

Fibroblast growth factors, their receptors and signaling

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Abstract

Fibroblast growth factors (FGFs) are small polypeptide growth factors, all of whom share in common certain structural characteristics, and most of whom bind heparin avidly. Many FGFs contain signal peptides for secretion and are secreted into the extracellular environment, where they can bind to the heparan-like glycosaminoglycans (HLGAGs) of the extracellular matrix (ECM). From this reservoir, FGFs may act directly on target cells, or they can be released through digestion of the ECM or the activity of a carrier protein, a secreted FGF binding protein. FGFs bind specific receptor tyrosine kinases in the context of HLGAGs and this binding induces receptor dimerization and activation, ultimately resulting in the activation of various signal transduction cascades. Some FGFs are potent angiogenic factors and most play important roles in embryonic development and wound healing. FGF signaling also appears to play a role in tumor growth and angiogenesis, and autocrine FGF signaling may be particularly important in the progression of steroid hormone-dependent cancers to a hormone-independent state.

Introduction

The pathogenesis of tumor growth results from the dysregulation of the normal mechanisms for cellular homeostasis in the context of the larger multicellular organism. Indeed, neoplasia by its very definition refers to cellular growth heedless to the signals provided by other, non-neoplastic cells that would normally maintain the balance of cellular proliferation and death. Consequently, an understanding of the signaling pathways important for regulating homeostasis will be necessary in order to understand how dysregulation of such pathways may contribute to tumorigenesis. Such an understanding will also be necessary for the rational design of therapeutics targeting these signaling pathways.

Fibroblast growth factors (FGFs) and the FGF signaling pathway appear to play significant roles not only in normal development and wound healing, but also in tumor development and progression. The FGF signaling pathway has been the subject of intense investigation in light of its interaction with the heparan-like glycosaminoglycans (HLGAGs) of the extracellular matrix (ECM), as well as its potential role in the progression of some cancers from a hormone-dependent to a hormone-independent growth phenotype. However, there remain a number of unresolved

issues regarding how some FGFs are released from the cells that produce them or from the ECM to which they are bound in order to act on their target cells. A clearer understanding of the mechanism by which FGF signaling is regulated and how this signaling contributes to embryonic development, wound healing and tumor growth will facilitate the development of cancer therapies to target this signaling pathway.

The FGF family of polypeptide growth factors

To date, twenty distinct FGFs have been discovered, numbered consecutively from 1 to 20. FGFs induce mitogenic, chemotactic and angiogenic activity in cells of mesodermal and neuroectodermal origin (Basilico & Moscatelli 1992). Defining features of the FGF family are a strong affinity for heparin and HLGAGs (Burgess & Maciag 1989), as well as a central core of 140 amino acids that is highly homologous between different family members. This central core folds into twelve antiparallel β -strands that together form a cylindrical barrel closed by the more variable amino- and carboxy-terminal stretches (Ago *et al.* 1991, Zhang *et al.* 1991). Interestingly, this structure is

Table 1 Characteristics of the members of the FGF family

| Name | Synonym(s) | Signaling through high-affinity receptors† | Comments* |
|------------|-------------------------|---|--|
| FGF-1 | Acidic FGF, aFGF | FGFR-1, IIIb & IIIc; FGFR-2, IIIb & IIIc; FGFR-3, IIIb & IIIc; FGFR-4 | 1 mRNA form, no signal sequence, nuclear localization motif |
| FGF-2 | Basic FGF, bFGF | FGFR-1, IIIb & IIIc; FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4 | 4 protein isoforms through the use of alternate start codons, no signal sequence, some isoforms have nuclear localization motifs |
| FGF-3 | Int-2 | FGFR-1, IIIb; FGFR-2, IIIb | Site of MMTV integration in mouse genome, signal sequence, nuclear localization motif |
| FGF-4 | kFGF, kaposi FGF, hst-1 | FGFR-1, IIIc; FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4 | Identified by screening stomach tumors and Kaposi's sarcoma, signal sequence |
| FGF-5 | | FGFR-1, IIIc; FGFR-2, IIIc | Signal sequence |
| FGF-6 | hst-2 | FGFR-1, IIIc; FGFR-2, IIIc, FGFR-4 | Signal sequence |
| FGF-7 | KGF | FGFR-2, IIIb | Specific for epithelial cells, signal sequence |
| FGF-8 | AIGF | FGFR-1,‡ FGFR-2, IIIc; FGFR-3, IIIc, FGFR-4 | 7 isoforms, all with signal sequences |
| FGF-9 | GAF | FGFR-2, IIIc; FGFR-3, IIIb & IIIc, FGFR-4 | No signal sequence, not angiogenic |
| FGF-10 | KGF-2 | FGFR-1, IIIb; FGFR-2, IIIb§ | Signal sequence, similar in structure and function to FGF-7 |
| FGFs 11–14 | FGFs | Unknown? | All contain nuclear localization motifs, none contains signal sequence |
| FGF-15 | | Unknown? | Gene is activated by E2A-Pbx1 |
| FGFs 16–19 | | FGF-17; FGFR-1, IIIc; FGFR-2, IIIc¶ | All have signal sequence |
| FGF-20 | XFGF-20 | Unknown? | Sequence homology to FGF-9 |

*Referenced in text. †From Ornitz *et al.* (1996), except where stated; ‡From Koga *et al.* (1995); §From Miralles *et al.* (1999); ¶From Xu *et al.* (1999).

topologically identical to interleukin-1 β (IL-1 β) (Zhu *et al.* 1991), with which some members also share the feature of secretion by an endoplasmic reticulum (ER)-Golgi-independent mechanism. Although structure, and not specificity of growth-promoting activity, is the defining feature of the FGF family, the historical nomenclature of the first of these proteins was based on their biological activity and by convention these molecules are now described as 'FGFs', followed by a numerical designation (Baird & Klagsbrun 1991). The use of these initials is not meant to imply that all of these factors have fibroblast stimulating activities (indeed, FGF-7 does not stimulate fibroblasts) but rather that they belong to the same family because they are structurally related. We will now consider each FGF in turn, focusing on isolation and relevant features of protein structure and sequence information. Table 1 contains a summary of this discussion and also includes relevant information on specific FGF receptor binding.

