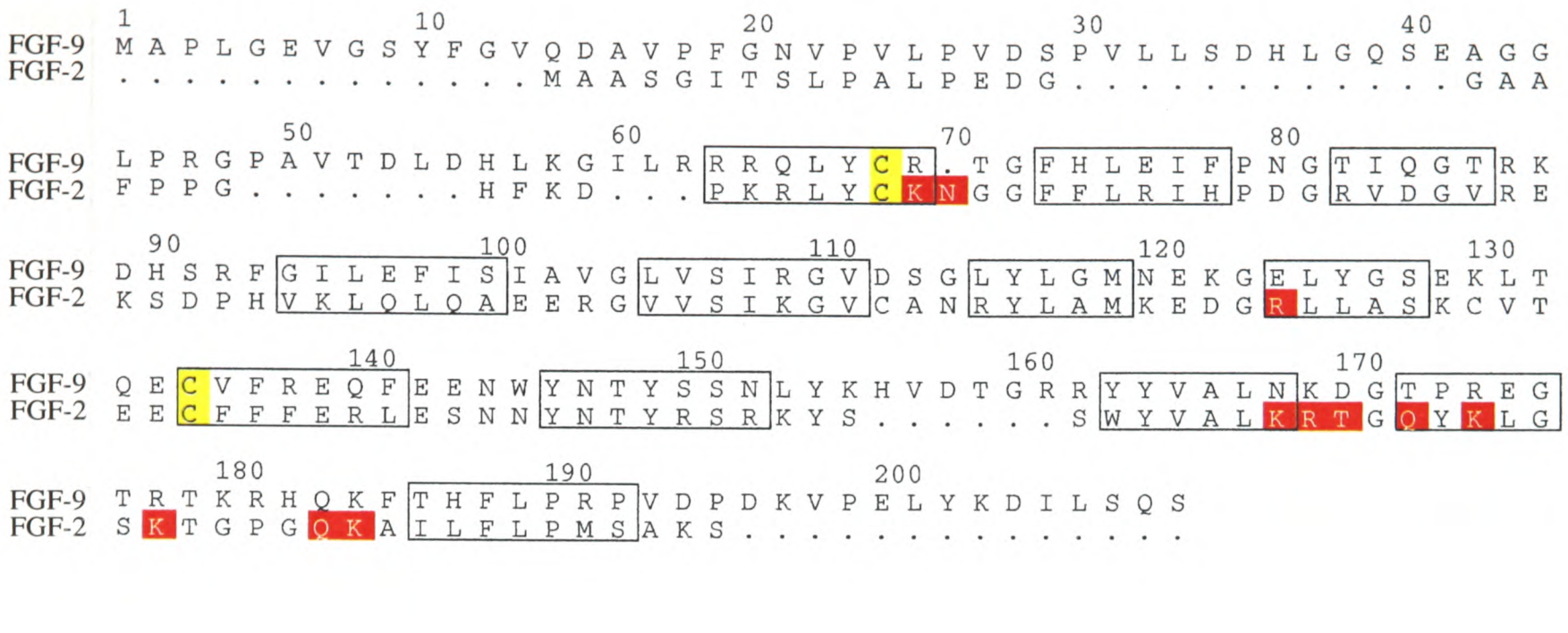
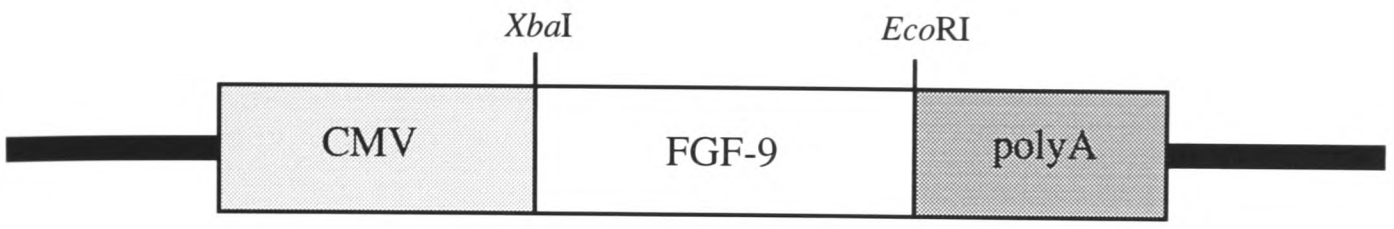


Figure 3.7. Alignment of Amino Acid Sequences of FGF-9 and FGF-2.
Residues forming the twelve antiparallel ß-strands of the FGF-2 molecule are boxed. Conserved cysteine residues are coloured yellow while key amino acids identified to form the heparin-binding domain of FGF-2 are in red. The specificity of interactions between FGF-2 and FGFRs is thought to involve recognition of residues in the loop between the 9th and 10th ß-strands of FGF-2.

A



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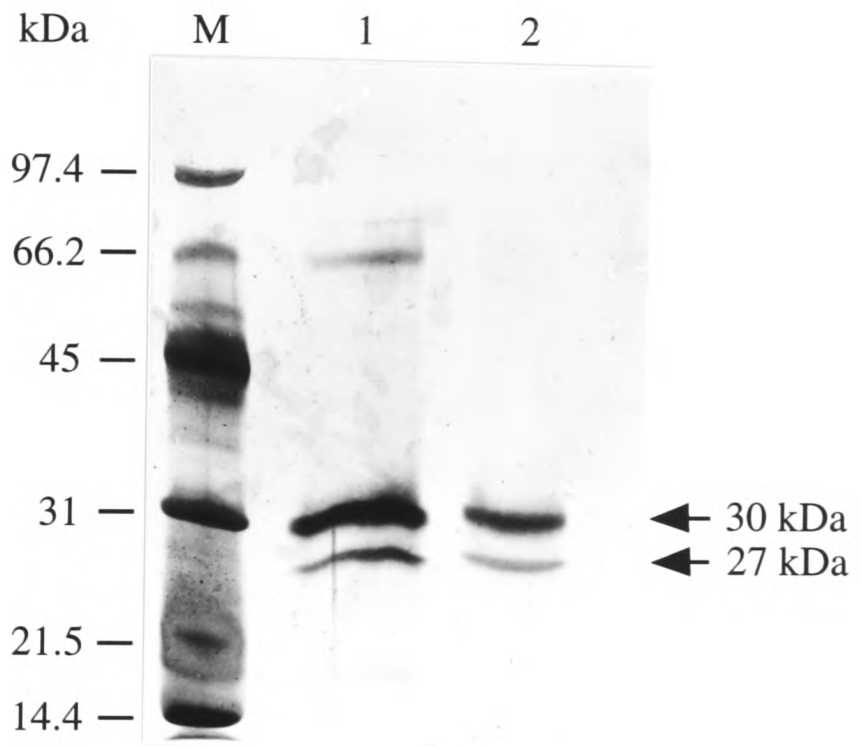


Figure 3.8. Protein Expression of Mouse FGF-9 in Mammalian Cells.
(A) Schematic Diagram of the pEE14mFGF-9 Expression Vector. CMV refers to the human cytomegalovirus promoter/enhancer of the vector pEE14 used for expression of the inserted cDNA encoding for mouse FGF-9. The transcription unit is completed with a polyadenylation signal from SV40 (polyA). The solid bar represents the rest of the pEE14 vector. (B) SDS-polyacrylamide gel analysis of FGF-9 purified from the culture supernatant of COS cells transfected with the pEE14mFGF-9 vector. Purification was achieved by passing the clarified supernatant through a Hitrap heparin affinity column (Pharmacia Biotech) and eluting the bound FGF-9 with 1 M to 2 M NaCl. The protein products were resolved as 30 and 27 kDa bands on a 12.5% polyacrylamide gel which was silver stained. Lane M, low range molecular weight standards (Bio-Rad) (phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa). Lanes 1 and 2, sample eluates from heparin affinity column.

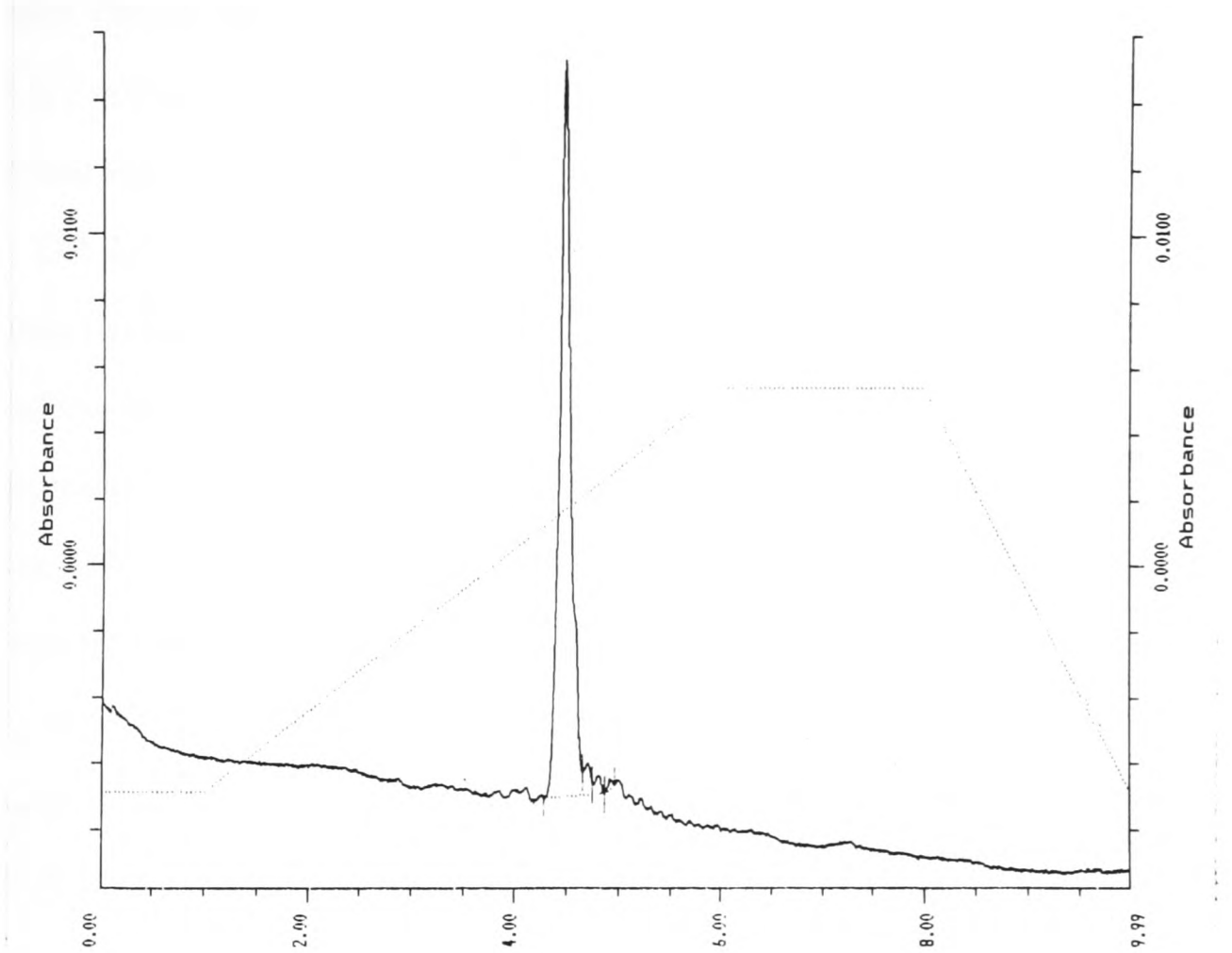


Figure 3.9. Reverse-Phase HPLC of FGF-9 Purified by Heparin Affinity.
Bound FGF-9 was eluted from a heparin affinity column and applied to a poros R6/8 column. The proteins were eluted with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10 - 60%, 2 ml/min) and detected by absorbance at 280 nm. FGF-9 was resolved as a single peak in the presence of 45% acetonitrile.

precipitate technique (Wigler et al., 1979). Protein products were purified from the culture supernatant in a similar manner as described for the COS cells and SDS-polyacrylamide gel analyses showed protein products of similar sizes. Again, at least 1.0 M NaCl was required for elution while 0.5 M NaCl failed to elute the protein from the heparin-Sepharose (Figure 3.10). Protein expression level in this cell line was found to be 2-fold higher than that for COS cells. Hence, this 293T protein expression system was advantageous for expressing FGF-9 in a eukaryotic background relative to the COS cell system in two ways: (1) ease of transfection and (2) greater efficiency of protein expression.

The data suggested that FGF-9 was secreted from the transfected COS and 293T cells since a significant quantity of the protein had accumulated in the culture supernatant of these cells to allow purification to be possible. This was also observed when human FGF-9 was expressed in COS-7 and CHO cells (Miyamoto et al., 1993). The results here, however, differ from the previous report by Miyamoto et al. (1993) in that three amino acids instead of just the initiating methionine were cleaved from the N-terminus of the purified 30 kDa protein. This observation is, on the other hand, in agreement with that for the native molecule purified from human glioma (Naruo et al., 1993). This apparently cleaved peptide of only three amino acids is not thought to be of sufficient length to serve as a secretion signal. In addition, the native FGF-9 molecule was found to contain a single sugar chain of 3 kDa (Naruo et al., 1993). Hence, the 30 kDa protein product is predicted to possess a sugar chain of a similar size.

This study showed that it is feasible to express FGF-9 in two mammalian cell lines. The level of protein expression in both cell lines were, however, low and the system will have to be scaled up many fold to obtain analytical quantities of the protein. In addition, as the purification method involves the use of heparin, a molecule known to interact with FGFs (Folkman and Klagsbrun, 1987; Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989), contamination with this molecule may interfere with biochemical analyses of FGF-9 activity. Hence, the feasibility of producing FGF-9 in a prokaryotic background and employing an alternative purification procedure was explored.

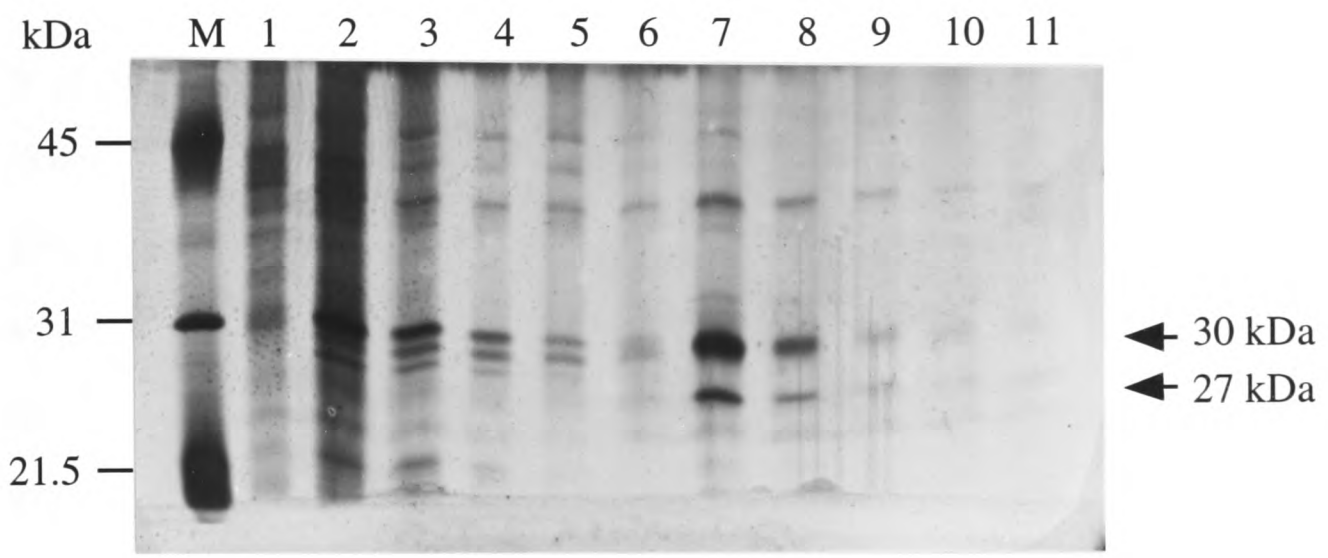


Figure 3.10. Heparin Affinity of FGF-9.
Culture supernatants of transfected 293T cells were pooled together and applied to a Hitrap heparin affinity column (Pharmacia Biotech). The column was washed with 50 mM sodium phosphate buffer, pH 7.0 and FGF-9 was eluted with increasing concentrations of NaCl, ranging from 0.5 to 2 M. Samples of the 1 ml eluates were analysed by SDS-polyacrylamide gel electrophoresis and silver staining. Little or none of the FGF-9 protein product was eluted with 0.5 M NaCl (lanes 1 - 5) while most of the product was recovered when 1 M NaCl was applied (lanes 6 - 10) leaving only a small amount on the column which was eluted with 2 M NaCl (lane 11). Lane M, low range molecular weight standards (Bio-Rad).

3.2.5 Expression of Mouse FGF-9 in Bacterial Cells

Expression of mouse FGF-9 in *E. coli* was achieved using the pGEX-2T bacterial expression system (Smith and Johnson, 1988). The *Fgf9* cDNA was inserted downstream from the glutathione *S*-transferase coding sequence and induction of the *tac* promoter with IPTG allows FGF-9 to be synthesized as a fusion product with glutathione *S*-transferase (GST) (Figure 3.11A). This facilitates purification of the protein product from the culture lysate by glutathione affinity chromatography. Furthermore, the fusion protein can be cleaved with thrombin and FGF-9 eluted easily from the affinity column.

The complete coding sequence of FGF-9 was cloned into the pGEX-2T vector. Difficulty, however, was encountered in expressing the full-length FGF-9 protein: there was either low or no response to induction with IPTG, as analysed by polyacrylamide gel electrophoresis of cell lysates. The protein synthesized may have been insoluble in aqueous solutions and cannot be easily purified. This could be attributed to the presence of the short stretch of hydrophobic residues in the N-terminus which was not thought to be sufficiently hydrophobic to serve as a secretory signal (Figure 3.6). To eliminate this potential problem, the feasibility of expressing an N-terminal-truncated but biologically-active form of FGF-9 was examined. Several lines of evidence suggest that several residues in the N-terminus of FGF molecules are not critical for biological activity. For instance, the three-dimensional structure of FGF-2 indicates that a stretch of seventeen residues at the N-terminus is flexible and apparently disordered, suggesting that it is not critical for the structural make-up of the molecule and hence predicted not to be essential for protein function (Zhu et al., 1990; Ago et al., 1991; Eriksson et al., 1991; Zhang et al., 1991). There are also reports that XeFGF and FGF-4 truncated with respect to several N-terminal residues are biologically active when synthesized *in vitro* (Isaacs et al., 1992; Niswander and Martin, 1992). Alignment of the FGF-2 and FGF-9 amino acid sequences suggests that the corresponding N-terminal 48 amino acid residues of FGF-9 may be deleted without adverse effects on protein structure or activity (Figure 3.7). The cDNA encoding FGF-9 truncated

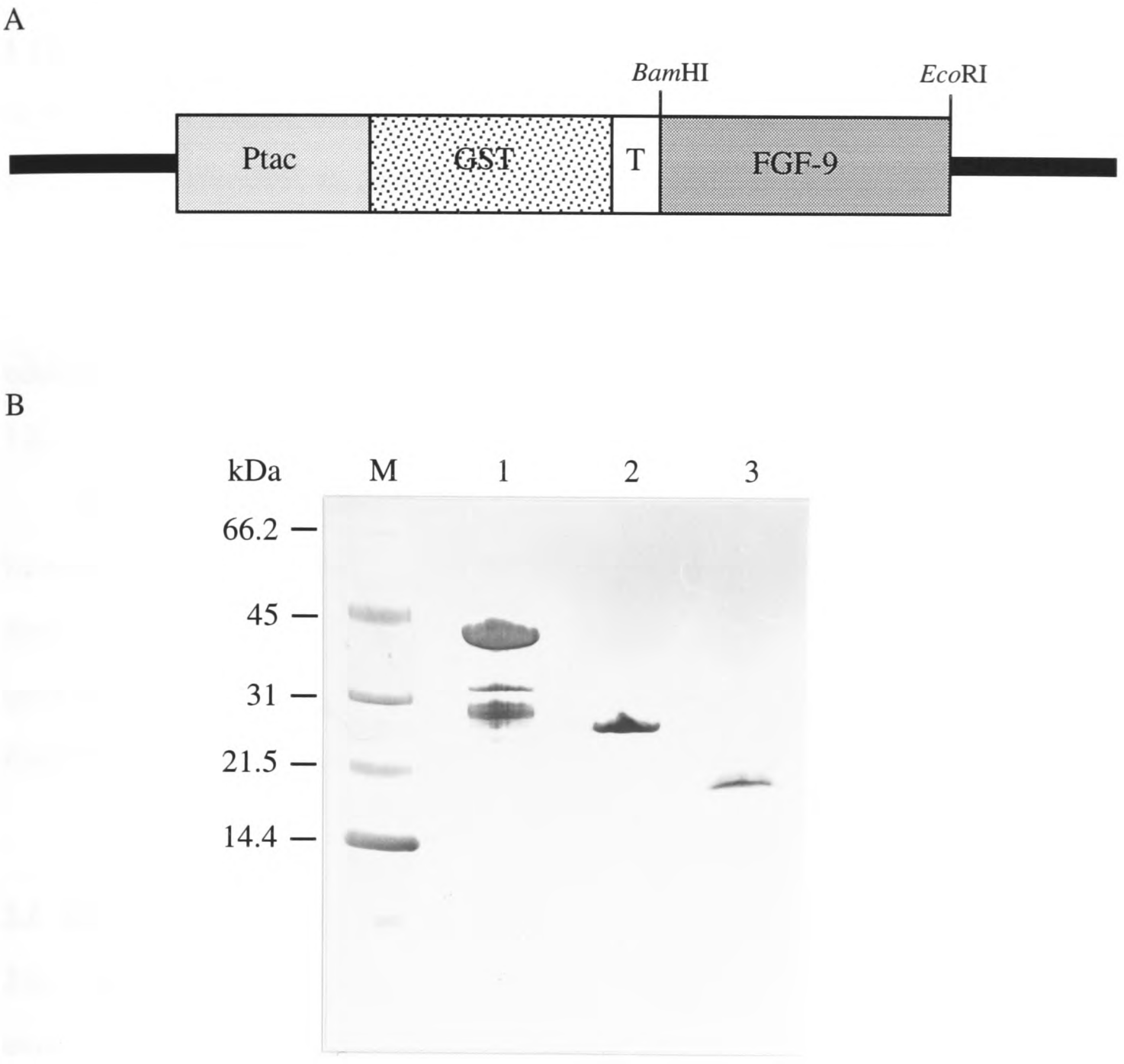


Figure 3.11. Protein Expression of Mouse FGF-9 in *E. coli*.
(A) Schematic Diagram of the pGEXmFGF-9 Expression Vector. The vector contains a *tac* promoter *(*Ptac) for chemically inducible high-level expression of the mouse FGF-9 gene in the presence of isopropylthiogalactoside (IPTG). The protein is expressed as a fusion product with *Schistosoma japonicum* glutathione *S*-transferase (GST) in prokaryotic cells. T refers to the recognition sequence for thrombin which allows cleavage of the 26 kDa glutathione *S*-transferase domain from the fusion protein. The solid bar represents the rest of the pGEX-2T vector. (B) SDS-polyacrylamide gel electrophoresis of the protein products derived from the pGEX-2T prokaryotic expression system. FGF-9, truncated with respect to the N-terminal 48 amino acids, was expressed in *E. coli* strain JM109 using the pGEXmFGF-9 vector. The GST fusion products (45 kDa, lane 1) were purified by passing the culture lysate through a glutathione sepharose affinity column (Pharmacia). Cleavage of FGF-9 from GST was effected by the addition of thrombin which resulted in the elution of FGF-9 as a 19 kDa protein product (lane 3). GST was left on the sepharose beads as a 26 kDa band as observed on the silver-stained 12.5% polyacrylamide gel (lane 2). Lane M, low range molecular weight standards (Bio-Rad).

with respect to the 48 amino acid residues at the N-terminus was accordingly generated by PCR, ligated to the pGEX-2T vector and used for transfection in *E. coli* (see section 2.1.11). This time, expression was observed. SDS-polyacrylamide gel analysis of the protein products (Figure 3.11B) showed the presence of the large 45 kDa GST-FGF-9 fusion protein absorbed to glutathione-agarose beads. On proteolytic cleavage with thrombin, FGF-9 was released as a product of approximately 19 kDa while glutathione *S*-transferase was left on the beads as a 26 kDa protein. Further analysis of the FGF-9 protein product by mass spectrometry indicated that the mass of the protein was 18.6 kDa (Figure 3.12).

Expression of FGF-9 in the *E. coli* system was advantageous in two ways: (1) the efficiency of protein production was approximately 1 mg of protein per 2 l of bacterial culture, indicating a 2.5-fold improvement from that attainable with the eukaryotic expression system; and (2) the protein was purified free from heparin which may potentially interfere with biochemical analyses of the protein.

3.2.6 Biological Activities of Mouse FGF-9

3.2.6.1 Mitogenic Activity on Balb/c 3T3 Fibroblasts

One of the many responses elicited by FGFs is the increase in DNA synthesis in 3T3 cells (Gospodarowicz, 1974). In this respect, human FGF-9 was reported to stimulate DNA synthesis of Balb/c 3T3 fibroblasts in a "hormonal dose" at a concentration of 1 pM (Naruo et al., 1993). Mouse FGF-9 produced in both mammalian and bacterial systems were tested for this property on Balb/c 3T3 fibroblasts. The assay was based on the method described previously (Sullivan and Klagsbrun, 1986) where the FGF was added to the fibroblasts previously rendered quiescent by serum starvation. The amount of tritiated thymidine incorporated was used as a measure of DNA synthesis.

In agreement with the previous report (Miyamoto et al., 1993), mouse FGF-9 expressed in both COS and 293T cells (which includes both the 30 and 27 kDa products) stimulated DNA synthesis of Balb/c 3T3 fibroblasts at concentrations over 5 pM (0.16

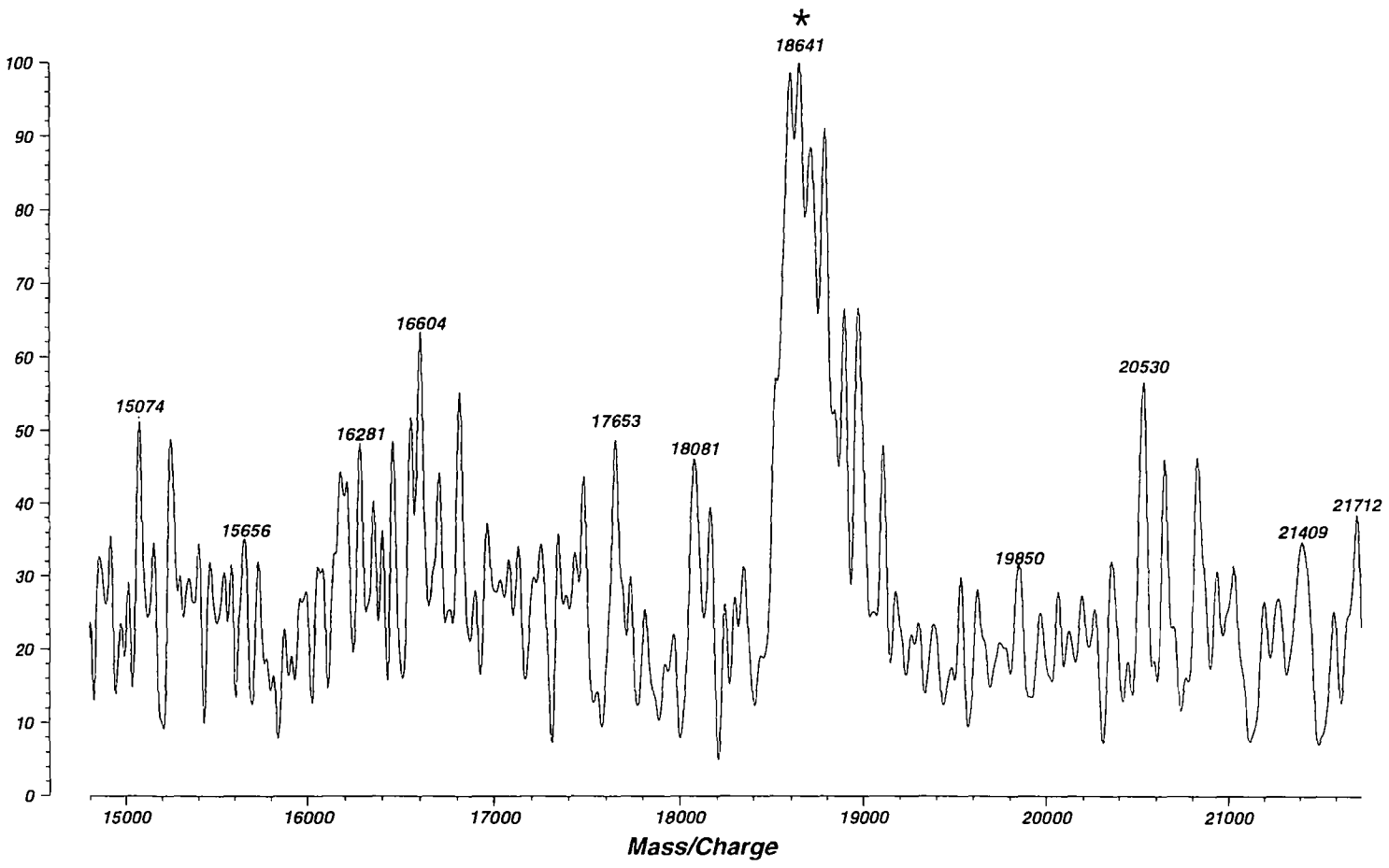


Figure 3.12. MALDI Spectra of Mouse FGF-9 Produced by the pGEX-2T Bacterial Expression System.
The mass of the N-terminal truncated form of FGF-9 expressed in *E. coli* (see text) was determined by matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry to be about 18.6 kDa (major peak marked *).

ng/ml), with the activity reaching a plateau at 40 pM (1.3 ng/ml) (Figure 3.13A). The concentration of FGF-9 required for the half-maximal stimulation of cell proliferation (ED_{50}) was determined to be ≈ 9 pM.

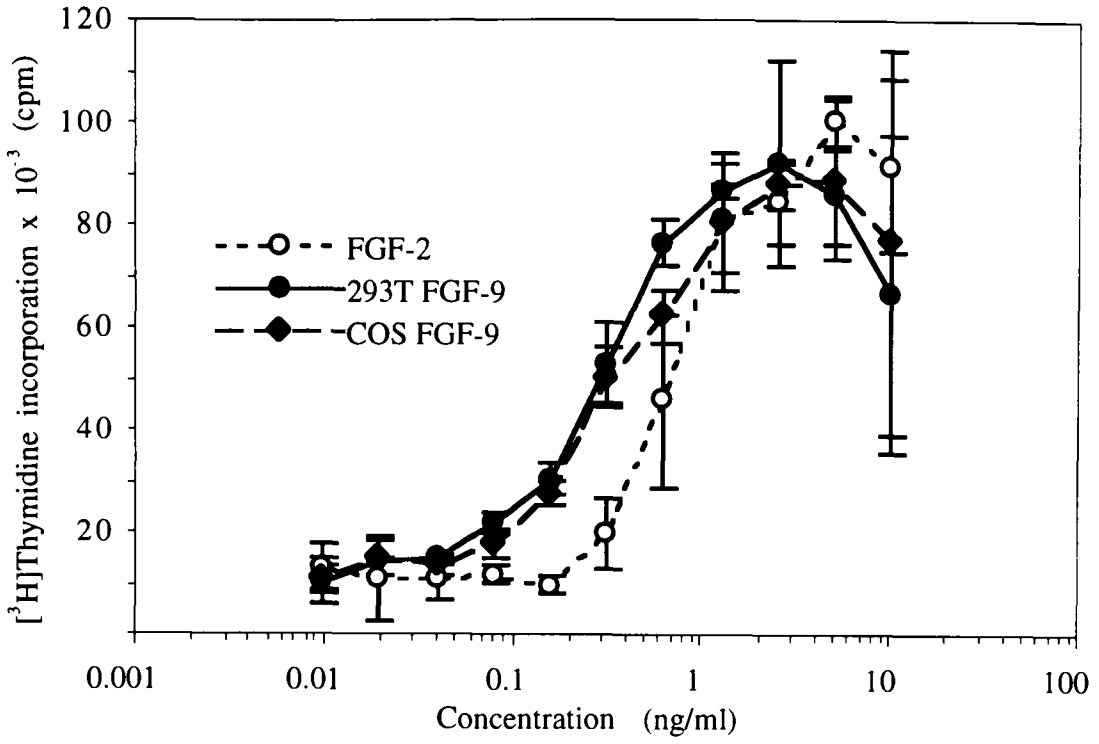
The ability of the truncated and non-glycosylated form of mouse FGF-9 to induce cell division of Balb/c 3T3 cells was also examined. In this instance, the smaller 18.6 kDa protein stimulated DNA synthesis only at concentrations over 30 pM (0.6 ng/ml) with the activity plateau at 270 pM (5 ng/ml) (Figure 3.13B). The ED_{50} of the bacteria-expressed mouse FGF-9 was ≈ 63 pM. The truncated protein thus appeared to be about 7-fold less potent than its full-length counterpart in stimulating cell division of Balb/c 3T3 cells. This decrease in mitogenic activity may be due to either N-terminal truncation of the protein or the lack of glycosylation, or both. It is also possible that the activity of the particular sample of FGF-9 tested might have decayed for some undefined reason as it was found that another sample from the same batch of protein preparation has mitogenic activity at as low as 15 pM (ED_{50} ≈ 50 pM; Figure 4.2C).

In summary, this assay indicated that both the full-length and truncated forms of mouse FGF-9 are biologically active in as far as the ability to stimulate DNA synthesis of Balb/c 3T3 fibroblasts is concerned. The truncated and non-glycosylated form may be slightly less potent than its full-length counterpart but this difference is not thought to be significant enough to preclude use of this smaller molecule for further biochemical studies.

3.2.6.2 Mesoderm Inducing Activity

Another property associated with the FGFs is their ability to induce ventro-vegetal differentiation (or mesoderm induction) in *Xenopus* embryos (Slack et al., 1987; Kimelman and Kirschner, 1987). The mesoderm-inducing factor (or MIF) assay was performed by exposing ectoderm explants of the *Xenopus* embryo to various concentrations of the protein samples and the response was measured by visual observation since induced explants were easily and clearly distinguishable from controls (Godsave et al., 1988). Explants placed in the presence of the protein being tested or in control medium initially round up so that the

A



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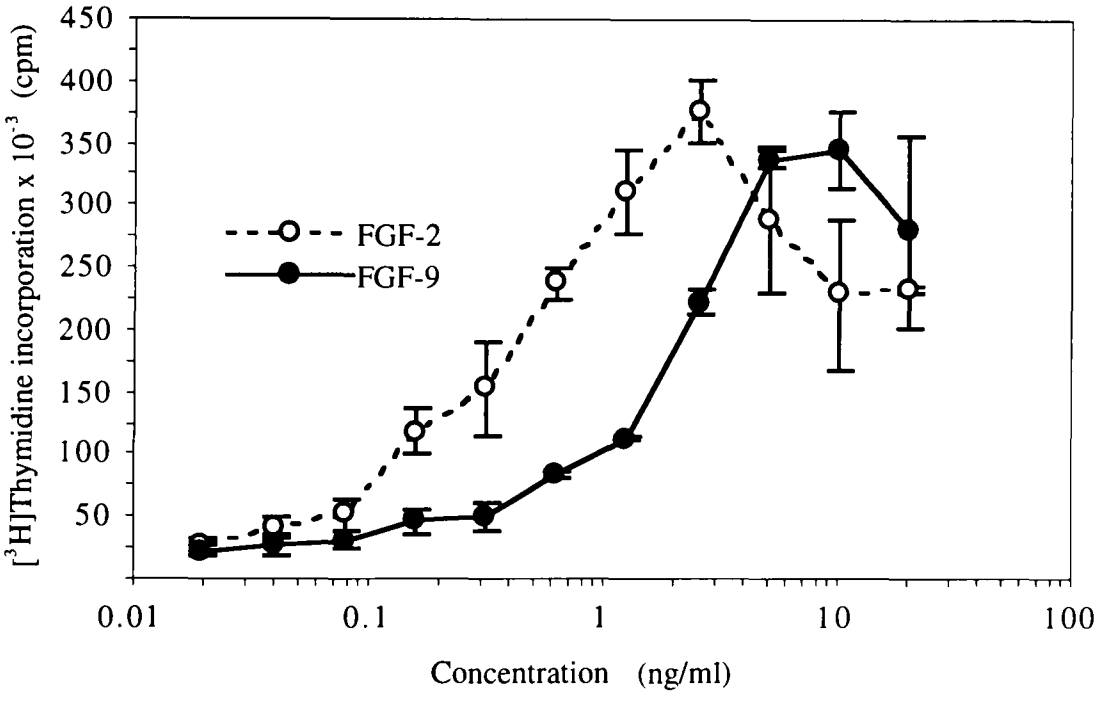


Figure 3.13. Mitogenic Activity of Mouse FGF-9.
(A) Samples of FGF-9 purified from the culture supernatants of transfected COS and 293T cells by heparin affinity were tested for mitogenic activity on quiescent Balb/c 3T3 cells. The ability of FGF-9 derived from both cell lines to stimulate DNA synthesis was comparable to that of recombinant FGF-2. (B) The N-terminal-truncated form of FGF-9 produced in *E. coli* was also able to induce DNA synthesis in Balb/c 3T3 cells. Mitogenic activity was measured as thymidine incorporation (y-axis). The x-axis indicate the final concentration of the FGFs added to the culture medium of quiescent Balb/c 3T3 cells.

blastocoelic surface is covered by the formerly outer layer of pigmented cells. In control or uninduced explants, little change in external appearance during a 3-day culture period is typically observed, with no differentiated tissues other than epidermis being formed (Godsave et al., 1988). On the other hand, explants treated with MIF becomes elongated, swollen and transparent, with the formation of translucent vesicles accompanied by a slight thickening of the epidermis at one end. This assay was kindly performed by Song, J.-H. at the ICRF laboratory, Department of Zoology, University of Oxford. The MIF assay clearly showed that both the full-length and truncated forms of mouse FGF-9 have mesoderm-inducing activity as measured by explant morphology (Figure 3.14). The activity of both forms of the protein was found to be 0.1 ng/ml. This observation clearly demonstrated two important points: firstly, this assay confirmed that truncation of FGF-9 did not compromise its mesoderm inducing activity in a significant way; secondly, FGF-9 shared a common property with other members of the FGF family such as FGF-1, FGF-2 and FGF-4 in the ability to induce mesoderm in *Xenopus* embryos (Kimelman and Kirschner, 1987; Paterno et al., 1989).

3.3 Discussion

The mouse *Fgf9* cDNA nucleotide sequence, as expressed in the mouse kidney, was cloned and analysed in this study. FGF-9 seems to have been extremely well conserved throughout evolution. For instance, mouse and human FGF-9 differ in only one of the 208 amino acids. This dramatic sequence conservation may indicate the specific requirement of FGF-9 in its interaction with a distinct set of target molecules, which include receptors and matrix components such as the heparan sulphate proteoglycans.

Like other members of the FGF family, FGF-9 is classified based on sequence conservation. It possesses the signature sequence diagnostic of the FGF family of proteins (Figure 3.5) while showing about 30% overall homology with each member of the family. With the exception of FGF-8, all members contain two conserved cysteine residues. The role of these cysteine residues have not been defined. The presence of these reduced

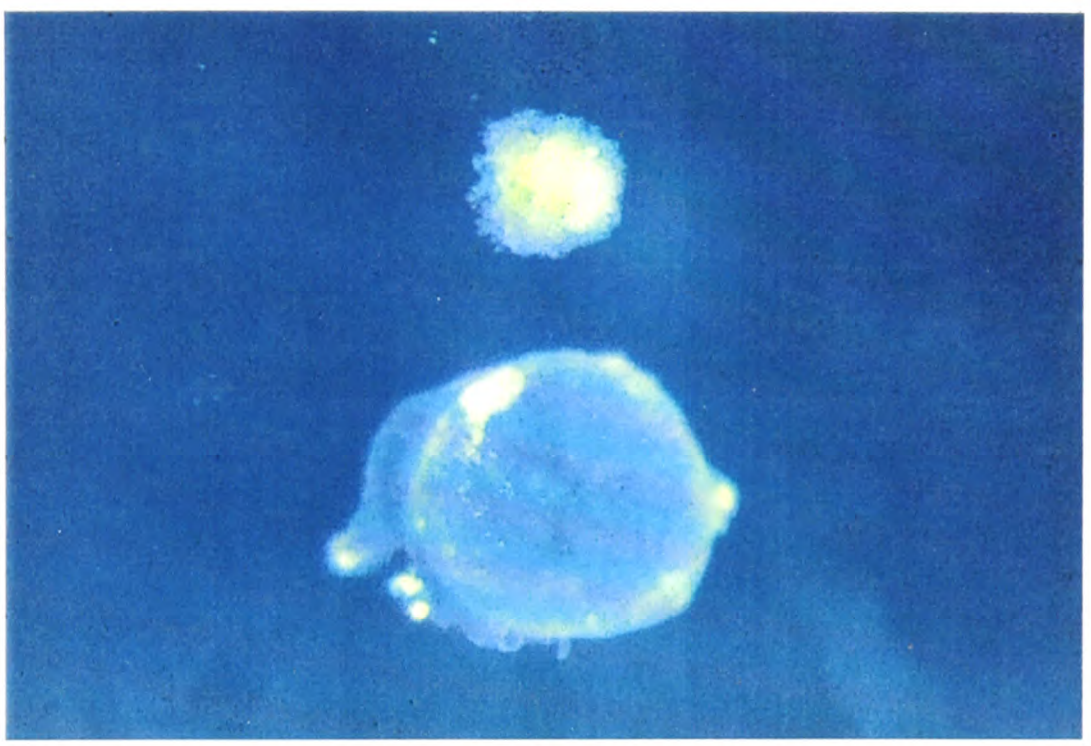


Figure 3.14. Mesoderm Induction by FGF-9.
The control *Xenopus* animal cap explant untreated with any mesoderm-inducing factor was rounded up and solid masses of epidermal cells had developed (top). In contrast, the explant treated with mouse FGF-9 was elongated and had swollen vesicles (bottom). The mesoderm induction response was observed at FGF-9 concentrations down to 0.1 ng/ml.

residues in the native molecule provides convenient sites for efficient labelling of the FGFs with markers such as biotin (Lee et al., 1989), as described in greater detail in Chapter 4.

FGF-9 lacks a typical signal sequence and this is reflected in the low hydrophobicity of the N-terminal 20 amino acids. In this respect, FGF-9 is similar to the two prototype FGFs, FGF-1 and FGF-2, while other members of the family have typical signal sequences and are thought to be secreted from cells. It is still a matter of contention as to whether FGF-1 and FGF-2 are indeed secreted from cells or if they are primarily intracellularly localized. Experiments using transient COS cell expression indicated that FGF-2 is translocated to the cell surface or the nucleus but not released into the surrounding culture media (Florkiewicz et al., 1991). There are, however, reports that indicated the association of FGF-2 with the extracellular matrix of cells producing the protein and there is evidence to suggest that it interacts with cell surface receptors (see section 1.7.1.1). It is possible then that FGF-2 may be released from cells by an as yet undefined novel pathway. A previous report has shown that human FGF-9 is secreted by transfected COS cells (Miyamoto et al., 1993). The results here also indicated that FGF-9 is likely to be secreted from both COS and 293T cells transfected with the murine *Fgf9* cDNA since significant quantities of the protein can be purified from the culture supernatants. N-terminal sequence analysis of the 30 kDa FGF-9 showed that a short peptide of three amino acids, including the initiating methionine was cleaved. This peptide cleavage was also observed for the native 30 and 29 kDa molecules purified from a human glioma conditioned medium (Naruo et al., 1993). It is not clear whether these truncated forms occur *in vivo* or are products of proteolysis that occurs during the extraction procedure (Klagsbrun et al., 1987). Nevertheless, this peptide is probably too short to work as a secretion signal. Hydropathy plot analysis of the protein also indicated the absence of a sufficiently hydrophobic sequence in the N-terminus that could serve as a typical signal peptide. It, however, highlighted a stretch of amino acids in the middle portion of the protein unique to FGF-9. This sequence may potentially serve as a novel secretory signal, allowing the channelling of FGF-9 to the endoplasmic reticulum/Golgi secretion pathway where it may also be

modified by glycosylation. FGF-9 has been determined to be a glycosylated protein (Naruo et al., 1993).

Higher molecular weight forms of FGF-2 and FGF-3 have been shown to arise from the use of upstream CUG codons as alternate initiation codons for translation (Florkiewicz et al., 1989; Prats et al., 1989; Acland et al., 1990). These different forms seem to correlate with differences in subcellular distribution to the nuclear or ribosomal fractions (Basilico and Moscatelli, 1992). There exists an in-frame CUG codon just adjacent to the initiation codon of *Fgf9* (Figure 3.3) but this, by itself, could not be utilized to produce a mitogenically active product (Miyamoto et al., 1993). Hence, translation of FGF-9 was thought to start exclusively at the ATG codon in the open reading frame identified.

FGF-9 appears to be subjected to post-translational regulation. This is reflected in the three forms of the protein of sizes 30, 29 and 25 kDa isolated from human glioma (Naruo et al., 1993). The difference between the three proteins appears to be due to N-terminal truncation of the predicted amino acid sequence. The 30, 29 and 25 kDa forms start at the 4th, 13th and 34th residues from the initiation methionine respectively. The smaller 29 and 25 kDa forms might have originated from the 30 kDa form by proteolytic cleavage. FGF-9 is also post-translationally modified by glycosylation. When the mouse protein was produced in COS or 293T cells via cDNA transfection, the smaller protein species of 27 kDa is thought to be a non-glycosylated form of the larger 30 kDa molecule rather than a proteolytically-derived product, as observed for the human homologue expressed in CHO cells (Miyamoto et al., 1993). In the latter report, N-terminal sequence analysis of the smaller 27 kDa form showed that it shares a similar sequence as the larger 30 kDa protein. In addition, it has been previously shown that a sugar chain of 3 kDa is present in the native molecules purified from a human glioma cell line (Naruo et al., 1993). These two observations led to the deduction that the 3 kDa N-linked oligosaccharide chain is absent from the 27 kDa product obtained here. This, however, could not be confirmed until this smaller product has been sequenced at the N-terminus or shown by other means to be non-truncated at any part of the protein sequence. With the exception of the prototypic

FGF-1 and FGF-2, modification by glycosylation is a feature shared by all other members of the FGF family (Delli-Bovi et al., 1988; Dixon et al., 1989; Miyagawa et al., 1988; Finch et al., 1989; Bates et al., 1991; Coulier et al., 1991; Zhan et al., 1988; Mason et al., 1994). The biological significance of the oligosaccharide moiety is unclear. It is not expected to be necessary for protein activity as FGF-9 produced in *E. coli* is shown here to be biologically active. It follows then that the presence of the sugar chain is not necessary for maintaining the structure or stability of the protein or for the interaction of FGF-9 with its high-affinity cell surface receptor(s).

The results obtained here and elsewhere (Miyamoto et al., 1993; Naruo et al., 1993) have demonstrated that FGF-9 displays properties diagnostic for FGFs. A characteristic feature is their ability to strongly bind to the glycosaminoglycan heparin (Paterno et al., 1989). The FGFs generally require NaCl concentrations of 1.0 M or greater for elution from heparin columns, a property which distinguishes them from other heparin-binding molecules such as the platelet-derived growth factor (or PDGF), a basic protein which binds heparin via weaker ionic interactions and requires only 0.5 M NaCl for elution from heparin affinity resins (Vlodavsky et al., 1987). FGF-9 produced from the culture medium of cDNA-transfected 293T cells was also found to bind strongly to heparin-Sepharose and required at least 1.0 M NaCl for efficient elution, as analysed by SDS-polyacrylamide gel analysis. Hence, FGF-9 binds quantitatively to heparin-Sepharose and is eluted at salt concentrations typical of other FGFs. This property facilitates its purification by heparin affinity chromatography. In addition, this implies that the protein is tightly associated with proteoglycans on the cell surface and extracellular matrix. FGF-9 is therefore predicted to be a short range effector, probably requiring intimate cell-to-cell contact to elicit a biological response.

Another property characteristic of FGFs is their broad spectrum of mitogenicity (Cross and Dexter, 1991). Both full-length and N-terminal-truncated forms of mouse FGF-9 have been shown to be able to stimulate DNA synthesis in quiescent Balb/c 3T3 cells. By implication then, FGF-9 can trigger the FGF receptors expressed by this cell line, with a binding affinity comparable to that of FGF-2 and its receptor(s) (Figure 3.12A, B). Four

genes encoding distinct FGF receptors have been identified (reviewed by Johnson and Williams, 1993) and different mouse tissues and cell lines were found to express varied complements of FGF receptors (Bernard et al., 1991; Olwin and Hauschka, 1986). As it is not clear which of the FGF receptors are expressed in Balb/c 3T3 cells, the identity of the receptor(s) to which FGF-9 binds cannot be defined from this assay. On this issue, a point of interest is the report that FGF-9 has no stimulatory effect on human umbilical vein endothelial cells (Naruo et al., 1993). It is likely then that this cell line does not express the receptor(s) that recognizes FGF-9 as a ligand. In addition, this result confirms that up to 48 of the N-terminal residues are not required to for the biological function of the molecule. This is in agreement with the deduction derived from the three-dimensional data of FGF-2 which showed that the N-terminal seventeen residues were not involved in the structural make-up (Zhu et al., 1990; Zhang et al., 1991).

A further property demonstrated by some members of the FGF family is their ability to induce mesoderm in the animal cap assay. For instance, FGF-1, FGF-2, FGF-4 and FGF-5 can, with different potencies, induce mesoderm formation in isolated *Xenopus laevis* animal pole explants (Slack et al., 1987; Kimelman & Kirschner, 1987; Slack et al., 1988; Paterno et al., 1989; Slack et al., 1989). The data here show that FGF-9 also has mesoderm-inducing capabilities. This property has also been shown for the *Xenopus* homologue of FGF-9 (Song, 1996). This observation suggests that FGF-9 may function as an embryonic morphogen. Further, this result implies that receptor(s) for FGF-9 exists in the *Xenopus* blastocysts. To date, five FGFRs expressed in *Xenopus* embryos have been identified. They include XFGFR1 (Musci et al., 1990), XFGFRA1 (Friesel and Dawid, 1991), XFGFR2 (Friesel and Brown, 1992), XFGFR4 (Shiozaki et al., 1995) and XFGFR4B (Riou et al., 1996). XFGFR1 is 95% homologous to XFGFRA1 and both are homologous to FGFR1 (Friesel and Dawid, 1991). Similarly, XFGFR4 and XFGFR4B are 95% identical and structurally related to FGFR4 (Riou et al., 1996). Each pair of homologous receptors may represent products of a pair of genes that is thought to have arisen from the genome duplication that occurred during the evolution of *Xenopus laevis* (Bisbee et al., 1977). XFGFR1, XFGFRA1, XFGFR4 and XFGFR4B are expressed

throughout embryo development while XFGFR2 expression commences during gastrulation (Musci et al., 1990; Friesel and Dawid, 1991; Friesel and Brown, 1992; Shiozaki et al., 1995; Riou et al., 1996). Hence, XFGFR2 is not thought to be involved in the primary stages of mesoderm induction since the mesoderm induction process begins well before the onset of zygotic transcription and all components involved in the process should be maternally transcribed. FGF-9 may thus interact with one or more of the other four receptors in mediating mesoderm induction in the *Xenopus* embryo. In support of the involvement of this molecule in embryonic development, *Xenopus Fgf9* has been shown to be expressed throughout embryonic development in the *Xenopus* embryo (Song, 1996).

In summary, FGF-9 shows both structural and functional similarities to other fibroblast growth factors. This protein is mitogenic towards fibroblasts and causes the induction of mesoderm in *Xenopus* explants. It is as yet unknown if these activities reflect functions normally performed by FGF-9 *in vivo*. Its ability to induce mesoderm in *Xenopus* embryos suggests that FGF-9 may be involved in embryonic development. To understand the normal roles of the *Fgf9* gene, its receptor binding specificity as well as its temporal and spatial expression patterns must be defined. These issues were addressed by using the N-terminal-truncated, bacterial-expressed form of mouse FGF-9 in receptor binding assays (Chapter 4) and both the genomic and cDNA sequences for probe preparations in RNA expression analyses (Chapter 5).

Chapter 4

Receptor Binding Specificity of FGF-9 & the Effect of Heparin

Summary

The receptor specificity of the murine homologue of FGF-9 was evaluated in this study using a simple plate binding assay based on a soluble dimeric form of the FGF receptor (FGFR) expressed as a fusion protein with the human IgG1 Fc peptide. Six of the seven currently identified receptor isoforms, namely murine FGFR1c, FGFR2b, FGFR2c, FGFR3c and FGFR4 and human FGFR1b were examined in this way. All the receptors tested consisted of three Ig-like domains in the extracellular region fused with the Fc peptide. Recombinant FGF-9 was shown to interact with the "b' splice form of FGFR2 and the "c" splice form of FGFR3. The specificity of the interaction of FGF-9 with FGFR3c was further confirmed by surface plasmon resonance analysis. In addition, the heparin requirement of FGF binding to FGFRs was also examined using the dimeric receptor system. FGF-9 binding to either FGFR2b or FGFR3c does not appear to require the presence of heparin. The same observation was made for FGF-1 in its binding interaction with both splice forms of FGFR1 and FGFR2 and for FGF-4 in its interaction with the "c" splice forms of FGFR1, FGFR2, and FGFR3. On the other hand, FGF-2 binding to FGFR1b was shown to require the presence of heparin. The reason for this discrimination is not clear but may indicate specific interaction of heparin with FGF-2

and/or with FGFR1b. Preliminary surface plasmon data suggests that, in this instance, heparin served mainly to increase the association rate of the FGF-2-FGFR1b complex.

4.1 Introduction

The biological effect of fibroblast growth factors is thought to be elicited through the binding and activation of specific cell surface signalling receptors. Four distinct FGF receptor genes, named numerically from FGFR1 to FGFR4, have been identified (Table 1.2; reviewed by Johnson and Williams, 1993). These high affinity, low capacity receptors bind FGFs with a K_D of 20-600 pM and are characterized by alternative splicing in the extracellular Ig-like domain III which yield receptor isoforms with different ligand specificities. In addition, the different receptor isoforms were shown to have distinct but overlapping expression patterns and implicated to have roles in development. Thus, a knowledge of the receptor specificity for each FGF is critical to an insight towards the biological role of the growth factor, both in development as well as in adult tissues.

Each member of the FGF family displays different receptor specificities. The receptor specificities of the prototype FGFs are the most studied. They showed different but overlapping binding abilities for the different receptors as pointed out above. The receptor specificities of the other FGFs, FGF-3, FGF-4, FGF-5, FGF-6, FGF-7 and recently FGF-8 have also been determined (Johnson et al., 1991; Miki et al., 1991; Mansukhani et al., 1992; Ornitz and Leder, 1992; Vainikka et al., 1992; MacArthur et al., 1995; Mathieu et al., 1995a, b). In this study, the receptor specificity of FGF-9 is examined based on a plate binding assay that incorporates soluble dimeric forms of the FGFRs (containing three extracellular Ig-like domains) synthesized as fusion proteins with the Fc peptide of human IgG1 as the solid-phase reagents. In this way, FGF-9 was found to interact with two receptor isoforms: FGFR2b, an epithelial-localized splice form of FGFR2 (Orr-Urtreger et al., 1993) and FGFR3c, a mesenchymal splice form of FGFR3

(Peters et al., 1993). This receptor binding specificity suggests a role for FGF-9 in the development of both ectodermally- and mesenchymally-derived tissues. It also implicates a role for FGF-9 in the central nervous system as both FGF-9 and FGFR3c expressions have been localized to the adult brain (Peters et al., 1993; Tagashira et al., 1995).

In addition to the requirement for high affinity receptors, FGF activity has been shown to be potentiated by the presence of low affinity receptors (reviewed by Schlessinger et al., 1995). These low affinity binding sites bind FGFs with 10- to 100-fold lower affinity than the FGFRs ($K_D \approx 2$ to 200 nM) and were identified as heparan sulphate proteoglycans located in the extracellular matrix or on the cell surface (Kiefer et al., 1990; Sakaguchi et al., 1991). Heparin, a highly sulphated glycosaminoglycan synthesized by mast cells, is highly similar to heparan sulphate in structure. It also binds to FGFs (reviewed by Burgess and Maciag, 1989) and has the ability to potentiate the biological activity of FGF-1 (Thornton et al., 1983; Lobb et al., 1986). In addition, heparin has been shown to be required for high affinity binding of FGF to its receptor (Ornitz and Leder, 1992; Ornitz et al., 1992). This observation is, however, not supported by other studies which indicated that the FGF-FGFR interaction is not dependent on heparin which appears only to have an enhancing effect on the binding interaction (Roghani et al., 1994). Furthermore, heparin has been reported to bind directly to FGFR1 (Kan et al., 1993; Pantoliano et al., 1994; Wang et al., 1995a). There is, however, also evidence to the contrary where heparin fails to interact with FGFR in binding assays (Ornitz et al., 1992; Spivak-Kroizman et al., 1994).

In this study, the requirement for heparin in the binding interaction between ligand and receptor *in vitro* was studied. For this purpose, the soluble dimeric forms of FGFRs was applied in plate binding assays as mentioned previously for defining the receptor specificity of FGF-9. Heparin was found to have no general effect on the binding interactions between different FGFs and their receptors. There is, however, a significant effect exerted by heparin on the interaction between FGF-2 and FGFR1b, indicating a requirement for heparin in the binding of FGF-2 to this receptor. The binding interactions of FGF-2 with FGFR1c and FGFR2b seemed to be moderately increased by heparin but it

has no apparent effect on the binding of FGF-1 and FGF-4 with any of the receptors examined so far. The binding of FGF-9 with either FGFR2b or FGFR3c also does not appear to require heparin.

In addition to *in vitro* plate binding assays, the method of total internal reflection or surface plasmon resonance (SPR, reviewed by Chaiken et al., 1992) was also applied to confirm the receptor specificity of FGF-9 and heparin requirement of FGF binding to FGFR. The advent of the Biosensor system (Pharmacia Biosensor, Uppsala) allows the detection of binding interactions between ligands immobilized on a sensor chip and macromolecular interactors in the solution phase flowing over the sensor chip. In this report, the method of surface plasmon resonance was used to confirm that FGF-9 does indeed interact with FGFR3c, in agreement with the data obtained from the plate binding assays. Furthermore, preliminary real-time kinetic measurements of the binding interaction indicates that FGF-9 binds to FGFR3c with high affinity. The SPR method was also applied to the study of heparin requirement for FGF and FGFR interactions. The kinetic measurements indicated that heparin served chiefly to increase the association rate of FGFR1b-Fc dimers with FGF-2.

4.2 Results

4.2.1 Construction of FGF Receptor Expression Vector

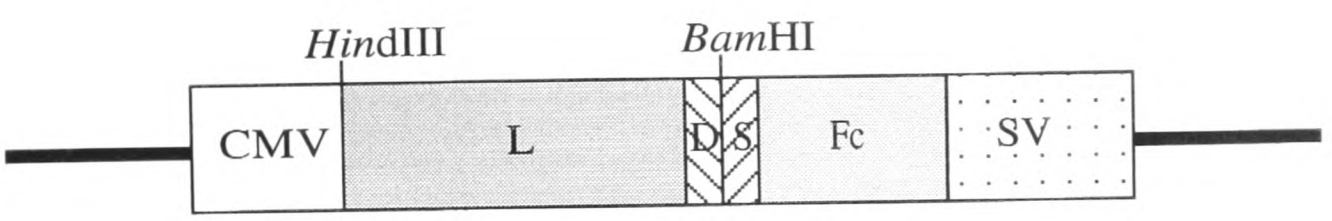
A cell-free system was adopted for the study of the receptor-binding specificity of FGF-9. As in the system described for studying the ligand-binding properties of FGFR1 and FGFR3 (Ornitz et al., 1992; Ornitz and Leder, 1992), the advantage of using soluble receptors is the ability to analyse interacting molecules in the absence of other potentially interfering cell surface molecules.

The mouse homologues of FGFR1c, FGFR2b, FGFR2c, FGFR3c and FGFR4 as well as the human homologue of FGFR1b were used in this study. These receptors were synthesized using a transient mammalian expression vector system based on the pIG vector which contains the coding sequence for human IgG1 Fc. As the Fc fragment includes the

hinge region, protein products are produced as dimers. Soluble FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3c and FGFR4 were therefore produced by fusing the 5' coding regions of the receptors, which stretched from the leader peptide, up to but not including the transmembrane domain, to the Fc-coding cDNA of IgG1 (Figure 4.1). The resulting constructs were transfected into 293T cells by the calcium phosphate DNA precipitate technique (Wigler et al., 1979) and protein expression allowed to occur for up to 5 days in the presence of serum-free chemically-defined UltraCHO medium (Biowhittaker). The fusion proteins secreted into the culture supernatants were detected by exploiting the affinity of the Fc fragment of the fusion protein for protein A. Fusion products bound to protein A-Sepharose were analysed by SDS-polyacrylamide gel electrophoresis as predominant bands of about Mr 100 000 (Figure 4.1B). In addition, monoclonal antibodies that recognize the human IgG1 Fc fragment are available such that easy and efficient analysis of the receptor-fusion products by enzyme-labelled immunosorbent assay (or ELISA) was possible. The concentrations of the fusion products in the culture supernatants of transfected 293T cells were estimated by this method which incorporates an enzyme colour reaction as the detection system (see section 2.2.3). Expression level in this system was typically about 10 μg protein per millilitre of transfected culture supernatant except for FGFR2b, the yield of which was generally low, at about 2 μg/ml. Receptor fusion proteins contained in the conditioned media were used directly in the plate binding assays.

4.2.2 Labelling of FGFs with Biotin

To conjugate biotin to FGF-1, FGF-2, FGF-4 and FGF-9, a maleimide derivative of biotin (biotin-BMCC, Mr 533.69) which reacts with free sulfhydryls on the proteins was used. Since there are at least two cysteine residues in the FGFs not involved in disulphide linkages (Zhu et al., 1990; Ago et al., 1991; Eriksson et al., 1991 ; Zhang et al., 1991), it is possible to couple biotin to the FGF molecules via the free sulfhydryl groups. On removal of the unreacted biotin molecules by dialysis, the reaction products were assessed by coating the proteins on 96-well microtiter plates and detecting for the presence of the



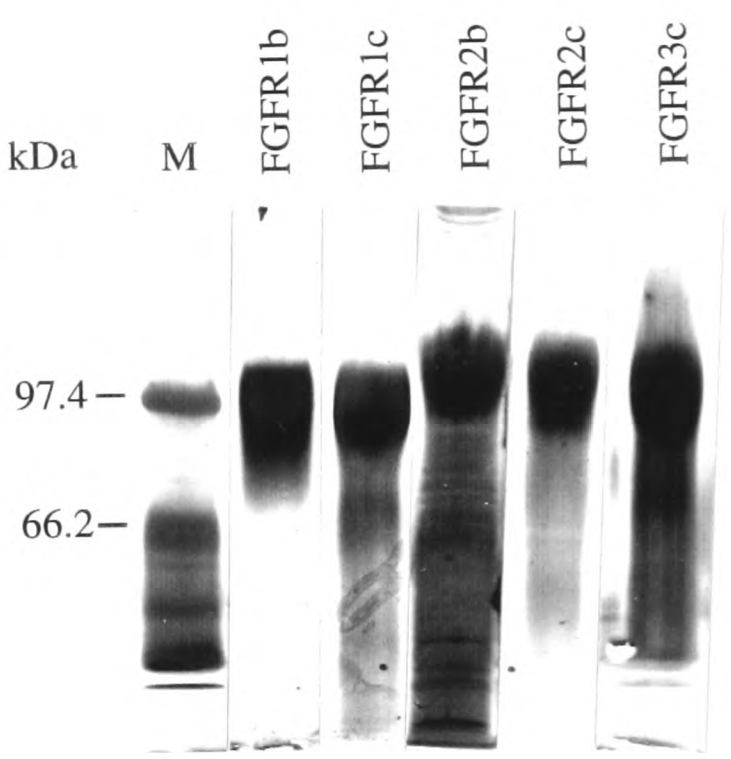


Figure 4.1. Schematic Diagram of the FGF Receptor Expression Vector and SDS-Polyacrylamide Gel Analysis of Receptor-Fc fusion products.
(A) The pIG expression system was used for the synthesis of soluble ectodomains of the FGFRs in 293T cells. The vector contains a cytomegalovirus (CMV) enhancer and promoter. The extracellular ligand binding domain of FGFR (L) includes a splice donor site (D) at its 3' end just upstream of the *Bam*HI cloning site, allowing in-frame fusion with the human IgG1 Fc peptide (Fc) which contains a complementary splice acceptor site (S) at its 5' end. Downstream transcript processing is provided by an SV40 intron and polyadenylation sequence (SV). (B) FGFR-Fc chimeras were produced in the expression system above as dimers via association of the hinge regions of the Fc peptide monomers. The FGFR-Fc fusion proteins bound to protein A-Sepharose migrated on 7.5% SDS-polyacrylamide gels as bands of about 100 kDa, as revealed by silver-staining. FGFR4-Fc protein has a similar migration profile on 7.5% SDS-polyacrylamide gels as a band of approximately 100 kDa (data not shown). Lane M, low range molecular weight standards (Bio-Rad) (phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

biotin moiety via streptavidin coupled to the enzyme horseradish peroxidase. The intensities of the colour reactions obtained were used to estimate the efficiencies of the labelling reaction. In general, the concentration of FGF-biotin that gave an optical reading of 0.5 units was used for the binding assays described below. The effect of the biotin label on the mitogenic activities of biotinylated FGF-2, FGF-4 and FGF-9 were examined based on the measurement of DNA synthesis in quiescent Balb/c 3T3 cells (see section 3.2.6.1) and compared to that of their unmodified counterparts (Figure 4.2). The presence of the biotin label did not appear to affect the biological activities of FGF-2 and FGF-9 but slightly reduced the activity of FGF-4 when compared to its unmodified counterpart. Half-stimulations by FGF-2-biotin and FGF-9-biotin were reached at 1 ng/ml (55 pM) and 1.5 ng/ml (80 pM) respectively while that for FGF-4-biotin was achieved at 3 ng/ml (170 pM), an approximately 4-fold reduction in activity relative to unlabelled FGF-4. It is thus feasible to label various members of the FGF family with biotin and not destroy their biological activities.

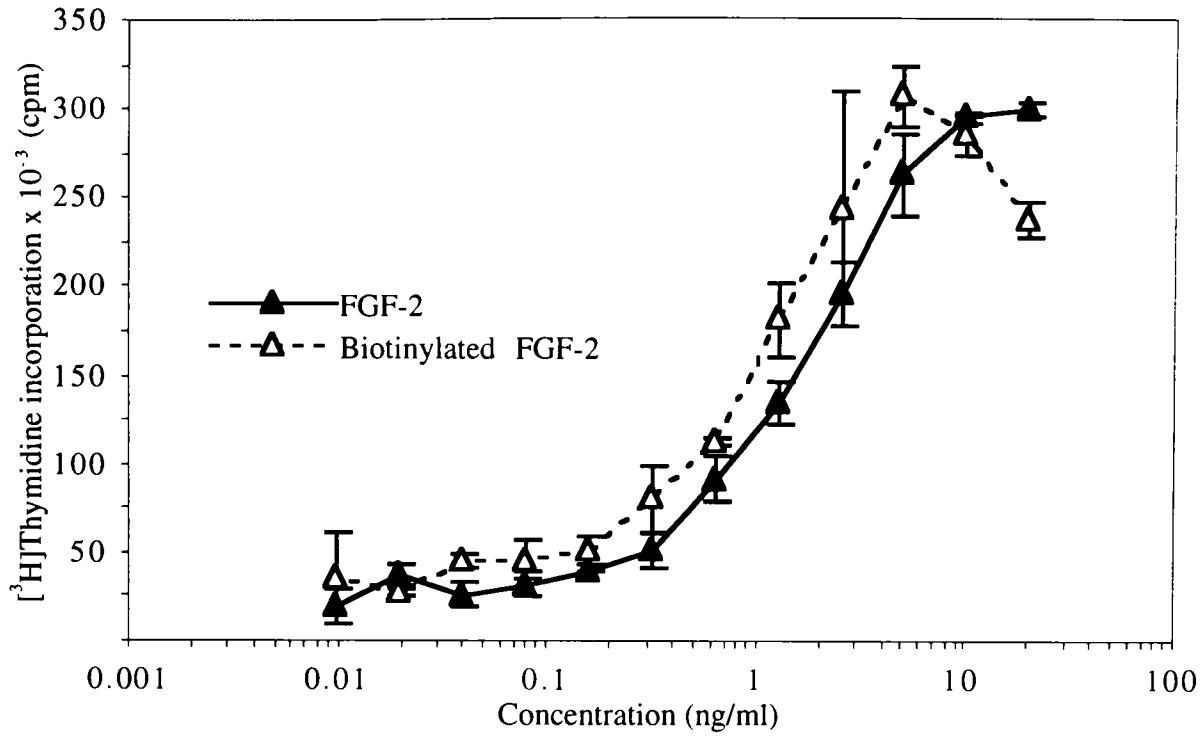
4.2.3 Direct Solid-Phase Receptor Binding Assays

As a first step towards determining the receptor specificity of FGF-9, the ability of FGF-9-biotin to bind to various FGFR-Fc fusion proteins immobilized to a solid support (96-well Maxisorp plates) was assayed (Figure 4.3). In this assay, binding medium (DMEM/0.5% crystalline BSA) containing increasing concentrations of FGF-9-biotin and a fixed concentration of heparin were added to a fixed amount of FGFR-Fc proteins coated onto individual wells of a microtiter plate.

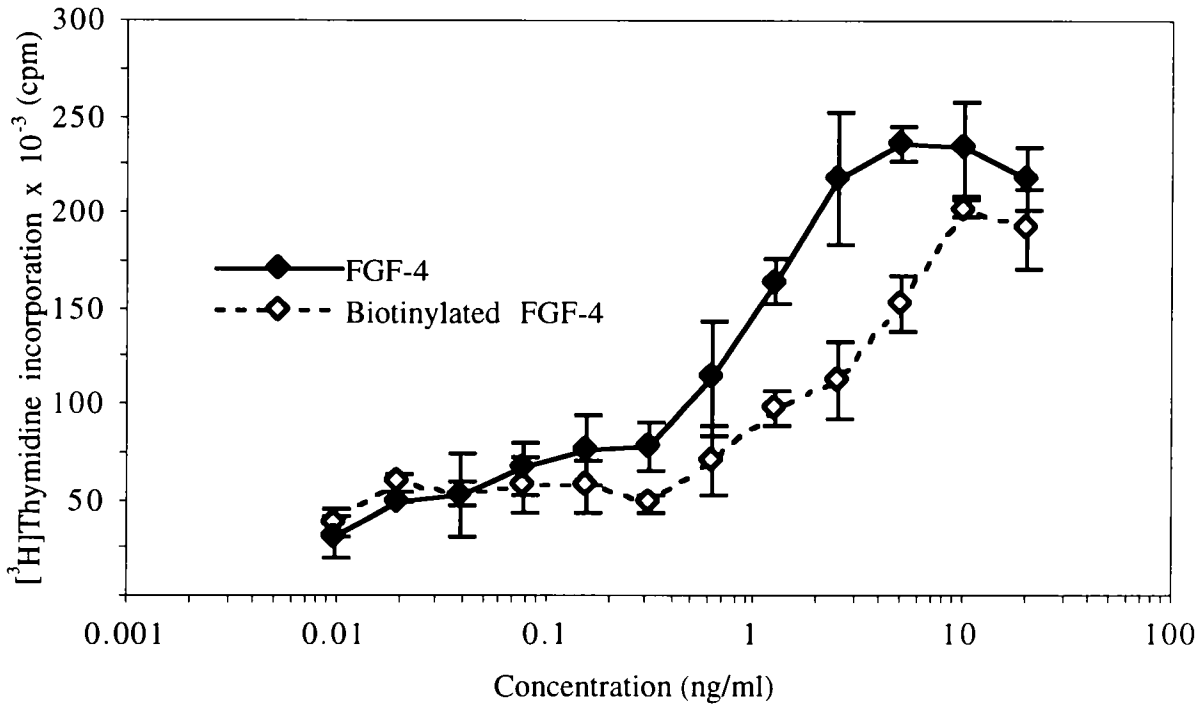
FGF-9 interactions with FGFR1c and FGFR2c were compared with that of FGF-2 which is known to bind to these receptors with high affinity (Mansukhani et al., 1990; Ornitz and Leder, 1992; Vainikka et al., 1992; Werner et al., 1992). FGF-9-biotin appeared to be unable to interact with these two receptors while FGF-2-biotin showed a positive response (Figure 4.4B and D). These assays also confirmed that the soluble FGFR1c- and

Figure 4.2. Mitogenic Activity of Biotinylated FGFs on Balb/c 3T3 cells.
The mitogenic activity of FGF labelled with biotin was compared with its unmodified counterpart. FGF or FGF-biotin was added to the Balb/c 3T3 cell cultures at varying concentrations as represented by the x-axis and the induction of DNA synthesis was measured as thymidine incorporation (y-axis). The activity of (A) FGF-2-biotin was compared with that of unlabelled FGF-2; (B) FGF-4-biotin with unlabelled FGF-4; and (C) FGF-9-biotin with unlabelled FGF-9. Each point is the mean of triplicate measurements ± SD.

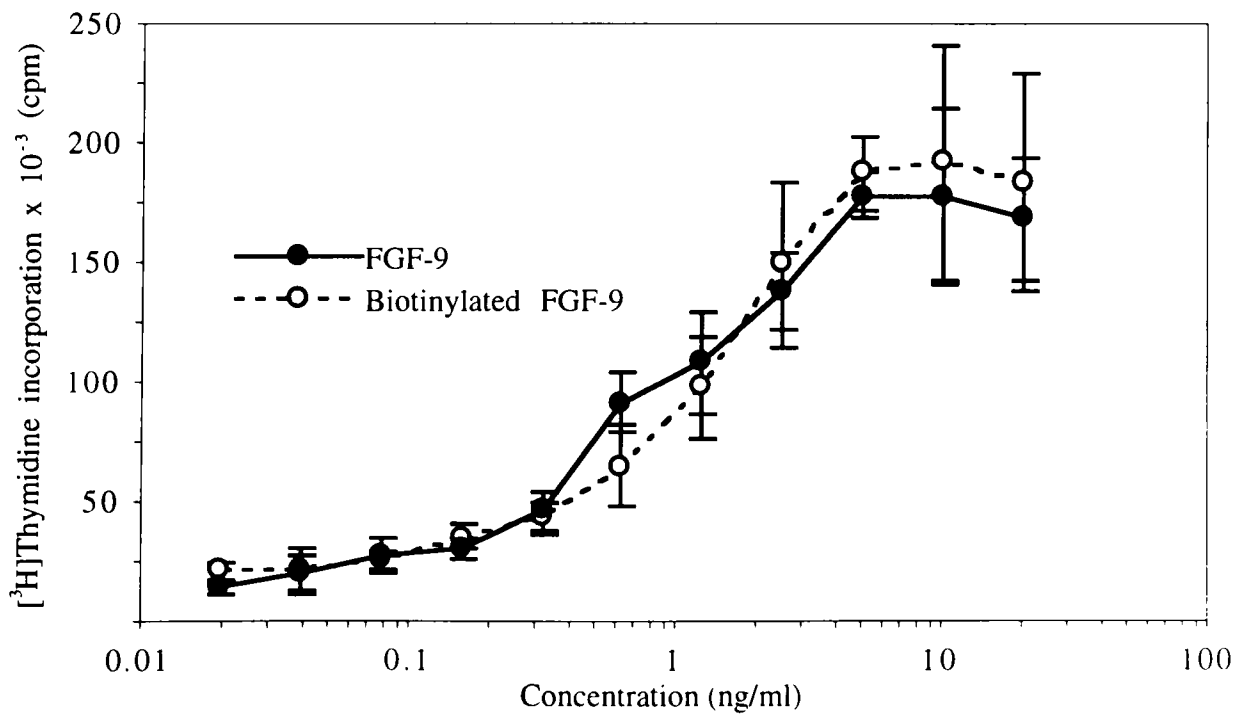
A


350
300
250
200
150
100
50
[3HJThymidine incorporation x 10-3 (cpm)
FGF-2
Biotinylated FGF-2
0.001
0.01
0.1
1
10
100
Concentration (ng/ml)

B


300
250
200
150
100
50
[3H]Thymidine incorporation x 10-3 (cpm)
FGF-4
Biotinylated FGF-4
0.001
0.01
0.1
1
10
100
Concentration (ng/ml)

C


250
200
150
100
50
[3H]Thymidine incorporation x 10-3 (cpm)
FGF-9
Biotinylated FGF-9
0.01
0.1
1
10
100
Concentration (ng/ml)

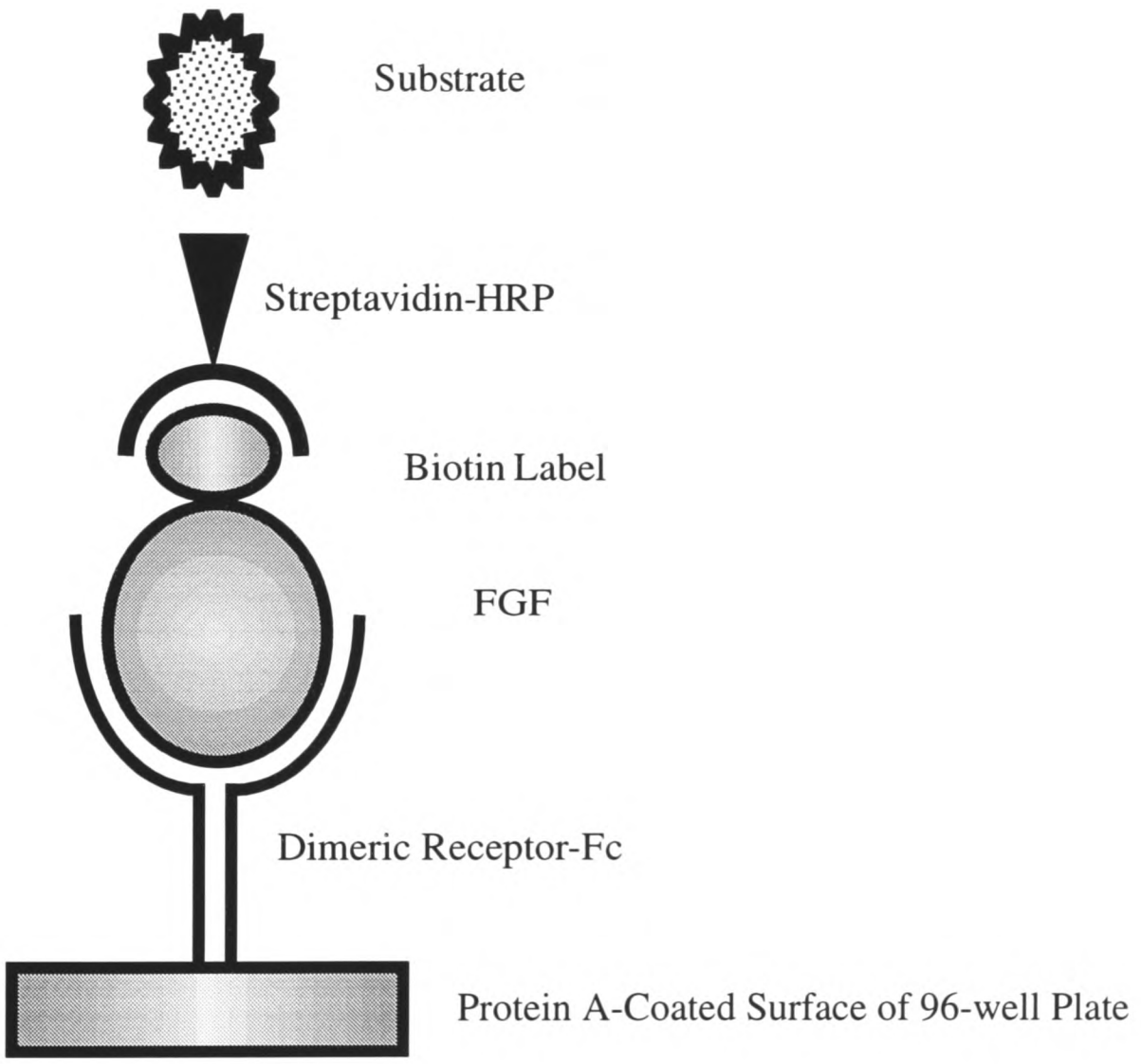
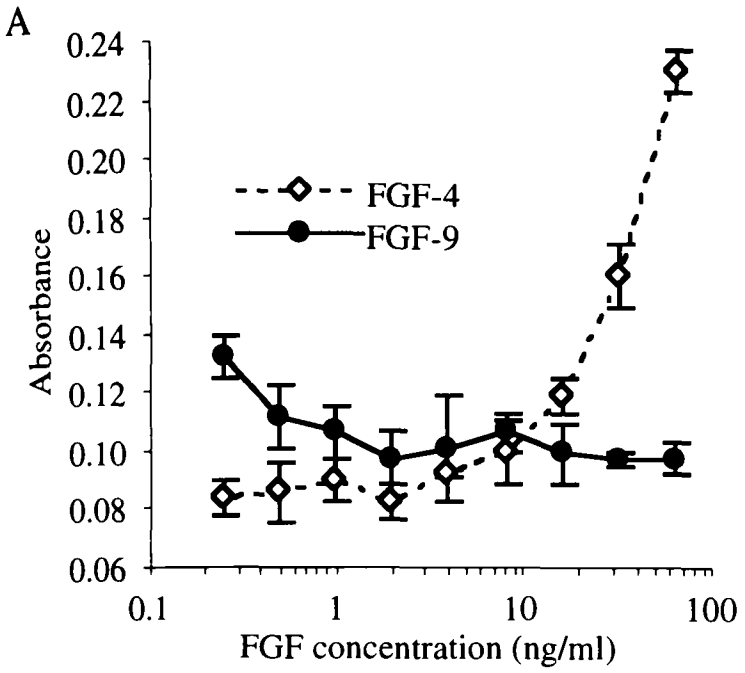
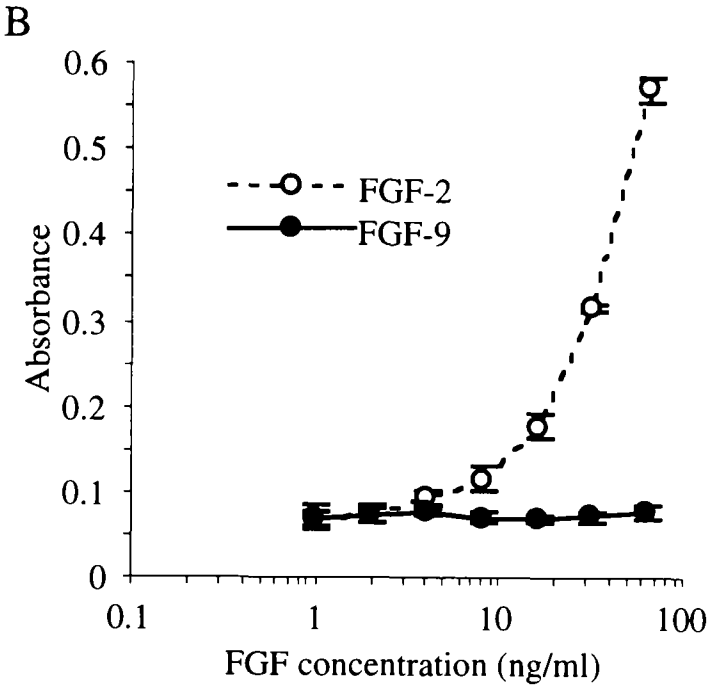
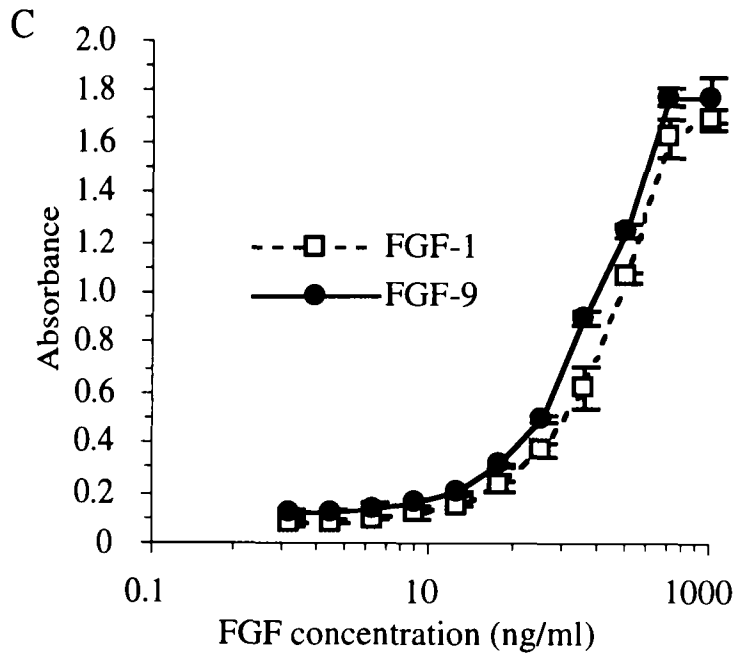


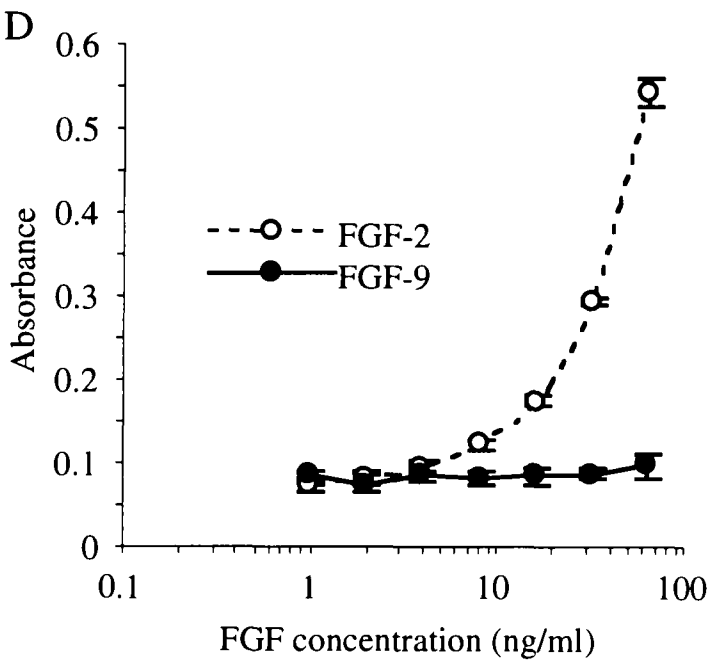
Figure 4.3. Schematic Diagram of the Dimeric Receptor Binding Assay System.
The receptor-Fc chimeras are produced as dimers via association of the hinge regions of the Fc peptide monomers. The *in vitro* solid-phase receptor binding assay system involves immobilizing the dimeric FGFR-Fc fusion proteins onto the wells of protein-A coated 96-well Maxisorp plates. This is followed by addition of the biotin-labelled FGF ligand. The biotin label can be detected by streptavidin, which when coupled to the enzyme horseradish peroxidase (HRP), enables measurement of the binding reaction by a colour reaction procedure (see section 2.2.5).

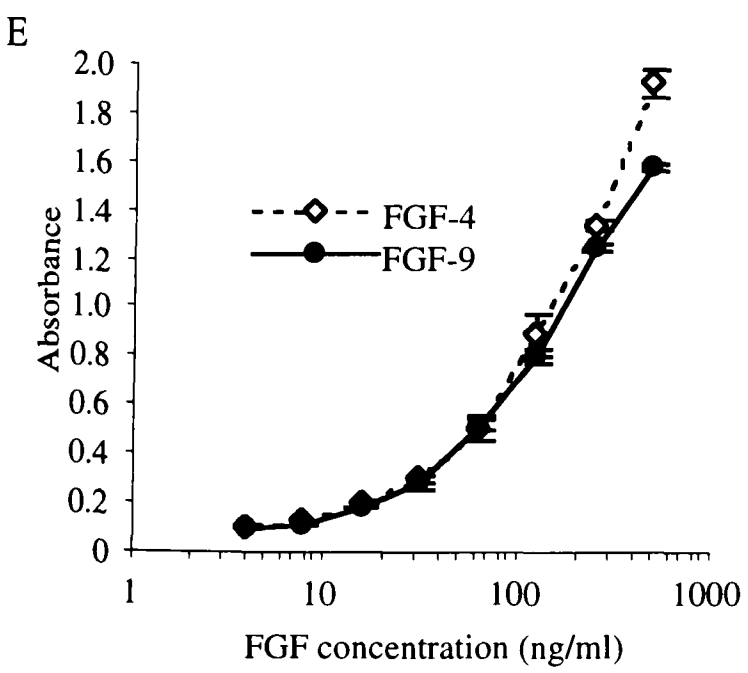
Figure 4.4. Direct Binding of Biotinylated FGFs to Soluble Receptor-Fc Fusion Proteins. Increasing concentrations of biotinylated FGF-9 were added to FGFR-Fc fusion protein immobilized on 96-well Maxisorp plates. (A) FGFR1b-Fc, (B) FGFR1c-Fc, (C) FGFR2b-Fc, (D) FGFR2c-Fc, (E) FGFR3c-Fc and (F) FGFR4-Fc. FGFR1b-Fc, FGFR1c-Fc, FGFR2c-Fc, FGFR3c-Fc, and FGFR4-Fc were immobilized to the plates at 10 μg/ml per well while FGFR2b-Fc was coated at a concentration of 2 μg/ml per plate. Binding experiments with each FGFR-Fc were performed in parallel with known ligands. The binding of labelled FGF-9 to either FGFR1b-Fc or FGFR3c-Fc was compared with that of similarly labelled FGF-4 while binding to FGFR1c-Fc and FGFR2c-Fc was compared with that of FGF-2-biotin; FGF-1-biotin was used as the control for analysing the interaction of FGF-9-biotin with FGFR2b-Fc and FGFR4-Fc. Binding reactions for (A), (B), (D) and (E) were performed in the presence of 10 μg/ml heparin while that for (C) and (F) were carried out with 1 μg/ml heparin in the binding medium (DMEM/0.5% BSA). The amount of horseradish peroxidase activity bound to the wells was used as a measure of the level of bound FGF, the reaction being read as absorbance at 492 nm. Each point is the average of triplicate readings ± SD, and each experiment is representative of at least three separate assays. In addition to analysing the binding specificity of FGF-9, this data also confirmed that the ligand specificities of the various receptor fusion proteins were intact when expressed and used in this manner.

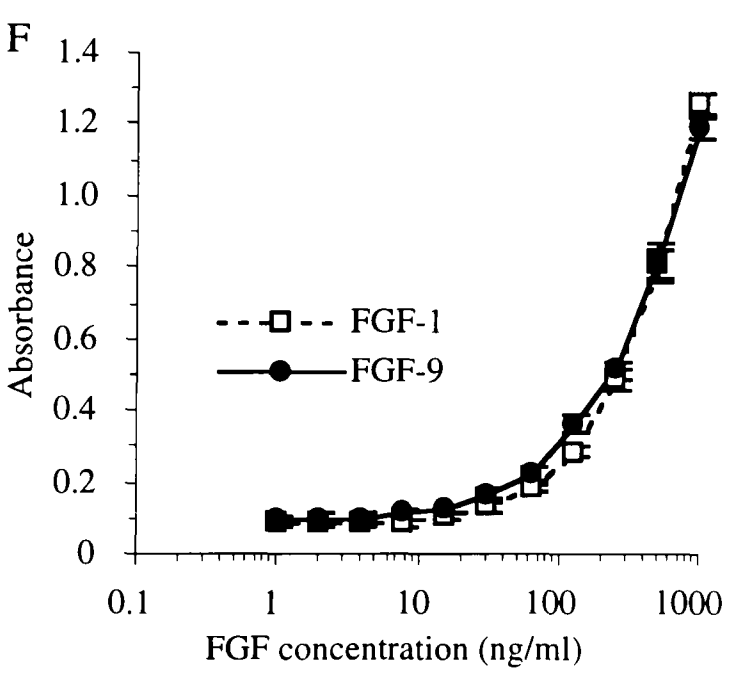

A
Absorbance
FGF-4
FGF-9
FGF concentration (ng/ml)


B
Absorbance
FGF-2
FGF-9
FGF concentration (ng/ml)


C
Absorbance
FGF-1
FGF-9
FGF concentration (ng/ml)


D
Absorbance
FGF-2
FGF-9
FGF concentration (ng/ml)


E
Absorbance
FGF-4
FGF-9
FGF concentration (ng/ml)


F
Absorbance
FGF-1
FGF-9
FGF concentration (ng/ml)

FGFR2c-Fc proteins have retained their ligand binding capacities and specificities when expressed as genetically-engineered fusion products.

Similarly, the interactions of FGF-9-biotin with FGFR1b and FGFR3c were examined relative to that of FGF-4-biotin. Increasing concentrations of FGF-4-biotin were correlated with increasing binding to these two receptors. FGF-9-biotin displayed a similar effect on FGFR3c but not towards FGFR1b (Figure 4.4A and E). Hence, FGF-9 appears to have the capacity to bind FGFR3c. These assays also confirmed that both FGFR1b- and FGFR3c-Fc fusion proteins are capable of recognizing the known ligand, FGF-4 (Ornitz and Leder, 1992).

Finally, the interactions between FGF-9-biotin and FGFR2b as well as FGFR4 were tested based on FGF-1-biotin as the control ligand. Increasing binding of FGF-1-biotin to FGFR2b and FGFR4 were shown at increasing concentrations of the ligand (Figure 4.4C and F). Likewise, FGF-9-biotin was found to bind to both of these receptors, indicating that FGF-9 may serve as a ligand for both FGFR2b and FGFR4.

These preliminary assays thus indicated that FGF-9 may interact with three FGFRs, namely, FGFR2b, FGFR3c and FGFR4.