FGF-1 (acidic FGF)

Both FGF-1 and FGF-2 were initially isolated from bovine pituitary extracts based on their stimulation of [3 H]thymidine incorporation in 3T3 fibroblasts (Armelin 1973, Gospodarowicz 1974). In humans, FGF-1 is a 155 amino

acid protein and the *fgf-1* open reading frame is flanked by stop codons resulting in only one protein form (Jaye *et al.* 1986). Like FGF-2, FGF-1 does not have a signal peptide for channeling through the classical secretory pathway (Jaye *et al.* 1986); however, it does possess a nuclear localization motif (Imamura *et al.* 1990) and has been found associated with the nucleus (Sano *et al.* 1990, Speir *et al.* 1991). The presence of a nuclear localization motif appears to be important for FGF-1-induced mitogenesis and removal has been shown to abrogate FGF-1's mitogenic effect (Imamura *et al.* 1990), whereas replacement of the nuclear localization motif with that of yeast histone 2B restores FGF-1's activity. This modular nature of the FGF-1 nuclear localization signal is consistent with the three-dimensional model of the FGF β -barrel, as this amino-terminal region does not participate in the formation of the β -barrel itself (Zhu *et al.* 1991). FGF-1 has also been shown to stimulate DNA synthesis without signaling through a cell-surface FGF receptor (Wiedlocha *et al.* 1994), suggesting that the nuclear localization signal may allow FGF-1 to act through an intracrine mechanism. However, the nuclear translocation motif, specifically lysine and leucine residues within it, may actually promote the mitogenic capacity of FGF-1 by stabilizing the FGF-1 receptor binding domain, not through nuclear translocation (Luo *et al.* 1996). The amino terminus

of FGF-1 is acetylated in mammalian cells (Crabb *et al.* 1986); however, as recombinant FGF-1 is equally mitogenic as that produced in mammalian or yeast systems (Jaye *et al.* 1987), this acetylation is probably not relevant for FGF-1 activity.

FGF-2 (basic FGF)

The 18-kDa form of FGF-2 has a 55% sequence identity with FGF-1 (Bohlen *et al.* 1985, Gimenez-Gallego *et al.* 1985). Four different FGF-2 polypeptides can be formed from the one *fgf-2* gene: in addition to the 18-kDa form, 22.5-, 23.1- and 24.2-kDa forms have also been identified (Florkiewicz & Sommer 1989). The 18-kDa form is a result of translational initiation at the 5' AUG start codon, while the others are a result of translation beginning at upstream, in-frame, CUG codons (Florkiewicz & Sommer 1989, Prats *et al.* 1989), thus the larger forms are co-linear amino-terminal extensions of the 18-kDa form. This situation is similar to that of the myc proto-oncogene, which can also use alternate non-AUG codons for translational initiation (Hann *et al.* 1988).

Like FGF-1, FGF-2 does not contain a signal sequence for secretion. In addition, a nuclear localization sequence has been identified upstream of the AUG start codon (Bugler *et al.* 1991), and larger forms of FGF-2 associate with the nucleus. However, the role of nuclear localization in the activity of FGF-2 remains unclear.

FGF-2 contains four cysteine residues at amino acids 26, 70, 88 and 93. While the cysteines at 26 and 93 are conserved, those at 70 and 88 are absent or located elsewhere in other FGFs (Arakawa *et al.* 1989). Mutation of all four cysteines to serines results in a protein with the same secondary structure and equally mitogenic for 3T3 cells as the wild-type FGF-2 (Fox *et al.* 1988), suggesting that the formation of disulfide bridges is not important for the secondary structure and mitogenic activity of FGF-2 (Arakawa *et al.* 1989).

It has also been observed that FGF-2 is a substrate for phosphorylation by protein kinase C (PKC) and protein kinase A (PKA). PKC phosphorylates FGF-2 at Ser⁶⁴; however this has no effect on biological activity, heparin-binding capacity or receptor-binding capacity (Feige & Baird 1989). On the other hand, PKA phosphorylates FGF-2 at Thr¹¹² in the FGF receptor binding domain, resulting in 3- to 8-fold better binding (Feige & Baird 1989b). It is unclear how phosphorylation of FGF-2 is regulated and what physiological role this may have for FGF-2 activity.

FGF-3 (Int-2)

FGF-3 is expressed primarily during development (Basilico & Moscatelli 1992) but it was first identified as an activated gene in mouse mammary carcinogenesis. *fgf-3* was

found to be a site in which the mouse mammary tumor virus (MMTV) often (50%) integrates and was named *int-2* (Dickson *et al.* 1984). Normally the FGF-3 promoter is silent in adult animals, but the long terminal repeat (LTR) of the proviral MMTV is a strong activator of the FGF-3 promoter (Grimm & Nordeen 1998), driving expression of a gene more properly expressed during development. Insertion of the provirus rarely occurs in the coding regions of *fgf-3* (Morris & Dutra 1997).

In the mouse, six different transcripts of the *fgf-3* gene are produced, all predicted to code for the same 245 amino acid protein based on a defined AUG start codon (Acland *et al.* 1990). The human *fgf-3* gene codes for a 239 amino acid protein (Brookes *et al.* 1989a) with 44% amino acid homology to FGF-2 in the core region (Dickson & Peters 1987). However, unlike FGF-2, FGF-3 has a defined amino-terminal signal sequence for secretion and also a C-terminal nuclear localization sequence (Antoine *et al.* 1997). As with FGF-2, translational initiation at CUG codons 5' to the AUG start codon results in larger polypeptide forms, often containing additional nuclear localization sequences (Kiefer *et al.* 1994). Consequently, localization of FGF-3 to the nucleus or to the secretory pathway is determined by competition between the signal sequence and the nuclear localization sequences (Kiefer *et al.* 1994). The role of localization to the nucleus or the secretory pathway in FGF-3 activity remains uncharacterized. However, exclusive production of a mutant lacking the signal sequence, yet containing the amino-terminal nuclear localization sequence, results in accumulation in the nucleus and inhibition of DNA synthesis and cell proliferation (Kiefer & Dickson 1995).