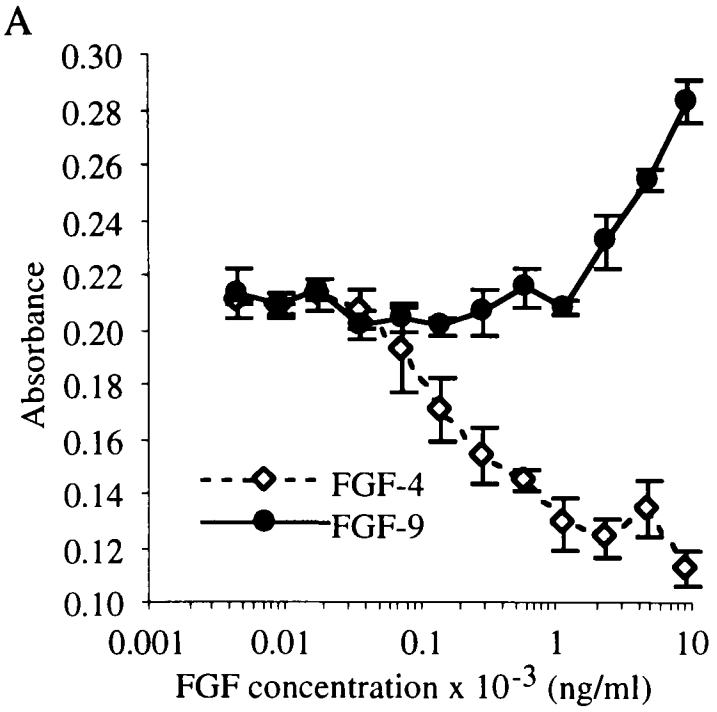
4.2.4 Competitive Solid-Phase Receptor Binding Assays

To further analyse and confirm the receptor binding specificity of FGF-9, the ability of FGF-9 to compete with other FGFs for binding to the six FGFR-Fc fusion proteins was tested. The competition assay was performed by adding increasing concentrations of unlabelled FGF to the binding medium containing a fixed concentration of FGF-biotin and heparin. This assay is thus, a measure of specific binding interactions to the exclusion of non-specific ones.

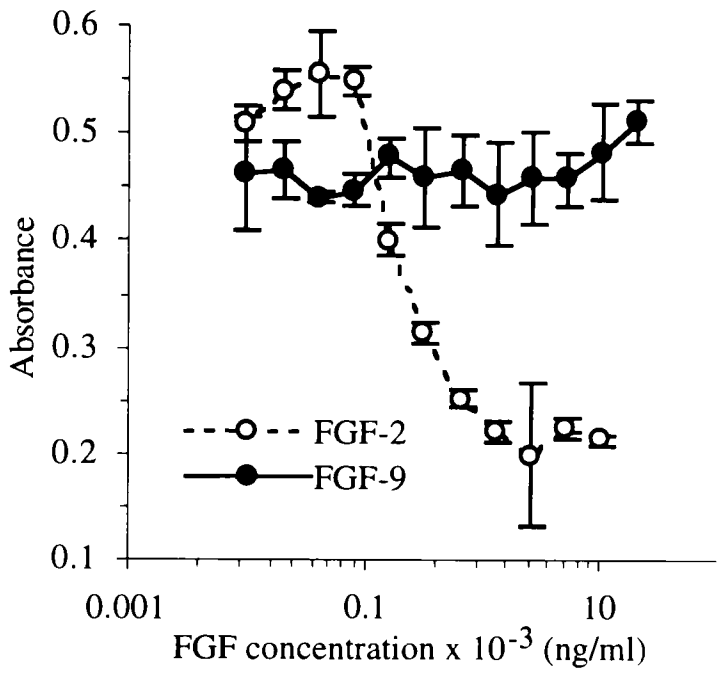
The interaction of FGF-9 with each of the following receptors: FGFR1c-, FGFR2b- and FGFR2c-Fc, was analysed by competition assays against biotinylated FGF-2 (Figure 4.5B, C, D). As predicted from the direct binding assay data (see section 4.2.3), FGF-9 could not compete with FGF-2-biotin for binding to FGFR1c or FGFR2c while

Figure 4.5. Competition Binding of Biotinylated FGFs to Soluble Receptor-Fc Fusion Proteins.
Competition binding was assayed for the following FGFR-Fc proteins immobilized on 96-well Maxisorp plates: (A) FGFR1b-Fc, (B) FGFR1c-Fc, (C) FGFR2b-Fc, (D) FGFR2c-Fc, (E) FGFR3c-Fc, and (F) FGFR4. Specific binding of biotinylated FGF-4 (150 ng/ml) to FGFR1b-Fc and FGFR3c-Fc was competed for by increasing concentrations of unlabelled FGF-4 or FGF-9. Similarly, specific binding of biotinylated FGF-2 (5 ng/ml) to FGFR1c-Fc, FGFR2b-Fc and FGFR2c-Fc was competed for by increasing concentrations of unlabelled FGF-2 or FGF-9. Finally, specific interaction between biotinylated FGF-1 (100 ng/ml) and FGFR4 was competed for by various concentrations of unlabelled FGF-1 or FGF-9. Competition binding reactions for (B), (C), (D) and (F) were carried out in the presence of 1 μg/ml heparin while (A) and (E) were performed in the presence of 25 μg/ml heparin. All FGFR-Fc proteins were immobilized on the Maxisorp plates at 10 μg/ml except for FGFR2b-Fc which was immobilized at a concentration of 2 μg/ml. Values shown are the average of triplicate samples ± SD. Each experiment is representative of at least three separate assays.

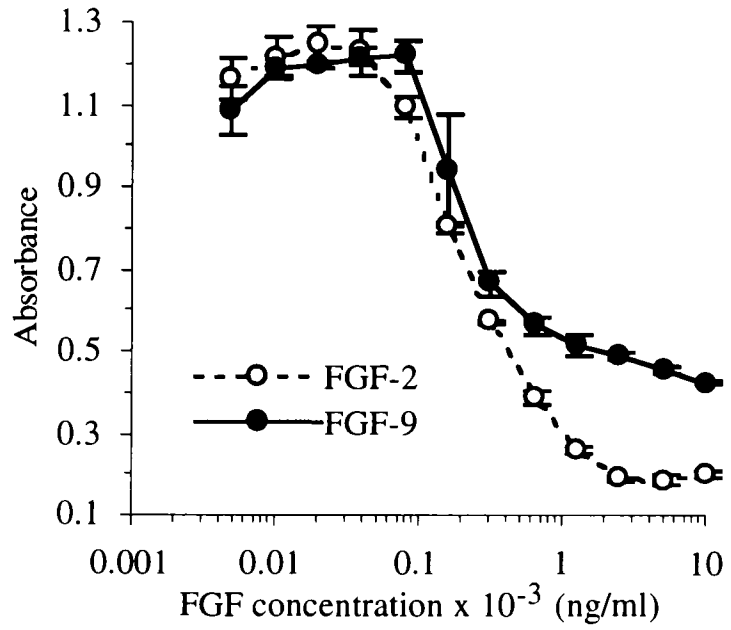
A


0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
Absorbance
FGF-4
FGF-9
0.001
0.01
0.1
1
10
FGF concentration x 10-3 (ng/ml)

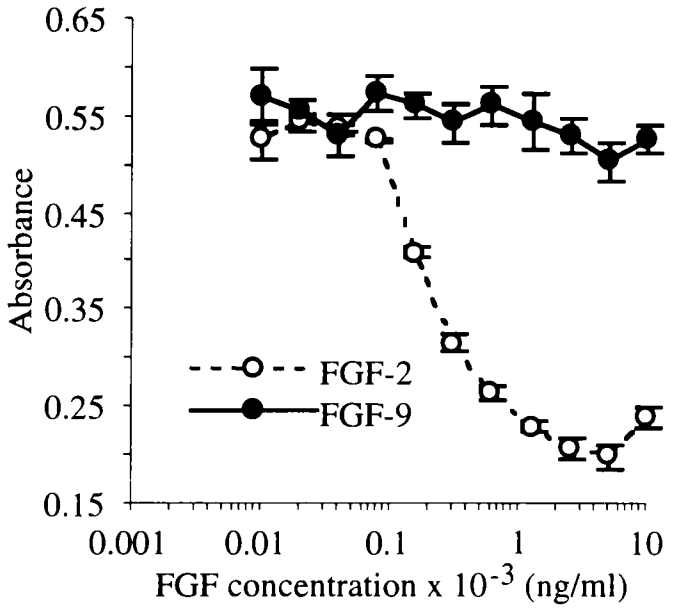
B


0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
FGF-2
FGF-9
0.001
0.1
10
FGF concentration x 10-3 (ng/ml)

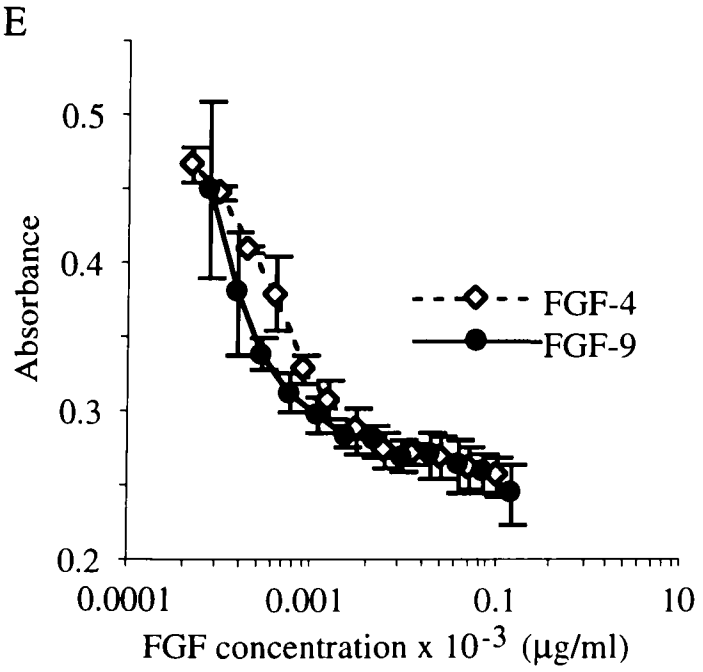
C


1.3
1.1
0.9
0.7
0.5
0.3
0.1
Absorbance
FGF-2
FGF-9
0.001
0.01
0.1
1
10
FGF concentration x 10-3 (ng/ml)

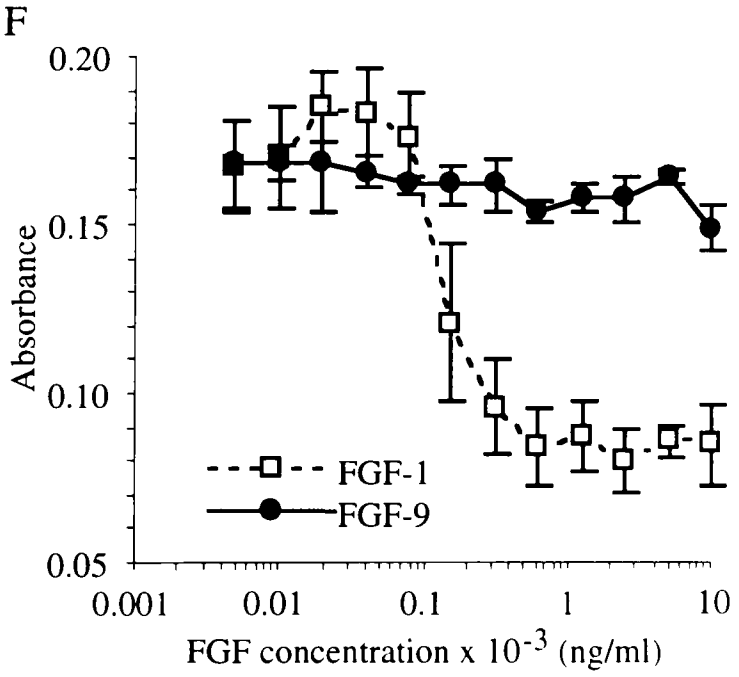
D


0.65
0.55
0.45
0.35
0.25
0.15
Absorbance
FGF-2
FGF-9
0.001
0.01
0.1
1
10
FGF concentration x 10-3 (ng/ml)

E


0.5
0.4
0.3
0.2
Absorbance
FGF-4
FGF-9
0.0001
0.001
0.1
10
FGF concentration x 10-3 (µg/ml)

F


0.20
0.15
0.10
0.05
Absorbance
FGF-1
FGF-9
0.001
0.01
0.1
1
10
FGF concentration x 10-3 (ng/ml)

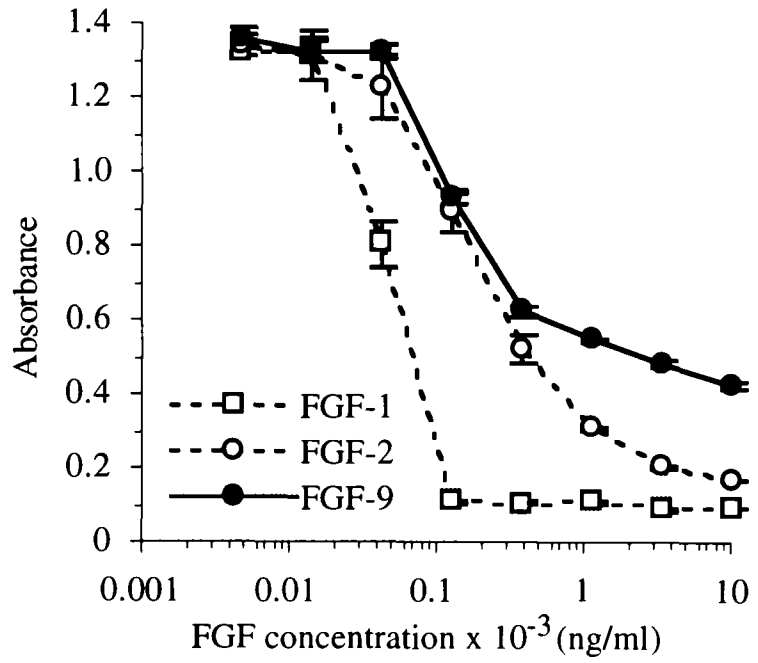
FGF-2 showed a positive competition effect as increasing concentrations of the unlabelled FGF-2 gradually inhibited binding of labelled FGF-2 to both these receptors (Figure 4.5B and D). On the other hand, in agreement with the direct binding assay result, FGF-9 was able to compete with biotinylated FGF-2 in binding to FGFR2b (Figure 4.5C). The ID_{50} (dose that inhibits binding by 50%) for the competition effect produced by FGF-9 against FGF-2-biotin with respect to FGFR2b was calculated from the binding curve to be ≈ 11 pM.

Similarly, the abilities of FGFR1b- and FGFR3c-Fc fusion proteins to recognize FGF-9 were tested by competition assays against biotinylated FGF-4. Again, as predicted from the direct binding assay (section 4.2.3), FGF-9 was unable to compete with FGF-4-biotin for binding to FGFR1b (Figure 4.5A) while unlabelled FGF-4 was able to do so. FGF-9 was, however, able to compete effectively with FGF-4-biotin in binding to FGFR3c (ID_{50} ≈ 27 pM; Figure 4.5E). This confirmed that FGF-9 does indeed bind specifically to FGFR3c.

Finally, the binding relationship between FGF-9 and FGFR4 was assayed by a competition binding assay against biotinylated FGF-1. FGF-9 was found to be unable to compete with FGF-1-biotin for binding to FGFR4 while FGF-1 was able to do so (Figure 4.5F). This result suggests that FGF-9 does not bind to the same site on FGFR4 as FGF-1. Hence, the binding phenomenon observed previously in the direct binding assay (see section 4.2.3) may reflect a non-specific interaction unique for FGFR4 since the binding data for the other FGFRs from the two binding assays corroborated one another.

In summary, the above binding reactions indicated that FGF-9 binds specifically to only two of the six receptors analysed, namely, FGFR2b and FGFR3c. The affinity of FGF-9 for either receptor was further compared relative to other FGFs. Both FGF-1 and FGF-2 are known to bind FGFR2b with different affinities (Bottaro et al., 1990; Miki et al., 1992). A comparison of the affinities of all three ligands, FGF-1, FGF-2 and FGF-9 for binding to FGFR2b was carried out by a competition binding assay against biotinylated FGF-2 (Figure 4.6A). It is observed that FGF-2-biotin binding to FGFR2b was competed for most efficiently by unlabelled FGF-1 followed by FGF-2 and FGF-9 (Table 4.1).

A



B

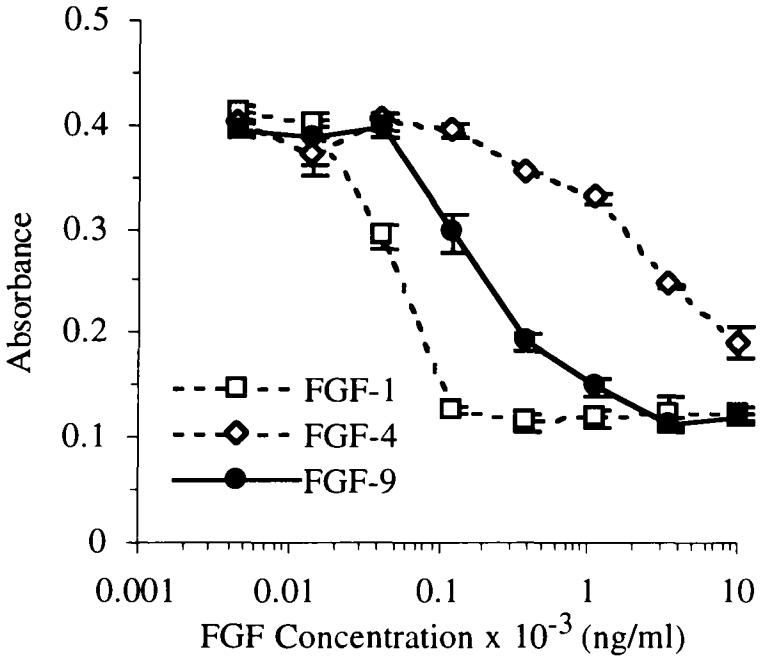


Figure 4.6. Affinity of FGF-9 for FGFR2b and FGFR3c Relative to Other FGF Ligands.
(A) Specific binding of biotinylated FGF-2 (10 ng/ml) to FGFR2b-Fc (2 μg/ml) immobilized on 96-well Maxisorp plates was competed for by increasing concentrations of unlabelled FGF-1, FGF-2 or FGF-9 in the presence of 1 μg/ml heparin. (B) Specific binding of biotinylated FGF-9 (100 ng/ml) to similarly immobilized FGFR3c-Fc (10 μg/ml) was competed for by increasing concentrations of unlabelled FGF-1, FGF-4 or FGF-9 in the presence of 25 μg/ml heparin. Values shown are the average of triplicate samples ± SD. Where error bars are not apparent, the error is less than the size of the symbol. Each experiment is representative of at least three separate assays.

Table 4.1. ID_{50} Values for the Binding of Various FGFs to FGFR-Fc proteins.

Receptor	**Ligand**				**Tracer**
	FGF-1	**FGF-2**	**FGF-4**	**FGF-9**	
FGFR2b	2.7	11	N.D.	11	FGF-2-biotin
FGFR3c	N.D.	N.D.	42	27	FGF-4-biotin
FGFR3c	2.9	N.D.	167	11	FGF-9-biotin

The ID_{50} values (in nM) were calculated from the competition binding data shown in Figures 4.5 and 4.6. The values are representative of more than one assay. N.D., not determined.

Relative to the concentration of FGF-2 required to effect 50% binding inhibition, 4-fold less FGF-1 and 1.5-fold more FGF-9 were required to compete for FGF-2-biotin binding to FGFR2b. Hence, of the three, FGF-9 appears to have the lowest affinity for FGFR2b.

Similarly, the binding affinity of FGF-9 for FGFR3c was compared with those of FGF-1 and FGF-4, FGF-1 being the ligand which binds to the receptor with a higher affinity than FGF-4 (Ornitz and Leder, 1992; Chellaiah et al., 1994). The competition assay, performed against a fixed concentration of biotinylated FGF-9, indicated that FGF-9-biotin binding to FGFR3c was most effectively competed for by unlabelled FGF-1, followed by FGF-9 itself and finally FGF-4 (Figure 4.6B; Table 4.1). Compared to the interaction between FGF-9 and its labelled counterpart, 4-fold less of unlabelled FGF-1 and 15-fold more of FGF-4 were required to elicit 50% binding inhibition. FGF-9 has, therefore, a substantially higher affinity for FGFR3c than FGF-4 but a lower affinity relative to FGF-1.

4.2.5 Heparin Dependence of FGF-Receptor Binding Interactions *in Vitro*

Heparin is a heterogeneous molecule consisting of repeating disaccharide subunits of hexuronic acid and D-glucosamine which are N-acetylated as well as N-sulphated (Kjellén and Lindahl, 1991). Heparin, being highly similar to endogenous heparan sulphate which represent the low-affinity binding receptor for FGF-2 (reviewed by Klagsbrun, 1990), is frequently used in binding experiments as representative of the endogenous low-affinity binding site for FGF. Interaction with this molecule, however, does not necessarily imply a biological function since it is the strongest negatively charged polyelectrolyte found in mammalian tissues and so has the ability to interact with positively charged regions in various proteins.

Heparin was first shown to be an absolute necessity for FGF-2 and FGFR1c binding interaction (Ornitz et al., 1992). Similarly, the binding of FGF-1 to FGFR1c or FGFR3c was found to be enhanced by heparin (Ornitz and Leder, 1992). Both reports used an assay system based on soluble forms of the receptors produced as fusion proteins with

alkaline phosphatase so that heparin requirement was evaluated in the absence of other interfering cell surface molecules, as in the assay system described here. The effect of heparin on the interactions of FGF-1, FGF-2 and FGF-4 with their respective sets of receptors was evaluated by adding biotinylated FGFs in the presence of varying concentrations of heparin to receptors immobilized on 96-well microtiter plates.

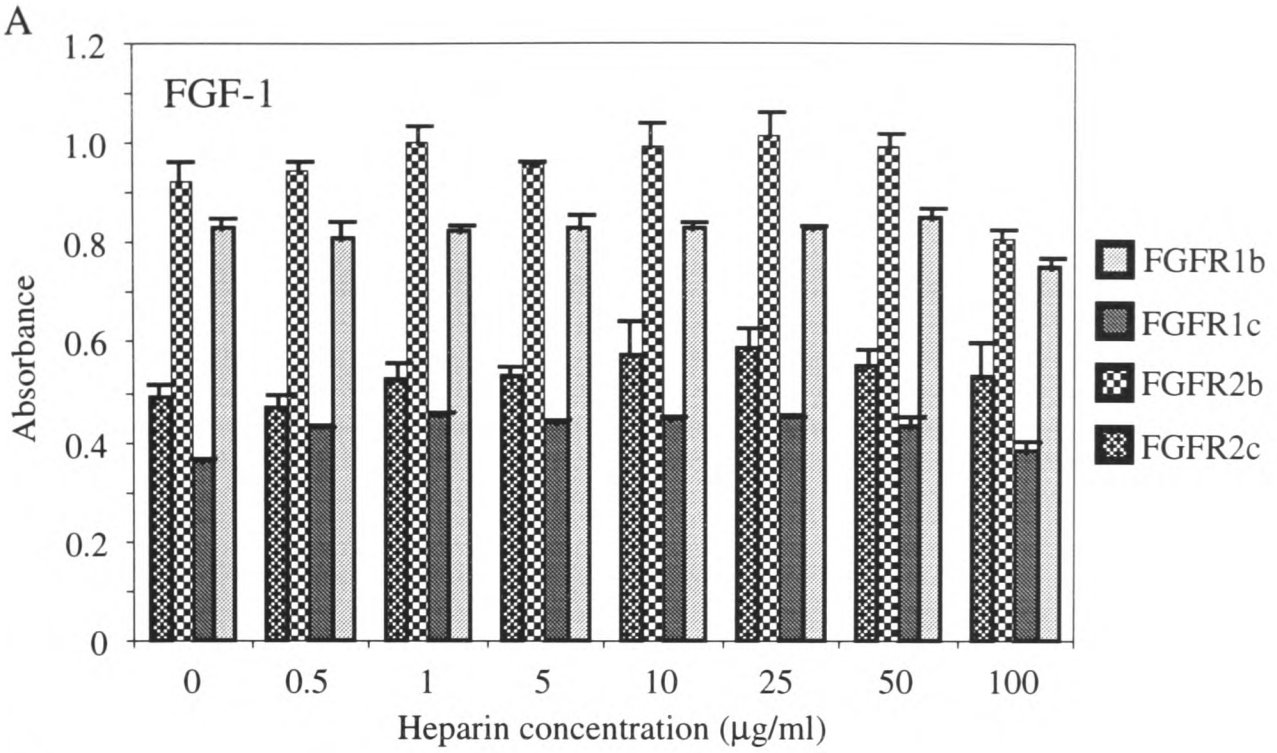
To assay for heparin requirement in the binding interactions of FGF-1 with FGFR1b, FGFR1c, FGFR2b and FGFR2c, binding medium containing a fixed concentration of FGF-1-biotin and various concentrations of heparin were added to the immobilized receptors. In contradiction to a previous report (Ornitz and Leder, 1992), no obvious requirement for heparin in the interaction of FGF-1 with any of the FGFRs was observed (Figure 4.7A). Indeed, FGF-1 bound to all the receptors tested just as well with or without heparin. Very high heparin concentrations appeared to slightly impede the interactions of FGF-1 with FGFR1c and FGFR2c.

Similarly, the effect of heparin in the binding interactions of FGF-2-biotin with its various receptors, FGFR1b, FGFR1c, FGFR2b and FGFR2c were examined. A significant effect imposed by heparin in the binding of FGF-2 with FGFR1b was observed; the effect of heparin on its interactions with FGFR1c and FGFR2b was less pronounced (Figure 4.7B). Relative to the absence of any heparin, the presence of 500 ng/ml heparin in the binding medium enhanced the binding of FGF-2 to FGFR1b by up to 7-fold while the increase in binding to FGFR1c and FGFR2b were 1.4- and 1.2-fold respectively. No effect was observed for the FGF-2-FGFR2c interaction. This differential effect of heparin suggest possible interactions between heparin and FGF-2 and/or FGFR1b. Maximal binding of FGF-2 with all the four receptors tested was observed at 1 μg/ml heparin while at heparin concentrations greater than 25 μg/ml, there appeared to be a decrease in binding interactions.

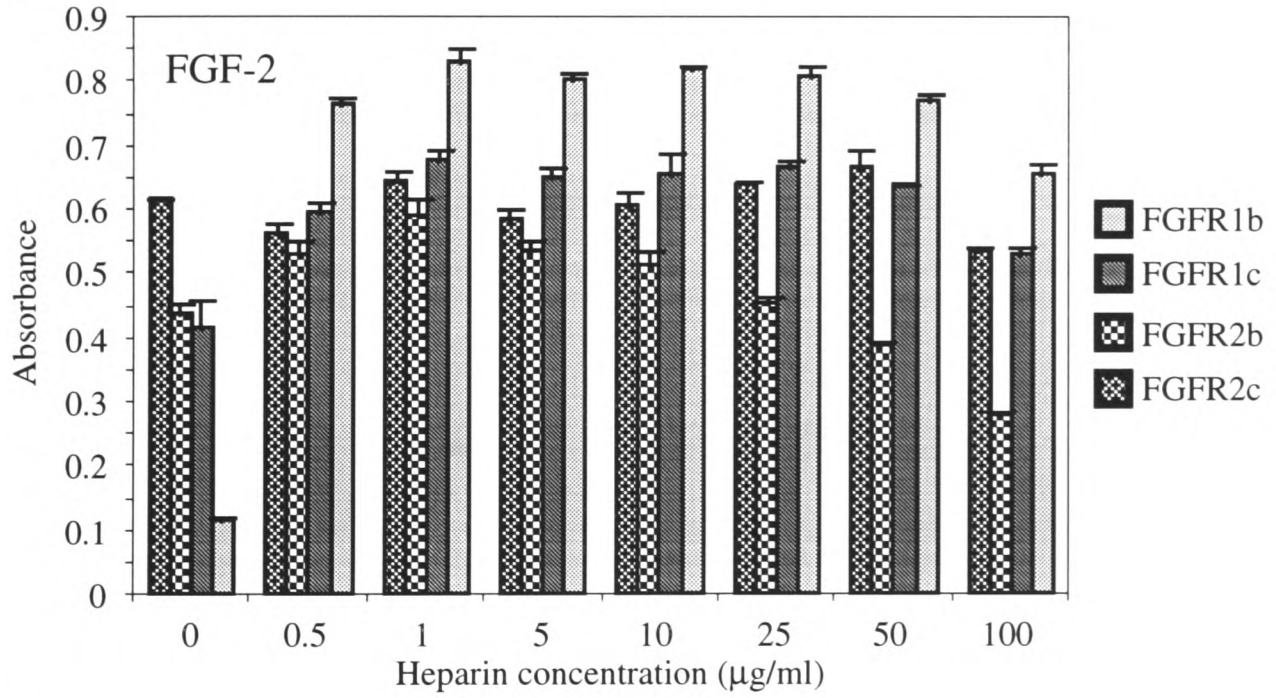
Finally, the involvement of heparin in FGF-4-biotin binding interactions with FGFR1c, FGFR2c and FGFR3c was tested as above. No significant effect was observed in the presence of heparin for any of the receptors tested (Figure 4.7C). There may be a slight increase in binding of FGF-4 with each of the receptors tested in the presence of

Figure 4.7. Heparin Dependence of FGF-Receptor Binding Interaction.
Direct binding of biotinylated FGFs to FGFR-Fc proteins immobilized on 96-well Maxisorp plates in the presence of increasing concentrations of heparin. (A) A fixed concentration of biotinylated FGF-1 (10 ng/ml) was bound to FGFR1b-Fc, FGFR1c-Fc, FGFR2b-Fc or FGFR2c-Fc (all immobilized at 10 μg/ml except for FGFR2b-Fc which was immobilized at 2 μg/ml). (B) Similarly, biotinylated FGF-2 (5 ng/ml) was bound to FGFR1b-Fc, FGFR1c-Fc, FGFR2b-Fc or FGFR2c-Fc. (C) Biotinylated FGF-4 (500 ng/ml) was bound to FGFR1c-Fc, FGFR2c-Fc or FGFR3c-Fc. The amount of biotinylated FGF bound was measured as absorbance at 492 nm. All values represent the mean of triplicate assays ± SD.

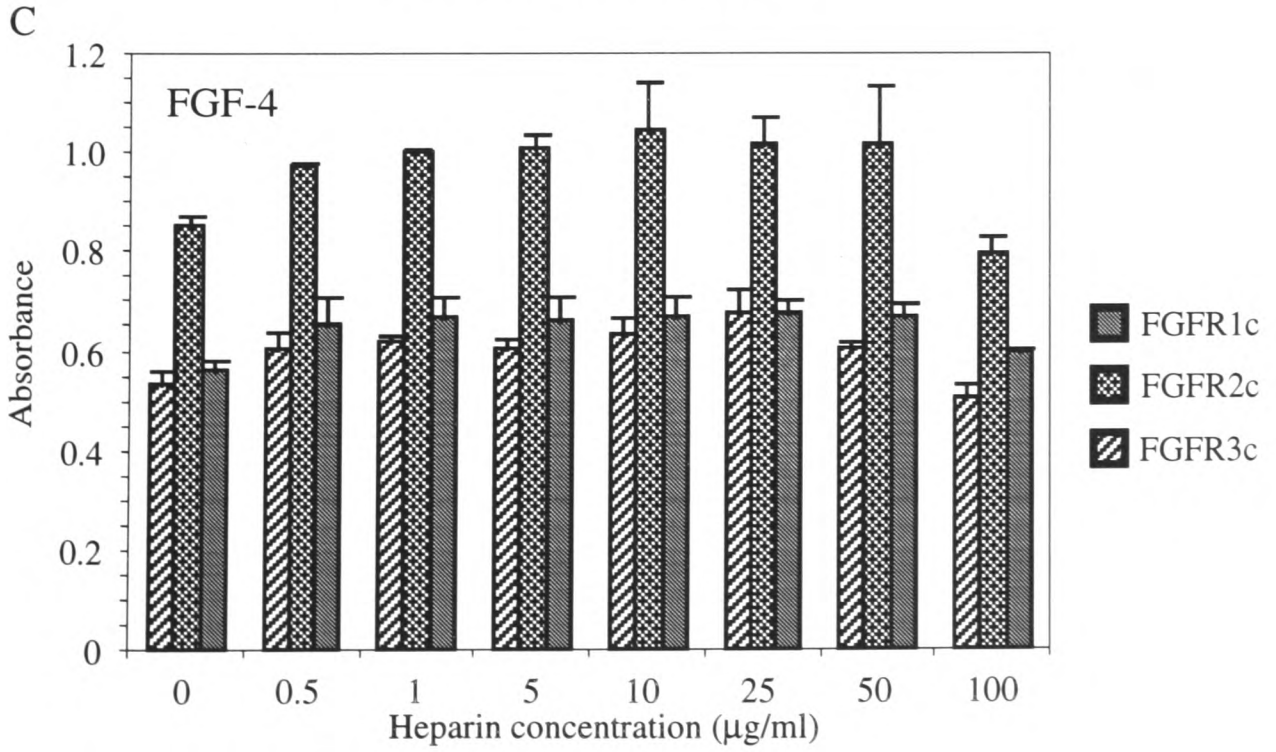
A


FGF-1
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1
5
10
25
50
100
Heparin concentration (μg/ml)
FGFR1b
FGFR1c
FGFR2b
FGFR2c

B


FGF-2
Absorbance
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
5
10
25
50
100
Heparin concentration (μg/ml)
FGFR1b
FGFR1c
FGFR2b
FGFR2c

C


FGF-4
Absorbance
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.5
1
5
10
25
50
100
Heparin concentration (μg/ml)
FGFR1c
FGFR2c
FGFR3c

heparin (Figure 4.7C). As observed previously for FGF-1 and FGF-2 interactions with various receptors (Figure 4.7A, B), high concentrations of heparin in excess of 25 μg/ml caused a decrease in the binding of FGF-4 to all the receptors tested.

The effect of heparin in the binding interaction of FGF-9 with either FGFR2b or FGFR3c was similarly assayed. No obvious effect on either binding interactions in the presence of heparin was observed (Figure 4.8).

4.2.6 Surface Plasmon Resonance Analysis

To confirm the affinity of FGF-9 for FGFR3c, the binding interaction was measured by surface plasmon resonance biosensor technology (Fägerstam, 1991; Jonsson et al., 1991).

The method consists of immobilizing protein molecules (ligands) onto a surface and allowing other interacting protein ligands (analytes) to pass over the surface. As the analyte binds to the surface, the concentration of molecules on the surface increases. This change in surface concentration can be measured optically, based on the principle of total internal reflection and hence the term surface plasmon resonance or SPR. The introduction of the Biosensor system (Pharmacia Biosensor, Uppsala) makes this measurement possible as it monitors the real-time binding of protein molecules to a sensor chip surface containing the immobilized ligand (Fägerstam, 1991; Jonsson et al., 1991; Chaiken et al., 1992). The sensor chip consists of a gold-coated glass surface to which a (carboxymethyl)dextran matrix is attached. This thin layer of gold metal provides the basis for the optical phenomenon of surface plasmon resonance to be observed as SPR arises under conditions of total internal reflection. Specifically, SPR occurs when monochromatic and p-polarized light is incident on a thin metal film interface between two media of different refractive index at a specific angle, resulting in a sharp dip in the intensity of reflected light (Figure 4.9). The incident light angle at which the dip is observed is the SPR angle. This angle depends on several factors including the refractive index of the medium close to the non-illuminated side of the gold film. Hence, when the refractive index in the vicinity of the gold surface is changed, the SPR angle is correspondingly modified. Since, refractive

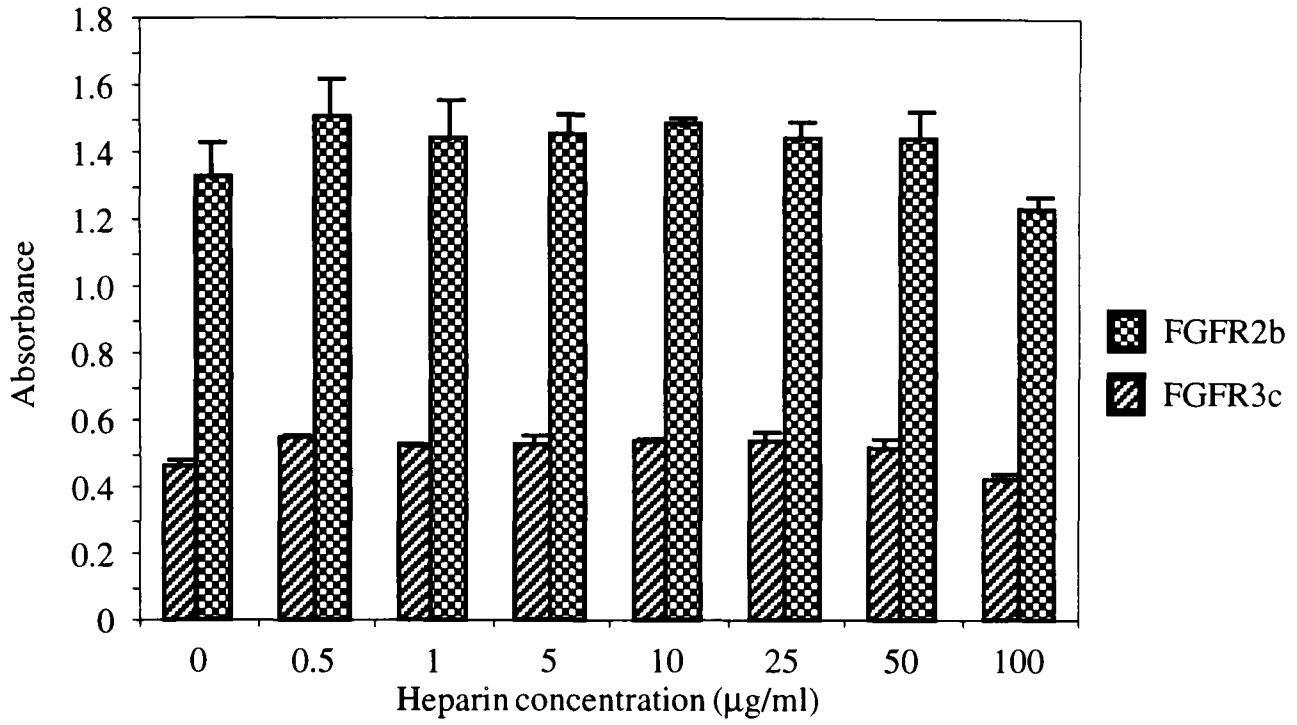


Figure 4.8. Heparin Dependence of FGF-9 Binding to FGFR2b and FGFR3c.
Biotinylated FGF-9 (250 ng/ml) was bound to FGFR2b-Fc or FGFR3c-Fc immobilized on 96-well Maxisorp plates in the presence of increasing concentrations of heparin. FGFR2b-Fc was immobilized at a concentration of 2 μg/ml while FGFR3c-Fc was immobilized at 10 μg/ml. Assays on the two receptors were done in parallel. The amount of biotinylated FGF bound was measured as absorbance at 492 nm. Values shown are the means of triplicate samples ± SD.

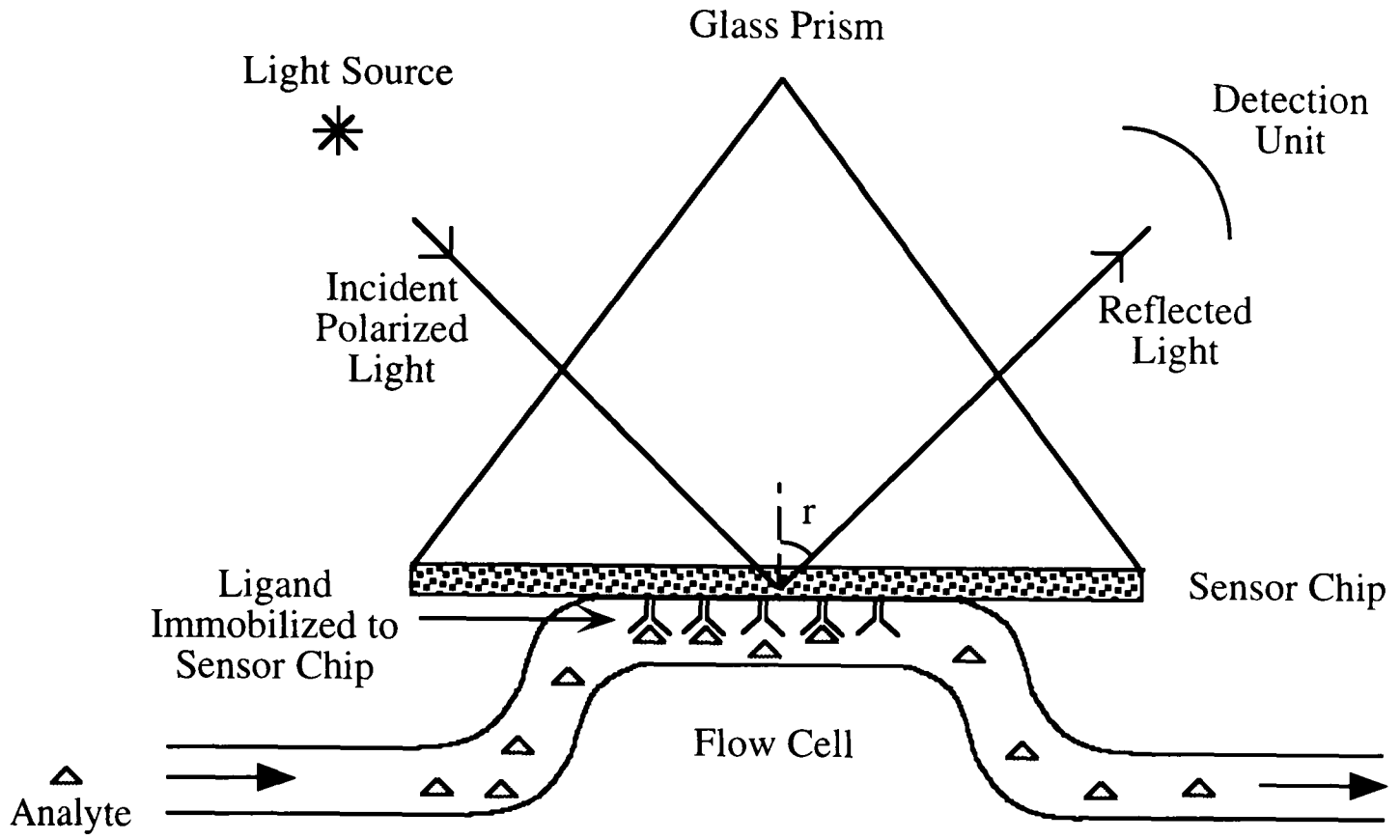


Figure 4.9. Schematic Illustration of the Principle of Surface Plasmon Resonance (SPR) for Analysis of Molecular Interactions.
SPR is based on an optical phenomenon operating at the interface between two media of different refractive index, for example, between glass and water. The optical phenomenon, total internal reflection is observed at a critical angle of incidence. In the presence of a thin layer of metal, such as gold, at the interface, the phenomenon of surface plasmon resonance occurs and is observed as a significant reduction in the intensity of the reflected light at a specific incident angle called the SPR angle. For analysis of biomolecular interaction, ligand is immobilized on the dextran matrix attached to the gold surface of the sensor chip, forming one wall of the micro-flow cells. Solution containing the interacting analyte is introduced in a continuous flow over the sensor surface. As the analyte binds to the immobilized ligand, the concentration of molecules on the sensor surface changes resulting in a corresponding change in the refractive index in the medium close to the non-illuminated side of the gold film. This in turn causes a change in the resonance angle, r, at which a sharp dip in reflected light intensity is observed. This shift in r is recorded instantly by the detection unit consisting of an array of light-sensitive diodes. Continuous monitoring of changes in the surface resonance angle therefore provides a measure of the real-time biomolecular interaction events.

index is directly correlated to the concentration of dissolved material in the medium, the SPR response can be used to measure changes in the concentration of molecules in a surface layer of solution in contact with the sensor surface. When the SPR is monitored continuously, real-time measurements of interactions between biomolecules can be obtained.

4.2.6.1 Kinetics of FGF-9-FGFR3c Binding Interaction

The FGFR3c-Fc fusion dimers, purified from the conditioned medium of cDNA-transfected 293T cells by protein A-affinity chromatography, were covalently coupled to the dextran surface of a sensor chip using the cross-linking agent N-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC). FGF-9, at varying concentrations ranging from 3 to 200 nM and resuspended in binding buffer containing heparin (25 μg/ml), was injected into the biosensor flow system and passed over the immobilized FGFR3-Fc attached to the sensor surface. The interaction was monitored for ~300 s. The resulting sensorgrams, plots of refractive index change (measured in resonance units or RU) against time, were overlayed on the same figure (Figure 4.10A). The sensorgrams represent real-time measurements of the association and dissociation phases of FGF-9 interaction with immobilized FGFR3. Heparin alone was also injected over the immobilized receptor but no association or dissociation response was observed, indicating the lack of a detectable interaction between these two molecules (data not shown).

The SPR data were fitted to kinetic expressions for the association and dissociation phases of the curves according to the biomolecular interaction model describing pseudo-first order interaction kinetics. Data analyses were aided by the BIAsimulation software program (Pharmacia). To obtain the k_d values of the dissociation phases, the real-time measurements at each injected FGF-9 concentration (Figure 4.10A) were subjected to analysis for first-order interaction kinetics. The corresponding k_a values were calculated by plotting the association phase data as ln(dRU/dt) against time, t (Figure 4.10C). The association phase of each sensorgram is characterized by an initial phase which is limited by

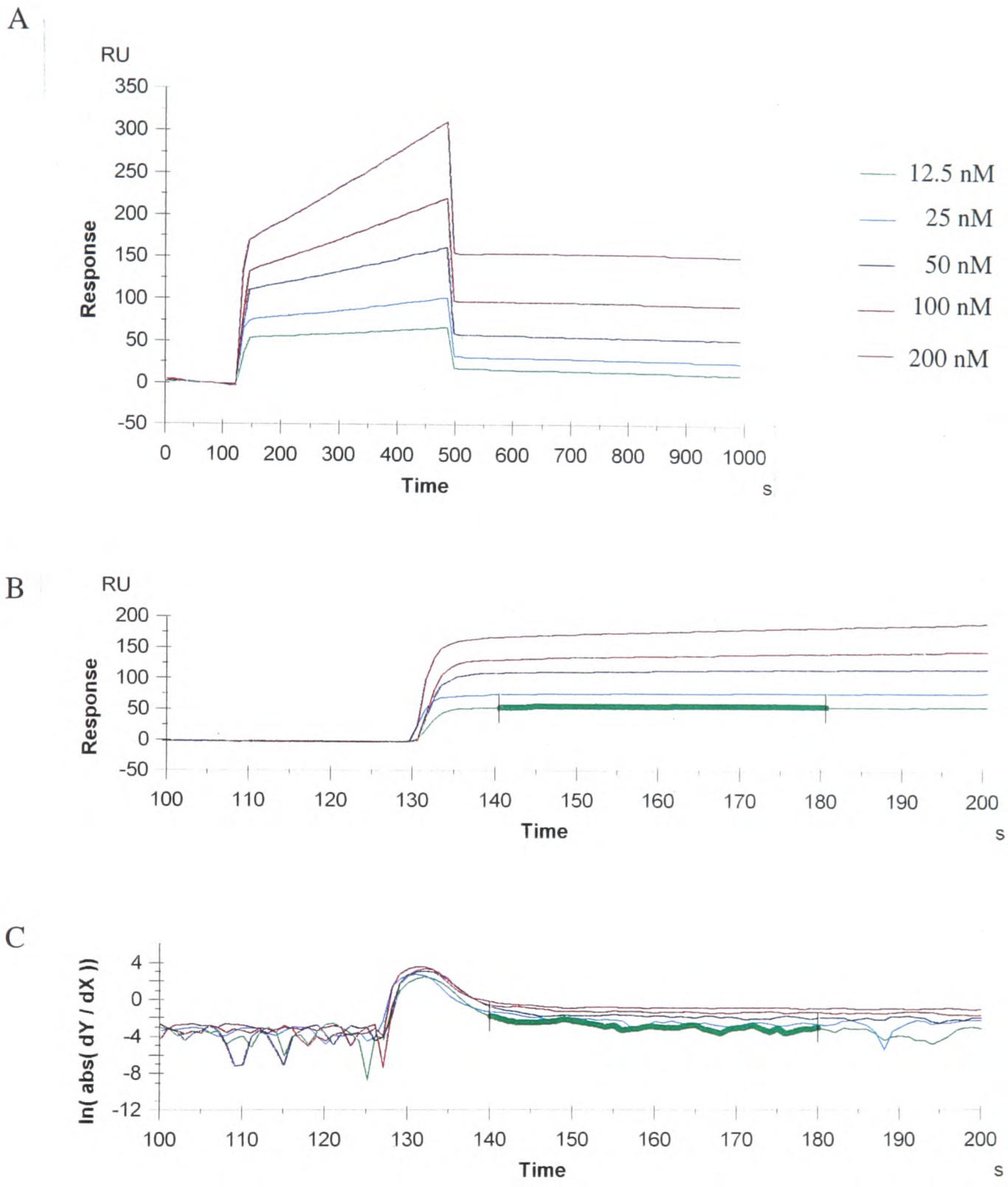


Figure 4.10. Kinetics of FGF-9 Binding to Immobilized FGFR3C-Fc.
(A) Overlay plot of the surface plasmon resonance sensorgrams (plots of RU versus time where RU represents resonance units) showing the binding of FGF-9 to immobilized FGFR3c-Fc when injected at 12.5, 25, 50, 100 and 200 nM for 6 min, in the presence of 25 µg/ml heparin. The resonance signal (RU) is a measure of the amount of protein associating or dissociating from the surface. (B) Amplified plot of (A) emphasizing the association phase. The region of the curves where binding was not limited by mass transport is highlighted and used for kinetic analysis. (C) Plots of the logarithmic form of the first derivative ln(dRU/dt) against time of the association phase data in (B) for the period 140 - 180 s when binding was not limited by mass transport.

mass transport of FGF-9 to the surface and represented as a constant binding rate (dRU/dt). This can be observed as a plateau on a plot of ln(dRU/dt) (Figure 4.10C). As FGF-9 binds to the FGFR3c immobilized on the surface, dRU/dt begins to reflect the binding rate between these two protein species and its value is observed to correspondingly decrease (Figure 4.10C). Increasing the concentration of FGF-9 produced a higher initial binding rate followed by a faster decrease in rate (Figure 4.10A). The best-fit kinetic parameters as well as the calculated equilibrium dissociation constants are shown in Table 4.2.

The average k_a and k_d values were calculated to be 8.64×10^5 $M^{-1}s^{-1}$ and 3.43×10^{-4} s^{-1} respectively. From these rate constants, the K_D of the interaction was calculated to be 630 pM. This value is close to the prediction made from the competition plate binding assays: the affinity of FGF-9 for FGFR3c was estimated to be about 4-fold less than that of FGF-1 (Figure 4.6B) which was previously determined to have a K_D of approximately 0.2 nM for binding to FGFR3 (Ornitz and Leder, 1992); hence, the K_D of FGF-9 for FGFR3 may be estimated to be about 800 nM.

4.2.6.2 Receptor Specificity of FGF-9

To further confirm the receptor specificity of FGF-9, the protein was immobilized to the sensorchip and analysed for binding to various injected receptors by measurements of the SPR signals. Biotinylated-FGF-9 was immobilized to the sensor chip matrix via streptavidin while soluble FGFR-Fc, purified from conditioned medium by protein A-affinity chromatography, was injected into the flow cell and passed over the FGF-9-coated sensor surface. The association of receptor with immobilized FGF-9-biotin was monitored for about 300 s. This was followed by buffer injection and the dissociation stage of the interaction was measured.

The sensorgrams derived from passing solutions of each of the four receptors over immobilized FGF-9 are plotted together (Figure 4.11). In agreement with the results obtained from the solid-phase binding experiments (sections 4.2.4 and 4.2.5), analyses of four of the receptors, namely FGFR3c-Fc, FGFR1c-Fc, FGFR2c-Fc and FGFR1b-Fc

Table 4.2. Kinetic and Calculated Equilibrium Constants for the Interaction of FGF-9 with Immobilized FGFR3.

[FGF-9] (nM)	k_a x 10^{-6} ($M^{-1}s^{-1}$)	k_d x 10^5 (s^{-1})	$K_{D,calc}$ (pM)
12.5	2.6	91.8	353
25	1.3	48.6	374
50	0.26	20.1	773
100	0.13	7.6	585
200	0.030	3.2	1077

The k_a and k_d values were estimated from fitting the association and dissociation kinetic data presented in Figure 4.10A. The k_a constants were calculated from plots of ln(dRU/dt) versus t (Figure 4.10C) for the periods when binding was not mass transport limited (Figure 4.10B). The average k_a value is 8.64 x 10^5 $M^{-1}s^{-1}$. The k_d constants were estimated from the dissociation phase for the period from t = 600 - 800 s and the average value is calculated to be 3.43 x 10^{-4} s^{-1}. $K_{D,calc}$ values are the equilibrium dissociation constants calculated directly from the kinetic constants (k_d/k_a). The average $K_{D,calc}$ value is 630 pM.

Table 4.3. Effect of Heparin on the Interaction Kinetics of Immobilized FGF-2 with FGFRs.

FGFR	Heparin					
	-			+		
	k_a x 10^{-5} ($M^{-1}s^{-1}$)	k_d x 10^4 (s^{-1})	$K_{D,calc}$ (nM)	k_a x 10^{-5} ($M^{-1}s^{-1}$)	k_d x 10^4 (s^{-1})	$K_{D,calc}$ (nM)
FGFR1b-Fc	2.98	13.3	4.46	4.31	12.2	2.83
FGFR1c-Fc	2.91	1.96	0.67	2.6	2.21	0.85
FGFR2c-Fc	2.48	3.25	1.31	2.88	5.05	1.75
FGFR3c-Fc	2.73	106	38.83	0.0034	N.D.	N.D.

All FGFR-Fcs were injected at a concentration of 2 μM except for FGFR1b-Fc which was injected at 1 μM concentration. - and + denotes the absence or presence of heparin (10 μg/ml) in the injected FGFR-Fc sample. The kinetic values are obtained from fitting the real-time data shown in Figure 4.12A to the kinetic expression for a first-order binding interaction. The k_a constants were calculated from plots of ln(dRU/dt) versus t for the periods when binding was not mass transport limited (Figure 4.12B-E). The k_d values are calculated for the dissociation phase between t = 600 - 800 s. The $K_{D,calc}$ values are calculated directly from the kinetic constants ($K_{D,calc} = k_d/k_a$). N.D., not determined.

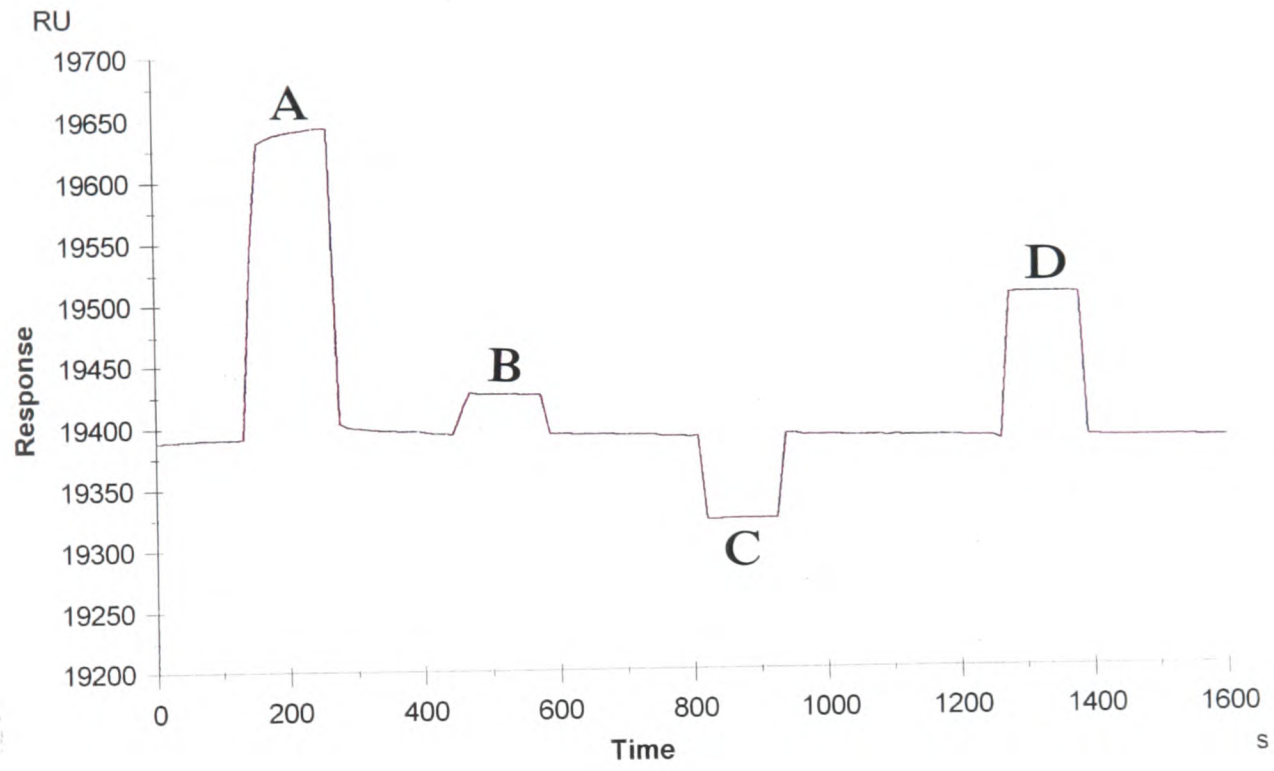


Figure 4.11. Receptor Binding Specificity by Surface Plasmon Resonance Analysis.
Biotinylated FGF-9 was challenged with four different FGFR-Fc proteins: (A) FGFR3c-Fc, (B) FGFR1c-Fc, (C) FGFR2c-Fc, and (D) FGFR1b-Fc. Each receptor (100 μg/ml) was injected separately over a sensorchip surface containing immobilized biotinylated FGF-9. The association/ dissociation from the surface over time was measured in resonance units (RU) as described in the text. Only FGFR3c-Fc showed an observable interaction, as indicated by the presence of the association phase.

showed that only FGFR3c-Fc interacted with biotinylated FGF-9. As mentioned before, several phases in a binding interaction between receptor and immobilized FGF-9 can be identified in the sensorgrams. The baseline level at ~19 590 RU reflects the effect of running buffer alone over the sensor surface. As buffer containing FGFR3c-Fc was passed over the sensor chip, the receptor dimers interacted with the surface in the association phase (~150 - 200 s) which was initially mass transport limited and later, rate limited (Figure 4.11A). The presence of an association phase in the binding interaction between FGFR3c-Fc and biotinylated FGF-9, observable as an increase in RU between 150 and 250 s, indicates a positive interaction between these two molecules. Running buffer reintroduced into the flow system at time beyond 250 s allows the dissociation phase to be monitored. A dissociation phase is observed for the binding interaction between FGF-9 and FGFR3 as a decrease in RU with time. As all the other receptors tested exhibited only bulk contribution and no association or dissociation phases in their sensorgrams (Figure 4.11B, C, D), the SPR results indicated clearly that only FGFR3c-Fc bound to FGF-9-biotin.

4.2.6.3 Heparin Dependence of FGF-2 Interaction with FGFR

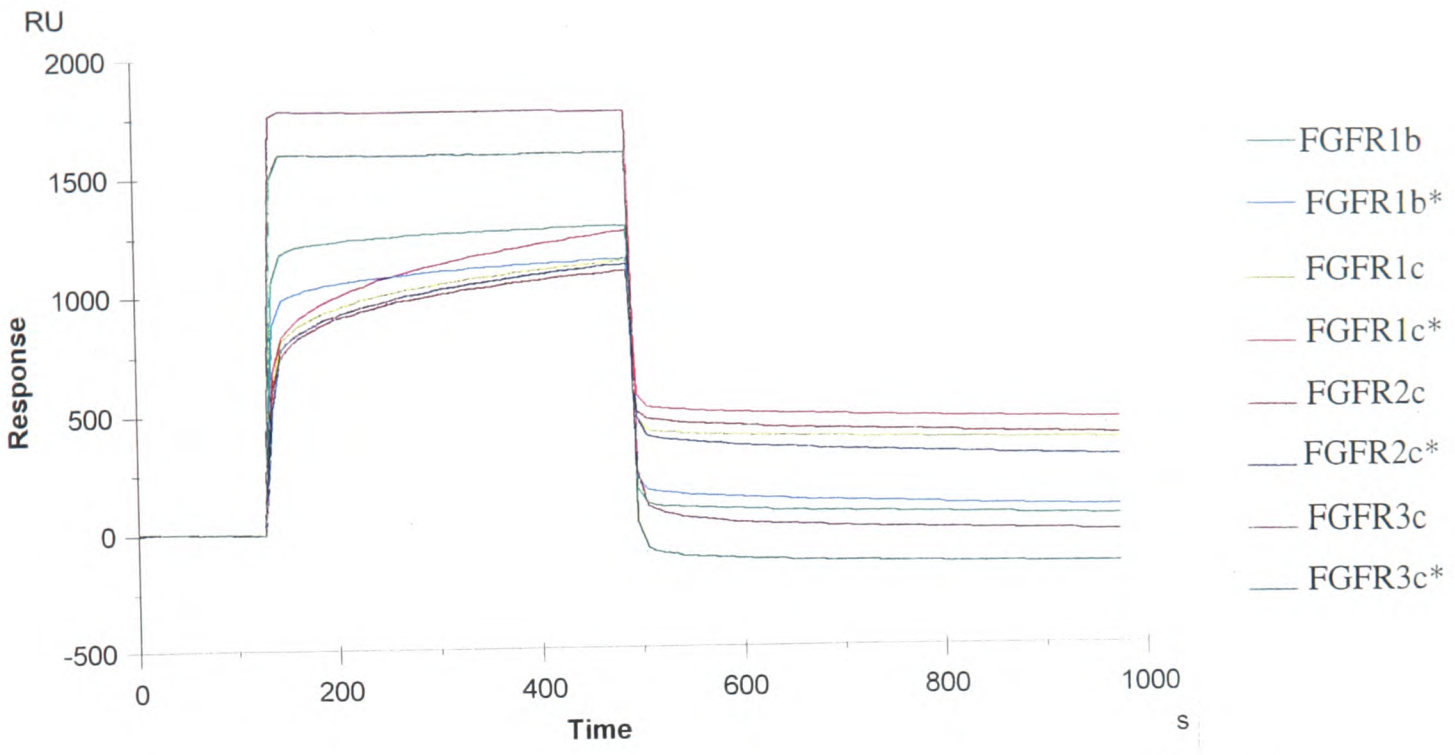
The effect of heparin on the binding interactions of FGF-2 with various receptors was also tested by SPR technology. In this study, biotinylated FGF-2 was immobilized to the sensorchip surface as described before for biotinylated FGF-9 (section 4.2.6.2).

The real-time data from the injections of soluble FGFR1b-Fc, FGFR1c-Fc, FGFR2c-Fc and FGFR3c-Fc in the presence or absence of heparin are overlayed in the same plot (Figure 4.12A). The best-fit kinetic values are presented in Table 4.3. Interestingly, the kinetics of the interactions of biotinylated FGF-2 with FGFR1c-Fc, FGFR2c-Fc and FGFR3c-Fc did not seem to be significantly affected by the presence of heparin while the association rate of FGFR1b-Fc with FGF-2 was enhanced 1.5-fold in the presence of heparin. No significant effect on the dissociation rate was observed. Taken together, this is reflected in an increase in the affinity of the interaction between FGF-2 and FGFR1b-Fc (K_D was reduced from 4.5 to 2.8 nM). These data thus support the results

Figure 4.12. Effect of Heparin on the Kinetics of FGFR-Fc Binding to Immobilized FGF-2.

(A) Overlay plot of the surface plasmon resonance sensorgrams showing the binding of FGFR-Fc to immobilized biotinylated FGF-2 in the absence or presence of heparin. The amount of biotinylated FGF-2 immobilized to the sensorchip was about 2100 RU and 30 µl of each receptor, with or without pre-mixed heparin, was injected at a concentration of 200 µg/ml (except for FGFR1b-Fc which was injected at 100 µg/ml). (B - E) Plots of the logarithmic form of the first derivative ln(dRU/dt) against time of the association phases data in (A) in the absence or presence of heparin. The association constant for each injection was calculated for periods when binding was not limited by mass transport (highlighted). (B) FGFR1b, (C) FGFR1c, (D) FGFR2c, and (E) FGFR3c. *, with addition of 10 µg/ml of heparin .

A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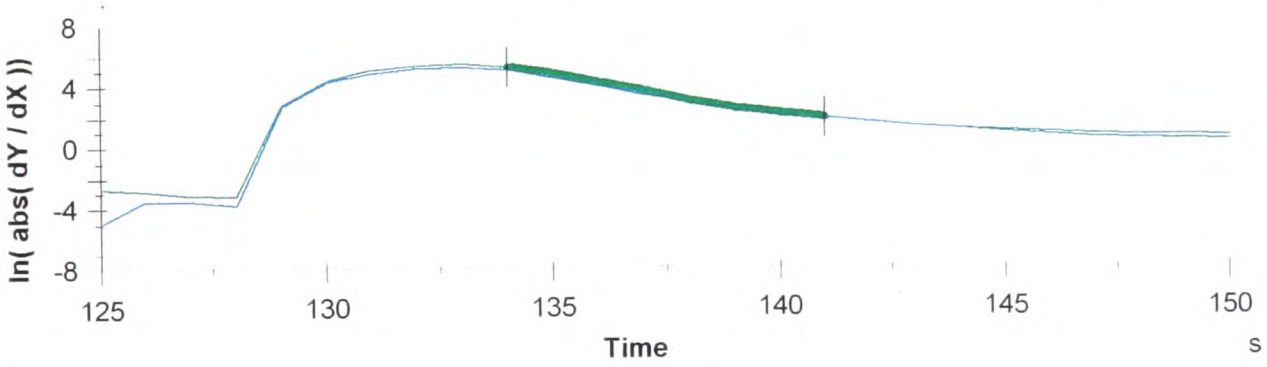
B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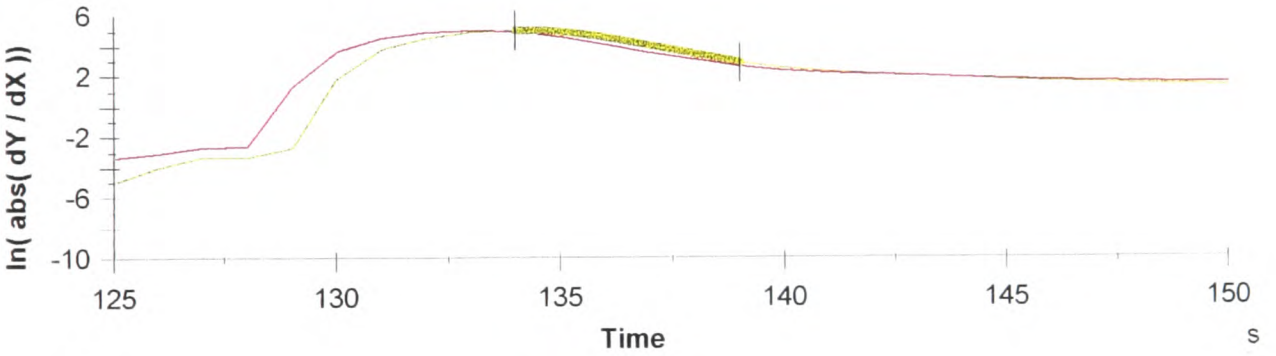
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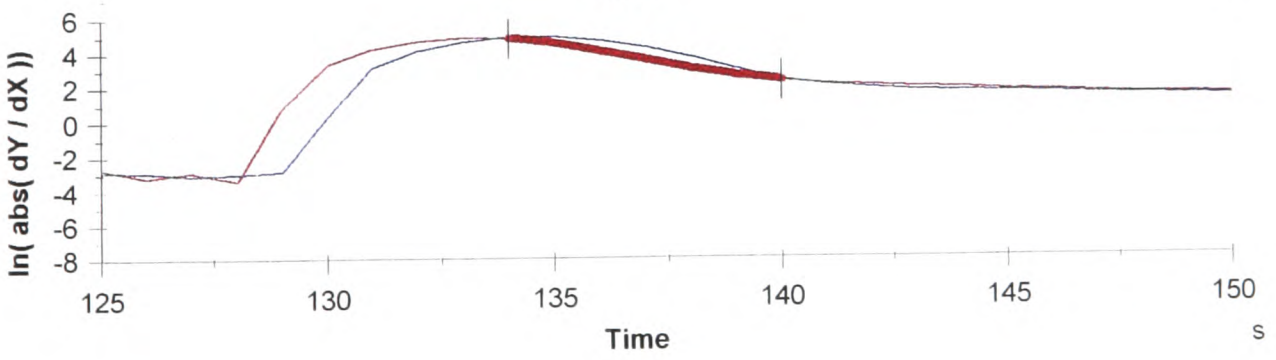
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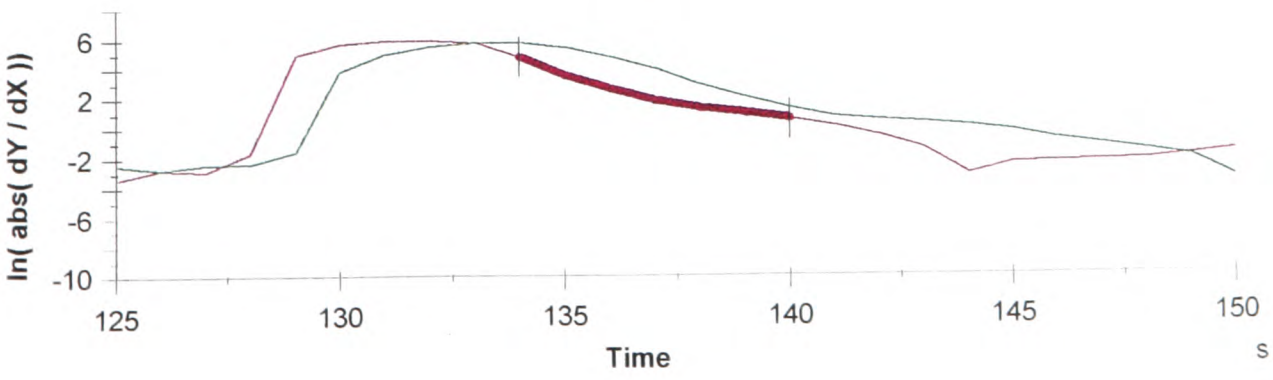
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obtained previously from the plate binding assays where the interaction between FGF-2-biotin and FGFR1b-Fc was shown to be affected by the presence of heparin. Hence, the predominant effect exerted by heparin on this interaction may be to increase the on-rate of the interaction.

4.3 Discussion

4.3.1 Receptor Binding Assays

In order to determine the receptor specificity of FGF-9, receptor binding assays based on a cell-free system in the absence of potentially interacting cell surface molecules were employed. An added advantage of this system was that it may be used for studying the effect of other molecules influencing the ligand-receptor complex formation. One such group of molecules is heparin which has been reported to influence receptor binding by the FGFs (Ornitz and Leder, 1992; Ornitz et al., 1992).

Ligand/receptor binding assays require sensitive and specific detection of the growth factor. The most widespread and common method for the direct detection of growth factors is based on the measurement of radioactivity. In this regard, ^{125}I is the most frequently used radioactive label. While imparting a high degree of sensitivity to the assay, the use of iodinated proteins has many drawbacks such as the requirement of a contained environment adapted to the manipulation of isotopic elements, the potential health hazards imposed by handling of volatile radioactive iodine, the low stability of the labelled protein due to the short half-life of radioactive iodine and radiolysis, and the difficulty of evaluating the exact quantity of the iodinated-product. For these reasons, non-radioactive modes of detection have been developed. FGF-2 has been successfully labelled with biotin (Lee et al., 1989) and digoxygenin, a 391 Da plant hapten (Gleizes et al., 1994) without lost of biological activity. In this report, the labelling of several FGFs with a maleimide derivative of biotin which is reactive towards the free thiols of FGFs was described. The advantages of this method are the ease and simplicity of the labelling procedure (see section 2.2.4), the high efficiency achievable (particularly for FGF-2 which has four free sulfhydryl groups),

and the adaptability to ELISA-like assays due to the high affinity of biotin for streptavidin and avidin. Most importantly, the labelling of recombinant FGFs with biotin did not modify their biological activities or binding properties (except perhaps for FGF-4-biotin which showed weaker biological activity but it's receptor binding specificity was retained). The receptor binding assays reported here were adapted for the detection of the biotin moiety on the modified FGFs.

Many different assays have been used to analyse the receptor specificities of the different FGFs or conversely, the binding properties of the individual FGFRs. Some assays exploit the fact that certain cell lines express specific endogenous receptors. For example, C57MG and NIH3T3 cells which are known to express FGFR1c and FGFR2c, have been used in direct binding assays for FGFs (Bottaro et al., 1990; Mathieu et al., 1995a). HC11 and Balb/MK keratinocytes have also been used in this fashion as they express the native FGFR2b (Bottaro et al., 1990; Cheon et al., 1994; Mathieu et al., 1995a). These cells have also been used in mitogenic assays for comparing the effects of different FGFs (Rubin et al., 1989). Other assays are based on the expression of specific receptors in cell lines that do not express endogenous FGFRs. Examples of these cell lines include the IL-3-dependent lymphoid cells (BaF3 cells, 32D and FDC-P1 myeloid cells) (Mathey-Prevot et al., 1986), L6 rat skeletal muscle myoblasts and Chinese Hamster Ovary (CHO) cells. BaF3 cells do not express detectable FGF receptors and only minimal extracellular matrix and cell surface HSPGs such as syndecan (Mathey-Prevot et al., 1986). Similarly, 32D and FDC-P1 myeloid cells are known not to express FGFR2c and FGFR1c respectively (Migliaccio et al., 1989). These cells can be transfected with specific receptors and subsequently induced by FGFs to elicit a mitogenic response, measurable as [^{3}H]thymidine incorporation into DNA. This assay is very sensitive and can detect FGF activity at concentrations as low as 20 pM (Bernard et al., 1991; Li and Bernard, 1992; Mansukhani et al., 1992; Ornitz and Leder, 1992; Ornitz et al., 1992; Gao and Goldfarb, 1995; Hecht et al., 1995; MacArthur et al., 1995a). The L6 myoblasts and mutant CHO cells have also been used to express specific FGFRs on the cell surface and direct binding assays performed with ^{125}I-FGFs (Mansukhani et al., 1990; Mansukhani et al., 1992;

Werner et al. 1992a). Other cell lines such as Rat-2 cells and COS cells which express low levels of FGFRs have also been applied to binding assays. Rat-2 cells express low levels of FGFR1 and when transfected with specific FGFRs, can be induced to produce focus formation on treatment with the appropriate ligand (Bernard et al., 1991). COS cells have been used to express specific FGFRs on the cell surface for direct binding assays with ^{125}I-FGFs (Partanen et al., 1991).

In addition to the above cell culture methods, *in vitro* cell-free binding assays have also been described by various groups. For example, FGFRs, genetically engineered as human placental alkaline phosphatase (AP) fusion proteins containing only the ectodomain of the FGFRs, can be immunoprecipitated with monoclonal antibodies that recognize placental AP so that the bound ^{125}I-FGFs can be isolated and counted in a gamma counter (Ornitz et al., 1992; Ornitz and Leder, 1992; Yayon et al., 1992; Hecht et al., 1995). Soluble FGFRs tagged with TPA have also been synthesized and used in binding assays on 96-well plates with ^{125}I-FGFs (Zhu et al., 1995). Besides these *in vitro* binding assays based on the isotopic detection of an interacting component, a method that requires no labelling of proteins is also available for studying ligand-receptor interactions. This technique is based on the measurement of surface plasmon resonance (SPR) on a sensorchip surface as the component immobilized to it interacts with soluble ligands passing over the surface (Fägerstam, 1991; Johnsson et al., 1991; Felder et al., 1993). A major advantage of this method is that real-time quantitation of the interaction kinetics can be obtained. Examples where this technique has been successfully applied are in the study of the interaction of heparin with FGF-1 (Mach et al., 1993), kinetic measurements of EGF binding to soluble EGR receptor monomers and dimers (Zhou et al., 1993), affinity and kinetic assays of the CD2 and CD48 cell adhesion molecules (van der Merwe et al., 1993) and the identification of B61 as a ligand for the ECK receptor (Bartley et al., 1994).

This work describes the analysis of the receptor specificity of FGF-9 by a combination of two methods: (1) plate binding assays based on non-isotopic detection of the FGF ligand and, (2) surface plasmon resonance technique. The plate binding assay described here was based on the synthesis of soluble FGFR-Fc chimera containing the

ectodomain of the FGFR attached to the Fc component of human IgG1. This fusion protein can bind to monoclonal antibodies and protein A that recognize the Fc domain. Similar FGFR2b-HFc chimeras have been described elsewhere but the binding assays were performed by precipitating the soluble receptors with protein A-Sepharose in the attempt to isolate the bound ^{125}I-FGF (Cheon et al., 1994). The binding assays described here, however, exploited the affinity of the Fc part of the receptor fusion proteins for protein A to coat 96-well plates with the receptors. Biotinylated FGFs could then be added directly to the receptors coated on the plates, washed and the bound FGFs detected by a simple colour reaction via the addition of streptavidin coupled to horseradish peroxidase. As such, laborious centrifugation steps as well as potentially hazardous handling of radioisotopes were eliminated. This method was used to obtain qualitative measurements of the receptor specificity of FGF-9 while quantitative data on the binding interaction was examined by the method of surface plasmon resonance.

4.3.2 Receptor Binding Specificity of FGF-9

The cell-free solid-phase binding assays showed that FGF-9 is selective in its receptor binding profile. FGF-9 was found to bind to FGFR2b with a moderate affinity comparable to that of FGF-2 and to FGFR3c with an affinity intermediate between that of FGF-1 and FGF-4. The specific binding of FGF-9 to FGFR3c was confirmed by SPR analysis. Thus, the receptor binding profile of FGF-9 presents yet another example of a ligand that discriminates between the IIIb and IIIc splice forms of FGFR2, a quality characteristic of FGF-7 (Bottaro et al., 1990; Miki et al., 1991; Miki et al., 1992).

At the time of writing, two other laboratories have also reported the receptor binding specificity of FGF-9 (Hecht et al., 1995; Santos-Ocampo et al., 1996). These workers described the specific binding of FGF-9 to FGFR2c and FGFR3c while its binding to FGFR2b was not detectable or extremely weak. This difference may have arisen as a result of the different methods employed to study ligand-receptor interactions. The contradicting reports were based on the use of soluble FGFRs synthesized as monomeric alkaline

phosphatase fusion proteins which were applied to FGF-9 immobilized to heparin-Sepharose or used in cross-linking experiments with radiolabelled FGF-9 (Hecht et al., 1995; Santos-Ocampo et al., 1996). Plate binding assays similar to those described here were also used to show specific and quantitative binding of iodinated FGF-9 to FGFR2c, which occurred at a significantly lower affinity ($K_D \approx 2.38$ nM) relative to FGFR3 ($K_D \approx 0.78$ nM) (Hecht et al., 1995). This low affinity may account for the apparent absence of binding to FGFR2c in the plate binding assays discussed here, indicating perhaps a lower detection sensitivity when biotinylated ligands were used instead of radiolabelled ligands. As a matter of fact, approximately 100-fold more biotinylated FGF was required for optimal detection compared to reports using iodinated ones. This, however, cannot be used to explain the SPR data that indicated the absence of FGF-9 binding to FGFR2c-Fc. The conflicting observations may also be accounted for by the fact that the receptor fusion proteins used in the study described here were presented to the FGF ligands in dimeric forms rather than as monomers. Each ectodomain of a dimeric FGFR may interact with each other to assume and/or stabilize a configuration distinct from that of the monomeric counterpart such that its binding characteristic is altered. As it is now well established that FGF activates FGFR by binding to and inducing FGFR dimerization (reviewed by Lemmon and Schlessinger, 1994), the issue of possible differences between monomeric and dimeric receptors for binding to FGF-9 may be addressed by expressing FGFR2b and FGFR2c in cell culture and measuring the physiological response to FGF-9. One such attempt has indeed been made, based on mitogenic assays using BaF3 cells expressing full-length FGFR2b and FGFR2c (Santos-Ocampo et al., 1996). FGF-9 was reported to be mitogenic toward cells expressing FGFR2c, activating the receptor efficiently while its effect toward cells expressing FGFR2b was not detectable. Hence, FGF-9 may indeed bind to FGFR2c which was not detected in the assays used here. The issue regarding FGFR2b is, however, less clear as FGF-9 may bind to this receptor and activate a unique physiological response that is unrelated to mitogenicity.

As FGF-9 does not bind to either FGFR2b or FGFR3c with an affinity higher than the prototype FGF-1, it is probable that FGF-9 may not represent the native ligand for

FGFR2b or FGFR3c. The pertinent question then, is: do FGF-9 and either receptor co-localize in the same temporal and spatial environment for interaction to be possible? This issue will be examined in the following Chapter 5.

4.3.3 Effect of Heparin on FGF-FGFR Binding Interaction

In addition to FGFRs, the role played by the low affinity receptors for FGFs, the heparan sulphate glycosaminoglycans, have also been extensively studied (reviewed by Ruoslahti and Yamaguchi, 1991). To determine the effect exerted by heparin in facilitating FGF action, both cell-free plate binding assays and SPR analysis have been used to examine the requirement and nature by which this heterogeneous molecule intervenes with FGF and FGFR interaction *in vitro*.

Two interesting observations were made regarding the effect of heparin on the interactions of FGFs with various FGFRs. The experimental data from the plate binding studies indicated that heparin has no effect on FGF-FGFR interactions in general, except in the case of the binding interaction between FGF-2 and FGFR1b-Fc. This requirement was not observed for FGF-2 interactions with the other receptors: FGFR1c-Fc, FGFR2b-Fc and FGFR2c-Fc nor for the receptor interactions of FGF-1, FGF-4 and FGF-9. There was, however, an enhancing effect produced by heparin in the interactions of FGF-2 with both FGFR1c-Fc and FGFR2b-Fc, in agreement with previous reports that heparin is not required for binding of FGF-2 to FGFR per sé, but merely increases the affinity of the ligand for the receptor (Pantoliano et al., 1994; Roghani et al., 1994). Conversely, there is also evidence to suggest that heparin is an absolute requirement for FGF-2 binding to FGFR1c (Yayon et al., 1991; Ornitz and Leder, 1992; Ornitz et al., 1992) and for FGF-9 binding to FGFR3 expressed on the surface of heparan sulphate-deficient cells (Hecht et al., 1995). The observation here that heparin is not required for these binding interactions may be due to the possible presence of sufficient heparan-sulphate-like molecules bound to the soluble receptor chimeras as they were synthesized in cell culture. It is also possible that the dimeric nature of the receptors used here are already in a high affinity conformation state

such that no extraneous molecules are required for high affinity interaction. On the other hand, it is equally likely that in general, heparin is not necessary for the binding interactions between FGF and its receptors, and that it functions only to concentrate the FGF ligands and to reduce ligand diffusion dimensionality (reviewed by Schlessinger et al., 1995).

The unique specificity of the heparin effect for FGF-2-biotin and not the other biotinylated FGFs suggests that the interaction of FGF-2 with the polyanionic heparin molecule may not be only electrostatic in nature but specific as well. An example of such a specific interaction is observed in the case of antithrombin which binds with high affinity to a unique pentasaccharide sequence in heparin or heparan sulphate (reviewed by Kjellén and Lindahl, 1991). The heparin-binding domain of FGF-2 has been delineated to key amino acids dispersed along the coding sequence (Thompson et al., 1994; Li et al., 1994) and evidence for the direct interaction between heparin and FGF is abundant (Habuchi et al., 1992; Maccarana et al., 1993; Mach et al., 1993; San Antonio et al., 1993; Pantoliano et al., 1994). A differential effect of heparin on different FGFs has been reported. For instance, heparin appeared to have a greater enhancing effect on FGF-2 binding to FGFR1 than FGF-1 (Ornitz and Leder, 1992). Consistent with this observation are the findings that binding to heparin induces a conformational change in FGF-2 (Prestrelski et al., 1992) but not FGF-1 (Copeland et al., 1991) and that the heparin structure that promotes FGF-2 signalling is different from that that promotes FGF-1 or FGF-4 activity (Guimond et al., 1993). Moreover, heparin was found to differentially modulate the mitogenic effect of FGF-1, FGF-3 and FGF-7 on the HC11 mammary cell line (Mathieu et al., 1995a). A relationship between the expression of specific HSPG populations and the differential promotion of FGF-1 and FGF-2 activities has also been reported (Nurcombe et al., 1993; Guillonneau et al., 1996).

The discriminatory nature of the heparin effect towards the "b" splice form of FGFR1-Fc suggests that a subset of heparin molecules may interact directly with this receptor to bring about a favourable conformational change for interaction with FGF-2. Conversely, binding of FGF-2 to the ectodomains of the "c" splice forms of both FGFR1 and FGFR2 may be passive interactions which are not as susceptible to conformational

changes (Seddon et al., 1995). There is evidence for the direct interaction between heparin and FGFR1. Heparin has been shown to protect a FGFR1 fragment from proteolysis and to bind to a sequence (K18K peptide) in the amino-terminus of Ig-like loop II of FGFR1 (Figure 4.13; Kan et al., 1993; Wang et al., 1995b). The interaction of FGFR1 with heparin has been measured by isothermal calorimetric methods (Pantoliano et al., 1994). Indirect evidence for this interaction also exists. For instance, cells overexpressing FGFR1 were reported to demonstrate significant mitogenic response to heparin in the absence of any FGFs, while parental cells or cells overexpressing FGFR3 showed no such response (Ornitz and Leder, 1992). There are, however, also reports that failed to detect direct interaction between heparin and soluble FGFR1 (Ornitz et al., 1992; Spivak-Kroizman et al., 1994).

Surface plasmon resonance analyses has permitted a closer study of the mechanism of action of heparin on FGF-2 binding to FGFR1b. The data suggest that heparin acts chiefly to increase the association rate of FGF-2 and FGFR1b, an observation that is in conflict with demonstrations that suggest that the role of heparin is not to increase the rate of formation of the FGF-receptor complex but rather, to reduce the dissociation rate of the complex (Moscatelli, 1992; Nugent and Edelman, 1992). The results here imply that heparin may promote the association of FGF-2 with FGFR1b by modifying the conformation(s) of either or both molecules to a form(s) that is more favourable for interaction to occur. Alternatively, heparin may serve to orientate the FGF-2 ligand for binding to FGFR1b immobilized on the chip surface.

A mechanism by which binding to heparan sulphates modifies the conformation of FGF-2 and thereby increases its affinity for binding to the receptor may be postulated from the observation that the binding of heparin to FGF-2 exposes a second lower affinity receptor binding site on the molecule (Springer et al., 1994). This modification may induce the FGF molecule to adopt a favourable conformation for the binding of a second molecule of FGFR, as proposed in a previous report (Pantoliano et al., 1994). It is thus possible that heparin may interact with FGF-2-biotin to expose a site on the molecule that is recognized by the ligand-binding domain of FGFR1b, which normally binds FGF-2 with 50-fold

Receptor	Peptide Sequence	Reference
FGFR1	NH_2-K*ME*K*KLHAVPAAKTVKFK-COOH	(Kan et al., 1993)
FGFR	-KMEKRLHAVPAANTVKFR-	(Dionne et al., 1990)
FGFR3	-RMDKKLLAVPAANTVRFR-	(Keegan et al., 1991)
FGFR4	-RMEKKLHAVPAGNTVKFR-	(Partanen et al., 1991)

Figure 4.13. Heparin Binding Domain of the FGFR Family.
The figure shows a comparison of the putative heparin binding sequences of the four known members of the tyrosine kinase FGFR family, the amino acid residues being represented by the one-letter code. The 18-residue putative heparin binding domain (also known as the K18K peptide) located at the amino-terminal region of Ig-like loop II, was determined previously for FGFR1 by Kan et al. (1993). Lysine residues shown to be critical for heparin binding in FGFR1 are indicated by *. Note the conserved HAV tripeptide (boxed) that is implicated in protein-protein interactions (Byers et al., 1992).

lower affinity relative to the "c" splice form (Werner et al., 1992a). In addition, heparin may also interact specifically with the Ig-like II domain of FGFR1 (Kan et al., 1993) and influence the structural fold of the Ig-like III domain, particularly with respect to the carboxy-terminal half which is known to define the ligand-binding specificity of the receptor (Yayon et al., 1992; Wang et al., 1995a). Indirect support for this possibility come from studies where replacing the variant region of Ig III of FGFR1 with that from FGFR2b was insufficient to abolish FGF-2 binding; complete loss of FGF-2 binding capacity was observed only when the Ig II domain of FGFR1 was also replaced by that of FGFR2b (Zimmer et al., 1993). The Ig II domain of FGFR1 is thus expected to contribute to structural conformations that favour receptor interaction with FGF-2. It is proposed here that this function of the Ig II loop may be partly mediated by specific interaction with heparin. The specificity for the exon IIIb splice form of only FGFR1 is, however, unclear as the heparin-binding domain is conserved between different FGFRs (Figure 4.13).

Heparin was shown to have a slight enhancing effect on the binding interaction between FGF-2 and either FGFR1c or FGFR2b. This observation may be accounted for by certain properties associated with heparin. Firstly, heparin, being a highly negatively charged molecule, can bind to the positively charged FGF-2 with a high association rate and at the same time dissociate from the bound ligand at a high rate (Tartaglia et al., 1993). Secondly, heparin is known to facilitate multivalent binding of FGF molecules (Ornitz et al., 1992; Spivak-Kroizman et al., 1994). Hence, heparin may increase receptor binding by FGF-2 by presenting the ligand to the receptors in a multivalent manner. In addition, the heparin-FGF complex, being larger and denser, reduces the rate of diffusion of the ligand and thereby concentrates the FGF molecules in the vicinity of the receptors where it quickly releases the ligand to the high affinity binding sites of the receptors.

Very high concentrations of heparin appear to reduce binding of biotinylated FGFs to their receptors, a phenomenon which was also observed previously for the binding of iodinated FGF-2 to soluble FGFR1 at a heparin concentration greater than 5 μg/ml (Ornitz et al., 1992). This observation can be explained by the fact that though heparin binds to the FGFs in a multivalent manner and effectively induces their oligomerization (Ornitz et al.,

1992; Mach et al., 1993; Spivak-Kroizman et al., 1994), the presence of a large molar excess of free heparin over FGF will result in every FGF molecule binding to a single heparin molecule. The outcome is the formation of a monovalent FGF-heparin complex. This is inhibitory to FGF-FGFR interaction as, instead of increasing the local concentration of FGF in the vicinity of the receptors, the heparin-bound ligands are sterically inhibited by the relatively large heparin molecules from binding to the surface layer of receptor molecules in an optimal fashion.

In summary, this work has established the receptor binding specificity of FGF-9. FGF-9 interacts selectively with FGFR2b and FGFR3c with no apparent requirement for accessory molecules such as heparin. Heparin is, however, required for the interaction of FGF-2 with FGFR1b. The next chapter will focus on the biological implications of the FGF-9-FGFR3 interaction based on the overlapping expression patterns of these two molecules.

Chapter 5

Expression Pattern of *Fgf9*

Summary

Members of the fibroblast growth factor (FGF) family have unique and overlapping patterns of expression during embryonic development. In this study, the expression of *Fgf9* during mouse embryonic development was examined by both RNase protection assays and RNA *in situ* hybridization analysis. Sites of *Fgf9* RNA expression include the germinal epithelium of the central nervous system, the gut primordia, the mesonephric vesicles and tubules, the optic cup, the otic vesicle and the somites, suggesting that *Fgf9* has multiple roles during the growth and differentiation of embryonic tissues. As FGFR3 has previously been established to be a putative receptor for FGF-9 (see Chapter 4), the potential co-localization of both FGF-9 and FGFR3 in the adult mouse brain was examined by double RNA *in situ* hybridization analysis. *Fgf9* and *Fgfr3* transcripts were found to co-localize in certain regions of the brain, including the olfactory bulb and cerebral cortex, suggesting a potential functional interaction between these two gene products in the brain.

5.1 Introduction

FGFs are known to display a variety of biological properties *in vitro*, such as influencing cell survival, proliferation, differentiation and migration (reviewed by Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989). One of the most interesting of these properties is the ability of these growth factors to affect the growth and development of the embryo. Members of the FGF family have been implicated to be required for mammalian development by evidence obtained from three main lines of studies: (1) *in vitro* biological assays that demonstrate the ability of FGF to influence the growth and/or differentiation effects of embryo cultures; (2) Northern, RNase protection, *in situ* hybridization and immunohistochemical analyses that detect and locate areas of FGF protein or RNA expression in embryo tissues; and (3) gene targeting and ectopic over-expression experiments that result in the creation of animal models exhibiting growth abnormalities. These studies have been corroborated by the observation that the high affinity FGF receptors are also expressed during embryogenesis and that inactivation of FGFR signalling results in developmental defects (Amaya et al., 1991; Deng et al., 1994; Yamaguchi et al., 1994).

The involvement of members of the FGF family in embryonic development is supported by evidence derived from a variety of *in vitro* assays based on the use of specific embryo cultures. One of the most important finding is the demonstration that FGFs can mimic the effect of the ventrovegetal signal necessary for the early patterning event in the *Xenopus* embryo. In this respect, FGF-1, FGF-2, FGF-4 and FGF-9 have been shown to be effective in inducing mesoderm in cultured animal caps of the early *Xenopus* blastulae (Slack et al., 1987; Kimelman and Kirscher, 1987; Paterno et al., 1989; Slack et al., 1989; Song, 1996). Other studies attempt to identify specific functions performed by FGFs during organogenesis. For example, FGF-2 has been implicated in cardiogenesis based on the observation that chick precardiac mesodermal cell proliferation was inhibited by oligonucleotides antisense to *Fgf2*, an effect that could be rescued by the addition of

exogenous FGF-2 (Sugi et al., 1993). Likewise, FGF-1 is implicated in myogenesis by a similar study using antisense *Fgf1* RNA which resulted in precocious myogenic differentiation (Fox et al., 1994). The normal development of smooth muscle cells in the *Xenopus* has also been shown to be influenced by FGF-2 (Saint-Jeannet et al., 1994). In addition, the FGFs are thought to be involved in the normal outgrowth and patterning of the embryonic limb. For instance, FGF-2 can induce a regenerative response in amputated chick limb buds (Taylor et al., 1994), regulate the polarizing potential of both anterior and posterior limb bud cells *in vitro* (Anderson et al., 1994) and maintain the outgrowth of limb buds that have been stripped of the apical ectodermal ridge (or AER) (Fallon et al., 1994). FGF-4 can also induce the outgrowth and patterning of cultured limb buds (Niswander and Martin, 1992a; Niswander et al., 1993) and maintain the polarizing activity of chick wing buds lacking the AER (Vogel and Tickle, 1993). FGF-8 also has the ability to replace the AER in maintaining limb bud outgrowth (Mahmood et al., 1995a). Recent studies indicate that the FGFs may be additionally involved in the initiation of limb bud outgrowth from the embryonic flank (Cohn et al., 1995). Another area where FGF function has been extensively studied is in the development of neural tissues. The FGFs have been implicated to have neurotrophic roles due to their activities on a variety of cultured neural or glial progenitor cells (Baird, 1994).