FGF-4 (hst-1/kFGF)

fgf-4 was identified by the screening of human stomach tumors and samples of Kaposi's sarcoma for genes capable of transforming 3T3 fibroblasts (Sakamoto *et al.* 1986, Delli Bovi & Basilico 1987). Initially, there were thought to be two different genes responsible for this activity, *hst* and the *KS* oncogene, but, based on homology to each other and to other *fgfs*, they were found to be one gene, *fgf-4* (Delli Bovi *et al.* 1987, Yoshida *et al.* 1987). As with *fgf-3*, expression of murine *fgf-4* has been shown to be upregulated by insertion of the MMTV provirus. In fact, because both murine genes are located within twenty kilobases of each other it has been suggested that they have evolved as a result of tandem duplication of a common ancestral gene (Brookes *et al.* 1989b). Human *fgf-4* codes for a 206 amino acid protein with a 42% homology in the core regions to FGF-2 (Taira *et al.* 1987). Newly translated FGF-4 contains a signal sequence as well as an N-glycosylation site (Miyagawa *et al.* 1991). Deletion of the signal sequence suppresses *fgf-4*'s ability to transform 3T3 cells suggesting that FGF-4 acts predominantly through cell surface receptors (Talarico &

Basilico 1991). On the other hand, not only has the N-glycosylation site been shown to be irrelevant for FGF-4-induced angiogenesis (Yoshida *et al.* 1994), but overexpression of a mutant form of the protein which cannot be glycosylated results in the production of a more active factor, suggesting that glycosylation may actually negatively regulate FGF-4 activity (Bellosta *et al.* 1993).

FGF-5

fgf-5 was originally identified by screening genes recovered from human tumor cell lines for their ability to promote 3T3 fibroblast growth in the absence of serum growth factors (Zhan *et al.* 1987). The FGF-5 protein is 267 amino acids long and has 40% and 50% homology in the core region to FGF-1 and -2 respectively (Zhan *et al.* 1988, Bates *et al.* 1991, Goldfarb *et al.* 1991). FGF-5 is also secreted as a glycoprotein (Bates *et al.* 1991), although glycosylation has not been shown to potentiate its activity (Clements *et al.* 1993).

FGF-6 (hst-2)

Unlike most other early *fgf* genes, which were identified by screening tumor genes for a mitogenic effect on 3T3 fibroblasts, *fgf-6* was first isolated based on its sequence similarity to *fgf-4* (Sakamoto *et al.* 1988, Marics *et al.* 1989). FGF-6 is a 198 amino acid protein containing a signal sequence (Iida *et al.* 1992) and glycosylation site. However, glycosylation does not seem to be important for the FGF-6-stimulated [³H]thymidine incorporation in 3T3 fibroblasts (Pizette *et al.* 1991).

FGF-7 (KGF)

FGF-7 was initially isolated as a growth factor specific for epithelial cells from the conditioned medium of a human fibroblast cell line and named keratinocyte growth factor (KGF) (Rubin *et al.* 1989). Because FGF-7 is produced by fibroblasts yet is only mitogenic for epithelial cells and not for fibroblasts or endothelial cells (Rubin *et al.* 1989), it has been suggested that FGF-7 is a unique stromal mediator of epithelial proliferation (Finch *et al.* 1989). *fgf-7* codes for a 194 amino acid protein containing a signal sequence and an N-linked glycosylation site (Aaronson *et al.* 1991).

FGF-8 (AIGF)

FGF-8 was initially identified as androgen-induced growth factor (AIGF) found in the conditioned medium of the androgen-dependent mouse mammary carcinoma cell line SC-3 (Tanaka *et al.* 1992). FGF-8 was found to be expressed and secreted in response to treatment with androgens in both the human breast cancer cell line MDA-MB-231 and the

SC-3 cell lines (Payson *et al.* 1996). *fgf-8* was also identified as a frequently activated gene in tumors from MMTV-infected Wnt-1 transgenic mice, much like *fgf-3* and *fgf-4* (MacArthur *et al.* 1995b). Murine *fgf-8* contains six exons and alternate splicing has been shown to result in at least seven isoforms with different efficiencies of colony formation in soft agar (MacArthur *et al.* 1995a). These isoforms differ at their amino-termini; however, the signal sequence is not altered. Regulation of isoform production has been proposed as a post-transcriptional mechanism for control of FGF-8 activity (MacArthur *et al.* 1995a).

FGF-9 (GAF)

FGF-9 was originally purified from the conditioned medium of the glial cell line NMC-G1 and, based on its activity, named glia-activating factor (GAF) (Miyamoto *et al.* 1993). FGF-9 is mitogenic for glial cells and fibroblasts, but has no effect on endothelial cells. *fgf-9* codes for a 208 amino acid protein which does not contain an amino-terminal signal sequence, like FGF-1 and FGF-2. Nevertheless, FGF-9 is still efficiently secreted, suggesting that it utilizes an alternate ER-Golgi-independent pathway for secretion.

FGF-10

fgf-10 was initially identified from rat embryos by homology-based PCR (Yamasaki *et al.* 1996). In fact, with the exception of FGF-15, all FGFs beyond FGF-9 have been isolated based on sequence information rather than the isolation of growth-promoting activity from tumors or tumor cell lines. FGF-10 is a 208 amino acid glycoprotein with a signal sequence (Emoto *et al.* 1997). FGF-10 has a high protein sequence similarity to FGF-7 and they are both mitogenic for keratinocytes (Emoto *et al.* 1997). Unlike FGF-7, however, FGF-10 in high concentrations is capable of stimulating fibroblasts (Igarashi *et al.* 1998). FGF-10 also has a higher affinity for heparin than FGF-7 (Lu *et al.* 1999) which may, in part, explain both why FGF-10 is associated with the cell matrix while FGF-7 is freely secreted, and why heparin inhibits the activity of FGF-7 yet potentiates FGF-10 (Igarashi *et al.* 1998). Like FGF-7, FGF-10 is expressed in stromal cells, especially those of muscle origin (Lu *et al.* 1999).

FGF-11, -12, -13, -14 (FHF's)

The FGF homology factors (FHF's) were identified together by random cDNA sequencing, database searches and degenerate PCR of human retinal tissues (Smallwood *et al.* 1996). The FHF's share between 58% and 71% amino acid identity between themselves, yet have less than 30% amino acid identity with other FGFs (Smallwood *et al.* 1996). This divergence is not surprising, considering that the early FGFs

are generally quite distinct from each other at the amino acid level, perhaps because, with the exception of FGF-8, they were isolated based on activity rather than sequence homology. FGF-11, -12, -13, -14 all contain nuclear localization signals and FGF-11 has been definitely identified as accumulating in the nucleus; however, none has been shown to possess a signal sequence for secretion (Smallwood *et al.* 1996). A novel isoform of FGF-13 by alternate splicing of the first exon has been shown to result in a protein with a unique amino-terminus (Munoz-Sanjuan *et al.* 1999); however, any effect this may have on activity has not yet been determined.