Analyses of FGF expression indicate that these molecules are expressed during development. Several FGFs are known to be expressed in embryonic stem (ES) or embryonic carcinoma (EC) cell lines. The *Fgf3* gene is expressed at low levels in F9 EC cells (Jakobovits et al., 1986; Smith et al., 1988; Yoshida et al., 1988b) while the *Fgf4* gene is expressed in both EC and ES cells as well as their differentiated derivatives (Yoshida et al., 1988b, c; Heath et al., 1989; Velcich et al., 1989). *Fgf5* is distinct in that its expression is induced when ES or EC cells undergo differentiation to form embryoid bodies *in vitro* (Hébert et al., 1990; Hébert et al., 1991). As the ES and EC cell lines resemble the pluripotent cells of the inner cell mass of the mouse blastocyst both morphologically and biochemically, they are widely employed for the study of early embryonic developmental events (Martin, 1980; Strickland, 1981; Robertson, 1987). The

pluripotentiality of ES cells in continuous culture can be maintained by the use of leukaemia inhibitory factor (LIF) (reviewed by Heath et al., 1990). On the other hand, in the presence of chemicals such as retinoic acid (RA), these cells can differentiate into a variety of cell types, mimicking some of the early events of embryonic development (Rudnicki et al., 1989). For example, 1009 EC cells differentiate into neural cell types in a manner resembling the early stages of neural development when treated with retinoic acid (Pfeiffer et al., 1981). As a first indication of the possible involvement of *Fgf9* in development, the expression of this gene was analysed in both ES and EC cells, and their differentiated derivatives.

The temporal and spatial regulations of FGF expression during development have been examined at either the protein or RNA level in species ranging from the mouse to *Xenopus* (Table 5.1). *Fgf1* gene expression in the rat embryo and foetus appears to be widespread, notably in areas such as the brain and connective tissues of developing organs (Fu et al., 1991; Wilcox and Unnerstall, 1991). *Fgf2* expression is primarily studied in the avian embryos, with transcripts detected in the developing nervous and muscular systems, limb mesenchyme and kidney epithelial cells (Joseph-Silverstein et al., 1989; Kalcheim and Neufeld, 1990; Dono and Zeller, 1994). In the *Xenopus* , the FGF-2 homologue has been localized to the oocytes, anterior and posterior regions of the zygote and later, in most parts of the head and muscle cells (Song and Slack, 1994). The next three members, FGF-3, FGF-4 and FGF-5 have been reported to be expressed prior to and during gastrulation (Wilkinson et al., 1990; Haub and Goldfarb, 1991; Niswander and Martin, 1992b) while at later stages of mouse development, these three molecules showed distinct expression patterns, particularly in the areas of the ear, tooth and limbs (Wilkinson et al., 1988; Wilkinson et al., 1989; Haub and Goldfarb, 1991; Niswander and Martin, 1992b). The *Xenopus* homologue of *Fgf3* has an amphibian expression pattern that shows some similarity with that of the mouse, particularly in the region of the developing otocyst (Tanahill et al., 1992). *Fgf6* expression appears to be restricted primarily to developing muscle cells (de Lapeyrière et al., 1993) while *Fgf7* RNA is predominantly found in the mesenchymal mucosal layers of organs (Mason et al., 1994). *Fgf8* is another member that

Table 5.1. Expression of Members of the FGF Family In Development.

FGF	Organism	Expression Pattern (Day Post-Coitum)†	References
FGF-1*	Rat	Developing brain: cerebral cortex, hippocampus, olfactory bulb and spinal cord Optic nerve Heart mesenchyme Connective tissue of lung, kidney, oesophagus and thymus Somites Ossification center of limbs Skeletal muscle precursors	Wilcox and Unnerstall, 1991; Fu et al., 1991
FGF-2*	Chick	Skeletal muscle myoblasts Developing head and brain Heart myocardium Myotomes Notochord Neural tube All dorsal ganglia Roots of spinal nerves Epithelia lining gut Ectoderm and mesenchyme of limb buds Kidney epithelial cells	Joseph-Silverstein et al., 1989 Dono and Zeller, 1994
	Quail	Spinal cord and ganglionic neurons Limb buds Mesenchyme dorsal to neural tube	Kalcheim and Neufeld, 1990
FGF-3	Mouse	Primitive streak (7.5-9.5) Neuroepithelium of myelencephalon adjacent to otic epthelium (8.5-9.5) Pharyngeal pouch endoderm (8.5-9.5) Sensory region of otic vesicle (10.5-17.5) Neuroblastic layer of retina (14.5-17.5) Tooth bud mesenchyme(14.5-18.5) Purkinjé layer of cerebellum (14.5-newborn)	Wilkinson et al., 1988; Wilkinson et al., 1989; Niswander and Martin, 1992; Drucker and Goldfarb, 1993
FGF-4	Mouse	Embryonic ectoderm of blastocyst (4.5) Primitive streak (7.5-8.5) Presomitic mesoderm in trunk (7.5-11.5) Primitive neuroectoderm (8-8.5) Tail bud (7-11.5) Branchial arch ectoderm and endoderm(8.5-10.5) Foregut endoderm (8.5-10.5) Myotome (9.25-14.5) Skeletal myoblasts (9.5-13.5) Posterior limb bud AER (10-12) Tooth bud ectoderm (14.5)	Niswander and Martin, 1992; Suzuki et al., 1992; Drucker and Goldfarb, 1993
FGF-5	Mouse	Epiblast (5.5-7.5) Head process (7.5-7.75) Lateral splachnic (E9.5-10.5) and somatic (10.5-12.5) mesoderm Myotomes (10.5-12.5) Mastication muscle (11.5-14.5) Limb mesenchyme (12.5-14.5) Acoustic ganglion (12.5-14.5)	Haub and Goldfarb, 1991; Hébert et al., 1991

FGF	Organism	Expression Pattern (Day Post-Coitum)†	References
FGF-6	Mouse	Somitic myotome (9.5-12.5) Skeletal muscles (13.5-14.5) Muscle masses, myotubes (15.5)	de Lapeyrière et al., 1990; de Lapeyrière et al., 1993
FGF-7	Mouse	Heart (8.5) Myotome (9.5-14.5) Limb bud (11) Mesenchyme of connective tissues (14.5) Ventricular layer of forebrain (14.5) Capsule surrounding muscles (16.5) Inner mucosal layer of ureter (16.5) Dermis (from 15.5)	Mason et al., 1994
FGF-8	Mouse	Pre-streak ectoderm and visceral endoderm (5.75-6.25) Primitive streak (7.5-9.75) Precursors of heart and associated vessels (7.75-8) Prospective cranial region (8): prechordal neuroepithelium, floorplate and cardiogenic plate, cranial paraxial mesoderm and overlying ectoderm Cephalic neural folds (8-8.5) Branchial arch ectoderm (8.5-10.5) Tail bud (8-10.5) Somitic myotome Frontonasal prominence, forebrain, midbrain, midbrain-hindbrain junction (8.75-10.5) Forelimb and hindlimb ectoderm, AER (9.25-14.5) Nephrogenic cords (9.5-10) Alar plate of ventral thalamus (9.5-12.5) Optic recess and optic stalk (9.5-14.5) Ectoderm overlying genital tubercle (10.5) Nasal pit ectoderm (10.5) Roofplate of diencephalon rostral to developing pineal gland (12.5-14.5) Developing labyrinth of inner ear (14.5) Bowman's capsules/renal tubule complexes (14.5-15.5)	Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995 Mahmood et al., 1995
FGF-8b		Surface of branchial arches (10.5) Nasal pits (10.5) AER of forelimb and hindlimb (10.5) Tail (10.5) Neuroepithelium of infundibulum, telencephalon, dicephalon, midbrain-hindbrain junction (10.5)	MacArthur et al., 1995
FGF-8c, FGF-8d, FGF-8g		Neuroepithelium of telencephalon, dicephalon, mesencephalon-metencephalon junction and infundibulum (10.5) Branchial arch ectoderm (10.5) Limb AER (10.5)	

†, stages of embryonic development are only indicated for mouse embryos. *In the event that the expression patterns of FGF-1 and FGF-2 have not been described for the mouse, the distribution of the prototype FGF-1 and FGF-2 in the developing rat and chicken embryos respectively were shown instead.

is expressed during gastrulation (Crossley and Martin, 1995). The expression patterns of four of the isoforms, *Fgf8b*, *Fgf8c, Fgf8d* and *Fgf8g*, appear to overlap, with transcripts detected in the developing central nervous system and surface ectoderm of the branchial arches and limb (or AER) (Ohuchi et al., 1994; MacArthur et al., 1995a; Mahmood et al., 1995a). The developmental expression pattern of *Fgf9* was examined in this study. This molecule appears to be transcribed in the central nervous system, the developing sense organs (eye and ear), the developing kidney and the somites.

The relevance of FGFs in embryonic development is strengthened by the discovery that the high affinity FGF receptors are also expressed during embryogenesis (Table 5.2). Members of the FGFR family have also been shown to be expressed in a regulated manner in ES and EC cells. While *Fgfr1* and *Fgfr4* are expressed constitutively in both differentiated and undifferentiated ES and EC cells, *Fgfr2* and *Fgfr3* expressions were found to be induced upon ES and EC cell differentiation, with the "b" and "c" splice forms showing differential regulation of expression (Campbell et al., 1992; McDonald and Heath, 1994). During gastrulation, RNA *in situ* hybridization studies indicated expression of both *Fgfr1* and *Fgfr2* ("b" and "c" splice forms inclusive) genes (Orr-Urtreger et al., 1991; Yamaguchi et al., 1992; Orr-Urtreger et al., 1993). During organogenesis, the expression of both receptors have been observed in most of the developing organs, including the brain, head and face, respiratory system, digestive system, limbs, muscles, bone and urogenital system. The striking difference between the expression patterns is that *Fgfr1* RNA is predominantly localized in the mesenchymal layer while *Fgfr2* RNA is found primarily in the surface ectoderm and epithelial layer of the developing organs. A subsequent study of the differential expression patterns of the "b" and "c" splice forms of *Fgfr2* indicates that in most cases, *Fgfr2b* transcripts are limited to the surface ectoderm and epithelia of the internal spaces of developing organs while *Fgfr2c* may be found in the immediately underlying superficial mesenchyme (Orr-Urtreger et al., 1993). The third receptor, *Fgfr3* ("b" and "c" splice forms inclusive) is transcribed in a more restricted manner, with expression detected in the developing brain and spinal cord, sensory hair cells of the inner ear, lens of the eye, bones, resting cartilage and at low levels in the fetal lung and kidney

Table 5.2. Expression of Members of the FGFR Family In Mouse Development.

FGFR	Expression Pattern (Day Post-Coitum)	References
FGFR1 (Not distinguish-ing for splice variants)	Primitive ectoderm (gastrulation) Migrating embryonic mesoderm (gastrulation) Presomitic mesoderm (7-8) Somites: dermatome and sclerotome (8.5-10.5) Brain: neural ectoderm (8); forebrain (8.5); diencephalon (9.5); spinal cord (11); maturing neurons in dentate gyrus, hippocampus, brainstem, cerebellum and trigeminal ganglia (16.5) Bone: cartilage blastema (12.5), long bones and mandible, hypertrophied cartilage, osteocytes and osteoblasts Craniofacial mesenchyme (12.5) Ear: otic vesicle (10.5) Eye: cornea and sclera Gut: surrounding mesenchyme of developing oesophagus, stomach, intestine and mesocolon Hair: dermal mesenchymal cells; dermal pilla of hair follicles (16.5) Heart: endocardial cushions, myocardium (E9.5-12.5); endothelium and smooth muscle (12.5-16.5) Kidney: nephrogenic cords (E9.5); renal tube and Bowman's capsule epithelium; cortical mesenchyme (12.5) Limb bud: premuscle mesenchyme (9.5-12.5) Liver (12.5) Lung: tracheal and lung bud mesenchyme (12.5) Muscle: differentiated skeletal muscle (16.5) Vertebral column: perichondrium and osteoblasts (11.5-12.5) Tooth: ameloblast layer of enamel; odontoblast layer and underlying mesenchyme of dental papilla	Safran et al., 1990; Orr-Urtreger et al., 1992; Peters et al., 1992; Yamaguchi et al., 1992
FGFR2 (Not distinguish-ing for splice variants)	Primitive ectoderm (inner cell mass) Bone: precartilage blastema of vertebrae; long bones and mandible; periostium, perichondrium; chondrification centers (12.5); body of distal bones (14.5) Brain: cranial and dorsal neural tube (9.5); mesencephalon and myelencephalon (9.5-16); ventricular layer of spinal cord; choroid plexes in glia (16.5) Ear: otic vesicle (10.5); ossicles of middle ear (12.5-14.5); semi-circular and cochlear ducts (14.5) Eye: corneal epithelium Gut: epithelium of oesophagus and stomach; mesenchyme of intestine Hair: hair bulb and dermal papilla (16.5) Heart: heart primordium (8); endocardial cushions and endothelium (9.5-12.5); developing cardiac skeleton and atrioventricular and semilunar valves (16.5) Kidney: nephrogenic cords (9.5); metanephric collecting duct epithelium (14.5) Limb: limb ectoderm (AER) (9.5-10.5); mesenchymal aggregates corresponding to future bones of limb (11.5); cartilage blastema (12.5) Lung: tracheal epithelium (9.5-16.5); lung bud epithelium (12.5); terminal bronchiols (16.5) Skin: epidermis (14.5); proliferating basal layer (16.5) Somites: rostral half of perichordal sclerotome (12.5)	Orr-Urtreger et al., 1992 Peters et al., 1992

FGFR	Expression Pattern (Day Post-Coitum)	References
FGFR2b	Primitive streak (7) Neuroectoderm Somites Lateral mesoderm (8.5) Bone: vertebral column, illum, long bones of limbs, ribs, skull, bones of face and nasal cavity Ear: cochlear duct (cochlear endothelium) Eye: cornea Gut: hindgut endoderm (10.5); endothelium of gut and stomach Kidney: nephrogenic cords (10.5); mesonephric vesicles Liver Ranke's pouch Respiratory system: epithelium of pharynx, nasal cavity; periderm of thoracic and nasal areas; alveolar epithelium of lung Salivary gland (close to lumen) Surface ectoderm (10-14.5) Spinal cord	Orr-Urtreger et al., 1993
FGFR2c	Primitive streak (7) Adrenal gland Bone: vertebral column, illum, long bones of limbs, ribs, skull, bones of face and nasal cavity Brain: neuroectoderm (8.5); cortical neuroepithelium (caudate putamen) Ear: otic vesicle (10.5-14.5); bony part of inner ear and auditory ossicles Eye: cornea mesenchyme and lens epithelium Hair: outer hair root sheath of vibrissae Heart: atrioventricular heart valve Limb: mesenchyme in posterior proximal region (9.5); mesenchyme aggregates corresponding to future bones of limb (11.5) Liver Skin: of nasal and thoracic areas; dermis and loose mesenchyme	Orr-Urtreger et al., 1993
FGFR3 (Not distinguish-ing for splice variants)	Bone: pre-bone cartilage rudiments of all bones including vertebral bodies (9.5-14.5), flat bones of skull and long bones, resting cartilage Brain: germinal epithelium of fore- and hindbrain (9.5-14.5) Ear: underlying support cells of developing cochlear duct (16.5) Eye: lens (14.5) Hair: differentiating hair cells (16.5) Liver (very low levels, 16.5) Lung (very low levels, 16.5) Spinal cord: germinal epithelium (ventral cord, including floor plate)	Peters et al., 1993
FGFR4	Definitive endoderm of developing gut (8.5-13) Endodermal component of yolk sac (8.5-14.5) Developing gut (9.5) Somites (9.5) Mesenchyme of first pharyngeal pouch (12) Mesenchyme of pre-vertebrae (myotomal portion) (12) Chondrocytes in prevertebrae (12) Adrenal gland: cortex (14.5) Cartilage: endochondral cartilage of ribs, developing cartilage of olfactory and auditory regions (14.5) Gut: epithelial cells (13); endoderm of intestine (14.5) Kidney: collecting tubules (14.5) Liver: epithelia (14.5) Lung: mesenchymal and epithelial components (14.5) Pancreas: endodermal epithelium (14.5) Skeletal muscles (14.5)	Stark et al., 1991; Korhonen et al., 1992

(Peters et al., 1993). Finally, *Fgfr4* RNA has been detected in the developing lung, gut, liver, pancreas, intestine, kidney, adrenal gland, endochondral cartilage and in the developing skeletal muscles (Stark et al., 1991; Korhonen et al., 1992).

Analyses of FGF and FGFR expression patterns are important first steps towards elucidating the developmental role of the FGFs. Indeed, data from these studies may be correlated with results derived from gene targeting experiments which confirm that the FGFs are indeed involved in embryonic development. For example, deletion of the *Fgf3* allele resulted in mice with ear defects (Mansour et al., 1993). This is probably related to the lack of expression of this gene in the sensory layer of the otic vesicle where it is normally transcribed (Wilkinson et al., 1989). Similarly, deletion of the *Fgf4* allele led to early postimplantation death (Feldman et al., 1995), confirming that expression of this gene prior to and during gastrulation serves a critical function. On the level of the FGFR, embryos homozygous for a null allele of *Fgfr1* underwent abnormal mesodermal patterning (Deng et al., 1994; Yamaguchi et al., 1994), indicating yet again that expression of this gene during gastrulation is important. This latter result is further strengthened by the demonstration that inhibition of *Xenopus* FGFR function led to abnormal development during gastrulation (Amaya et al., 1991). There is, however, an instance where expression pattern does not obviously correlate with function. This is seen in mice homozygous for the null allele of *Fgf5* where the most apparent defect appears to be related to hair growth (Hébert et al., 1994). Nevertheless, expression data are extremely useful in giving a first clue to the embryonic role of FGF.

In view of the neurotrophic role of FGF-9 on primary rat glial cells (Naruo et al., 1993) and the presence of both *Fgf9* and *Fgfr3* transcripts in the adult rat or mouse brain (Peters et al., 1993; Tagashira et al., 1995), the expression patterns of both of these transcripts in the adult mouse brain were examined by *in situ* hybridization analyses to identify areas where FGFR3 may be required to transduce signals responsible for normal functions of the brain via interaction with FGF-9. The expression of FGF-9 in the adult central nervous system is not unique among the members of the FGF family as FGF-1, FGF-2 and FGF-5 are also found to be expressed in the adult central nervous system

(Pettmann et al., 1986; Emoto et al., 1989; Haub et al., 1990; Bean et al., 1991; Fu et al., 1991; Wilcox and Unnerstall, 1991) and shown to have neurotrophic activities *in vitro* (reviewed by Wagner, 1991). FGF-9 thus represent another member of the family predicted to serve a neurotrophic function in the adult brain, probably by interacting with FGFR3.

5.2 Results

5.2.1 RNase Protection Analysis

The template for the antisense riboprobe used to determine the expression pattern of *Fgf9* by RNase protection analysis was derived from the mouse *Fgf9* genomic clone (section 3.3.2). The probe contained all of the exon III sequence as well as 76 bp of the adjoining intron II sequence (Figure 5.1). In the presence of complementary *Fgf9* mRNA, the 417 nt riboprobe protects an RNA fragment of 274 nt corresponding to the exon III sequence against RNase degradation. The control probe used in this experiment was derived from the constitutively expressed mouse ß-actin gene. The latter control probe (350 nt) protects a 250 nt fragment in the presence of complementary ß-actin RNA.

5.2.1.1 Expression of Fgf9 during Development

To determine the pattern of *Fgf9* expression during development, RNase protection assays were performed using total RNA isolated from mouse embryos at 9.5, 10.5, 11.5, 13.5, 14.5, 15.5, 16.5, 17.5, 18.5 days post coitus (d.p.c.) and from newborn mice (Figure 5.2). Protected fragments were resolved on a 5% polyacrylamide gel which was then exposed to X-ray film (see 2.3.2). Expression levels were found to be generally low since signals were only obtained after prolonged exposure of the gel to X-ray films. *Fgf9* transcripts were detected during embryonic development from 10.5 to 14.5 d.p.c. and during fetal growth from 14.5 to 18.5 d.p.c.. Doublet bands produced by the ß-*actin* probe are probably RNase digestion artefacts.

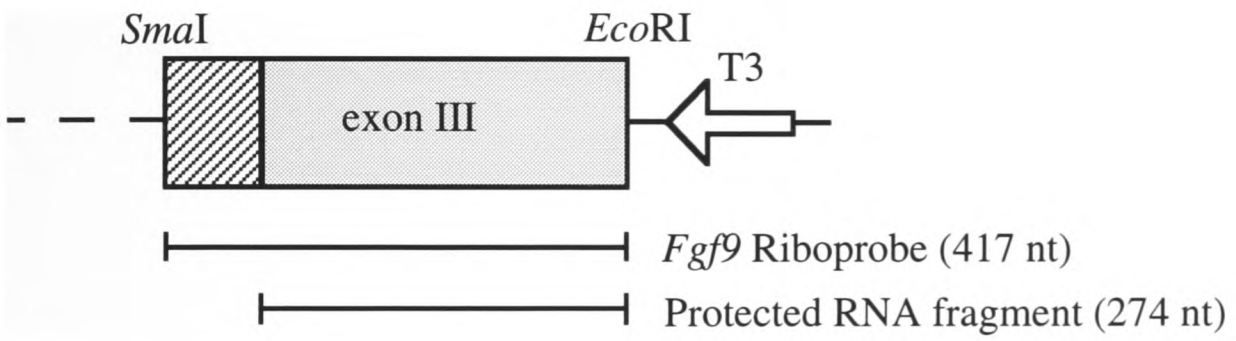
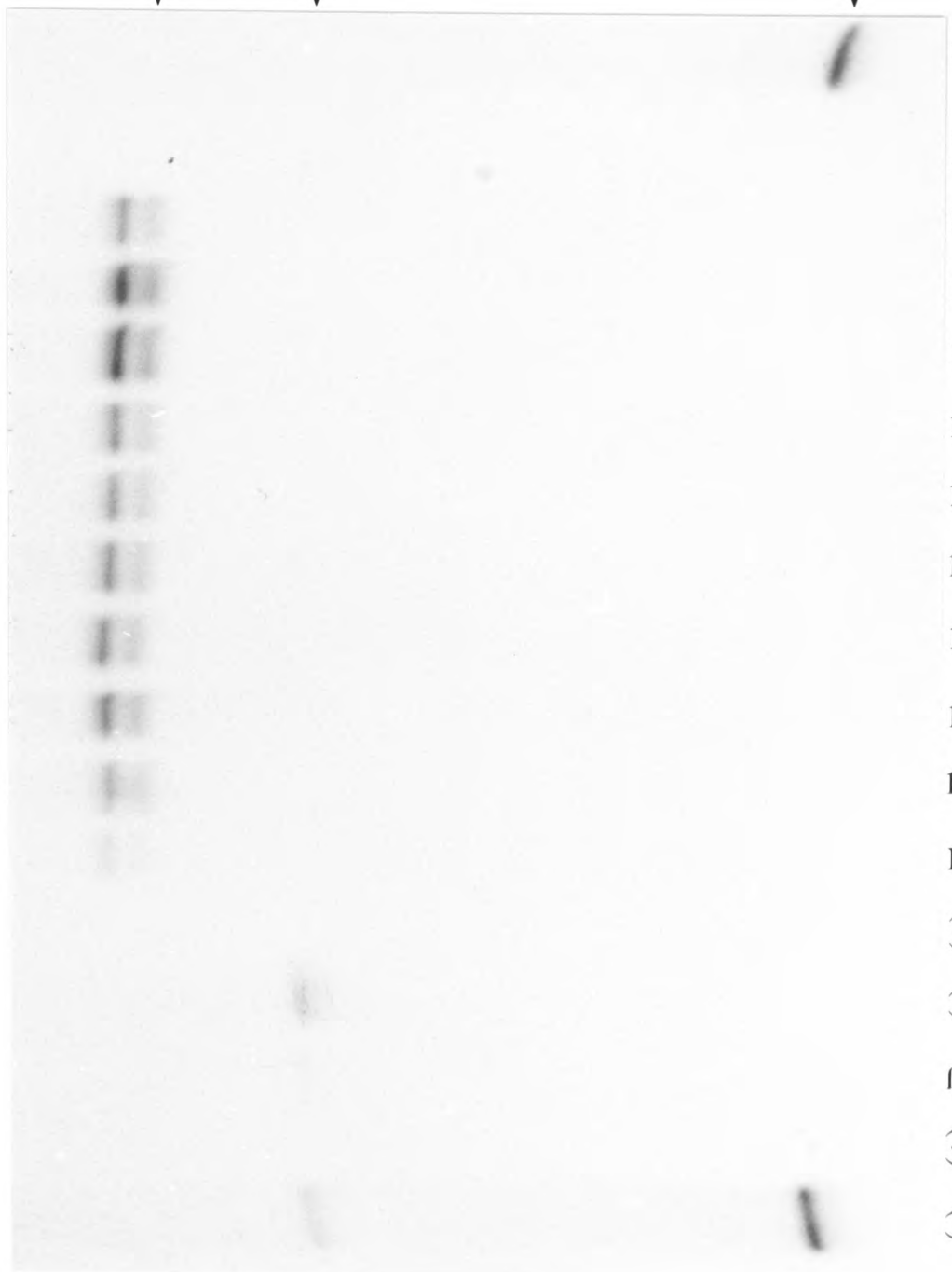


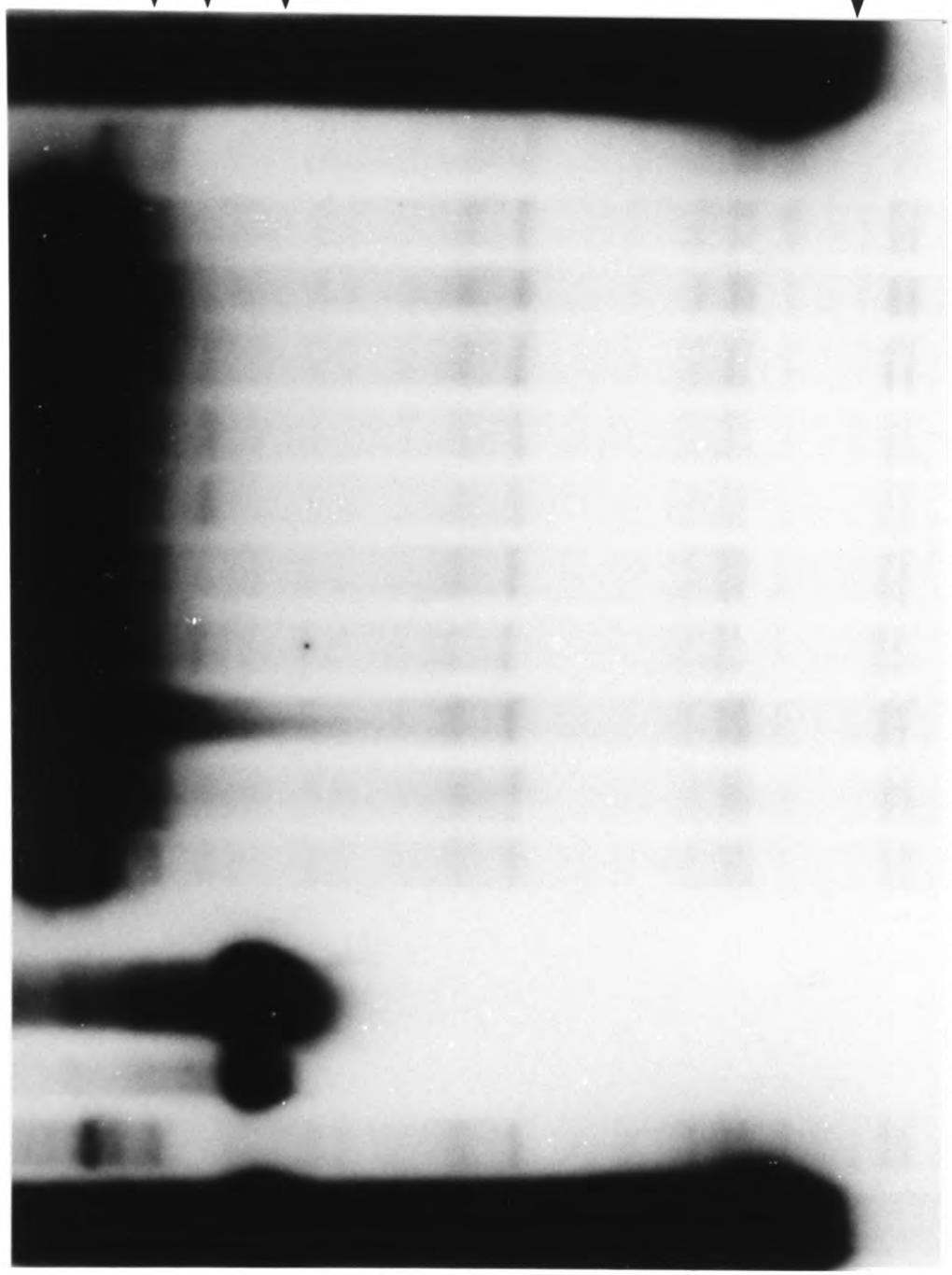
Figure 5.1. Template for preparation of the *Fgf9* antisense riboprobe.
The mouse *Fgf9* genomic sequence containing 76 bp of the 3' end of intron II (hatched box) and all of exon III (stippled box) was sub-cloned into the pBluescript vector (dashed line) (see section 2.3.3). This plasmid was used to generate the antisense radiolabelled *Fgf9* riboprobe on linearization with *Sma*I followed by *in vitro* transcription with T3 RNA polymerase. In the presence of *Fgf9* transcripts, the mouse *Fgf9* antisense riboprobe (417 nt) protects a fragment of 274 nt corresponding to the size of the coding exon III against digestion by RNase.

Figure 5.2. Ribonuclease Protection Analysis of *Fgf9* Expression During Development. The *Fgf9* antisense riboprobe was hybridized with total RNA (50 µg) prepared from mouse embryos at 9.5, 10.5, 11.5, 13.5, 14.5, 15.5, 16.7, 17.5 and 18.5 d.p.c. and from newborn mice. The radiolabelled mouse ß-*actin* riboprobe (350 nt) was included in each sample as a control for RNA equivalency between samples. The RNA fragment protected by the ß-*actin* probe is resolved on a 5% polyacrylamide gel as a 250 nt band. Further controls were carried out by hybridization of the probes with yeast tRNA (lanes marked (+) and (-)). The integrity of both riboprobes was verified by hybridization of the probes with yeast tRNA and omitting the RNase treatment step (lanes marked (-)). RNase activity and assay specificity were confirmed by treatment of the probes with RNase (lanes marked (+)) in the presence of yeast tRNA. The absence of cross-hybridization between the *Fgf9* and ß-*actin* antisense riboprobes was also checked by hybridization of both probes with yeast tRNA followed by treatment with RNase. The autoradiogram was exposed for 3 h (A) and up to 2 weeks (B). The *Fgf9*-protected band was apparent only after prolonged exposure (B).

(A)


FGF-9 Probe (417 nt)
ß-Actin Probe (350 nt)
ß-Actin
(-) Fgf9
(+) Fgf9
9.5 d.p.c.
10.5 d.p.c.
11.5 d.p.c.
13.5 d.p.c.
14.5 d.p.c.
15.5 d.p.c.
16.5 d.p.c.
17.5 d.p.c.
18.5 d.p.c.
Newborn
(+) ß-actin
(-) ß-actin
ß-actin
(+) Fgf9 + ß-actin
(-) Fgf9 + ß-actin

(B)


FGF-9 Probe (417 nt)
ß-Actin Probe (350 nt)
FGF-9
ß-Actin
(-) Fgf9
(+) Fgf9
9.5 d.p.c.
10.5 d.p.c.
11.5 d.p.c.
13.5 d.p.c.
14.5 d.p.c.
15.5 d.p.c.
16.5 d.p.c.
17.5 d.p.c.
18.5 d.p.c.
Newborn
(+) ß-actin
(-) ß-actin
ß-actin
(+) Fgf9 + ß-actin
(-) Fgf9 + ß-actin

5.2.1.2 Expression of Fgf9 in ES and EC Cells

To further verify the expression of *Fgf9* during development, embryonal cells were similarly examined using mRNAs isolated from embryonal stem (ES) cells (Evans and Kaufman, 1981) and embryonal carcinoma (EC) cells (Figure 5.3). As *Fgf9* is expressed in the rat C6 glioma cell line (see section 3.3.1), mRNA obtained from these cells was used as a positive control for the presence of *Fgf9* transcripts. This control is, however, not ideal as the rat *Fgf9* cDNA sequence deviates from the mouse homologue at several positions (Miyamoto et al., 1993; GENBANK, D14839) with the result that shorter fragments of ~117 nt were protected from RNase cleavage as the enzyme acts on regions of non-homology. This control nevertheless confirmed the high level of specificity that this method confers.

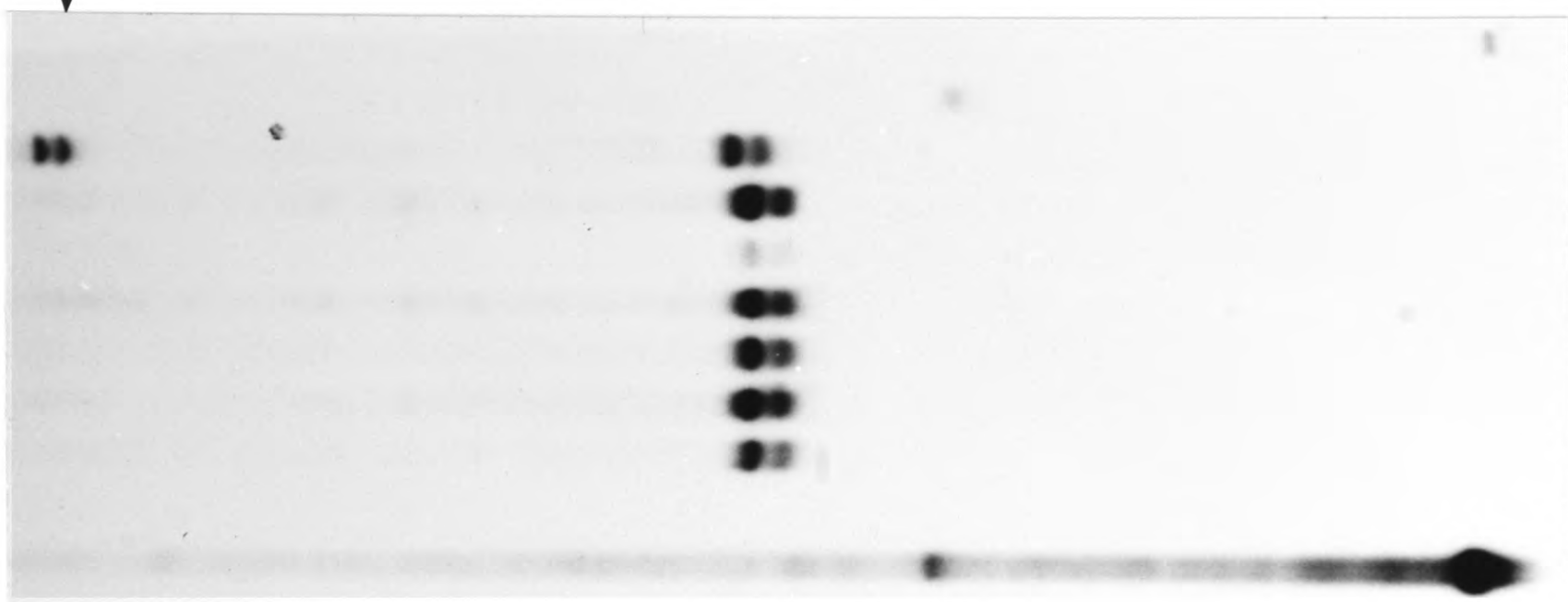
The RNase protection assays on embryonal stem or carcinoma cells showed that *Fgf9* transcripts are not detectably present in the uninduced E14 ES or 1009 EC cells. Examination of E14 ES cells induced to differentiate by: (a) exposure to retinoic acid in the presence of LIF, (b) exposure to retinoic acid in the absence of LIF, or (c) withdrawal of LIF, also revealed no detectable *Fgf9* transcripts. On the other hand, 1009 cells differentiated by induction with retinoic acid showed the presence of *Fgf9* RNA. This observation is significant since these differentiated cells consist predominantly of pre-neural and neural cell types (Pfeiffer et al., 1981). Hence, the results suggest that *Fgf9* is not expressed in the early postimplantation stages of normal mouse development and that the predominant expression of *Fgf9* in the early embryo may be restricted to the developing nervous system.

5.2.1.3 Expression of Fgf9 in Mouse Adult Tissues

The expression of *Fgf9* mRNA in various tissues of the adult mouse was also determined by RNase protection (Figure 5.4). *Fgf9* transcripts were detected in the brain, heart, kidney and ovary but not in the liver, lung or spleen. Expression levels appeared to be relatively

Figure 5.3. Ribonuclease Protection Analysis of *Fgf9* Expression in Murine Embryonic Stem (ES) Cells, Embryonal Carcinoma (EC) Cells and Their Differentiated Derivatives. E14 ES and 1009 EC cells were maintained in a morphologically undifferentiated state in the presence of LIF. Differentiated E14 cells were generated by the addition of RA to the cell culture in the presence or absence of LIF or, by withdrawal of LIF while differentiated 1009 EC cells were obtained by exposing the cells to RA for 6 days (see section 2.3.1). Poly(A)+ RNA (8 μg) isolated from both undifferentiated and differentiated ES and EC cells were hybridized to the radiolabelled *Fgf9* riboprobe derived from the template shown in Figure 5.1. Control hybridizations were performed as in Figure 5.2, with one addition: poly(A)+ RNA (8 μg) isolated from rat C6 glioma cells known to express FGF-9 (see section 3.2.1) was hybridized to the antisense *Fgf9* riboprobe as a postiive control. The protected fragments obtained from the latter sample were observed as smaller bands (*) due to sequence heterogeneity in the third exon between the rat and mouse *Fgf9* coding sequences which were recognized and cleaved by RNase. The autoradiogram was exposed for 3 h (A) and up to 2 weeks (B) when FGF-9 expression is detected only in differentiated 1009 EC cells.

(A)


FGF-9*
Fgf9 Probe
ß-actin Probe
C6
E14
E14 + LIF + RA
E14 - LIF + RA
E14 - LIF
1009
1009 + RA
(+) *Fgf9* + ß-actin Probes
(-) *Fgf9* + ß-actin Probes

(B)

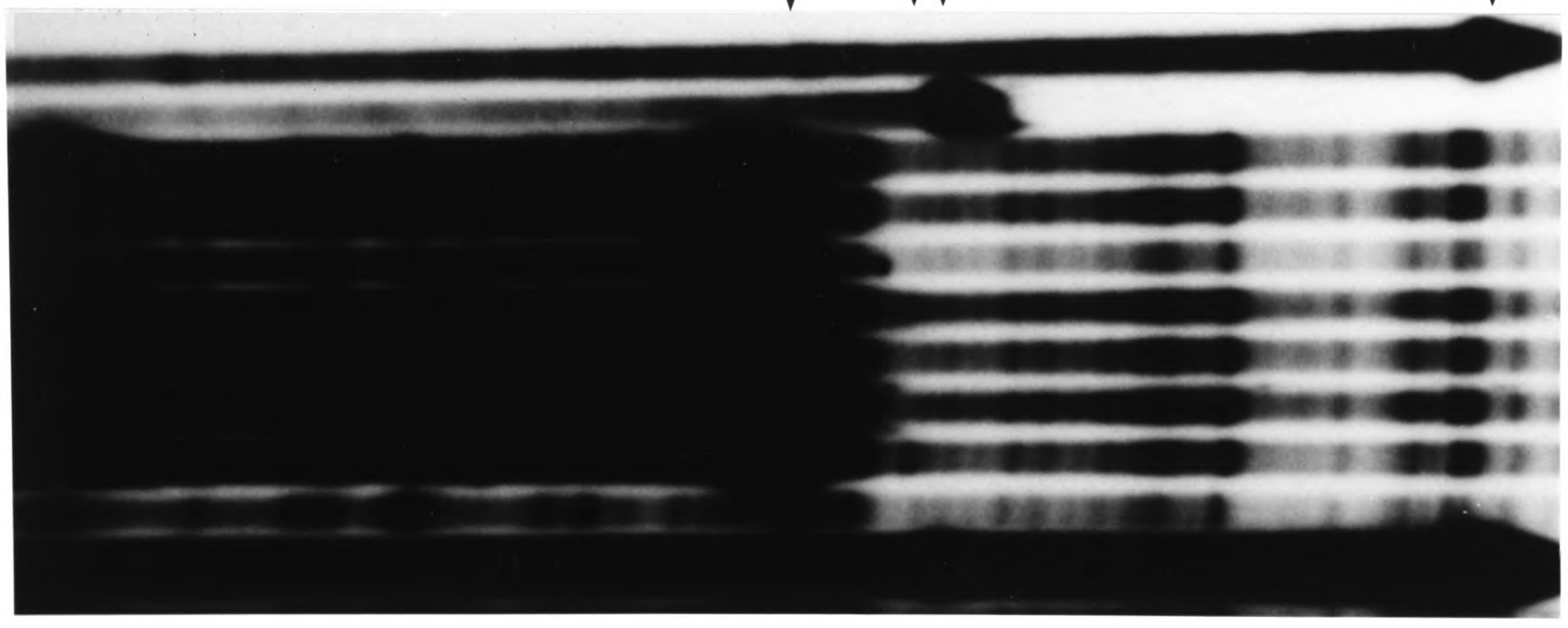

ß-Actin
ß-Actin Probe (350 nt)
FGF-9
FGF-9 Probe (417 nt)
Fgf9 Probe
ß-actin Probe
C6
E14
E14 + LIF + RA
E14 - LIF + RA
E14 - LIF
1009
1009 + RA
(+) *Fgf9* + ß-actin Probes
(-) *Fgf9* + ß-actin Probes

Figure 5.4. Ribonuclease Protection Analysis of *Fgf9* Expression in Adult Mouse Tissues.
Samples of total RNA (50 µg) obtained form mouse brain, heart, kidney, liver, lung, ovary and spleen were analysed by RNase protection assay as in Figure 5.2. Control hybridizations were carried out as before (Figure 5.2). The autoradiogram was exposed for 2.5 h (A) and up to 2 weeks (B). The specific protected fragments of *Fgf9* transcripts were detected in the brain, heart, kidney and ovary of the adult mouse.

(A)

Fgf9 Probe
(-) *Fgf9* Probe
(+) *Fgf9* Probe
Brain
Heart
Kidney
Liver
Lung
Ovary
Spleen
(+) ß-actin Probe
ß-actin Probe
(+) *Fgf9* + ß-actin Probes
(-) ß-actin Probe
(-) *Fgf9* + ß-actin Probes

FGF-9 Probe (417 nt)

ß-Actin Probe (350 nt)

FGF-9

ß-Actin

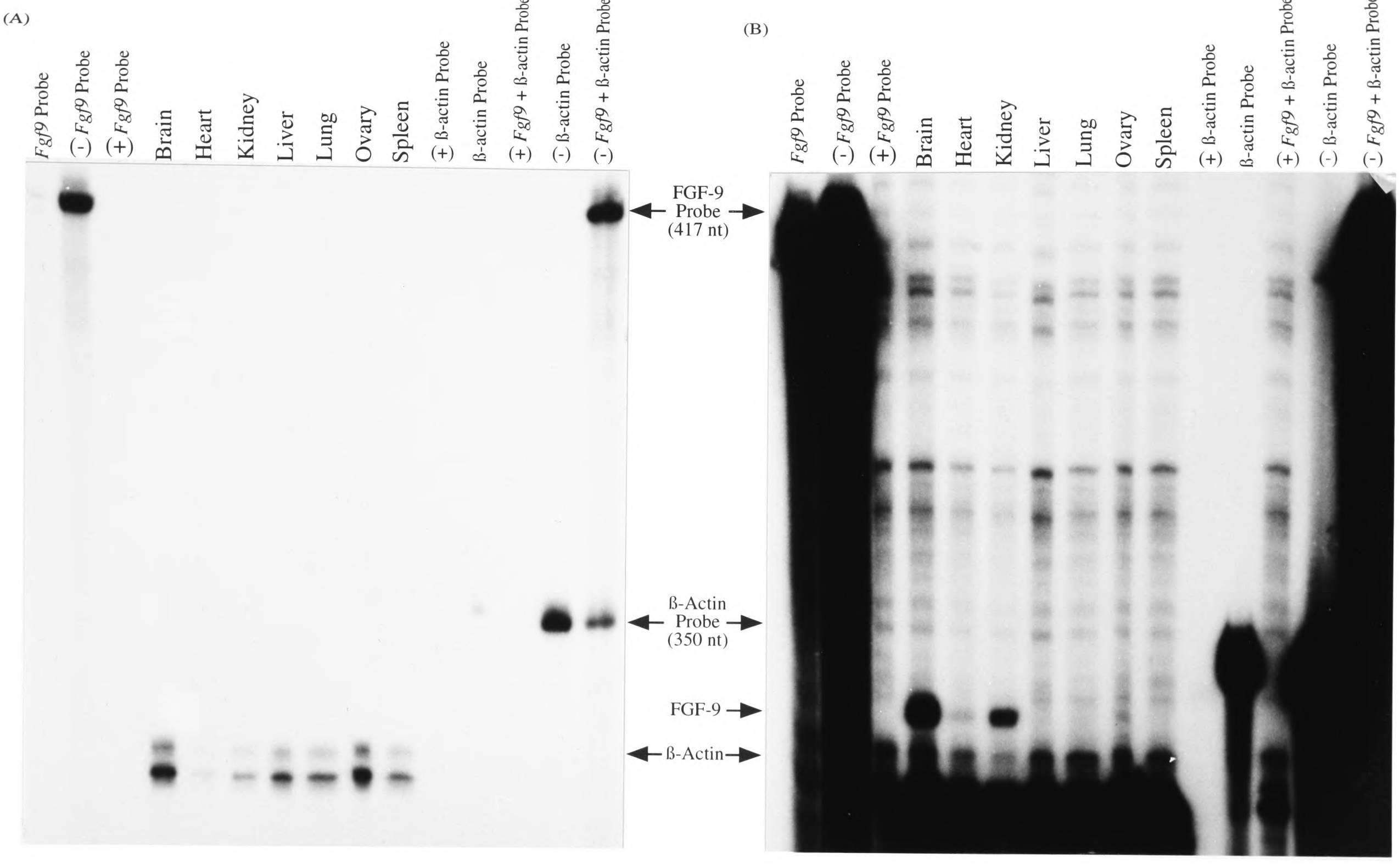
(B)

Fgf9 Probe
(-) *Fgf9* Probe
(+) *Fgf9* Probe
Brain
Heart
Kidney
Liver
Lung
Ovary
Spleen
(+) ß-actin Probe
ß-actin Probe
(+) *Fgf9* + ß-actin Probes
(-) ß-actin Probe
(-) *Fgf9* + ß-actin Probes

Figure 5.4. Ribonuclease Protection Analysis of *Fgf9* Expression in Adult Mouse Tissues.
Samples of total RNA (50 μg) obtained form mouse brain, heart, kidney, liver, lung, ovary and spleen were analysed by RNase protection assay as in Figure 5.2. Control hybridizations were carried out as before (Figure 5.2). The autoradiogram was exposed for 2.5 h (A) and up to 2 weeks (B). The specific protected fragments of *Fgf9* transcripts were detected in the brain, heart, kidney and ovary of the adult mouse.

(A)

(B)


Fgf9 Probe
(-) Fgf9 Probe
(+) Fgf9 Probe
Brain
Heart
Kidney
Liver
Lung
Ovary
Spleen
(+) ß-actin Probe
ß-actin Probe
(+) Fgf9 + ß-actin Probes
(-) ß-actin Probe
(-) Fgf9 + ß-actin Probes
FGF-9 Probe (417 nt)
ß-Actin Probe (350 nt)
FGF-9
ß-Actin

high in the brain and kidney. The result here differs from the previously reported Northern blot analysis on adult rat tissues where expression was detected only in the brain and kidney (Miyamoto et al., 1993). The identification of *Fgf9* mRNA in additional tissues probably reflects the increased sensitivity of the RNase protection method relative to the Northern blot method in detecting gene expression, particularly where the expression level is low.

5.2.2 *In Situ* Hybridization Analysis

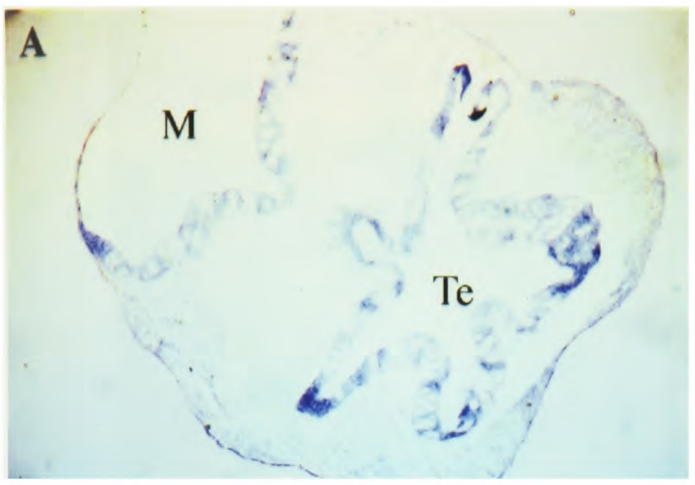
5.2.2.1 Developmental Localization of Fgf9 RNA

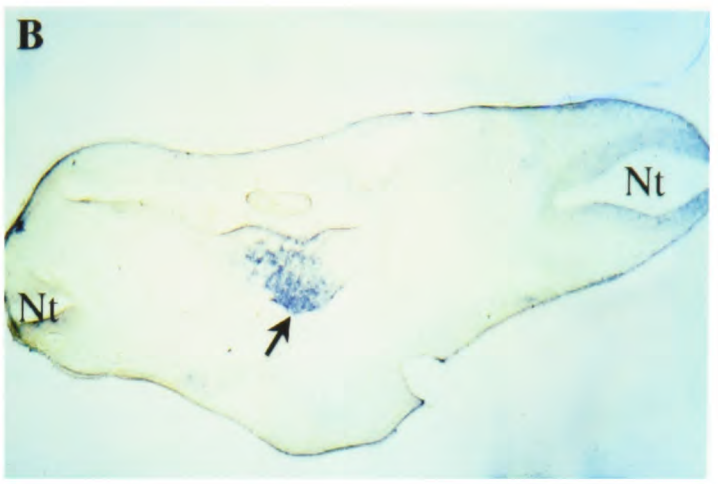
To determine the tissue distribution of *Fgf9* RNA during development, whole mount *in situ* hybridizations were carried out on 6.5 to 11.5 days p.c. mouse embryos using digoxygenin-labelled *Fgf9* probe. The full length 1.2 kb antisense riboprobe for murine *Fgf9* was transcribed from pBSmFGF-9 (section 3.3.3). Parallel experiments performed in the absence of any probe served as negative controls. No significant signals above background were detected in all the embryo stages studied (data not shown). It is possible that the levels of *Fgf9* RNA expression are below the threshold detectable by this method and more sensitive detection assays may be required.

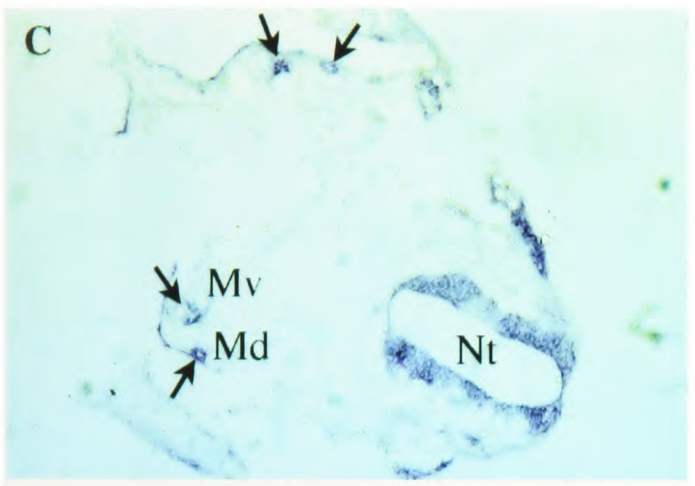
RNA *in situ* hybridizations were also carried out on embryo cryosections. Mouse embryos at 10.5 d.p.c. were sectioned and hybridized *in situ* to either antisense or sense *Fgf9* probes labelled with digoxygenin. The sense riboprobe, also transcribed from the plasmid pBSmFGF-9 and yielding a shorter 490 bp transcript, was used in parallel experiments to determine the specificity of the hybridization signals.

Prominent *Fgf9* RNA expression was detected in the germinal epithelium of the fore- and hindbrains (Figure 5.5A). This localization is significant since the putative receptor for FGF-9, FGFR3c has been reported to be expressed in this region (Peters et al., 1993). *Fgf9* transcripts were also found in the gut primordia (Figure 5.5B) and the lateral region of the pharyngeal pouch (Figure 5.5G). More detailed studies are required to determine the possible regional or cell type-specific distribution of *Fgf9* expression in these

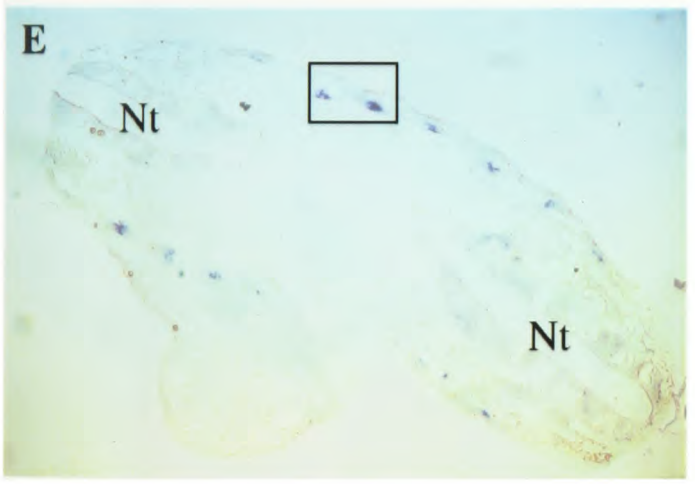
Figure 5.5. *Fgf9* Expression in 10.5 d.p.c. Mouse Embryo.
In situ hybridization of transverse tissue sections of 10.5 d.p.c. mouse embryos with sense or antisense probes for *Fgf9*. *Fgf9* expression is observed in the neuroepithelium of the mid- and fore-brain (A), gut primordia (B, arrow), mesonephric vesicle and duct (C, arrows), somites (E, F) and lateral region of the pharyngeal pouch (G, arrow). (F) is a magnified view of the boxed section in (E). (D) Section of embryo comparable to (C) but hybridized with sense *Fgf9* probe. Abbreviations: M, midbrain; Te, telencephalon; Md, mesonephric duct: Mv, mesonephric vesicle; S, somite; Hb, hindbrain; Nt, neural tube. Magnifications: A, B, E and G, 50x; C - D, 100x; F, 400x.

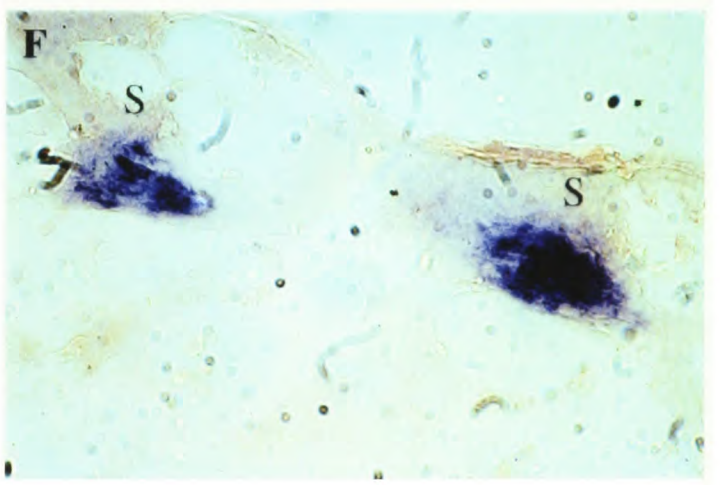

A
M
Te

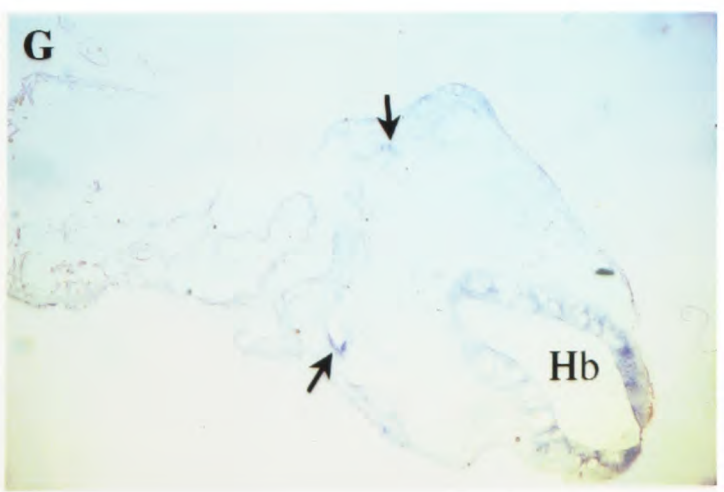

B
Nt
Nt


C
Mv
Md
Nt


D
Nt


E
Nt
Nt


F
S
S


G
Hb

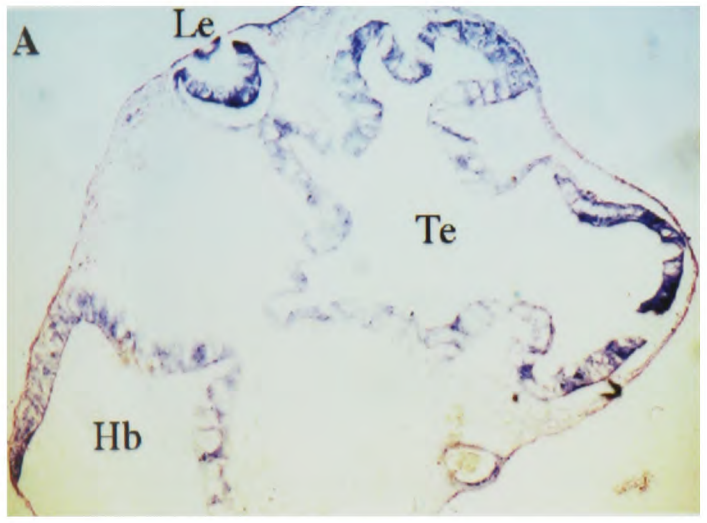
two areas. Three other FGF members, namely, *Fgf3, Fgf4* and *Fgf8* are also expressed in the pharyngeal pouch where they exhibit a similar spatial and temporal distribution pattern (Wilkinson et al., 1988; Niswander and Martin, 1992; Drucker and Goldfarb, 1993; Mahmood et al., 1995a). Other areas of *Fgf9* expression include the mesonephric vesicles and mesonephric ducts (Figure 5.5C) and the somites (Figure 5.5E). At 10.5 days p.c., the mature somites, which arise by segmentation of paraxial mesoderm in an anterior-to-posterior sequence at 8.5 days p.c., have already differentiated to form the three cell layers, namely the sclerotome, dermatome and myotome (Ott and Buckingham, 1992). It is not clear which cell type is staining positive for *Fgf9* RNA, but based on the anatomical position of the signals and the morphology of the labelled cells, *Fgf9* transcripts might be restricted to the myotome. Further studies with markers for skeletal myoblasts such as α-cardiac actin (Sassoon et al., 1988) will be required to confirm the localization of *Fgf9* RNA to this compartment.

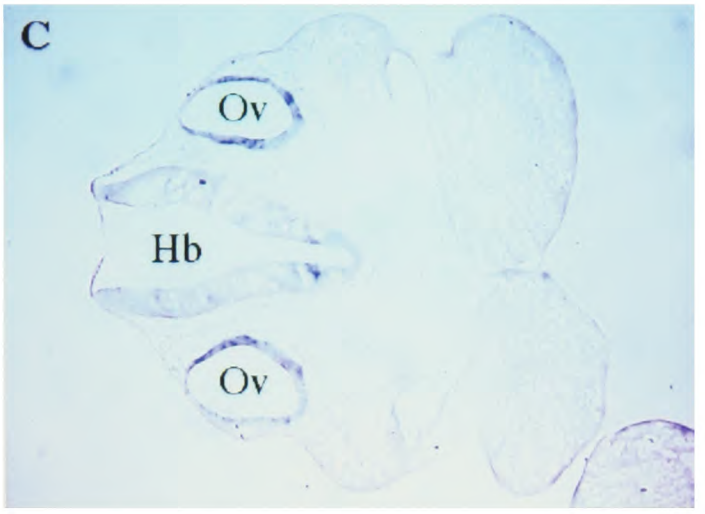
Fgf9 expression was also detected in the developing sense organs (Figure 5.6). *Fgf9* transcripts were observed in the inner layer of the optic cup that gives rise to the future nervous layer of the retina and in the lens epithelium (5.6A-B). Signals corresponding to *Fgf9* RNA expression were also detected in the otic vesicle (Figure 5.6C-D).

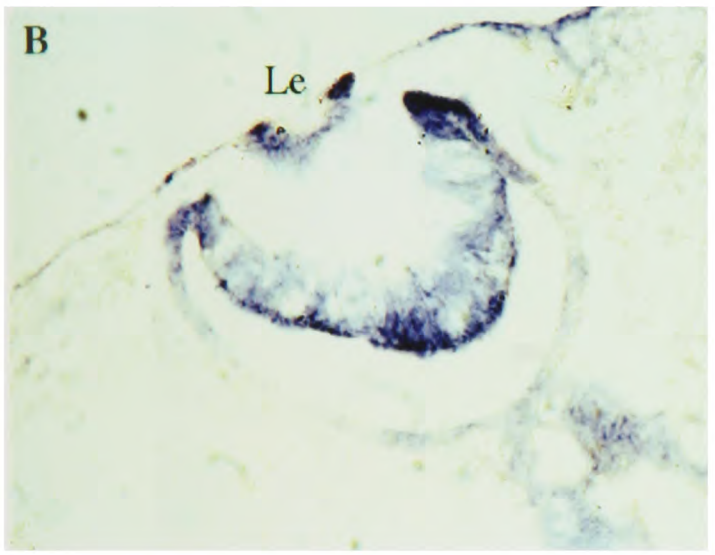
5.2.2.2 Co-localization of Fgf9 and Fgfr3 RNAs in the Adult Mouse Brain

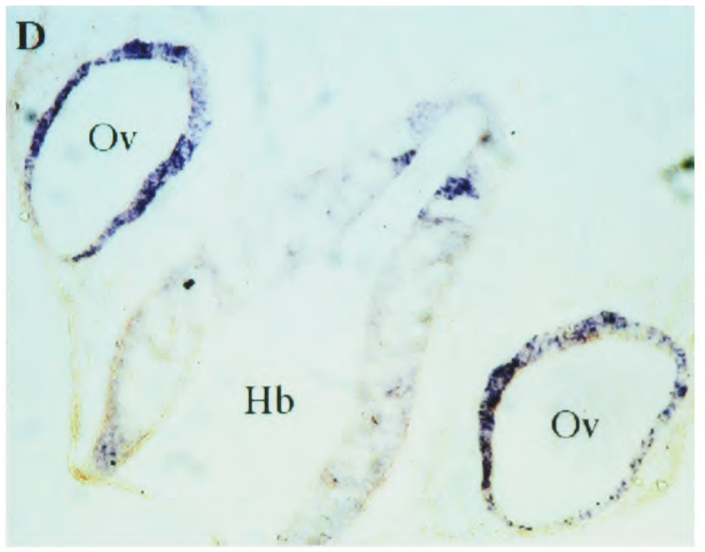
The expression pattern of *Fgf9* RNA in the adult rat brain has been described (Tagashira et al., 1995). FGFR3c previously shown to be a putative receptor for FGF-9 (see Chapter 4), is also expressed in the adult brain (Peters et al., 1993). To investigate the relationship between *Fgf9* and *FGFR3* localization in the brain, cryosections of adult mouse brains were hybridized simultaneously with *Fgf9* and *Fgfr3* antisense riboprobes labelled with digoxygenin and fluorescein respectively (or vice versa) in co-localization or double *in situ* hybridization experiments. The antisense probe for *Fgf9* was derived as described above (sections 2.3.3.2 and 5.2.2.1) while that for *Fgfr3* was derived from the cDNA template spanning the transmembrane and juxtamembrane portions of the gene, as described

Figure 5.6. *Fgf9* Expression in the Developing Eye and Ear.
Sections of a 10.5 d.p.c. mouse embryo were hybridized to antisense *Fgf9* probe. Expression is observed in the lens epithelium (Le) and inner layer of the optic cup (A, B), and in the epithelium of the otic vesicle (C, D). Panel B is a magnified view of panel A, emphasizing the eye region. Likewise, panel D is a magnified version of panel C, showing the otic vesicles. Abbreviations: Hb, hindbrain; Te, telecephalon; Le, lens epithelium; Ov, otic vesicle. Magnifications: A and C, 50x; B, 200x; D, 100x.


A
Le
Te
Hb


C
Ov
Hb
Ov


B
Le


D
Ov
Hb
Ov

previously (see section 2.3.3.4; Peters et al., 1993). The specificity of the hybridization signals was determined in parallel experiments with sense riboprobes derived from the same templates and labelled with the same markers.

Both *Fgf9* and *Fgfr3* were expressed in a diffuse pattern in the adult mouse brain. Co-localization of *Fgf9* and *Fgfr3* RNAs is most apparent in the olfactory bulb and cerebral cortex. In contrast, they exhibit distinct distribution patterns in the region of the cerebellum.

5.2.2.2.1 Olfactory Bulb

Both *Fgf9* and *Fgfr3* RNAs were detected in similar regions of the olfactory bulb (Figure 5.7). The labelled layers include the outermost glomerular layer, the mitral cell layer, and the ependyma and subependymal layers.

5.2.2.2.2 Cerebral Cortex

The cerebral cortex may be divided into six histological layers (Zilles and Wree, 1995). The *Fgf9* antisense probe indicated diffuse patterns of expression of the transcript throughout the layers of the cerebral cortex (Figure 5.8A) while *Fgfr3* transcripts appeared to show a more layered distribution with highest labelling intensity in a region that corresponds anatomically to the cortical layer II. The piriform cortex also exhibited the co-localization of high levels of both transcripts (Figure 5.8C-F).

5.2.2.2.3 Cerebellum

Fgf9 and *Fgfr3* showed distinct patterns of RNA expression in the cerebellum. The cerebellar cortex is divided into three histological layers: the molecular layer, the Purkinje cell or ganglionic layer and the granular layer (Ito, 1984). Highest level of *Fgf9* transcripts appeared to reside in the white matter while highest labelling intensity for *Fgfr3* RNA appeared to localize within the pyramidal cell layer of the cerebellum, as shown by both NBT and fluorescein precipitates (Figure 5.9). In addition to labelling the Purkinje neuronal

Figure 5.7. *Fgf9* and *Fgfr3* Expression in the Olfactory Bulb.
Double *in situ* hybridization of horizontal sections of the adult mouse brain with *in vitro* synthesized fluorescein-labelled *Fgf9* and DIG-labelled *Fgfr3* antisense riboprobes. The *Fgf9* probe was detected using the ELF substrate which generates fluorescent green precipitates that are observable by UV microscopy (see section 2.3.4.4). On the other hand, areas corresponding to *Fgfr3* probe hybridization were detected by the NBT-BCIP colour reaction which allows signals to be observed under the light microscope as blue precipitates (see section 2.3.4.4). (A) Photograph obtained using UV microscopy showing the distribution of *Fgf9* transcripts in the olfactory bulb. Signals were observed in the glomerular cell layer, mitral cell layer and in the ependymal and subependymal layer. (B) Photograph of (A) obtained using light microscopy which shows the localization of *Fgfr3* RNA in similar areas of the olfactory bulb. (C) High magnification of (B) with low-level transmitted light so that simultaneous visulalization of both the silver grains and blue precipitates is obtained; the section shows co-localization of both *Fgf9* and *Fgfr3* transcripts in the mitral and ependymal layers. Abbreviations: Gl, glomerular cell layer; Epx, external plexiform layer; Ml, mitral cell layer; Gr, granule cell layer; Ep, ependymal layer. Magnifications: A-B, 50x; C, 250x.

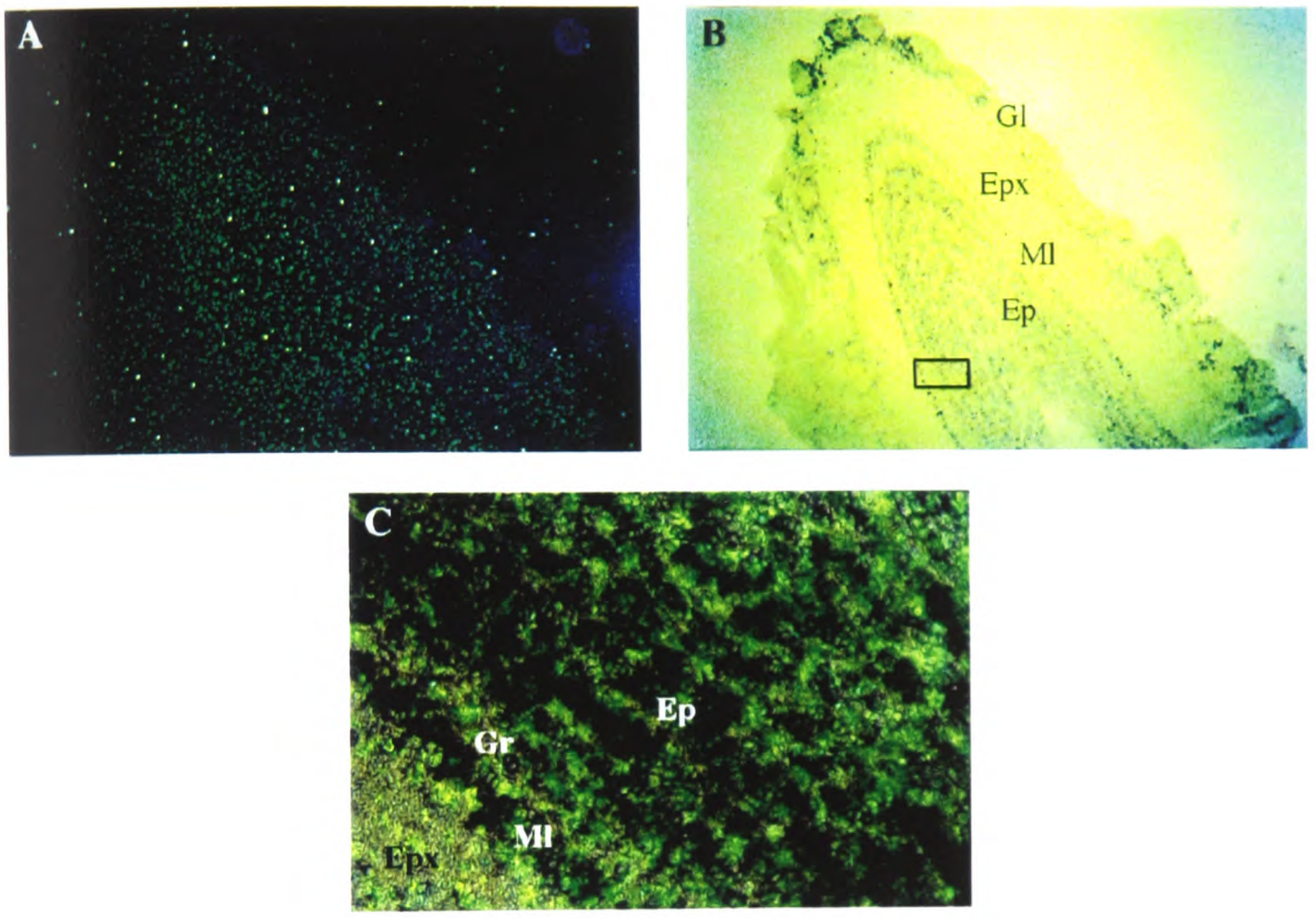
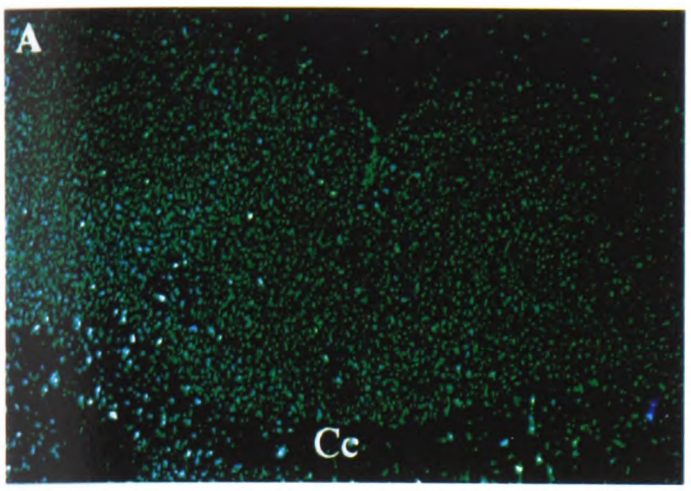
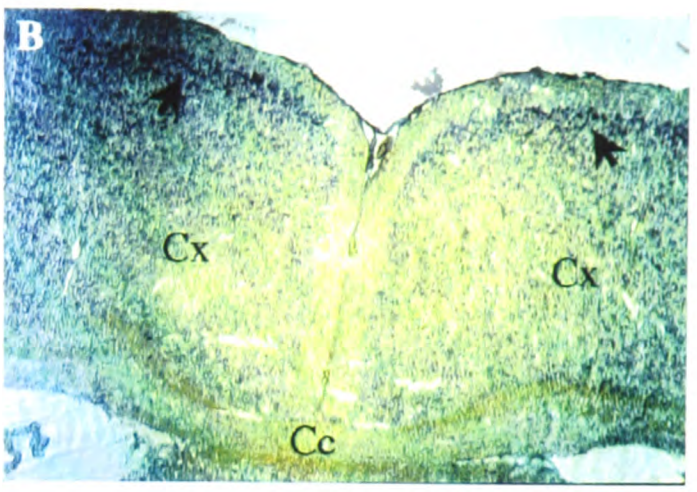
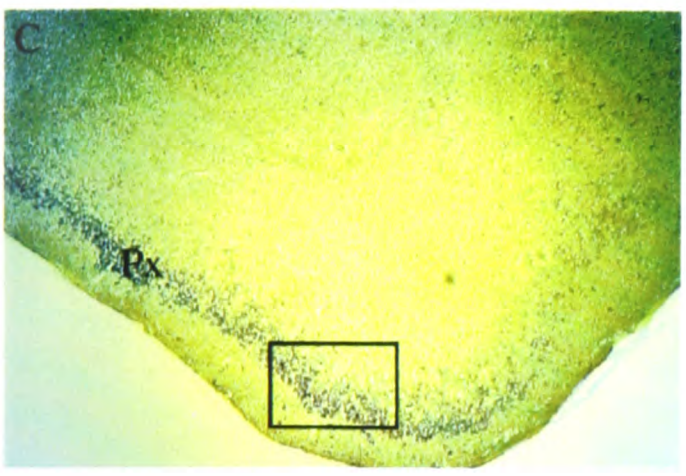
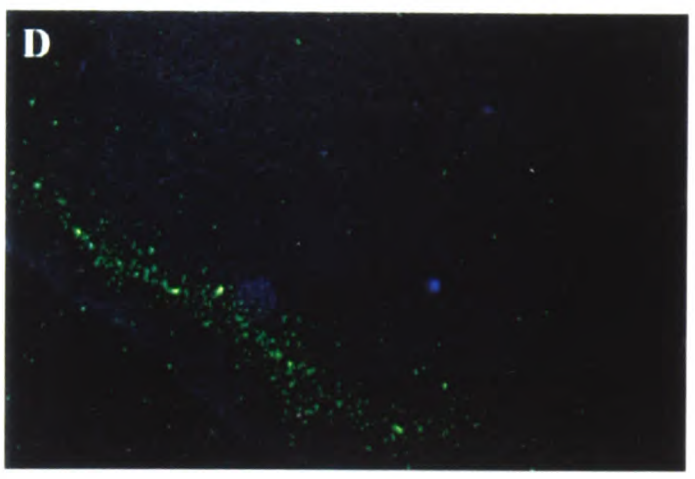

A
B
Gl
Epx
Ml
Ep
C
Ep
Gr
Ml
Epx

Figure 5.8. *Fgf9* and *Fgfr3* Expression in the Cerebral Cortex.
Double *in situ* hybridization was carried out on adult mouse brain sections with differentially labelled *Fgf9* and *Fgfr3* riboprobes as in Figure 5.7. (A) Coronal section of the cerebral cortex co-hybridized with fluorescein-lablled *Fgf9* and DIG-labelled *Fgfr3* probes, showing *Fgf9* transcripts widely distributed in the cerebral cortex when viewed under UV illumination. (B) Same view of (A) under bright-field illumination, revealing the more layered distribution of *Fgfr3*, with the most intensely staining layer indicated by arrows. (C) Coronal section on th level of the piriform cortex, demonstrating *Fgf9* expression which is observed as blue precipitates. The section was hybridized simultaneously with DIG-labelled *Fgf9* and fluorescein-labelled *Fgfr3* riboprobes. (D) The same section as in (C) photographed under UV illumination for visualization of *Fgfr3* expression. (E) Higher magnification of the boxed region from (C) and photographed under conditions of low-level transmitted light. Both types of transcripts show co-localization in the piriform cortex. (F) Horizontal section at the level of the neocortex viewed under low-level transmitted light shows the co-expression of *Fgf9* and *Fgfr3* transcripts in the neocortex. This section was hybridized with fluorescein-labelled *Fgf9* and DIG-labelled *Fgfr3* riboprobes; *Fgf9* expression is represented by green fluorecent precipitates and *Fgfr3* expression is observable as blue precipitates. Abbreviations: Cc, corpus callosum; Cx, cerebral cortex; Px, piriform cortex. Magnifications: A-D, 50x; E-F, 250x.


A
Cc


B
Cx
Cx
Cc


C
Rx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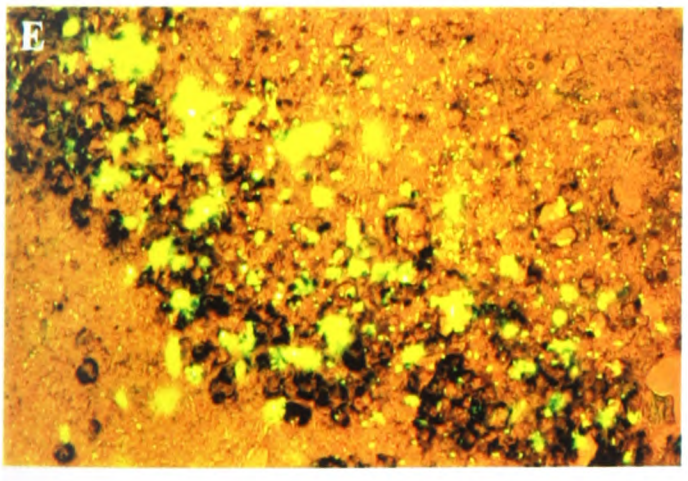
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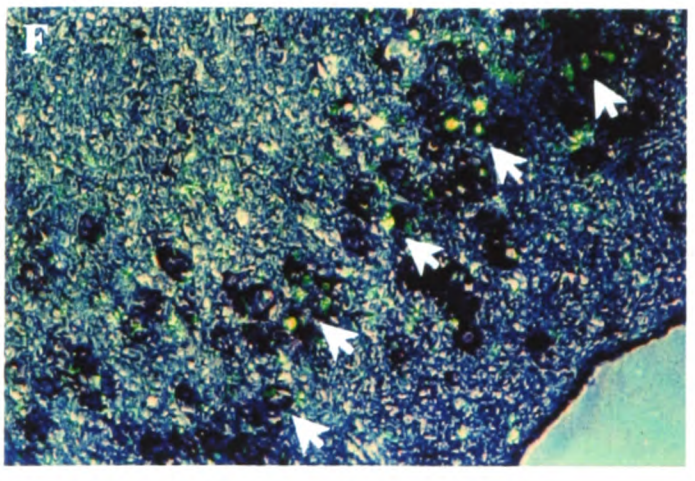
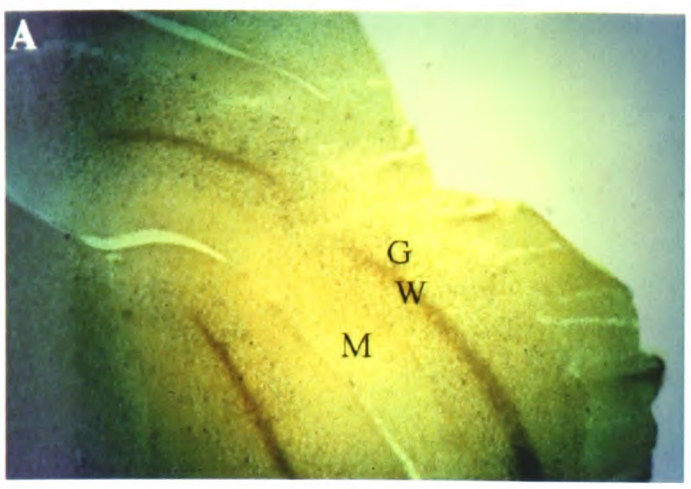
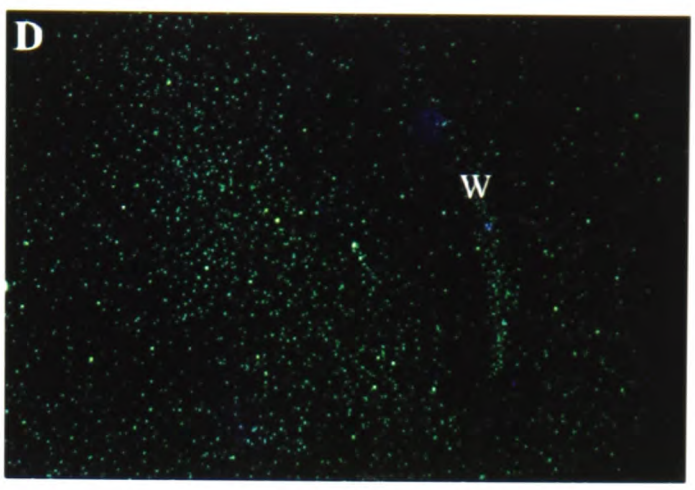
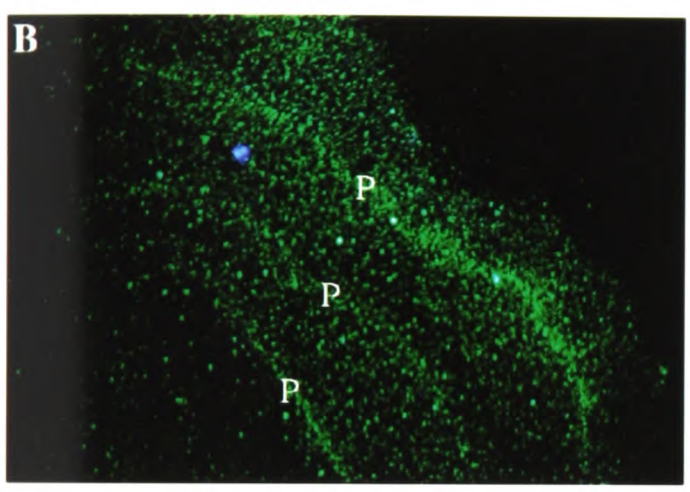

F

Figure 5.9. *Fgf9* and *Fgfr3* Expression in the Cerebellum.
Double *in situ* hybridization of horizontal sections of the adult mouse brain were performed as in Figure 5.7. (A) Section of cerebellar lobules co-hybridized with DIG-labelled *Fgf9* and fluorescein-labelled *Fgfr3*. *Fgf9* expression is detected in the granule cell layer and white matter under bright-field illumination. (B) The same section as in (A) viewed under UV illumination shows high *Fgfr3* expression in the Purkinje cell layer. (C) The same section as in (A) photographed under conditions of low-level transmitted light demonstrating the distinct expression patterns of the two types of transcripts. Localization of the fluorecent precipitates corresponding to *Fgfr3* expression is indicated by arrows. (D) Cerebellum section hybridized simultaneously with fluorescein-labelled *Fgf9* and DIG-labelled *Fgfr3*. *Fgf9* expression is observed as fluorescent green precipitates under UV illumination; the signal in the white matter is relatively high. (E) The same section as in (D) viewed under bright-field illumination shows *Fgfr3* expression as blue precipitates. Signal is again distinctly high in the Purkinje cell layer. (F) Higher magnification of the boxed region in (E), photographed using low-level transmitted light so that simultaneous visualization of the two types of signals was possible; the Purkinje cell layer was labelled by the *Fgfr3* probe (arrows point to the relatively large pear-shaped Purkinje cells) while the *Fgf9* probe labelled the white matter strongly. Abbreviations: G, granule cell layer; M, molecular layer; P, Purkinje cell layer; W, white matter. Magnifications: A-E, 50x; F, 250x.


A
G
W
M


D
W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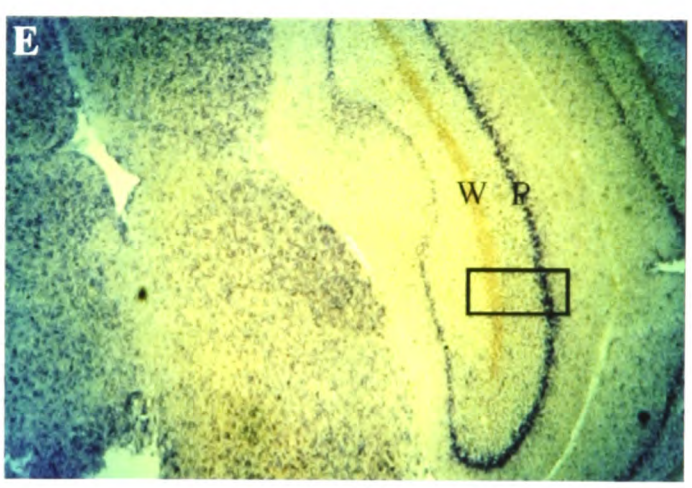
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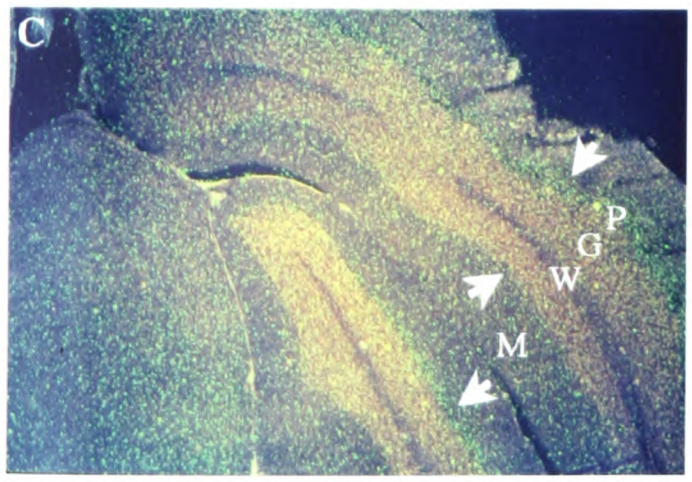
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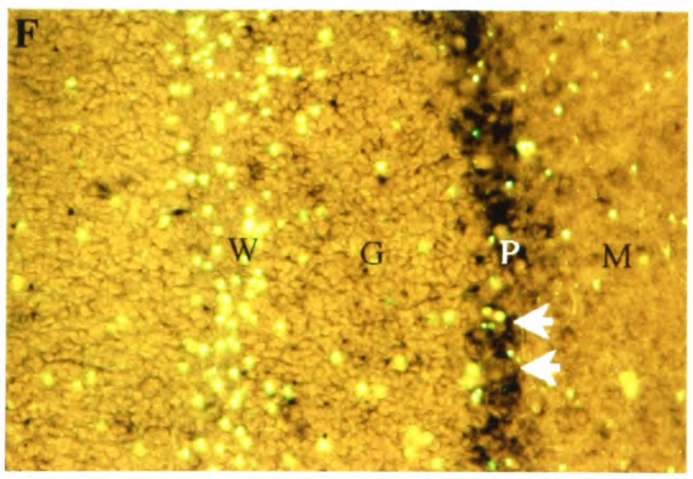
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cells, as found for *Fgfr3* expression in the monkey brain (Thompson et al., 1991), closer examination at higher magnification of the Purkinje cell layer labelled by the *Fgfr3* riboprobe revealed that some of the labelled cells may include Golgi epithelial cell bodies which lie slightly above or below the Purkinje layer (Figure 5.9F). These glial cells extend parallel extensions known as Bergmann fibers through the molecular layer. This observation is consistent with a previous report that *Fgfr3* transcripts are found in the Golgi epithelial cells in the cerebellum (Peters et al., 1993).

5.3 Discussion

5.3.1 *Fgf9* Expression during Embryogenesis

Embryonic expression of the *Fgf9* gene is regulated both temporally and spatially. The expression levels are low even at stages where expression can be detected by RNase protection analysis. Embryonic stem (ES) cells in culture that retain the pluripotential characteristics of normal pluripotent stem cells and their immediate differentiated progenies were shown not to express *Fgf9* transcripts. Hence, *Fgf9* may not be expressed in the early postimplantation stages of normal mouse development. This prediction is supported by the observation that 1009 embryonic carcinoma cells, another cell line derived from pluripotential embryonic ectoderm, do not appear to express *Fgf9* mRNA. These cells, however, do express the transcript on differentiation to a population of neuronal cells representative of the neuroectodermal cell types of the early embryo (Pfeiffer et al., 1981). It may be deduced then that *Fgf9* expression during early embryogenesis is restricted to the developing nervous system where it may function in the regulation of neuroectodermal proliferation and differentiation.

RNase protection analysis of embryonic RNA detected *Fgf9* expression during mouse development from 10.5 to 18.5 d.p.c.. In addition, RNA *in situ* hybridization analysis indicates that *Fgf9* expression is spatially restricted, allowing for attempts to speculate on the potential functions of FGF-9 during embryogenesis.

At 10.5 d.p.c., the brain may be subdivided by the trigeminal ganglion, the cranial ganglia and the acoustic (VIII) nerve (Rugh, 1993). At this stage of development, the neural components of the brain are rapidly proliferating and differentiating. FGF-9 expressed in the germinal epithelium of the fore-, mid- and hindbrain may thus be involved in one or both of these biological events. It may then share a common property with the prototypic FGF-1 and FGF-2 in possessing a neurotrophic function. Numerous studies have shown that the FGFs have neurotrophic activities on cells derived from embryonic neural tissues. For instance, FGF-1 and/or FGF-2 can enhance the survival of embryonic ciliary ganglion, spinal cord neurons and neuroepithelial cells (Unsicker et al., 1987; Murphy et al., 1990) as well as stimulate the proliferation of oligodendrocyte-type-2 (O-2A) progenitor cells (Bögler et al., 1990; McKinnon et al., 1990), cerebrum multipotential precursor cells (Kilpatrick and Bartlett, 1995) and neural crest cells (Murphy et al., 1994). The prototype FGFs can also promote the differentiation of 10 d.p.c. embryonic neuroepithelial cells into mature neurons and glia at high concentrations (Murphy et al., 1990) and induce the differentiation of neuronal cells such as neonatal adrenal chromaffin cells (Stemple et al., 1988) and sympathoadrenal progenitor cells (Birren and Anderson, 1990). Another role for the FGFs in neurodevelopment is uncovered by the finding that FGF-2 can effect anterior-posterior neural patterning in the *Xenopus* embryo (Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Lamb and Harland, 1995). FGF-9 may thus contribute to the development of the central nervous system by promoting the survival and proliferation of neuronal and glial cells, or by regulating the differentiation of specific neuronal or glial cell populations. Another possible role for FGF-9 may be to render competence to the neuroectoderm cells to respond to signals that specify positional values or cell lineages. This is an extension from the observation that FGF-2 is able to render chromaffin cells competent to respond to nerve growth factor, a molecule required for their differentiation into sympathetic neurons (Stemple et al., 1988; Birren and Anderson, 1990).

The transcription of *Fgf9* in the germinal neuroepithelium is striking as it coincides with that of *Fgfr3* (Peters et al., 1993). This suggests that a functional interaction between

FGF-9 and FGFR3 occurs during the development of the central nervous system. On the other hand, other putative ligands of FGFR3, namely, *Fgf1, Fgf2* (Ornitz and Leder, 1992) and *Fgf8c* (MacArthur et al., 1995a) have also been localized in the germinal neuroepithelium of the mouse, rat or chicken embryos (Wilcox and Unnerstall, 1991; Dono and Zeller, 1994; Heikinheimo et al., 1994; Ohuchi et al., 1994; Crossley and Martin, 1995; MacArthur et al., 1995a; Mahmood et al., 1995a, b). The issue is further complicated by the observation that FGFR1 and FGFR2, the other putative receptors for both FGF-1 and FGF-2 (Dionne et al., 1990; Mansukhani et al., 1990; Ornitz and Leder, 1992; Vainikka et al., 1992) are also transcribed in the epithelia of the developing central nervous system at this stage of development (Orr-Urtreger et al., 1991; Peters et al., 1992b; Yamaguchi et al., 1992). The biological significance of the potential FGF-9 and FGFR3c interaction in the developing central nervous system is thus unclear and requires further studies to define the specific contribution made by FGF-9 during neural development. This issue may be made more complicated by the possibility that FGFR3 signalling in the central nervous system is non-critical or redundant, as suggested by a recent study of FGFR3-deficient mice which displayed no apparent histological abnormalities in the brain (Deng et al., 1996),

The eye begins as an invagination of the optic cup, which at 10.5 d.p.c., consists of two layers of cells: the inner is destined to form the neural part of the retina while the outer layer forms the pigment layer of the retina. At this stage of development, the inner layer is about three to four times the thickness of the outer layer. The results in this study showed that *Fgf9* RNA is expressed in the lens epithelium and in the inner neural layer of the embryonic retina, a region characterized by cell proliferation at this stage of development. As the retina undergoes differentiation into inner and outer nuclear layers only at about 15 d.p.c. (Rugh, 1993), the role of FGF-9 at 10.5 d.p.c. is likely to be associated with retinal cell proliferation. Other FGFs have also been reported in the lens epithelium and retina. The lens epithelium have been found to transcribe the RNAs for FGF-1 and FGF-2 (Fu et al., 1991) while the embryonic retina has been reported to be a source of FGF-1 and FGF-2 (Mascarelli et al., 1987). Both prototypic FGFs have also been localized in late stages of

retina development in the rat embryo (Fu et al., 1991), suggesting that they participate in retinal differentiation. *Fgf3* transcripts were also described in the neuroblastic layer of the developing retina, but at later stages of development (14.5 to 16.5 d.p.c.) (Wilkinson et al., 1989).