FGF-15

fgf-15 was identified as a downstream target of E2A-Pbx1, a homeodomain transcription factor fused by the t(1;19) chromosomal translocation in pre-B cell leukemias to the activation domain of the basic helix-loop-helix (bHLH) transcription factor E2A (McWhirter *et al.* 1997). Because E2A-Pbx1 is thought to aberrantly activate genes normally regulated by Pbx1 during development, *fgf-15* is thought to play some role during embryogenesis.

FGF-16, -17, -18, -19

As with *fgf-10*, the cDNAs for these FGFs were isolated from rat tissues by homology-based PCR (Hoshikawa *et al.* 1998, Miyake *et al.* 1998, Ohbayashi *et al.* 1998, Nishimura *et al.* 1999). These cDNAs code for proteins which range in size from 207 to 216 amino acids and all are thought to play some, as yet uncharacterized, role in development.

FGF-20 (XFGF-20)

Recently, the latest member of the FGF family, FGF-20, has been identified by degenerate PCR-based screening of a *Xenopus* cDNA library with a probe based on the mammalian FGF-9 (Koga *et al.* 1999). The FGF-20 cDNA contains a single open reading frame coding for a 208 amino acid protein. Because FGF-20 is expressed in early stages of embryonic development, as determined by RT-PCR, and because overexpression of FGF-20 mRNA results in abnormal gastrulation, it is likely that FGF-20 plays a role in development.

FGF secretion

It is clear that most FGFs act extracellularly, not only because family members are present in the extracellular matrix, most noticeably FGF-2 (DiMario *et al.* 1989), but also because of their activity through high-affinity extracellular receptors (Safran *et al.* 1990). It is puzzling then that three of the FGF family members, FGF-1, FGF-2 and FGF-9, lack

classical leader sequences. The leader sequence is a stretch of hydrophobic amino acids located at the amino terminus of newly synthesized polypeptide chains which plays a key role in the classical polypeptide secretion pathway (Blobel 1980). The leader sequence is recognized by a signal recognition particle (SRP) which halts translation and trafficks the translation complex to the ER. At the ER, translation of the peptide resumes with vectorial transport of the nascent polypeptide chain into the ER lumen. Secretory proteins can be completely released into the ER lumen while transmembrane proteins become anchored to the membrane by an additional hydrophobic sequence. From the ER, proteins destined for secretion are transported to the Golgi apparatus and are eventually packaged into vesicles for secretion from the cell surface.

Because the large (greater than 18 kDa) forms of FGF-2 contain different 5' sequences (due to the use of upstream, non-AUG codons for translational initiation), Florkiewicz & Sommer (1989) have hypothesized that these larger forms of FGF-2 may contain signal sequences in their amino-terminal extensions. However, this hypothesis is inconsistent with the observation that, while the 18-kDa form remains cytoplasmic, the larger forms associate with the nucleus instead of with secretory vesicles (Renko *et al.* 1990). The nuclear trafficking of larger forms of FGF-2 is due to the addition of a nuclear localization signal in the 37 amino acids upstream of the AUG start site (Bugler *et al.* 1991), and it is now clear that no FGF-2 species contain classical leader sequences. The fact that three of the FGF family members, FGF-1, FGF-2 and FGF-9, lack classical leader sequences implies that they may be transported to the extracellular space by some other mechanism.

There have been various mechanisms proposed for the release of FGF family members lacking leader sequences. Mechanical damage has been proposed as one such mechanism for release of biologically active FGF-2 from endothelial cells (McNeil *et al.* 1989). While cell damage may provide a physiologically appealing mechanism for FGF-2 release in wound repair, it could hardly serve as a regulatable mechanism for FGF-2 release during development. Heat-shock has been found to trigger FGF-1 release; unfortunately the released form of FGF-1 is neither mitogenic nor does it bind heparin (Jackson *et al.* 1992). However, FGF-1 release following heat-shock is inhibited by cycloheximide and potentiated by treatment with brefeldin A, a Golgi inhibitor (Jackson *et al.* 1995), implying that new protein synthesis is required, perhaps of some chaperone protein(s), which facilitates the transport of FGF-1 from a cytoplasmic pool separate from the Golgi-derived secretory pool. Components of this FGF-1 chaperone complex may include synaptotagmin-1 and the calcium-binding protein S100A13. Synaptotagmin-1 is a 65-kDa vesicular protein that acts as a calcium sensor for neurotransmitter release (Kelly 1995). LaVallee *et al.* (1998) have shown that a

40-kDa proteolytic fragment of synaptotagmin is released along with FGF-1 following heat-shock and Tarantini *et al.* (1998) have shown that expression of an antisense synaptotagmin-1 gene represses this release. S100A13, another component of this release mechanism, binds the anti-inflammatory compound amlexanox. Carreira *et al.* (1998) have shown that amlexanox represses the heat-shock-induced release of FGF-1 and synaptotagmin-1 in a dose-dependent manner. Together, this is consistent with a model in which the FGF-1:synaptotagmin-1 (and perhaps also S100A13) complex at the cytosolic face of exocytotic vesicles trafficks to the cytoplasmic membrane and from there to the extracellular space in response to heat-shock and perhaps some other as yet unidentified stimulus.

By use of a phagokinetic assay (Albrecht-Buehler 1977), Mignatti *et al.* (1992) has demonstrated two characteristics of FGF-2 secretion. The migration of individual 3T3 cells in the phagokinetic assay is enhanced by a calcium ionophore, yet is not affected by drugs that block ER-Golgi-mediated secretion. This implies that FGF-2 is released by an ER-Golgi-independent mechanism. In addition, the motility of individual cells is inhibited by antibodies to FGF-2 added to the culture medium, which presumably block the extracellular ligand-receptor interaction (Mignatti *et al.* 1991). If FGF-2 were signaling by an intracrine mechanism to promote cell migration, this migration should not be affected by the extracellular antibody. Consequently, this implies that FGF-2 acts in an autocrine, not intracrine, fashion to promote fibroblast motility.

In the classical secretion pathway, the signal sequence is cleaved from the polypeptide in the ER; consequently, if there were some other domain of FGF that fulfils the role of the signal sequence it might be reasonable to expect that this sequence may be removed during transport as well. Amino-terminal degradation has been found to occur with FGF-2, by comparing the predicted cDNA sequence to that of the secreted protein. However, the portion of FGF-2 removed, while being slightly hydrophobic, does not contain a known signal sequence motif (Klagsbrun *et al.* 1987). The situation is different with FGF-9, as almost the full-length FGF-9 protein is secreted from COS cells following transfection of the FGF-9 cDNA (Miyamoto *et al.* 1993). Using amino- and carboxy-terminal peptide antibodies as well as amino-terminal sequence analysis, it has been determined that only the amino-terminal methionine of FGF-9 is cleaved between translation and secretion. This provides still further evidence that secretion of FGF family members lacking classical leader sequences is through some novel secretory pathway that is probably independent of the ER and the Golgi apparatus.