The putative receptors for FGF-9, FGFR2b and FGFR3c have been reported to be expressed in the developing eye. *Fgfr2b* transcripts were detected in the cornea with weaker levels in the lens epithelium at 14.5 d.p.c. (Orr-Urtreger et al., 1993) while *Fgfr3* RNA was localized in the lens of the developing eye at 14.5 d.p.c. (Peters et al., 1993). The localizations of these receptors are more in line with a role for the FGFs in directing the growth and differentiation of the lens, rather than that of the nervous layer of the retina. Hence, *Fgf9* expressed in the lens epithelium may be involved in lens development. On the other hand, as *Fgf9* expressed in the embryonic retina is likely to localize to the vicinity of its transcription, it being expected to associate with the HSPGs of the intercellular matrix, it is possible that FGF-9 expressed here may interact with another as yet unidentified receptor. Alternatively, *Fgf9* expressed in the neural layer of the retina may have no function.

The inner ear develops as a thickening of the ectoderm in a region lateral to the presumptive hindbrain which eventually invaginates to form the otic vesicle at 8.5 d.p.c. By 9 d.p.c., the vesicle has deepened rapidly and a closed otic vesicle, also known as the otocyst, is formed (Kaufman, 1992). Analysis of the otic vesicle at 10.5 d.p.c. showed the presence of *Fgf9* transcripts in the otic epithelium. This expression pattern is distinct from that of *Fgf3* which was found to be transcribed in the sensory cell precursors at 10.5 d.p.c. (Wilkinson et al., 1989) and from that of *Fgf5* which was only detected specifically in the acoustic ganglion in the vestibular system at 12.5 d.p.c. (Haub and Goldfarb, 1991). To determine if these differential expression patterns can be correlated with those of the putative receptors, the reported localizations of *Fgfr1*, *Fgfr2b* and *Fgfr3* in the developing ear were compared. The putative receptors for FGF-3 include FGFR1b, FGFR2b and FGFR2c (Mathieu et al., 1995a), all of which are expressed in the otic vesicle at 10.5 d.p.c. (Orr-Urtreger et al., 1991; Peters et al., 1992b; Orr-Urtreger et al., 1993). As mice

homozygous for a null mutation at the *Fgf3* allele feature inner ear defects that arose from a failure to induce the endolymphatic duct during development (Mansour et al., 1993), FGF-3 may interact with one or more of these putative receptors to effect proper development of the inner ear. FGF-5 has been found to bind only FGFR1c (Ornitz and Leder, 1992) which was detected in the otic vesicle from 10.5 to 14.5 d.p.c. (Orr-Urtreger et al., 1991). The expression of FGF-5 between 12.5 and 14.5 d.p.c. in the acoustic ganglion located at the posterior region of the otic vesicle suggests a potential role of this molecule in imposing an inductive effect on the adjacent otic epithelium within this time frame. Mice deficient for FGF-5, however, showed no ear defect (Hébert et al., 1994), indicating that expression here is not critical or redundant for normal ear development. *Fgfr2b*, one of the putative receptors for FGF-9, is expressed in the cochlear endothelium at 14.5 d.p.c. (Orr-Urtreger et al., 1993) while *Fgfr3*, the other putative receptor, is transcribed in the differentiating sensory hair cells and underlying supporting cells of the developing inner ear at 16.5 d.p.c. (Peters et al., 1993). As the expression patterns of these two receptors in this organ at earlier stages of development have not been described, it is not possible to define the target cells of FGF-9 in the vestibular system at 10.5 d.p.c.. As FGF-9 may be secreted from cells, it may be released from the epithelial cells of the otic vesicle and induces mesenchyme surrounding the otic capsules. Alternatively, FGF-9 may simply contribute to the proliferative aspect of the development of the otic vesicle during early organogenesis. Further analysis of the temporal and spatial regulation of *Fgf9* expression in the developing ear will provide more information on the potential role(s) of FGF-9 in subsequent stages of ear development.

Fgf9 transcripts were also detected in the somites. Transcription appeared to be localized preferentially in the myotomal compartment which gives rise to the skeletal muscles of the trunk and limbs. This expression in the myotome is not exclusive for *Fgf9*. For instance, FGF-1 expression has been detected by immunohistochemistry to the somites of the rat embryo (Fu et al., 1991). FGF-2 protein or transcript is similarly found in the myotomal region of the somites in the chicken embryo (Joseph-Silverstein et al., 1989; Savage et al., 1993; Dono and Zeller, 1994). *Fgf4* RNA bas been reported in the myotome

from 9.25 to 14.5 d.p.c. (Suzuki et al., 1992; Drucker and Goldfarb, 1993) while *Fgf5* transcription in the myotome occurs at least one day later (Haub and Goldfarb, 1991). Both *Fgf6* and *Fgf7* transcripts have also been localized to the somitic myotome in the mouse embryo from 9.5 d.p.c. (de Lapeyrière et al., 1993; Han and Martin, 1993; Mason et al., 1994) and *Fgf8* expression in the somitic myotome is restricted to the rostral and caudal regions in the mouse embryo (Mahmood et al., 1995a). By 10.5 d.p.c., the myotome has been committed to the myogenesis pathway based on the appearance of transcripts of myogenic genes such as *Myf5, Myf6*, myogenin and *MyoD1* (Sassoon et al., 1989). Expression in the myotomes is frequently correlated with subsequent expression in the developing skeletal muscles. For example, FGF-1 proteins can be detected in the skeletal muscle precursors and myoblasts in the rat embryo (Fu et al., 1991) while FGF-2 proteins were found not only in the skeletal muscle myoblasts in the chick embryo and rat foetus (Joseph-Silverstein et al., 1989; Gonzalez et al., 1990), but also in the skeletal muscles of the adult mouse (DiMario et al., 1989). *Fgf4* transcription was subsequently found in skeletal myoblasts of the mouse embryo (Drucker and Goldfarb, 1993) and *Fgf5* expression was localized to the mastication muscle (Haub and Goldfarb, 1991). The participation of both *Fgf6* and *Fgf7* in myogenesis is even more apparent. *Fgf6* RNA is expressed in most of the skeletal musculature of the embryo between 13.5 and 14.5 d.p.c. (de Lapeyrière et al., 1993; Han and Martin, 1993) while that for *Fgf7* has been localized to the developing axial, facial and tongue muscles as well as limb muscles (Mason et al., 1994).

The co-expression of several members of the FGF family in the early somitic myotome suggests possible functional redundancy among the FGFs during myogenesis, at least during the initial stages. This view may be supported by the expression patterns of the FGFRs in the somite. Neither FGFR1 nor FGFR2 expression was detected in the myotome. *Fgfr1* RNA was localized to the dermatome and sclerotome while *Fgfr2* transcription was detected predominantly in the perichordal sclerotome (Orr-Urtreger et al., 1991; Peters et al., 1992b; Yamaguchi et al., 1992). The sclerotome is the part of the somite that contributes to the vertebral column and ribs. *Fgfr2b* RNA was found in the

somites at 8.5 d.p.c. but expression at later stages was not reported (Orr-Urtreger et al., 1993). Similarly, transcripts of *Fgfr3* have not been reported in the somites. The only FGFR gene known to be expressed in the myotome appears to be *Fgfr4* (Stark et al., 1991). As FGF-1, FGF-2, FGF-4 and FGF-6 have all been shown to bind to FGFR4 (Vainikka et al., 1992), the autocrine action of these FGFs in the somitic myotome may be elicited through this receptor. FGF-5, FGF-7 and FGF-9, on the other hand, do not appear to interact with FGFR4. They may interact with an as yet unidentified receptor(s) expressed in the myotome.

The roles for FGFs in myogenesis have been substantiated by various *in vitro* studies. For example, the prototypic FGFs have been shown to have the ability to promote the proliferation and inhibit the differentiation of myoblasts (Lathrop et al., 1985; Clegg et al., 1987; Fox et al., 1994). Hence, *Fgf9* expression in the myotome suggests that this molecule may also have a role in the regulation of myoblast division and differentiation. In addition, a recent report indicated that *Myf-5*-deficient mice, which exhibit the lack of distal rib structures, do not appear to express *Fgf4* and *Fgf6* RNA in the somites from 9.5 d.p.c. to 10.5 d.p.c., suggesting the potential involvement of these growth factors in mediating myotomal-sclerotomal interactions during chondrogenesis (Grass et al., 1996). Hence, a paracrine function for FGF-9 may also be hypothesized. Analysis of *Fgf9* expression at earlier and subsequent stages of muscle development may provide a further indication as to the nature of the role of this molecule in the development of the skeletal musculature.

The kidney is an example of an organ that arises by reciprocal epithelial-mesenchymal inductive interactions. The nephrogenic mesenchyme surrounding the metanephric duct epithelium has the ability to induce the latter to branch and elongate. In turn, the terminal duct epithelium can induce the mesenchyme to form renal tubule and Bowman's capsule epithelium (Ekblom, 1989). Expression of *Fgf9* RNA in the mesonephric ducts and tubules suggest that it may be involved in epithelial-mesenchymal interactions during morphogenesis of the kidney. In addition, the expression of *Fgf9* transcripts in this location appears to be coincidental with the reported expression of *Fgfr2b* in the nephrogenic cords at 10.5 d.p.c. (Orr-Urtreger et al., 1993). Hence, the interaction

between FGF-9 and FGFR2b may be functionally important during development of the kidney. Another member of the family, *Fgf8*, has also been detected in the developing kidney (12.5 d.p.c. to 16.5 d.p.c.) at the site of the primitive glomeruli (Mahmood et al., 1995a) where it may coincide with the expression pattern of *Fgfr1* (Peters et al., 1992b). The potential involvement of FGFs in kidney development is supported by a recent finding that FGF-2 can induce the early events of renal tubulogenesis by promoting mesenchyme condensation though it cannot promote the epithelial conversion associated with tubule formation in the metanephrogenic mesenchyme (Perantoni et al., 1995).

5.3.2 *Fgf9* Expression in the Adult Mouse Brain

The results obtained here confirmed data from a previous report (Tagashira et al., 1995) that *Fgf9* RNA is localized in widespread regions of the adult murine brain. The *in situ* hybridization assays showed that *Fgf9* is expressed in a diffuse manner in many areas of the brain.

Other members of the FGF family, namely *Fgf1, Fgf2* and *Fgf5* have also been found to be expressed in the adult brain (Table 5.3). *Fgf1* transcripts were observed mainly in neuronal cells (Fallon et al., 1992). In contrast to *Fgf1*, there is evidence that *Fgf2* is expressed mainly in glial cells, as shown by immunohistochemical methods (Gómez-Pinilla et al., 1992; Woodward et al., 1992). The expression of *Fgf3* in the mature adult brain has not been described but it is known to be expressed in the Purkinje cell layer of the developing cerebellum and in the newborn (Wilkinson et al., 1989). The remaining known *Fgfs*, including *Fgf4, Fgf6, Fgf7* and *Fgf8* appear not to be expressed in the adult brain. From the available data, it is apparent that only specific members of the FGF family are expressed in the brain and where found, the distribution patterns of the different members are distinct and overlapping. Hence, each member may have unique roles in the brain.

Within the FGFR family, all four members are expressed in the adult brain (Table 5.4). *In situ* hybridization analyses of expression patterns of the individual members of the FGFR family showed that while *Fgfr1* transcripts are expressed in specific neuronal

Table 5.3. Expression of the FGFs in the Adult Brain.

FGF	Organism	Expression Pattern	References
FGF-1	Rat	Hippocampus: CA1, CA3 regions; dentate gyrus Cerebral cortex: pyramidal cell layer Ventromedial hypothalamus Midbrain: substantia nigra pars compactar; oculomotor nucleus; mesencephalic nucleus of 5th nerve; lateral mammillary nucleus; ventral tegmental area Locus ceruleus Nucleus accumbens Caudate nucleus Cerebellum: external and internal granule cell layers	Bean et al., 1991; Wilcox and Unnerstall, 1991
	Mouse	Olfactory bulb: mitral cell layer Basal ganglia: amygdala and septum Hippocampus: CA1-3 neurons Neocortex: layers II, III, V Cingulate and retrosplenial cortices: layers II-V Perirhinal, piriform and periamygdaloid cortices Thalamus: thalamic, subthalamic and epithalamic nuclei Hypothalamus: lateral and medial regions, periventricular nucleus; tuberal nuclei; mammillary nuclei Striatum Pallidum Midbrain: mesodiencephalic border, prerubral field, mesencephalic reticular formation, oculomotor nucleus; cuneiform nucleus; superior colliculus Brainstem: vestibular nuclei; gigantocellular reticular nucleus alpha; laterodorsal tegmental nucleus; facial motor nucleus; superior olivary and periolivary areas; ventral cochlear nucleus; A1-5 regions; nucleus ambiguus; dorsal motor nucleus of vagus; hypoglossal nucleus; locus coeruleus; pedunculopontine region Cerebellum: Purkinje cells	Fallon et al., 1992

FGF	Organism	Expression Pattern	References
FGF-2	Rat	Hippocampus: CA2 neurons; dentate gyrus Primary olfactory cortex Cortex: frontal, parietal and occipital cortices; neuronal somata, axons and proximal part of some dendrites Cingulate cortex: layers II and VI Indusium griseum Fasciola cinereum Hypothalamus Striatum Midbrain: substantia nigra Brainstem: facial nerve nucleus; motor and spinal subdivisions of trigeminal nucleus and facial nerve nucleus Cerebellum: Purkinje, granule, stellate, basket cells and external germinal layer cells Mainly glial cells (nucleus and cytoplasm); also microglial cells	Pettmann et al., 1986; Emoto et al., 1989; Bean et al., 1991; Woodward et al., 1992; Gómez-Pinilla et al., 1992
FGF-5	Rat	Olfactory bulb: periglomerular elements; mitral cell layer Hippocampus: CA1-4 regions; dentate gyrus Primary olfactory cortex: layer II Cerebral cortex: layers I, II, III and VI Entorhinal cortex: layer II	Gómez-Pinilla and Cotman, 1993
	Mouse	Hippocampus Cerebral cortex: layer VI Thalamus Cerebellum: Purkinje and granule cells	Haub et al., 1990
FGF-9	Mouse	Olfactory bulb: mitral cell, external plexiform and internal granular layers Caudate putamen Cerebral cortex: layers III and V Hippocampus: CA1-3 regions; dentate gyrus Thalamus: anterodorsal thalamic nucleus; most other regions Hypothalamus: ventromedial hypothalamic nucleus; dorsomeidal part; ventromeidal hypothalamic nucleus; ventrolateral part; most other regions Midbrain: red nucleus, magnocellular and parvocellular parts; oculomotor nucleus; most other regions Brainstem: vestibular nucleus; facial nucleus; reticular nucleus; spinal trigeminal nucleus; vestibulocochlear nerve Cerebellum: medial cerebellar nucleus; interposed cerebellar nucleus; lateral cerebellar nucleus; granule cell layer; Purkinje cell layer	Tagashira et al., 1995

FGF-1 and FGF-2 distributions in the brain have been determined on both the RNA (Bean et al., 1991; Wilcox and Unnerstall, 1991; Emoto et al., 1989; Gomez-Pinilla et al., 1992; Woodward et al., 1992) and protein (Pettmann et al., 1986; Fallon et al., 1992) levels. The expression patterns of othe FGFs were examined on the RNA level by *in situ* hybridization techniques.

Table 5.4. Expression of the FGFRs in the Adult Brain.

FGFR	Organism	Expression Pattern	References
FGFR1	Rat	Olfactory bulb: inner granular and mitral layers Tenia tecta Islands of Calleja Primary olfactory cortex Bed nucelus of stria terminalis (medial part) Hippocampus: CA1-4 regions; dentate gyrus; subiculum Cerebral cortex: layers I - VI Cingulate and entorhinal cortices Amydaloid complex: central, medial, cortical Diencephalon: medial preoptic area; supraoptic nucleus; paraventricular hypothalamic nucleus; dorsomedial hypothalamic nucleus; median eminence; arcuate nucleus; medial habenular nucleus; lateral mammillary nucleus; medial mammillary nucleus (medial part) Midbrain: substantia nigra; ventral tegmental area; interpeduncular nucleus (central); oculomotor nucleus; dorsal raphe nucleus; pontine nucleus; trochlear nucleus; parabigeminal nucleus; laterodorsal tegmental nucleus; pedunculopontine tegmental nucleus Motor trigeminal nucleus Principal sensory trigeminal nucleus Locus ceruleus Cerebellum: granule and Purkinje cell layers Deep cerebellar nuclei Ventral cochlear nucleus Vestibular nuclei Facial nucleus Spinal trigeminal nucleus Abducens nucleus Prepositus hypoglossal nucleus Ambiguus nucleus Inferior olive Solitary tract nucleus External cuneate nucleus Dorsal motor nucleus of vagus nerve Hypoglossal nucleus Lateral reticular nucleus	Wanaka et al., 1990; Yazaki et al., 1994

FGFR	Organism	Expression Pattern	References
FGFR2	Rat	Lateral olfactory tract Fimbria hippocampus Corpus callosum Stria medularis of thalamus Stria terminalis Optic tract External capsule Inner capsule Anterior commissure Cerebellar peduncle, white matter Choroid plexus	Asai et al., 1993; Yazaki et al., 1994
FGFR3	Rat	Preferentially in glial cells	Yazaki et al., 1994
	Mouse	Diffuse expression patterns Cerebellum: pyramidal cell layer, probably Bergmann glial	Peters et al., 1993
FGFR4	Rat	Diencephalon: medial habenular nucleus neurons	Itoh et al., 1994

FGF-1 and FGF-2 distributions in the brain have been determined on both the RNA (Bean et al., 1991; Wilcox and Unnerstall, 1991; Emoto et al., 1989; Gomez-Pinilla et al., 1992; Woodward et al., 1992) and protein (Pettmann et al., 1986; Fallon et al., 1992) levels. The expression patterns of othe FGFs were examined on the RNA level by *in situ* hybridization techniques.

populations, *Fgfr2* RNA is localized predominantly in glial cells. *Fgfr3* RNA is characterized by diffuse expression in the brain, and like *Fgfr2*, has been found to localize preferentially in glial cells (Peters et al., 1993; Yazaki et al., 1994). Indeed, *Fgfr3* mRNA has not been detected in the hippocampus, cerebellar granule cells or brainstem. *Fgfr4* is distinct from all the above receptors in being expressed in the adult brain preferentially at one site - the medial habenular nucleus (Itoh et al., 1994).

In support of the expression data in the brain, the FGFs have been demonstrated to possess activities on cells derived from the central nervous system (reviewed by Baird, 1994). The prototype FGFs have been shown to stimulate neurite extension of cultured PC12 cells (Togari et al., 1983, 1985; Neufeld et al., 1987), and promote survival or fiber outgrowth of neurons isolated from the cerebral cortex (Morrison et al., 1986; Walicke, 1988), the hippocampus (Walicke et al., 1986; Walicke, 1988), cerebellum (Hatten et al., 1988), the septal area (Grothe et al., 1989), the ciliary ganglion (Schubert et al., 1987; Unsicker et al., 1987; Eckenstein et al., 1990), spinal cord (Unsicker et al., 1987), sympathetic and sensory ganglia (Eckenstein et al., 1990), and thalamus (Walicke, 1988). FGF-2 is also mitogenic towards astrocytes (Pettmann et al., 1985), oligodendrocytes (Eccleston and Silberberg, 1985), Schwann cells (Davis and Stroobant, 1990) as well as multipotential cells from adult rat forebrain (Gritti et al., 1996). In addition, the ability of FGF-2 to promote neuronal survival and neurite extension has also been shown *in vivo*. FGF-2 can promote the survival of basal forebrain cholinergic neurons after fimbria-fornix transections in the adult rat brain (Anderson et al., 1988) as well as protect entorhinal layer II glutamatergic neurons from axotomy-induced death (Peterson et al., 1996) and primary hippocampal neurons challenged with excitotoxins (Mattson et al., 1989). Thus, it is likely that the FGFs may play multifunctional roles in the brain, with particular emphasis on their neurotrophic, wound healing and neuromodulatory effects as the adult brain is not a site of active cell proliferation. FGF-9 is predicted to possess similar neurotrophic activity in the brain where regulation of the plasticity and maintenance of the brain structure is particularly important. As FGF-9 is believed to be secreted by cells that synthesize it (see Chapter 3; Miyamoto et al., 1993; Naruo et al., 1993), it is possible that FGF-9 may be released into

the extracellular milieu and act as a trophic factor for neighbouring neurons or glia. FGF-9 may also be secreted at synaptic intervals and modulate neuronal communication.

The activity of FGF-9 in the brain is dependent on its anatomical localization as well as that of its putative receptors. The specific localization of *Fgfr2b* RNA in the adult brain has not yet been documented. *Fgfr3* transcripts are distributed diffusely throughout the brain and thought to be mainly associated with glial elements (Peters et al., 1993; Yazaki et al., 1994). The target cells for FGF-9 action are then predicted to consist of non-neural astrocytes or oligodendrocytes. This is consistent with the ability of FGF-9 to stimulate growth of primary rat glial cells (Naruo et al., 1993). The results presented here, however, suggest that cells that are competent to respond to FGF-9 are not only limited to glial cells, but include neuronal cells as well. This is based on the observation that *Fgfr3* mRNA co-localizes with *Fgf9* transcripts in neuron-rich regions of the brain, such as the olfactory bulb and the cerebral cortex. Further examinations of the areas of co-localization at higher magnifications indicated that both transcripts may be present in the same cell populations. FGF-9 may therefore function in an autocrine and/or paracrine manner in these areas as it recognizes and binds to FGFR3 expressed in the same or neighbouring cells.

The function of the olfactory bulb is associated with smell sensation. Other FGFs are also expressed in this region, including FGF-1 and FGF-5 (Fallon et al., 1992; Gómez-Pinilla and Cotman, 1993). FGF-1 is expressed mainly in the mitral layer of the olfactory bulb while FGF-5 is localized mainly in the glomerular and mitral cell layers (Gómez-Pinilla and Cotman, 1993). FGFR1c, a potential receptor for FGF-1 and FGF-5 (Ornitz and Leder, 1992), is also expressed in the mitral cell layer of the olfactory bulb (Wanaka et al., 1990). In addition, it is shown here that FGFR3, the putative receptor for FGF-9, is expressed in the mitral and internal granular layers of the olfactory bulb suggesting that at least three members of the FGF family may play important roles in the olfactory bulb since cells in this region of the brain are competent to respond to these growth factors. The FGFs in the olfactory bulb may serve a trophic supporting role in maintaining the local connectivity of this highly plastic region particularly in areas such as the glomerular layer which is an area of dense synaptic contacts.

Another area of interest regarding FGF-9 action is the cerebellum, a part of the brain thought to be involved with such functions as elementary reflexes (both somatic and autonomic), posture and locomotion, and voluntary movements. High levels of *Fgf9* transcripts were found associated with the white matter of the cerebellar lobules, a region composed predominantly of astrocytes. *Fgfr3* mRNA, on the other hand, is concentrated within the Purkinje cell layer and may be localized in both the Purkinje neurons and cell bodies of Golgi epithelial cells that extend long processes known as Bergmann fibers through the molecular layer. Purkinje cells are implicated in the control of component movements. Indeed, protein kinase Cγ (PKCγ) mutant mice exhibiting abnormal multiple innervation of Purkinje cells by climbing fibers displayed impaired motor coordination (Chen et al., 1995; Kano et al., 1995). The Bergmann fibers are implicated in directing the migration of granule cells during development of the cerebellar cortex in addition to performing a laminating function on the dendritic synaptic sites of Purkinje cells and cell process of other neurons (Rakic, 1972). It is thus possible that FGF-9, expressed at relatively lower levels in the Purkinje cell layer, may be important for maintaining the integrity of such glial-neuronal interactions in the mature brain.

The overlapping expression patterns of members of the FGF family as well as other growth factors in the adult brain suggest that FGF-9 may represent yet another player in the general cascade of neurotrophic events that mediate plasticity or homeostasis of different cell populations in the brain. Indeed, a variety of *in vitro* studies have implicated the participation of FGFs in such cascades. FGF-2, for example, was found to influence the level of expression of platelet-derived growth factor (PDGF) receptors on oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells (McKinnon et al., 1990) and to cooperate with PDGF in promoting continuous division of O-2A cells in the absence of differentiation (Bögler et al., 1990). The expression of the nerve growth factor (NGF) receptor is also induced by FGF-2 as it brings about differentiation of a sympathoadrenal progenitor cell line (Birren and Anderson, 1990). In addition, FGF-2 has been shown to up-regulate nerve growth factor (NGF) synthesis by astrocytes (Yoshida and Gage, 1991) while at the same

time preserving the competence of neurons to respond to NGF by retaining the expression of NGF receptors on these cells (Gómez-Pinilla et al., 1992).

Taken together, the studies described here support the role of FGFR3 as an *in vivo* receptor for FGF-9. This supposition is based on two lines of evidence: firstly, FGF-9 has the capacity to bind FGFR3, as shown by *in vitro* competition binding experiments and, secondly, the expression pattern of *Fgfr3* overlaps that of *Fgf9* in specific areas of the developing embryo and the adult brain. This co-localization of *Fgf9* and *Fgfr3* transcripts imply that FGF-9 interaction with FGFR3 may be functional for the development of specific tissues in the embryo and for effecting complex brain functions such as maintaining neural plasticity and coordination of movements. It should, however, be noted that FGFR3 function during development and in the central nervous system may be redundant as mice deficient with respect to FGFR3 activity showed no apparent developmental or brain defects other than postnatal skeletal abnormalities (Deng et al., 1996).

Chapter 6

Targeted Disruption of the *Fgf4* Gene

Use of an Isogenic Construct

Summary

The *Fgf4* proto-oncogene is a member of the fibroblast growth factor (FGF) gene family showing mitogenic and mesoderm-inducing properties in a variety of *in vitro* studies. It also has an expression pattern suggestive of a role in early development in the mouse. In order to explore the physiological roles of FGF-4 *in vivo*, the principle of homologous recombination was used to generate embryonic stem (ES) cells harbouring a null mutation in the *Fgf4* gene. Previous attempts to target the gene were carried out with nonisogenic constructs which resulted in very low targeting efficiencies of less than 1% (McDonald, 1992). In this study, a targeting construct isogenic with an ES cell line derived from an identical mouse strain was used. The result is an impressive improvement in targeting efficiency of up to 20-fold, demonstrating the importance of employing isogenic DNA to achieve a reasonable targeting efficiency.

6.1 Introduction

FGF-4 is a well-studied member of the FGF family. It was originally isolated as a transforming gene present in DNA from human stomach cancer (Sakamoto et al., 1986) and Kaposi's sarcomas (Delli-Bovi and Basilico, 1987) as it caused NIH 3T3 cells to transform upon transfection. The proto-oncogene was thus given various names, such as *hst*, *KS3* and *kFgf*. Analysis of the protein sequence eventually led to its identification as a member of the fibroblast growth factor family (Yoshida et al., 1987). The gene maps to the human chromosome region 11q13 and to chromosome 7 in the mouse genome, both near *int-2* (Huebner et al., 1988; Yoshida et al., 1988a; Peters et al., 1989). The gene contains an open reading frame that codes for a protein of 206 amino acid. The protein includes potential binding sites for heparin (Yoshida et al., 1987) as well as a stretch of hydrophobic amino acids that could serve as a signal peptide (Delli-Bovi et al., 1987). Indeed, FGF-4 was observed to be cleaved after the signal peptide, glycosylated and efficiently secreted as a mature protein of 175-176 amino acids in COS-1 cells transfected with a plasmid encoding the *Fgf4* cDNA (Delli-Bovi et al., 1988). FGF-4 has also been purified from the culture medium of transfected silkworm-derived cells with the aid of heparin affinity chromatography (Miyagawa et al., 1988).

The biological properties of the gene has been studied extensively. *In vitro* cell culture experiments have established that FGF-4 is a mitogen for fibroblasts, NIH 3T3 cells and endothelial cells (Delli-Bovi et al., 1988) as well as human umbilical vein endothelial cells (Miyagawa et al., 1988). The presence of heparin appeared to potentiate the growth factor activity of FGF-4 (Delli-Bovi et al., 1988). The factor also exhibits transforming properties like supporting cell growth in soft agar and serum-free medium (Delli-Bovi et al., 1988) by an autocrine mechanism requiring activation of the mitogenic pathway at the cell surface (Talarico and Basilico, 1991; Fuller-Pace et al., 1991). An *in vivo* study using neural transplants in the mouse shows that FGF-4 possesses angiogenic activity (Brüstle et al., 1991). In addition, FGF-4 may have an active role during development. For instance,

the protein has been shown to be able to induce mesoderm formation in isolated *Xenopus laevis* animal pole explants (Paterno et al., 1989) and inhibit apoptosis in the developing dental mesenchyme (Vaahtokari et al., 1996). More interestingly, recent reports indicate that FGF-4 may have important influences on the development of the limb bud. The protein was reported to stimulate proliferation of the limb mesenchyme when the apical ectodermal ridge (AER), an area overlying the apical mesenchyme of the limb bud and required for the outgrowth and subsequent patterning of the limb bud, was removed in the mouse embryo (Niswander and Martin, 1992a). In addition, FGF-4 has the ability to maintain the polarizing activity of posterior limb bud cells *in vivo* and *in vitro* (Vogel and Tickle, 1993). In fact, it has been shown that FGF-4 can virtually replace the apical ectodermal ridge in directing the outgrowth and patterning of the limb (Niswander et al., 1993). Furthermore, FGF-4 may be involved in the initiation of limb bud formation (Abud, 1995; Cohn et al., 1995; Abud et al., 1996). The recent observation of ectopic expression of *Fgf4* in the anterior regions of the limb buds of *Strong's Luxoid* (lst^{D}) mutant mice presenting a limb polydactyly phenotype supports the role of this growth factor as a polarizing factor in limb development (Chan et al., 1995a). On the other hand, studies based on mice recessive for the mutation *limb deformity* (*Id*) where distal skeletal structures are still present in the deformed limbs, suggest that FGF-4, whose expression is depressed or absent in the *Id* limb AER, may not be essential for the formation of distal limb structure during normal limb development (Chan et al., 1995b).

The expression pattern of FGF-4 is also highly suggestive of a role of the protein in embryonic development. Its transcription was observed in some germ cell tumours and mouse embryos but not in postnatal mice (Yoshida et al., 1988b). Another experiment using a mouse teratocarcinoma cell line as a model system for early mammalian development showed that *Fgf4* is expressed in the undifferentiated cells but the amount of expression was drastically reduced (Yoshida et al., 1988c) or became undetectable (Velcich et al., 1989; Hébert et al., 1990) upon differentiation of these cells. *Fgf4* RNA was also detected in murine embryonic stem (ES) cells (Heath et al., 1989) which is also capable of differentiating *in vitro* in a manner reflecting the behaviour of early embryonic cells. These

data suggest that FGF-4 expression is important during early embryonic development. Subsequent Northern-blot and RNA *in-situ* hybridization analyses have detected *Fgf4* expression in a wide variety of sites in the developing mouse embryo from the period of fertilization to the 16th day of gestation, with first detection at the late blastocyst stage (Table 5.1; Niswander and Martin, 1992b; Drucker and Goldfarb, 1993). During the initial stages of gastrulation, *Fgf4* mRNA was detected in the primitive streak. As development proceeds, expression was detectable in the paraxial presomitic mesoderm in the trunk, the primitive neuroectoderm and the tail bud. In later embryos, after the three primary germ layers are established and organogenesis begins, *Fgf4* expression was detected in the pharyngeal pouch endoderm, skeletal myoblast groups (Drucker and Goldfarb, 1993), branchial arch units, the somitic myotome, the tooth bud, and the posterior apical ectodermal ridge of the developing limb bud (Niswander and Martin, 1992b; Suzuki et al., 1992). Further studies of *Fgf4* expression in the limb bud indicate that its expression is induced by Sonic hedgehog in a positive feedback loop (Laufer et al., 1994; Niswander et al., 1994). Taken together, both the expression data and the biological properties of FGF-4 suggest the likelihood that FGF-4 may have multiple roles during embryonic development.

This study aims to elucidate the *in vivo* biological function of FGF-4 by using homologous recombination in ES cells to generate mice carrying a targeted disruption in the *Fgf4* gene.

6.2 Results

6.2.1 Transformation Characteristics of the BL-6/III ES Cell Line

The plating efficiency of the BL-6/III ES cell line, grown on feeders, was determined to be approximately 10% (Table 6.1). The transfection efficiencies, as measured by staining for *lacz* activity, at various electroporation conditions were shown in Table 6.2 and Figure 6.1. The plasmid used for the experiment contains the *lacz* gene under the control of the *pgk* promoter (see section 2.4.3). As it was observed that only one or two cells per colony were stained, the decision was to consider each staining cell as the result of a successful

Table 6.1. Plating Efficiency of BL-6/III cells.

No. of cells plated	No. of colonies	Plating efficiency (%)
500	53	10.6
500	55	11
1000	87	8.7
1000	101	10.1

The plating efficiency (%) is the number of colonies obtained per cell plated multiplied by 100. The average plating efficiency is calculated to be 10.1%.

Table 6.2. Transfection Efficiency Under Different Electroporation Conditions.

Test	Voltage (V)	Capacitance (mF)	Time constant	No. of staining colonies	Transfection efficiency (%)
1	200	960	14.2	23	1.6×10^{-4}
2	240	250	3.0	23	1.6×10^{-4}
3	240	500	6.9	81	5.7×10^{-4}
4	240	960	12.9	164	11.6×10^{-4}
5	800	3	0.1	29	2.1×10^{-4}
Control	240	500	7.0	0	-

The transfection efficiency was measured as a function of the number of cells that stained positive for *lacz* activity. It is calculated as the number of staining colonies per number of cells electroporated. In each case, 1.41×10^7 BL6/III cells were electroporated with the plasmid expressing *lacz* under control of the *pgk* promoter. In the control, water instead of the plasmid was used for the electroporation to ensure that there was no nonspecific staining. The above data are transformed into a graph in Figure 6.1.

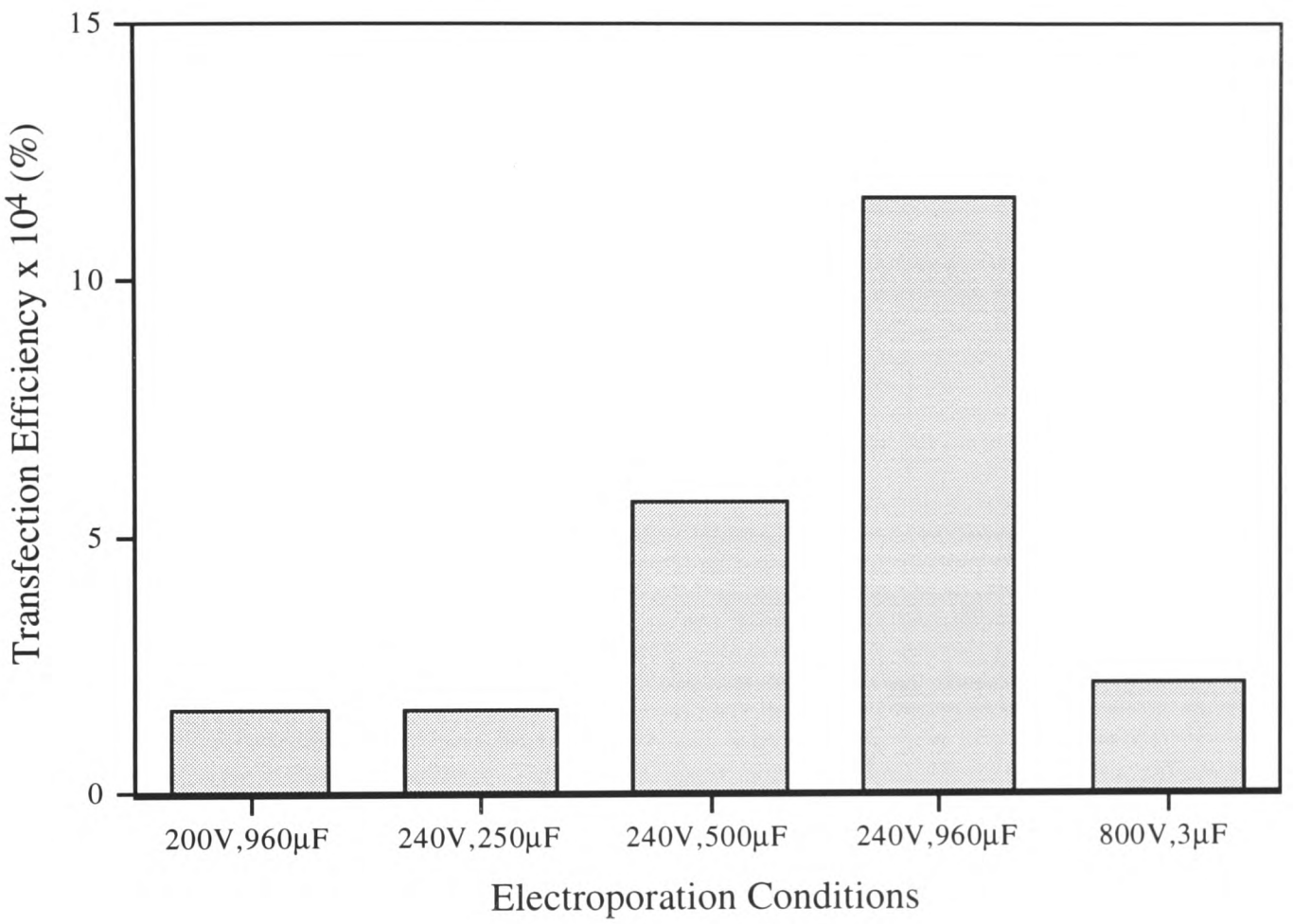


Figure 6.1. Transfection Efficiency of the BL-6/III ES Cell Line under Different Electroporation Conditions.
The transfection efficiency was scored on the basis of the expression of lacz activity. See Table 6.2 for details.

transformation event. Hence, each staining "colony" as such refers to one or a group of staining ES cells within a colony. The transfection efficiency represents the number of staining "colonies" per number of cells electroporated. The electroporation set-up of 240 V and 960 μF was found to be the most efficient in transforming the cells since it gave a two to seven-fold better transfection efficiency than the other electroporation conditions tested.

The minimum concentration of G418 required to kill the untransformed BL-6/III cells was determined to be 150 μg/ml of medium.

6.2.2 Targeting Efficiency at the Mouse *Fgf4* Locus in ES Cells

The strategy for inactivating the mouse *Fgf4* gene was to disrupt the second exon. To this end, a replacement-type targeting vector (Thomas and Capecchi, 1987) PS1 was constructed where part of exon 2 was deleted and replaced by a phosphoglycerate kinase (PGK)-neo expression cassette, as described by McDonald (1992) (Figure 6.2). The *Fgf4* sequence used in the vector was derived from C57BL/6 mouse genomic DNA (Brookes et al., 1989b).

Following digestion of the targeting vector at the unique *Kpn*I and *Not*I sites at either end of the insert, the vector was electroporated into the MBL-5 (129 strain; Pease et al., 1990; McDonald, 1992) and BL-6/III (C57BL/6 strain; Ledermann and Burki, 1991) embryonic cell lines. The latter cell line was used to maximise the degree of sequence homology between the targeting vector and the target gene since both the cell line and the targeting vector were derived from the same mouse strain (C57BL/6). This avoids a potential reduction in targeting efficiency due to genomic sequence polymorphisms between the vector and target DNA, as observed in comparative studies examining the relationship between targeting frequency and the use of homologous versus heterologous vectors in ES cells (Deng and Capecchi, 1992; te Riele et al., 1992; van Deursen and Wieringa, 1992).

With the BL-6/III cell line, a total of 33 clones resistant to G418 were isolated in one experiment and expanded for screening by Southern analysis of genomic DNA. The probe (probe A) used to identify homologous recombinants contained exon 3 sequences not

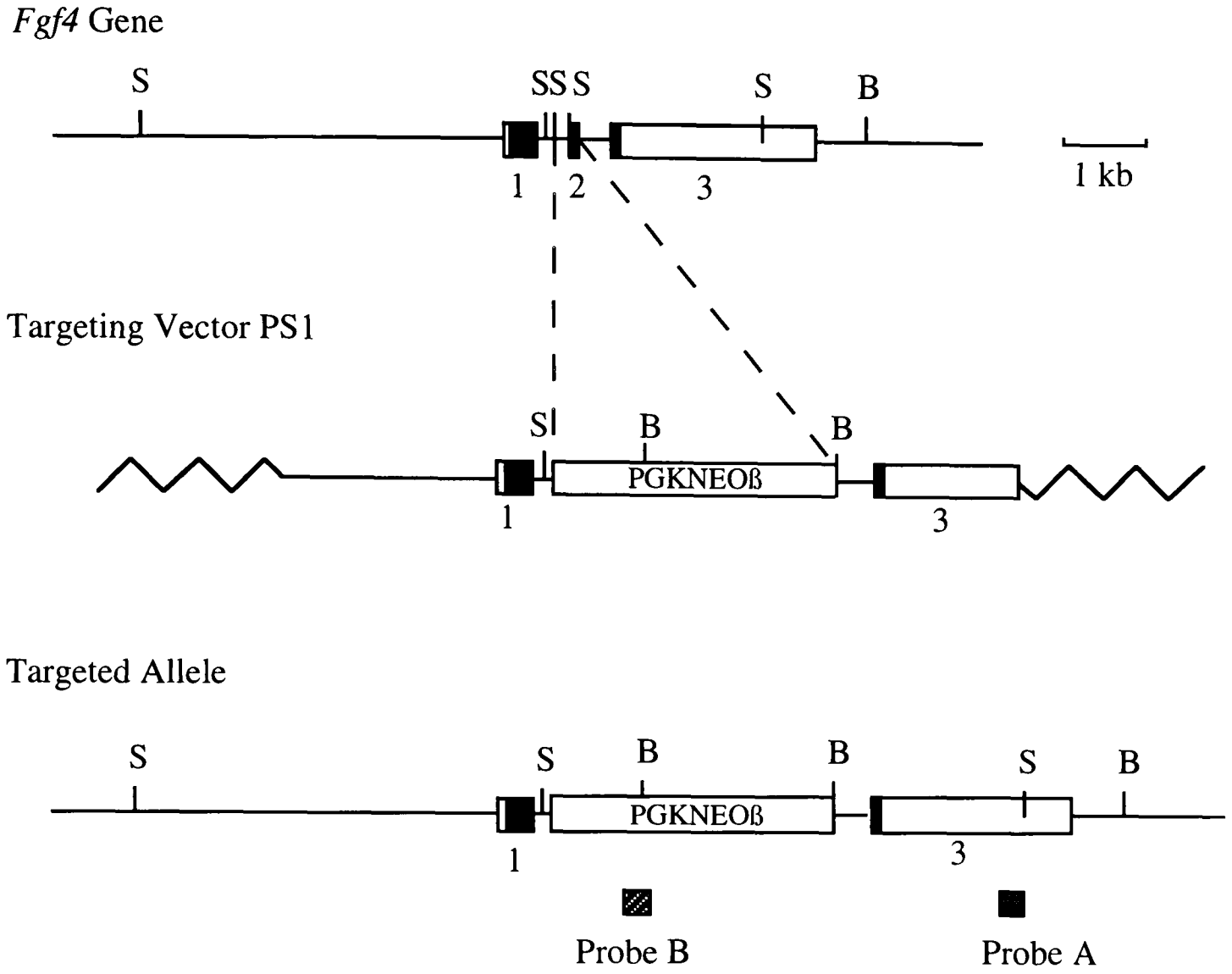


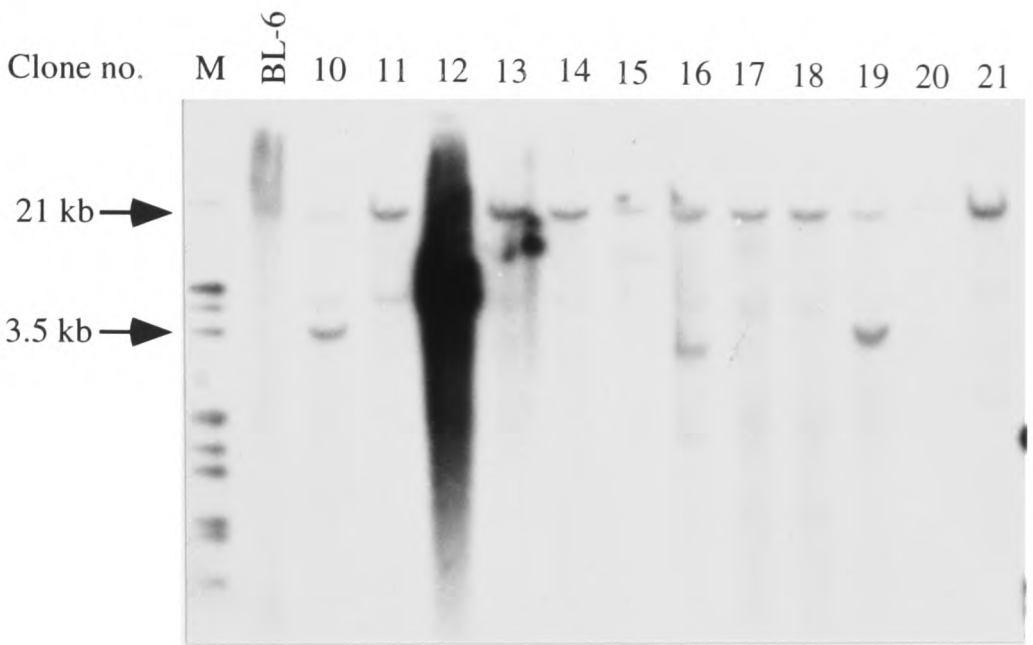
Figure 6.2. Strategy for Targeted Disruption of the Mouse *Fgf4* Gene.
The upper schematic depicts the organization of the three exons (numbered 1 to 3) of the mouse *Fgf4* gene. Shaded boxes represent the protein-coding domains. The vector PS1 was modified by the removal of most of exon 2 and the insertion of a *neo* cassette that is driven by the PGK promoter (middle schematic; McDonald, 1992). The bottom schematic shows the predicted structure of the disrupted gene together with the two probes used for Southern analyses. Probe A (a 327 bp *Sac*I-*Dra*I fragment) hybridizes on *Bam*HI-digested DNA to a 21 kb fragment from the wild-type allele and to a 3.5 kb fragment from the disrupted gene. Alternatively, probe A hybridizes on *Sac*I-digested DNA to a 2.2 kb fragment from the wild-type allele and to a 5.4 kb fragment from the targeted gene. Probe B (a 273 bp *Sph*I-*Hinc*II fragment) is the *neo* probe. B, *Bam*HI; S, *Sac*I.

included in the targeting vector (Figure 6.2). Hence, the probe is designed to hybridize to a 21 kb *Bam*HI fragment in wild-type DNA while in cell lines containing the targeted *neo* gene where two additional *Bam*HI sites are present, the probe hybridizes to a *Fgf4* fragment that is 3.5 kb in length (the size as predicted from mapping the 3' end of the *Fgf4* locus, data not shown). Hybridization of genomic DNAs from clones 10 to 33 (digested with *Bam*HI) with probe A revealed that clones 10, 19 and 22 carried the targeted *Fgf4* allele (Figure 6.3). Clone 10 was further verified by digesting the DNA with *Sac*I followed by hybridization with probe A (Figure 6.4). In this case, a fragment of predicted size 5.4 kb was observed to hybridize with probe A, in addition to a 2.2 kb fragment from the wild-type allele. Clones 1 to 9 were similarly analysed by digesting the genomic DNAs with *Sac*I. Clone 1 was subsequently found to carry the targeted *Fgf4* allele (data not shown). Hence, the targeting frequency at the *Fgf4* locus using the BL-6/III cell line was 12%.