It is possible that FGF family members lacking leader sequences may be secreted by the same secretory pathway as IL-1 β , which also lacks a signal sequence. Support for this hypothesis comes from the fact that neither IL-1 β nor FGF-2

are found in cytoplasmic vesicles (Rubartelli *et al.* 1990) and because IL-1 β has approximately a 30% homology with FGF-1 (Jaye *et al.* 1986). Rubartelli *et al.* (1990) have suggested that IL-1 β may avoid the classical secretory pathway for reasons that are applicable to the FGFs as well: first, the thiol groups in IL-1 β and the FGFs may require a reducing environment in order to be functional and must therefore avoid the oxidative environment in the ER, and secondly, perhaps IL-1 β and the FGFs utilize an alternative secretory pathway in order to compartmentalize ligands from receptors and avoid intracrine signaling. However, similarities between IL-1 β and the FGFs do not account for all the possible pathways of FGF secretion. IL-1 β secretion is not inhibited by cycloheximide while FGF-1 secretion following heat-shock is dependent on new protein synthesis and the synaptotagmin-1:S100A13 complex.

The secretion of FGF-3 provides a counter example to the secretory pathways of FGF-1, -2 and -9. Like many other FGF family members FGF-3 has a leader sequence; however, it is inefficiently secreted compared with FGF-4 and -5. FGF-3 accumulates in the Golgi apparatus after entering the secretory pathway and undergoing primary glycosylation. Kiefer *et al.* (1993) have proposed that FGF-3 is slowly secreted because of the unique character of its amino-terminal glycosylation site, which may sequester FGF-3 in the Golgi apparatus. Amino-terminal glycosylation is important for cell-surface transport (Guan *et al.* 1985) and substitution of the FGF-3 amino-terminus with that of FGF-5 resulted in efficient secretion of the hybrid protein. However, the reader should recall that, like FGF-2, there is a nuclear localization sequence 5' to the AUG start codon in FGF-3. Consequently, the improvement that Kiefer and coworkers observed may be due to altered competition between secretion and nuclear localization rather than the glycosylation effect resulting in Golgi retention.

Physical interactions between FGFs and heparin

While FGF family members can be quite divergent in their amino acid sequences and expression, binding to heparin and HLGAGs is a defining feature of the family. Armelin (1973) and Gospodarowicz (1974) were the first to isolate FGF-1 and -2. They partially purified a growth-promoting fraction of bovine pituitary extracts, containing both FGF-1 and -2, which was capable of stimulating [³H]thymidine uptake in 3T3 cells. Purer preparations of FGF-1 and -2 were made by utilizing the growth factors' intrinsic affinity for heparin binding. FGF-1 was found to elute from a heparin-Sepharose column with 1 M NaCl (Maciag *et al.* 1984, Thomas *et al.* 1984) while FGF-2 could be eluted with 1.5 M NaCl (Esch *et al.* 1985).

The heparin polymer is a disaccharide chain composed of alternating *l*-iduronic acid (Idu) and *d*-glucosamine (GlcN)

moieties joined by α (1-4) linkages (Faham *et al.* 1996). Each disaccharide unit may contain a total of three sulfate groups: one at the 2-hydroxyl group of Idu, one at the 2-amino group of GlcN, and one at the 6-hydroxyl group of GlcN. These sulfate groups impart a strong negative charge to the heparin chain. The glycosaminoglycan heparan sulfate is structurally similar to heparin; however, it also contains *D*-glucuronic acid moieties and is not as fully sulfated, resulting in less of a negative charge.

The strong binding to heparin requires the existence of specific heparin-binding domains in FGFs. Extensive research has gone into identifying these heparin-binding sites at both the functional level and the molecular level. Using reductive methylation, Harper and Lobb (1988) identified Lys¹¹⁸ as playing an important role in FGF-1 binding to heparin. Lobb (1988) determined that thrombin was able specifically to inhibit FGF-1's binding to heparin, but not that of FGF-2, by cleaving the protein between Arg¹²² and Thr¹²³. This cleavage removed eighteen carboxy-terminal amino acids, suggesting that the heparin-binding activity of FGF-1 resides in the carboxy-terminus of the full-length protein. There are three putative heparin-binding domains based on similarity to motifs proposed by Jackson *et al.* (1991). Site directed mutagenesis of these domains reveals that only the carboxy-terminal site between amino acids 122-137 is actually involved in heparin binding (Wong *et al.* 1995). Together these results indicate that the functional domain for heparin binding is dependent on residues at the carboxy terminus of FGF-1, probably between amino acids 122-137.

Efforts to identify the heparin-binding sites of FGF-2 resulted in the initial determination of two sites as ascertained by stoichiometric analysis of heparin-FGF-2 mixtures (Arakawa *et al.* 1994). Baird *et al.* (1988) characterized two functional heparin-binding domains in FGF-2 through the use of peptide blocking studies and localized these domains to amino acids 24-68 and 106-115. Not only did peptides corresponding to these two sequences inhibit binding of [¹²⁵I]FGF-2 to immobilized heparin, the peptides themselves were also capable of binding [³H]heparin. More recently, Faham *et al.* (1996) have used crystal structures of heparin-derived tetra- and hexasaccharides with FGF-2. Their paper also identified two sites of interaction, although the amino acids involved were not arranged consecutively in the primary structure of the polypeptide chain, as was the case in the peptide blocking studies of Baird. Instead, the binding sites were composed of groups of basic amino acids brought together by the secondary structure of the folded polypeptide. It is difficult to reconcile these two reports except to suppose that, while small peptides may contain amino acids capable of binding heparin, the functional heparin-binding sites of the larger protein can only be ascertained by an analysis of its secondary structure in the context of heparin binding.

This discussion of heparin binding now leads us to a more relevant question: is heparin binding just a convenient method for purification of FGFs or is it essential for the function of these growth factors? An interaction between FGF-2 and the ECM was suggested by the fact that FGF-2 regulates the interaction of bovine epithelial lens (BEL) cells with their ECM *in vitro* (Tassin *et al.* 1983). Treatment with FGF-2 alters the morphology of BEL cells by decreasing their production of ECM components such as laminin and fibronectin, such that they assume a more rounded shape because there are fewer sites of attachment. If FGFs were a component of the ECM itself we could explain this observation as part of a negative feedback loop which ordinarily keeps production of the ECM in check.

Jeanny *et al.* (1987) were the first to describe FGF-2 in the ECM and found that [¹²⁵I]FGF-1 and -2 bound specifically to the basement membranes in the mouse embryonic eye. Vlodavsky *et al.* (1987) showed that endothelial cells synthesize FGF-2 which is then deposited and sequestered in the subendothelial ECM, a major component of which is heparan sulfate proteoglycan. It was also determined that this binding of FGF-2 to the basement membrane was specific to HLGAGs and not other basement membrane components such as laminin or collagen type IV (Vigny *et al.* 1988). Folkman *et al.* (1988) hypothesized that the interaction with heparin is representative of an *in vivo* affinity of FGFs for heparin sulfate proteoglycans, and furthermore, that the storage of FGFs in the basement membrane may be a mechanism for regulating their accessibility to vascular endothelium. Neovascularization may therefore be the result of the release of angiogenic factors from their storage in the basement membrane.

Functional consequences of FGF-heparin binding

The binding of the FGFs to heparin or HLGAGs may serve two physiologically relevant goals: the protection of the FGFs from degradation and the creation of a local reservoir of growth factors. Early studies of the binding of FGF-1 and -2 to heparin showed that this interaction protected the growth factors from acid and heat (Gospodarowicz & Cheng 1986), to which they are extremely sensitive, and from degradation by aprotinin-sensitive proteases (Damon *et al.* 1989). However, these conclusions are obscured by the fact that in these studies, biological activity was used as an indicator of protection of FGFs instead of an analysis of the remaining protein itself. Because heparin alone did not have any biological activity in these studies, the conclusion was made that any mitogenic effect must be due to the FGFs themselves. However, a synergistic effect on mitogenicity by heparin with the FGFs may also have explained the observed protection of biological activity. Later studies did make this distinction and found that heparin or HLGAGs protect the

FGF-1 protein itself from proteolysis by thrombin (Lobb 1988), and the FGF-2 protein from trypsin (Sommer & Rifkin 1989) and plasmin (Saksela *et al.* 1988). Thus, one effect of HLGAG binding *in vivo* seems to be protection from circulating proteases.

The role of FGFs in development provides a clear example of the second physiologically relevant goal of HLGAG binding: the creation of a local reservoir of growth factors. This local reservoir allows for a strict spatial regulation of FGF signaling, as FGFs can only signal to those cells in contact with the ECM. In limb development the creation and maintenance of a concentration gradient of FGFs is crucial for the function of the apical ectodermal ridge (AER) in promoting limb outgrowth. That there must be a focal concentration of FGFs is demonstrated by the fact that beads soaked in FGFs can replace the AER, while exposure of the entire embryo to FGFs will not result in additional limb development (Cohn *et al.* 1995). FGFs must be localized to a particular population of cells in order to be useful in organized development and prevention of diffusion elsewhere can be achieved by the binding of FGFs to HLGAGs in the ECM. In addition, the regulated expression of cell surface HLGAGs could provide a mechanism for the regulated localization of FGFs (Gould *et al.* 1995).

The creation of a local reservoir of FGFs not only implies that FGF signaling may be spatially regulated, but also that a large supply of FGFs may be mobilized from this reservoir. In fact, the process of mobilizing FGFs from the ECM may itself be extensively regulated, allowing an indirect control of FGF signaling through regulation of its mobilization from the ECM to target cell surface receptors. This type of regulation may be particularly important in the formation of new blood vessels, a process that is known to be governed by a variety of positive and negative regulatory factors. That such a large functional reservoir of FGFs exists is clear from the observation that the EC₅₀ of FGF-2 for its receptor is approximately 1 ng/ml, while the tissue concentration of FGF-2 has been found to be between 10 and 500 ng/ml (Gospodarowicz 1987). There are currently two known mechanisms for the release of FGFs from this ECM reservoir: enzymatic cleavage of ECM components, by proteases or heparanases, resulting in the release of FGFs, or by the binding to a carrier protein, FGF binding protein (FGF-BP), which can then deliver FGFs to their receptors. We will address both of these mechanisms in greater detail below in the discussion of FGF signaling in cancer.

FGF receptors

Initial characterization of receptors

It is clear that FGFs produce their mitogenic and angiogenic effects in target cells by signaling through cell-surface,

tyrosine kinase receptors. That FGF signaling involves tyrosine phosphorylation was first suggested by a finding that FGF-1 and FGF-2 stimulated tyrosine phosphorylation in 3T3 fibroblasts, as detected by Western blots using phosphotyrosine antibodies (Coughlin *et al.* 1988). Based on these findings, it was proposed that the mitogenic effect of FGFs on fibroblasts was elicited, at least in part, by protein modification through tyrosine phosphorylation. The existence of FGF receptors themselves was supported by the early binding studies of Moscatelli (1987), who used [¹²⁵I]FGF-2 to find a high affinity ($K_D=20$ pM) FGF receptor on the surface of BHK cells. A series of crosslinking studies identified these high affinity sites as proteins between 125 and 160 kDa (Neufeld & Gospodarowicz 1985, 1986, Friesel *et al.* 1986, Moenner *et al.* 1986, Blanquet *et al.* 1989) which could bind both [¹²⁵I]FGF-1 and [¹²⁵I]FGF-2.

Isolation from the chicken cDNA of a receptor capable of binding FGF-1 provided valuable information on the structure of the FGF receptor protein (Lee *et al.* 1989). This first FGF receptor was found to be a transmembrane protein containing three extracellular immunoglobulin (Ig)-like domains (designated IgI, IgII and IgIII), an acidic region between IgI and IgII, a transmembrane domain, and an intracellular tyrosine kinase domain. This protein structure places the FGF receptor in the Ig superfamily of receptors, such as platelet-derived growth factor (PDGF)- α receptor (PDGF α R), PDGF β R and interleukin-1 receptor (IL-1R), which are also receptor tyrosine kinases containing Ig-like domains (Johnson *et al.* 1990). Cloning of the human FGF receptor (FGFR) genes identified the first two, *fgfr-1* and *fgfr-2*, as *flg* and *bek* (Dionne *et al.* 1990), both of which were previously identified tyrosine kinase proteins (Kornbluth *et al.* 1988, Ruta *et al.* 1989). There are now four known FGF receptors, FGFR-1 through FGFR-4, which share between 55% and 72% homology at the protein level (Johnson & Williams 1993) (see Fig. 1).