The targeting event was further verified by using a *neo* probe (probe B). The probe detects a 1.7 kb *Bam*HI fragment internal to the selectable marker (Figure 6.2). Additional fragments would indicate multiple random insertions of the vector into the ES cell genome. Hybridization of genomic DNAs from clones 10 to 33, digested with *Bam*HI, with probe B showed that the targeted clones 10, 19 and 22 contained only the targeted integration of the vector in the absence of any random integrations (Figure 6.5). Multiple random integrations were present in some of the non-targeted clones such as in clones 12, 13, 16, 17 and 21.

The above experiment was done with the electroporation conditions of 250 V and 500 μF. As this was not the optimal electroporation set-up as determined above, one attempt was made to transform the BL-6/III cells at 240V and 960 μF in the hope that more clones could be obtained. However, this experiment yielded only 4 clones and none of them appeared targeted by Southern blot analyses.

Clone 10 was subsequently injected into Balb/c mouse blastocysts, an experiment performed by H. Abud (CRC Growth Factor Group, Department of Biochemistry, University of Oxford). Two weak chimeras were obtained in separate injection experiments. At the time this result was obtained, it became known that Dr. M. Goldfarb's laboratory has succeeded in attaining germline transmission of a *Fgf4* null mutation



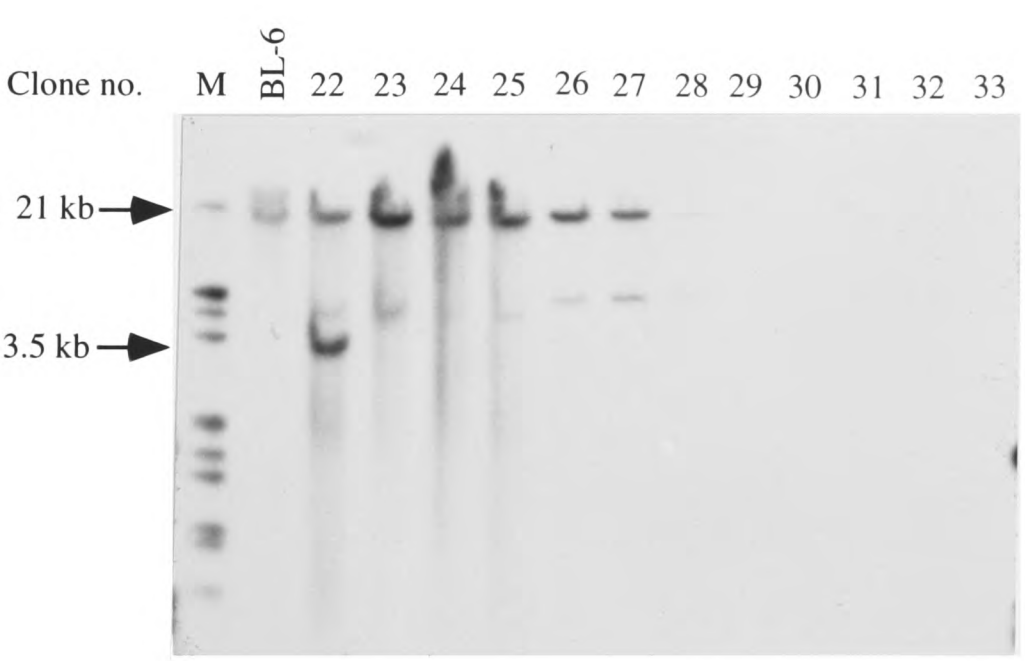


Figure 6.3. Genomic Southern Analysis of Cell Lines 10 to 33.
Southern blot of parental BL-6/III DNA and that of 24 of the 33 G418-resistant cell lines derived from a single transfection experiment, digested with *Bam*HI and probed with probe A (see Figure 6.2). The targeted allele gave rise to a 3.5 kb fragment versus a 21 kb fragment from the wild-type gene. Out of the 24 G418-resistant colonies examined, 3 (clones 10, 19 and 22) were found to be targeted. M, λ/*Eco*RI-*Hind*III marker.

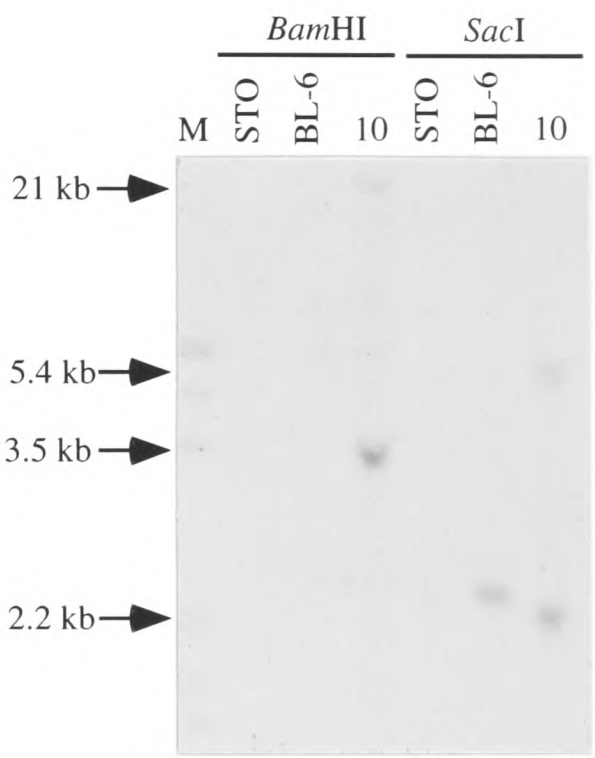
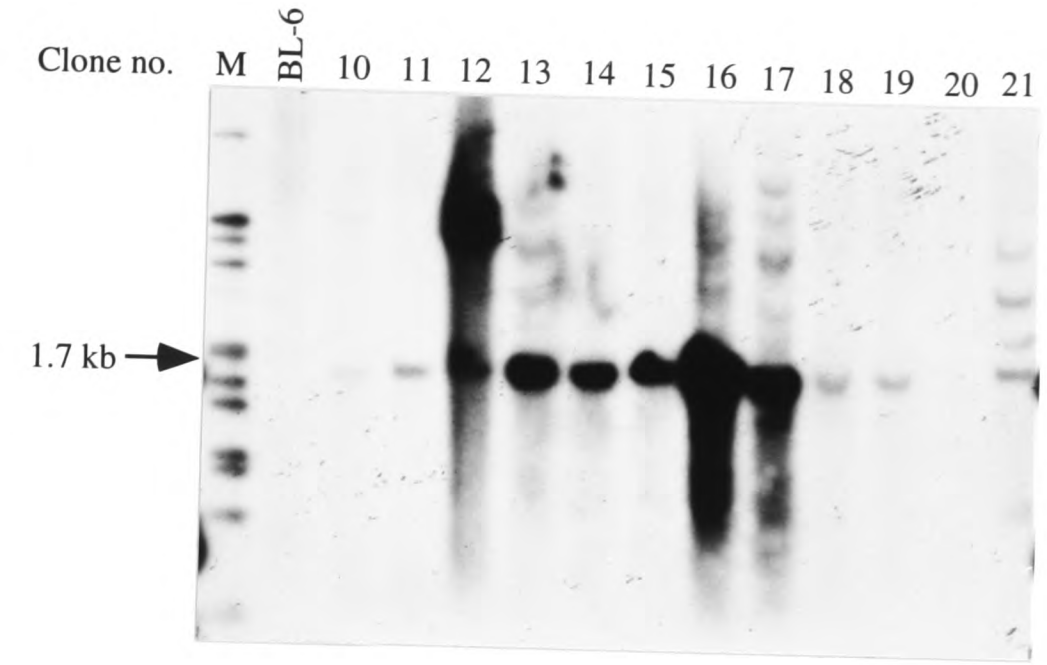


Figure 6.4. Genomic Southern Analysis of Cell Line 10.
Southern blot of STOOB500 fibroblast, parental BL-6/III and clone 10 genomic DNAs, digested with *Bam*HI or *Sac*I and probed with probe A (see Figure 6.2). For *Bam*HI-digested DNAs, the targeted allele gave rise to a 3.5 kb fragment versus a 21 kb fragment from the wild-type gene (as in Figure 6.3). As for *Sac*I-digested DNAs, the targeted allele gave rise to a 5.4 kb fragment versus a 2.2 kb fragment from the wild-type gene. M, λ/*Eco*RI-*Hind*III marker.



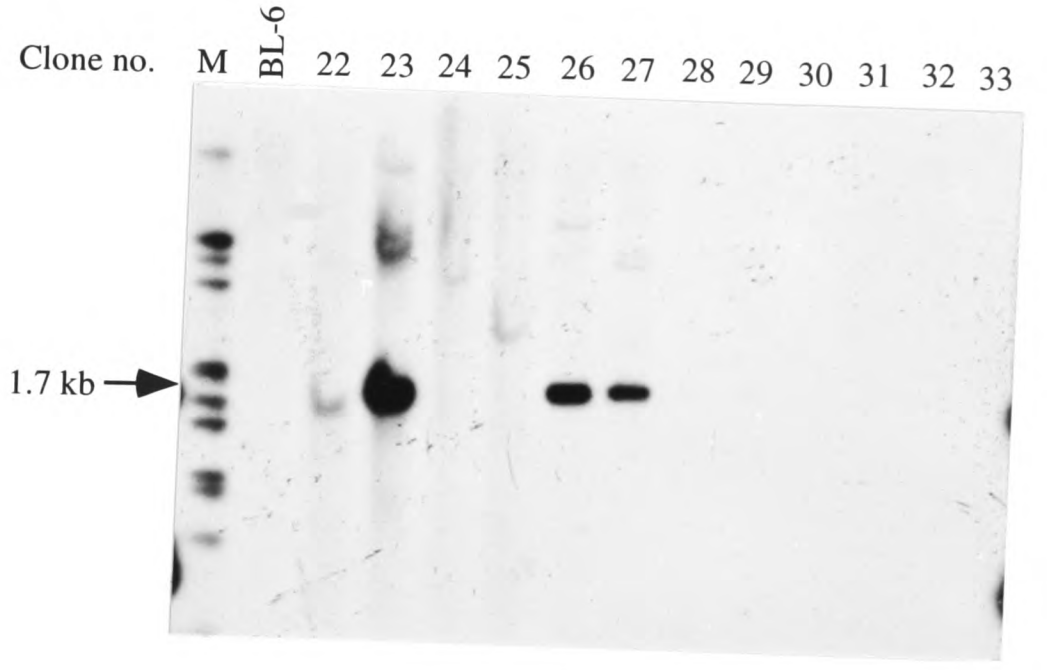


Figure 6.5. Genomic Southern Analysis of Cell Lines 10 to 33.
Southern blot of parental BL-6/III and that of 24 of the 33 G418-resistant cell lines derived from a single transfection experiment, digested with *Bam*HI and probed with probe B (*neo* probe, see Figure 6.2). The targeted clones 10, 19 and 22 do not appear to contain random insertions of the targeting vector. M, λ/*Eco*RI-*Hind*III marker.

(Feldman et al., 1995). Hence, the experiment was aborted at this stage and it is not known if the targeted ES cell clones have the ability to transmit the mutation to the germline.

6.2.3 Karyotype Analysis of Clones

A major factor influencing the potential of a targeted cell line to contribute to the germline may be the presence of chromosomal abnormalities in the transformed cells. To examine whether the ES cell lines possess any chromosomal aberrations after going through the transfection and selection processes, several cell lines were karyotyped. Clone 2 (non-targeted), clone 5 (non-targeted) and clone 10 (targeted) were analysed. Since the majority of embryo-derived stem cell lines has a euploid chromosome complement and a strong modal number of 40, a normal cell line may be considered to have a chromosomal count of 40 (Robertson, 1987). A Giemsa-stained mitotic spread displaying a modal number of chromosomes is shown (Figure 6.6). The results of the karyotype experiment are summarised in Figure 6.7: clone 2 appeared to have 42 chromosomes in about half of the cells counted. On the other hand, clone 5 and clone 10 appeared to have the modal chromosome number in about half of the cells counted. However, the sample size for the above experiment ranged only from 9 to 18. As a result, the counts may not be representative of the genotype of a big population of cells.

6.3 Discussion

The *Fgf4* gene has been implicated to be involved in a variety of developmental processes based on its expression pattern in the mouse embryo (Suzuki et al., 1992; Niswander and Martin, 1992; Drucker and Goldfarb, 1993). In particular, the role of FGF-4 on limb development has been of recent interest (Vogel and Tickle, 1993; Niswander and Martin, 1992a; Niswander et al., 1993; Abud, 1995; Cohn et al., 1995; Abud et al., 1996). As a step towards understanding the role of FGF-4 in development, the principle of homologous recombination was used to produce ES cells mutated at the *Fgf4* locus with the aim that a mouse model inactivated with respect to FGF-4 function may be generated.

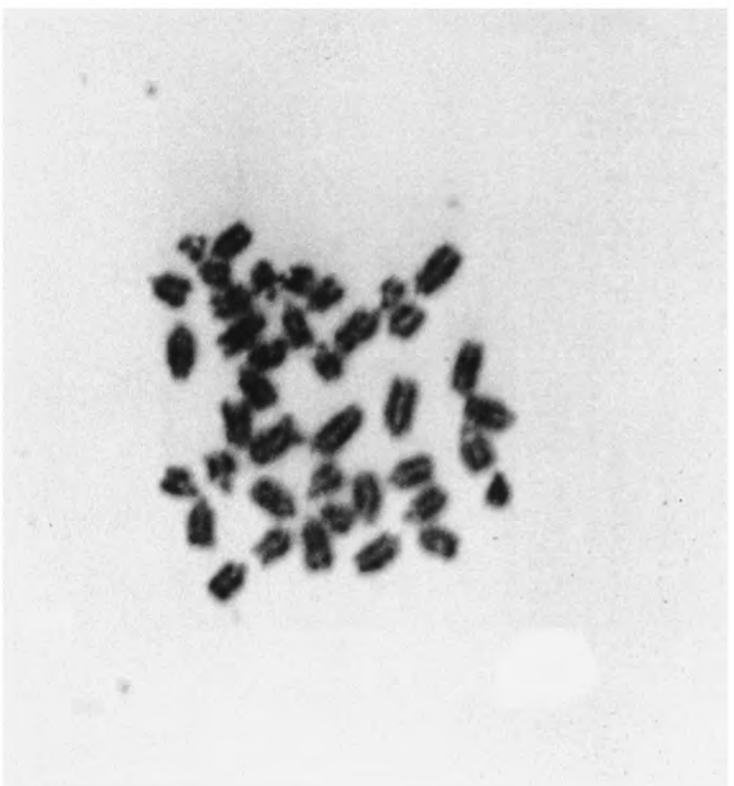
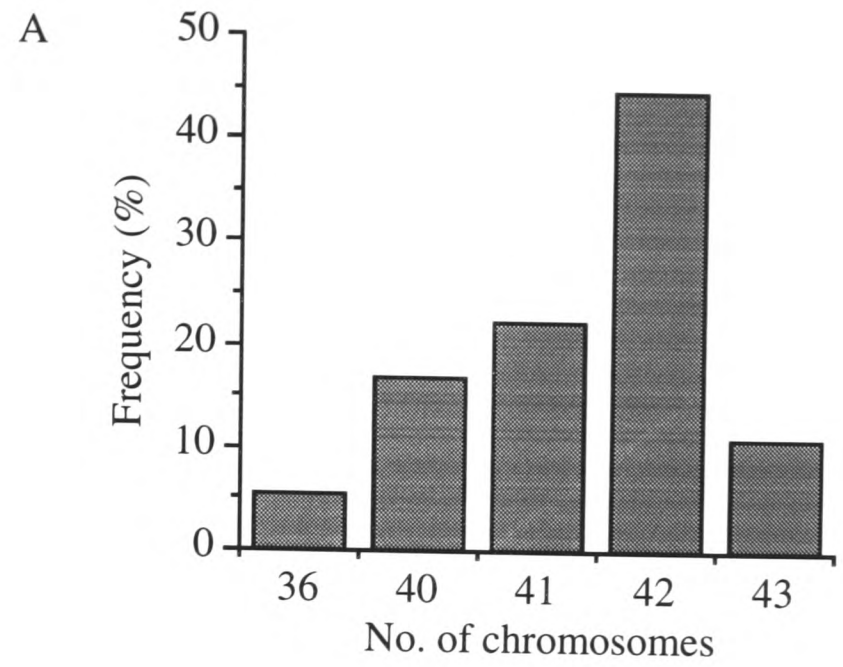
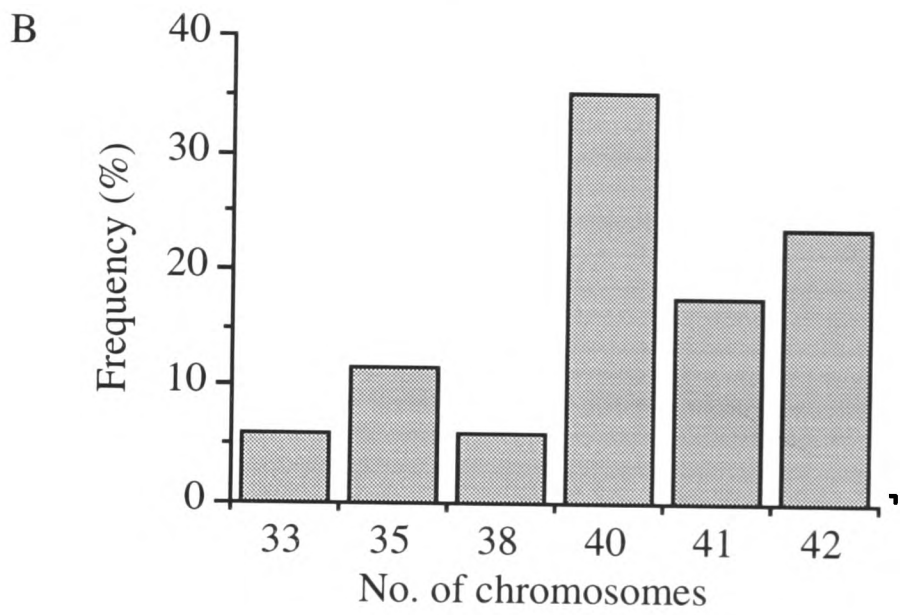


Figure 6.6. Karyotype Analysis of ES cells.
Photograph of a mitotic spread of an ES cell showing a modal chromosome number of 40. Stained with 3% Giemsa. Magnification: 1000x.





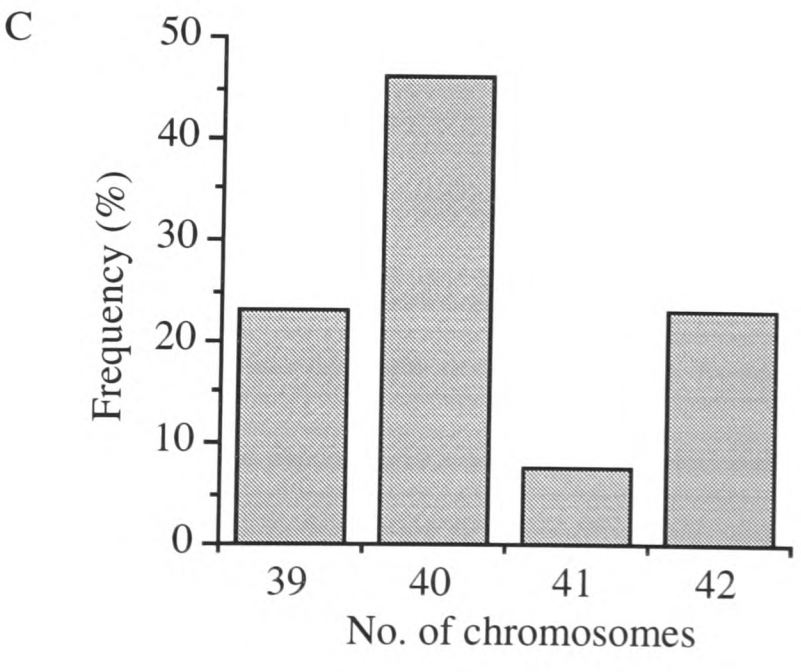


Figure 6.7. Karytoype Analysis of Targeted and Non-targeted Cell Lines.
The frequencies of the chromosome counts for the BL-6/III-derived clones: (A) clone 2 (non-targeted cell line), (B) clone 5 (non-targeted cell line) and (C) clone 10 (targeted cell line) are shown. Majority of the cells of clones 5 and 10 appeared to have the modal chromosome number while a significant number of cells of clone 2 appeared to have deviated from the normal genotype by having acquired two additional chromosomes.

6.3.1 Gene Targeting Frequency and Isogenic Constructs

Several targeting vectors have been constructed for creating a null mutation in the *Fgf4* gene (McDonald, 1992). None of the vectors appeared to produce a high targeting frequency which ranged from 0.09% to 0.7%. A critical assessment of the literature suggests that targeting frequency is dependent on several factors, including (i) the type (insertion or replacement) of vector used, (ii) the extent of homology between the targeting vector and the target locus, (iii) the degree of homology between the target and the source of vector DNA, and (iv) the locus being targeted. While it is impossible to alter potential targeting-resistant elements at the gene locus of interest, it is possible to improve on the other three factors by either changing the design of the vector, or changing the ES cell line used. The factors affecting optimal target vector design have been described previously (McDonald, 1992).

The effect of using an isogenic construct on gene targeting frequency was examined in this study. Sequence heterology may be encountered when vector DNA is derived from a mouse strain nonisogenic with that of the target line and this can vary in degree along the genome. Base-pair mismatches have been found to strongly influence the efficiency of genetic recombination in bacteria (Claverys et al., 1986; Radman, 1988; Rayssiguier et al., 1989) and of intrachromosomal recombination in mammalian cells (Bollag et al., 1989). The base pair heterology between sequences derived from the Swiss Webster and the CC1.2 strain was found to exceed 1% at the *hprt* gene (Deng and Capecchi, 1992) while that between 129 and Balb/c sequences averages at 1 nucleotide difference per 160 nucleotides at the retinoblastoma susceptibility gene (Rb) (te Riele et al., 1992). These data showed that alleles at certain gene loci can be significantly polymorphic and this may be reflected in the varying targeting efficiencies observed in the targeting of various genes. Indeed, isogenic vectors were found to be 4- to 5-fold more efficient in targeting the *hprt* locus than comparable ones prepared from nonisogenic DNA (Deng and Capecchi, 1992). In addition, it was reported that the use of an isogenic vector instead of a nonisogenic

vector resulted in a 20-fold increase in efficiency in targeting the *Rb* gene, with homologous recombination frequencies of up to 80% (te Riele et al., 1992). Thus, evidence for the importance of using perfect match targeting constructs to attain efficient gene disruption in ES cells is compelling. Studies of intrachromosomal recombination in mammalian cells have also suggested that the rate of homologous recombination appeared to be determined by the presence of uninterrupted homology of several hundred base pairs (Rubnitz and Subramani, 1984; Waldman and Liskay, 1988). Hence, depending on the distribution pattern of sequence heterology of a given gene over different mouse strains, the use of nonisogenic vectors may lead to varying targeting efficiencies.

The *Fgf4* gene may contain significant DNA polymorphisms between alleles since it is observed that the use of the BL-6/III cell line, derived from the C57BL/6 mouse strain, with the PS1 vector constructed with DNA derived from the same mouse strain, resulted in a substantial and significant increase in targeting efficiency of up to 20-fold compared with the case where a cell line derived from a different mouse strain 129 was used. In addition, the preliminary results obtained here indicated that random integrations in the targeted cells were absent, in agreement with a previous finding for the *hprt* gene targeted at high efficiency (te Riele et al., 1992). Therefore, the previously low targeting efficiencies reported at the *Fgf4* gene (McDonald, 1992) may be attributed to the use of nonisogenic constructs.

6.3.2 Germline Transmission of a Targeted Mutation

Having obtained ES cell lines carrying null mutations in the *Fgf4* gene, the next step is to obtain animals carrying the mutation (Figure 6.8). This final step in transmitting the mutation into mice may be the most difficult and likely to be the rate-limiting step.

The most critical parameter in the production of chimeras is the pluripotency of the ES cells, particularly that of sub-cloned cell lines that have been genetically manipulated *in vitro*. Ways of ensuring that ES cells remain in a pluripotent state include the rigorous adoption of a number of principles in ES cell-culture (such as the use of feeder layers

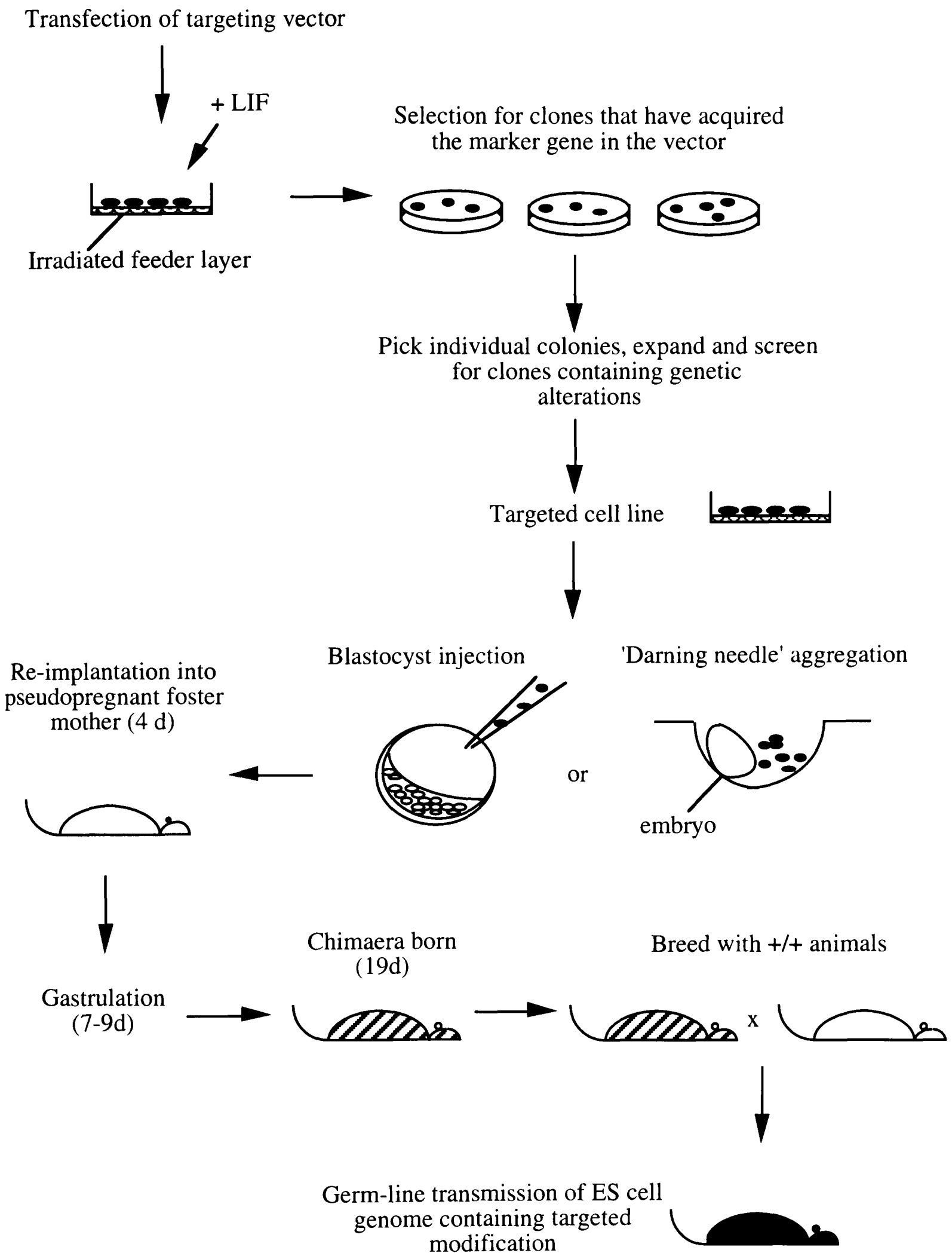


Figure 6.8. Gene Targeting and Production of Germline Chimaeras.
Schematic diagram showing the steps involved in the generation of mice carrying a desired mutation in the germline. The ES cells shown are derived from a mouse with a black coat colour while the host blastocysts are from a white mouse.

and/or LIF) (Robertson, 1987), using early passage cells and limiting the number of successive cloning steps. In addition, it is important to know that a particular cell line being used has a history of success in producing germline chimeras after being genetically manipulated. The parental BL-6/III cell line used in this experiment in targeting the *Fgf4* gene has not only been shown to be germline competent (Ledermann and Burki, 1991), it has also been used to produce mice harbouring targeted mutations at the *Igk* locus (Zou et al., 1993). This indicates the inherent potential of the BL-6/III ES cell lines carrying mutations at a specific gene to give rise to germline chimeras. Loss of pluripotentiality may, however, arise due to several factors, including karyotype changes, DNA mutations and secondary epigenetic changes such as methylation. Hence, cells should be karyotyped to check for potential chromosomal abnormalities or karyotype changes such as the loss of the Y chromosome from an XY ES cell line. The preliminary karyotyping results reported here suggest that the targeted cell line clone 10 appeared to be largely normal with respect to chromosomal count.

The source of the embryos, that is, the strains of mice from which they are derived, may greatly affect the probability of obtaining germline transmission with a given targeted ES cell line. Germline transmission of the modified *hprt* gene was obtained when the inbred mouse strain C57BL/6J was used instead of the ICR strain of albino mice as a source of recipient blastocysts for the injection of ES cells derived from strain 129 (Koller et al., 1989). Similarly, germline transmission of ES cells derived from strain 129 mice was obtained on using C57BL/6 blastocysts but not when blastocysts from the outbred albino CD-1 and MF1 mice were used (Schwartzberg et al., 1989). The BL-6/III ES cell line has been found to give better germline transmission in Balb/c host blastocysts but not in C57BL/6 blastocysts (B. Ledermann, personal communication). In our hands, however, Balb/c blastocysts exhibited poor reproductive performance and so limits the use of this cell line in gene targeting experiments.

6.3.3 FGF-4 is Required for Postimplantation Development

At the time this study was carried out, it became known that a mouse model inactivated with respect to FGF-4 function has been obtained elsewhere (Feldman et al., 1995). Hence, the experiment here was aborted. Mouse embryos homozygous for the null allele of the *Fgf4* gene were found to undergo uterine implantation and induce uterine decidualization but fail to develop thereafter (Feldman et al., 1995). The degeneration of the postimplantation embryo may be a reflection of the requirement for FGF-4 in promoting the survival and growth of the inner cell mass of the blastocysts (Feldman et al., 1995). This method of analysis thus provides little information regarding the function of FGF-4 later in embryogenesis, particularly with respect to its role in limb development. To this end, complementary studies based on ectopic expression or application of FGF-4 appeared to be most useful (Niswander et al., 1993; Abud, 1995; Cohn et al., 1995; Abud et al., 1996).

Chapter 7

Discussion & Future Directions

Members of the FGF family appear to have diverse biological functions. For example, FGF-4 activity is apparently important in early embryo development where it performs a function that cannot be compensated by any other molecules (Feldman et al., 1994). FGF-3 appears to be required for the proper development of the ear and tail (Mansour et al., 1993) while FGF-5 affects hair development (Hébert et al., 1994). This thesis aims to determine the specific biological properties associated with the ninth member of the FGF family, FGF-9. To this end, the mouse homologue of FGF-9 was cloned and expressed (see Chapter 3), its receptor binding specificity defined (see Chapter 4), and its *in vivo* expression pattern during mouse embryonic development and in the adult mouse brain examined (see Chapter 5). FGF-9 was found to bind to two known members of the FGFR family, namely FGFR2b and FGFR3c. Its expression was detected at various sites in the developing embryo, including the germinal epithelium of the central nervous system, the gut primordia, the mesonephric cords, the pharyngeal pouch, the ear and the eye. *Fgf9* RNA expression in the brain appears to co-localize with that of *Fgfr3*, particularly in the region of the olfactory bulb and the cerebral cortex while in the cerebellum, the two types of transcripts occupy distinct positions.

7.1 Structure of FGF-9

Members of the FGF family are characterized by conserved structural similarities. Some members are also highly conserved between species. The ninth member, FGF-9, is a case in point: the human form of the protein is 99.5% and 93% identical to the mouse and *Xenopus* homologues respectively (see Chapter 3). Hence, it may be predicted that FGF-9 performs an essential function(s) *in vivo*.

FGF-9 shows restricted expression patterns in both the adult animal and in the developing mouse embryo (see Chapter 5). To understand the mechanism by which regulation of FGF-9 expression is achieved and maintained, knowledge of the gene elements involved in controlling *Fgf9* transcription and translation is required. Analysis of the *Fgf9* genomic clone (see Chapter 3) will yield information regarding *cis*-acting regulatory elements as well as provide clues to the identity of potential *trans*-acting factors.

Like the prototype FGF-1 and FGF-2, the coding sequence of FGF-9 does not appear to contain a classical hydrophobic signal sequence for directing secretion of the protein (see Chapter 3). There is, however, a stretch of hydrophobic amino acids near the middle region of the FGF-9 coding sequence, a feature unique to this member of the FGF family. The possibility that this may serve as a novel secretory signal should be addressed. In this case, it is possible that an internal hydrophobic sequence may drive the protein through the endoplasmic reticulum processing pathway resulting in modification of the protein by glycosylation followed by its subsequent release from the cell (see Chapter 3).

7.2 Receptor Binding Specificity

The determination of the receptor binding profile of FGF-9 described here was based on *in vitro* binding assays involving the use of dimeric receptor fusion proteins (see Chapter 4). It has not been shown, however, that binding of FGF-9 with either of the two putative receptors is functional. Hence, assays that measure a biological response to FGF-9 binding to cell-surface receptors are necessary to confirm that the binding receptors are biologically

Table 7.1. Summary of FGF-9 Receptor Binding Data Obtained by Other Laboratories and in This Study.

Reference	Receptor Binding Profile	
	Specific Binding	**No Specific Binding**
Hecht et al., 1995	FGFR2c, FGFR3b, FGFR3c	FGFR1, FGFR2b, FGFR4
Santos-Ocampo et al., 1995	FGFR2b, FGFR2c, FGFR3b, FGFR3c	FGFR1c
This study	FGFR2b, FGFR3c	FGFR1b, FGFR1c, FGFR2c, FGFR4

relevant. Assays employed for this purpose typically involve over-expressing the receptor in question in a cell-line expressing very low or undetectable levels of the endogenous receptor followed by measuring a mitogenic or other observable physiological response in the presence of the ligand.

At the time of writing, two other laboratories have reported the receptor binding specificity of FGF-9 (Hecht et al., 1995; Santos-Ocampo et al., 1996). While all the studies agreed on the ability of FGF-9 to bind FGFR3, differences arise regarding the binding of FGF-9 to FGFR2b and FGFR2c (Table 7.1). The deviation in observations between these reports and the results reported here may originate from differences in the nature of the binding assays adopted: monomeric receptor forms were used in the above-mentioned reports while dimeric receptor forms were utilized here. It is important, therefore, to determine if dimerization of receptors does indeed alter or stabilize receptor interactions with the ligand. Analyses of potential differences in the ligand binding properties of monomeric versus dimeric FGFRs would help clarify the issue.

7.3 Requirement for Heparin in FGF-FGFR Interaction

The contentious issue relating to the requirement for heparin in FGF-receptor interaction was further complicated by the results presented in this thesis. The observations made in this study indicate that in general, heparin has no effect on receptor binding, as shown for several members of the FGF family, including FGF-1, FGF-2, FGF-4 and FGF-9. Heparin, however, appears to have a specific effect on the interaction between FGF-2 and FGFR1 expressing exon IIIb (see section 4.2.5). It is proposed here that heparin interacts with FGF-2 to increase its affinity for FGFR1b. Heparin may also bind FGFR1, probably at a site encompassing Ig-like loop II, the Ig II-Ig III interloop region and the amino-terminal portion of Ig-like loop III (Kan et al., 1993; Wang et al., 1995a), to alleviate a constraint on FGF-2 binding imposed by Ig IIIb (Wang et al., 1995a). This latter hypothesis may be tested by measuring the ability of peptide fragments bearing the extracellular ligand binding domain of FGFR1 (expressing exon IIIb) and of similar peptide

fragments mutated with respect to the putative heparin binding site (Kan et al., 1993), to bind FGF-2 in the presence or absence of heparin. The effect of heparin on the Ig IIIc splice form of FGFR1 peptide should similarly be addressed to confirm that the heparin effect is indeed specific for the exon IIIb splice form of this receptor. To eliminate other possibilities including receptor conformational changes due to dimerization and modification of FGF-2 by biotinylation, ligand binding differences arising from the use of receptor dimers or monomers, alternatively labelled or unmodified FGF-2, and potential ligand concentration effects should be tested. Furthermore, to eliminate the possibility that heparin may contribute to the binding interaction simply by oligomerization of FGF molecules, the effect of the heparin analogue, sucrose octasulphate, which has the property of binding to FGF in a monovalent manner (Spivak-Kroizman et al., 1994), should also be examined. In addition, as commercial preparations of heparin are heterogeneous, with components of molecular weights ranging from 3 000 to 30 000 (Hirsch, 1991), the oligosaccharide structure responsible for the effect of this molecule on FGF-2-FGFR1b binding is unknown. It is important, then, also to analyse the effect of oligosaccharides of known compositions on this binding interaction.

Most importantly, however, the issue of the requirement of heparin or heparan sulphate for receptor binding of FGF may only be satisfactorily resolved when the three-dimensional structure of the ligand-receptor complex is elucidated.

7.4 Expression Pattern of FGF-9

As part of the ongoing work on the analysis of FGF-9 function, preliminary data on the distribution of *Fgf9* mRNA in the mouse embryo at 10.5 d.p.c. and in the adult mouse brain as examined by *in situ* hybridization techniques is described in Chapter 5. *Fgf9* appears to show a distinct but overlapping expression pattern with other members of the family. It may be hypothesized then, that a certain degree of functional redundancy amongst members may exist in specific tissues. More detailed analysis of *Fgf9* expression at different stages of embryonic development is required to establish the temporal and spatial

distribution of the gene during embryogenesis. In addition, the potential involvement of FGF-9 function in the adult organs should also be examined by analysing the regional localization of this gene in the heart, kidney and ovary.

An understanding of the potential biological function of FGF-9 in a specific tissue involves establishing a correlation between its expression pattern and that of its putative receptors, i.e. FGFR2b and FGFR3c. To this end, double *in situ* hybridization experiments involving simultaneous analysis of the expression patterns of *Fgf9* and that of its putative receptor will be useful in delineating tissue areas where the ligand-receptor interaction may be biologically relevant, as described for the co-localization of *Fgf9* and *Fgfr3* in the adult mouse brain (see Chapter 5). Attempts to match the available reported expression data for both the ligand and its receptor indicate that there are instances where a relationship between the expression pattern of the ligand and that of its putative known receptor does not appear to exist. For example, *Fgf9* expression in the retina of the eye cannot be correlated with the reported distribution patterns of *Fgfr2b* or *Fgfr3c* (see Chapter 5). In these cases, several possibilities may be suggested. One possibility is that other unidentified receptors for FGF-9 may exist in the region of interest. For example, the little known cysteine-rich FGF receptor (CFR) (Burrus et al., 1992) in the chicken embryo is reported to be expressed in neural tissues, particularly in the retina where its expression pattern is complementary to that of FGFR1 and FGFR2, suggesting that it may have roles distinct from the tyrosine kinase family of FGF receptors (Fayein et al., 1996). Hence, elucidation of the distribution of CFR during mouse embryonic development and determination of its ligand binding profile and role in FGF signalling is crucial. Another possibility for the non-coincidental ligand and receptor distribution patterns may be that FGF expression in a specific region has no function. A third possibility remains that the receptor expression in specific regions may be below the threshold detectable by the methods employed.

Fgf9 expression level in most tissues examined so far is low, as determined by RNase protection analysis (Chapter 5). This low level in expression suggests that this protein may have potent activities. Thus, regulation of the dosage level of this growth factor may be critical. It follows then that over-expression of this protein may result in cellular

transformation, as observed for other FGFs. In regard to this, FGF-9 may contribute to the transformation of glia cells, as deduced from its presence in a human glioma-derived cell line (Naruo et al., 1993). Hence, examining the effect of high levels of FGF-9 in glia and other cell types would yield information relating to the transforming ability of this protein.

7.5 Analysis of Gene Function by Alteration of FGF Signalling

The functions of FGFs *in vivo* have been analysed by a variety of methods, including the generation of mouse models homozygous for null mutations of the gene in question, inactivation of FGFR signalling by use of dominant-negative receptors or other receptor mutants, and by activation of FGF signalling. An attempt to inactivate FGF-4 function in the mouse by the principle of homologous recombination is described in Chapter 6. It is found that a reasonable level of targeting efficiency with respect to the *Fgf4* allele in ES cell clones is achievable by the use of isogenic ES cell lines. The value of this technique for studying gene function is, however, limited in instances where inactivation of gene function is lethal for the animal early in development so that other functions of the gene later in embryogenesis or adulthood cannot be observed, as in the case for FGF-4 (Feldman et al., 1994). In the example of FGF-4, complementary studies that examined the effects of over-expressing FGF-4 have shown that FGF-4 is also involved in limb development (Abud, 1995; Cohn et al., 1995; Ohuchi et al., 1995; Abud et al., 1996).

As *Fgf9* mRNA expression was not detected in embryonic stem cells (Chapter 5), it is predicted here that inactivation of FGF-9 function is unlikely to yield an early embryonic lethal phenotype. Tissues predicted to require FGF-9 function include the central nervous system, the kidney and the developing ear and eye, as deduced from the expression pattern of this gene in the mouse embryo and adult tissues. Additional information may be obtained by complementary studies based on targeted over-expression of this gene in the above-mentioned tissues by use of tissue-specific promoters or by other methods including the implantations of FGF-coated beads or FGF-expressing cells in the chicken embryo, all of which have been used successfully in FGF-over-expression studies (Cohn et al., 1995;

Ohuchi et al., 1995; Robinson et al., 1995). Hence, to define the potential *in vivo* role of FGF-9, both gene targeting and tissue-specific over-expression analyses of this gene would be useful in providing valuable information.

7.6 Final Remarks

It has been twenty years since the purification of the first FGF, and much has been discovered about the biological properties of the FGFs. There are, however, many more issues that need to be resolved. For example, the stoichiometry of FGF-FGFR interaction has not yet been unequivocally determined and there is as yet no consensus on the mechanism by which heparan sulphate enhances FGF activity. On the other hand, some clues regarding the specific biological function(s) of each FGF are now available, much of which is derived from *in vivo* studies involving gene ablation or activation. The issue of the specificity of FGF function has been examined on many levels, including the receptor binding profile of each FGF member and the expression patterns of both the ligand and its receptor(s). In the case of FGF-9, both these factors have been the subject of study in this thesis. It is clear that this member of the family has unique properties, as characterized by a distinct receptor binding profile and expression pattern. The data presented here thus provide a framework for future studies aiming to elucidate the biological function of this protein.

Appendix I

Oligonucleotide Sequence

3'BEK	5'-ACGGATCCACTTACCTGTATCTGGGGAAGCCGT-3'
3'FGFR3	5'-ACGGATCCACTTACCTGTCTCATCAGTTTCCATCAGCTCCT-3'
3'FLG	5'-ACGGATCCACTTACCTGTCACAGCTGGTCTCTCTTCCAGG-3'
3'hFGFR1IIIb	5'-ACGGATCCACTTACCTGTCATCCTGCCGGCCTCTCTTCC-3'
3'KGFR	5'-ACGGATCCACTTACCTGTATAATCTGGGGAAGCCGTGATCT-3'
3'mFGFR4	5'-ACGGATCCACTTACCTGTTCTGGCCTCAGGGGTTGCT-3'
5'BEK	5'-CCCAAGCTTGCCACCATGGTCAGCTGGGGGCGCTTCA-3'
5'FGFR3	5'-CCCAAGCTTGCCACCATGGTAGTCCCGGCCTGCGTGCTA-3'
5'FLG	5'-CCCAAGCTTGCCACCATGTGGGGCTGGAAGTGCCTCC-3'
5'KGFR	5'-CCCAAGCTTGCCACCATGGTCAGCTGGGGGCGCTTCAT-3';
5'mFGFR4	5'-CCCAAGCTTGCCACCATGTGGCTGCTCTTGGCCCTGT-3'
A558	5'-TGATCCATACAGCTCCCCCTT-3'
A812	5'-AAGAATTCAAGGGCTCAAGTGAAGACACTGT-3'
A888	5'-GGCACTGCTAATCAATAAGAACCC-3'
BSX1	5'-GCTTCAAGAACTGGCTC-3'
BSX12	5'-AATCCCCATTATTCAAA-3'
BSX4	5'-ATTTTTCAATGTTGCTT-3'
BSXH260	5'-ATGGGCATTTTAATGAA-3'
BSXH280	5'-GAAACCGAATCTTATAG-3'
C20/1	5'-TTTAATGCAACATAGTA-3'
C20/2	5'-AGACCTTCTGCCTGCTG-3'
C20/3	5'-ATGGATTGAAGAAAAGA-3'

C20/4	5'-TCCAGTCCTGCAGTACA-3'
C20/5	5'-GCCGAGTCCTCTGATGG-3'
C20/6	5'-GCTCCCAACTTCACCTA-3'
EX2/3'	5'-ACTAGGTATGTCGAGGG-3'
hFGFR1IIIbIgI	5'-CCCAAGCTTGCCACCATGTGGAGCTGGAAGTGCCTCC-3'
I441	5'-TCCCCCGGGGGAATGGAGAATATTGCTTTTTAA-3'
L-42	5'-AAAGGATCCCCCGCAGTCACGGACTT-3'
mR3TJ3'	5'-TCCCCCGGGGGACATTGGCCAGAACAGGACCTTCTCC-3'
mR3TJ5'	5'-GGAATTCCCTGCAGGCGCTAACACCACCGACAAG-3'
S152	5'-CTTGTGATTAAAAGCCGAGTCCTC-3'
S178	5'-AAAGGATCCATGGCTCCCTTAGGTGAAGTTG-3'
T3	5'-ATTAACCCTCACTAAAG-3'
T7	5'-AATACGACTCACTATAG-3'
X178	5'-GCTCTAGAATGGCTCCCTTAGGTGAAGTTG-3'

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