FGFR diversity

Requirement for FGFR diversity

As we have seen, there are many different types of FGFs, and these different proteins have diverse effects (mitogenesis, angiogenesis, chemotaxis, etc.) on diverse target cells (fibroblasts, endothelial cells, keratinocytes, etc.). In order to achieve this kind of diversity, the FGF signaling system demands variation at the level of the receptors. The required diversity is typified with the case of FGF-7, which, unlike FGF-1 and FGF-2, is mitogenic for keratinocytes but not fibroblasts or endothelial cells (Rubin *et al.* 1989). The difference in cell response to these different FGFs implies that the different cells express different forms of the FGF receptor.

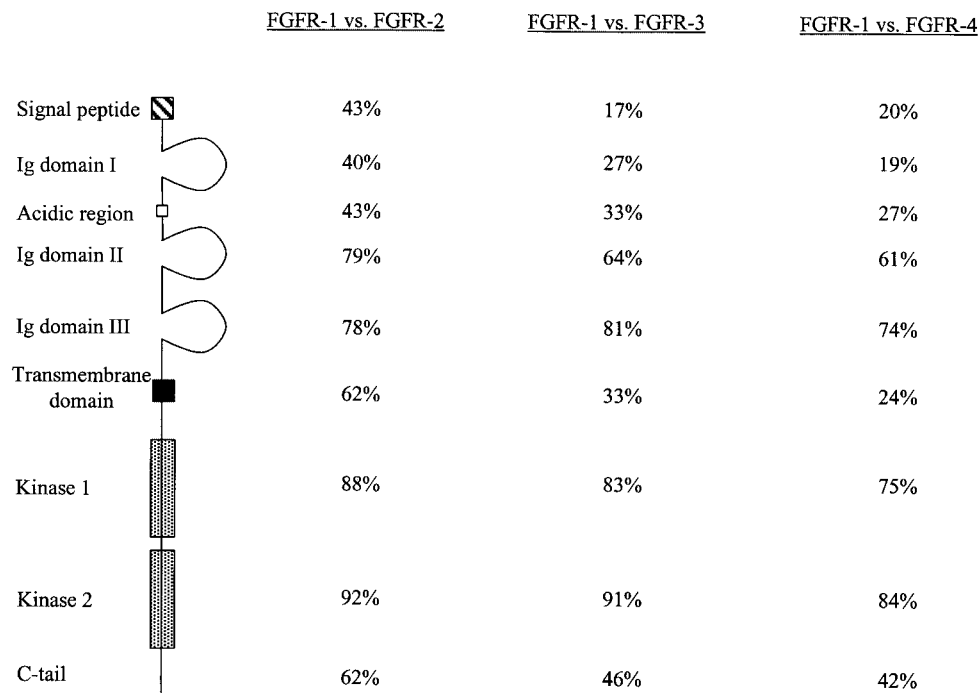


Figure 1 Comparison of human FGF receptors from different genes at the amino acid level. (Modified from Johnson & Williams 1993.)

Mechanisms for FGFR diversity

Different forms of the FGF receptor may be expressed in two possible ways: by the expression of splice variants of a given FGFR gene, or by the expression of different FGFR genes themselves (see Fig. 2).

Alternate splicing of the same gene

Through the use of splice variants it is possible for the same FGFR gene to code for a variety of different receptor protein isoforms. This kind of diversity is possible with FGF receptors because of the structure of the respective genes.

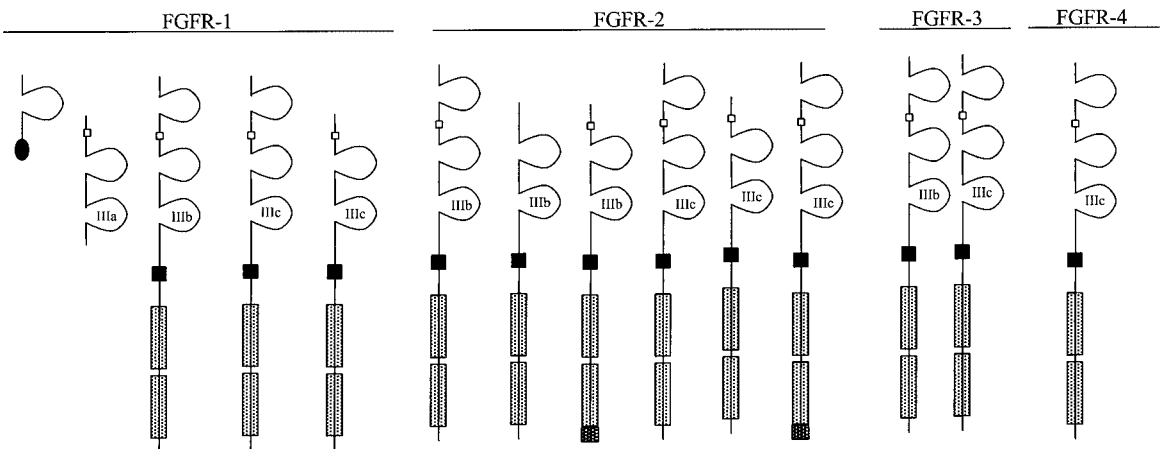


Figure 2 Representative variety of FGF receptors possible through the use of splice variants (Solid oval represents premature truncation and hatched boxes represent alternate c-termini, see Fig.1 for definition of other symbols). (Modified from Johnson & Williams 1993.)

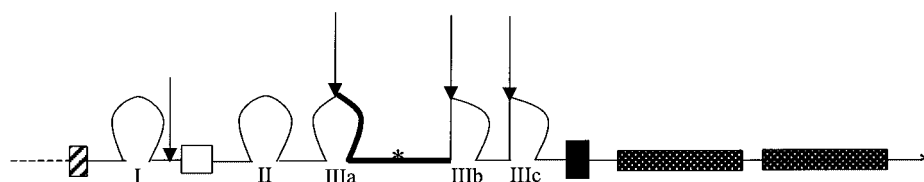


Figure 3 Structure of human FGFR-1 coding exons showing locations of regions coding for three possible IgIII domains. The dashed line is the 5' untranslated region (UTR), the striped box is the signal sequence, the open box is the acidic sequence, the black box is the transmembrane sequence and the stippled boxes are the kinase sequences. The asterisks indicate the stop codons and arrows indicate select locations of exon splicing. (Modified from Johnson *et al.* 1991.)

Using the FGFR-1 gene as a prototype, the following structural features are found in the mRNA prior to splicing: a 5' non-translated sequence, a hydrophobic signal sequence, the IgI and IgII sequences separated by an 'acid box', the 5' end of the IgIII sequence followed by three possible 3' ends of IgIII that are due to alternative splicing, the transmembrane domain, and finally the kinase domain (see Fig. 3). Analysis of the FGFR genes reveals that *fgfr-1*, *fgfr-2* and *fgfr-3* have a remarkable conservation of the arrangement of intron/exon boundaries (Ornitz *et al.* 1996). Different exon usage allows the translation of proteins which may be prematurely truncated, lack Ig-like domains, or utilize different coding regions for the same Ig-like domains (see Fig. 2). For example, variations in splicing have been shown to result in secreted receptors which are truncated after either IgI or IgIII by the introduction of early stop codons (Johnson & Williams 1993). Alternatively, differential splicing may result in the loss of IgI (Johnson *et al.* 1990), which, while not shown to significantly alter FGF-1 and FGF-2 binding to FGFR-1 (Johnson & Williams 1993), may explain the differences in receptor sizes found in the initial crosslinking studies described above. Finally, variations in splice site usage may result in the coding for one of three possible IgIII domains.

One of the most important mechanisms by which FGF receptors determine specificity for different FGFs is by alternate exon usage of the IgIII forms. The exons coding for the three possible IgIII domains, designated IgIIIa, IgIIIb and IgIIIc, are situated contiguously and in the same order in *fgfr-1*, *fgfr-2* (Johnson *et al.* 1991) and *fgfr-3* (Chellaiah *et al.* 1994). The *fgfr-4* gene is unique in that there is only one possible form of its IgIII domain (Vainikka *et al.* 1992). The IgIIIa splice variant codes for a truncated protein which, as it is secreted and is not a transmembrane protein, cannot independently transduce an extracellular signal, although it may act to sequester released FGFs and inhibit FGF signaling. However, differential expression of IgIIIb and IgIIIc is very important for determining FGF signaling specificity. Interestingly, the expression of FGFR-2 isoforms of IgIIIb and IgIIIc is restricted to cells of epithelial and mesenchymal lineages respectively (Orr-Urtreger *et al.* 1993, Yan *et al.* 1993, Alarid *et al.* 1994). Because FGF-7 is known

to bind FGFR-2(IIIb) but not FGFR-2(IIIc) (Ornitz *et al.* 1996), this may explain the selectivity of FGF-7 for keratinocytes over fibroblasts as due to the expression of these different splice variants.

Analogous splice variants of different genes

Because the IgIII domain seems to be so important for specificity of FGF binding, and because the three IgIII domains are more homologous between genes than between each other, one might conclude that the same IgIII domain would confer specificity of binding regardless of which *fgfr* gene is expressed. However, this is not the case. For example, FGF-7 binds FGFR-2(IIIb) but not FGFR-1(IIIb) or FGFR-3(IIIb), despite the fact that all three receptors have the same IgIIIb splice variant (Ornitz *et al.* 1996). This implies that there are other receptor domains besides IgIII which confer binding specificity, and these domains differ between the different *fgfr* genes.

The use of both mechanisms for receptor diversity, different IgIII splice variants of the same gene and different genes coding for the same IgIII splice variant, allows for seven different receptor possibilities (3 receptor genes \times 2 splice variants [IIIb and IIIc] each/*fgfr-4*=7 possibilities). Ornitz *et al.* (1996) determined the specificity of different FGFs for different receptor isoforms by overexpressing these isoforms in Baf3 cells, which do not normally express FGFRs, and assaying for [3 H]thymidine incorporation in these cells following treatment with different FGFs (see Table 2). Their results convincingly show that diversity in FGF signaling is achieved by different FGFs binding to different FGFR splice variants and different *fgfr* gene products.

Role of FGF-heparin interaction in FGFR activation

As part of the search for molecules involved in FGF signaling, low and high affinity binding sites for FGF were found on the surface of cells in culture. As mentioned above, Moscatelli (1987) performed a Scatchard analysis of the binding of [125 I]FGF-2 to baby hamster kidney (BHK) cells and found two binding sites for FGF: a high affinity binding

Table 2 Specificity of different FGFs for different receptor isoforms as determined by mitogenic stimulation. The relevant affinities of FGF-2 and FGF-7 for FGFR1(IIIc) and FGFR2(IIb) are in bold

| FGFR | FGF-1 | FGF-2 | FGF-3 | FGF-4 | FGF-5 | FGF-6 | FGF-7 | FGF-8 | FGF-9 |
|----------------|-------|------------|-------|-------|-------|-------|-----------|-------|-------|
| 1, IIIb | 100 | 60 | 34 | 16 | 4 | 5 | 6 | 4 | 4 |
| 1, IIIc | 100 | 104 | 0 | 102 | 59 | 55 | 0 | 1 | 21 |
| 2, IIb | 100 | 9 | 45 | 15 | 5 | 5 | 81 | 4 | 7 |
| 2, IIIc | 100 | 64 | 4 | 94 | 25 | 61 | 2.5 | 16 | 89 |
| 3, IIIb | 100 | 1 | 2 | 1 | 1 | 1 | 1 | 1 | 42 |
| 3, IIIc | 100 | 107 | 1 | 69 | 12 | 9 | 1 | 41 | 96 |
| 4 | 100 | 113 | 6 | 108 | 7 | 79 | 2 | 76 | 75 |

Modified from Ornitz *et al.* (1996).

($K_D=20$ pM) which represented binding to FGF receptors themselves, and a low affinity binding ($K_D=2$ nM). Because binding to the low affinity sites was competed for by heparin or heparan sulfate, but not by other glycosaminoglycans (GAGs) such as chondroitin sulfate and keratin sulfate, Moscatelli concluded that this low affinity binding represented the binding of FGF to cell surface heparin-like molecules. This conclusion was supported by the fact that treatment with heparinase abolished 62% of the low affinity binding. Moscatelli also concluded that binding to the low affinity sites was not physiologically relevant for FGF signaling because saturating FGF-2 with exogenous free heparin, such that there was none bound to the low affinity sites, had no effect on FGF-2 stimulation of plasminogen activator production, an induction mediated by binding to the high affinity receptor.

Yayon *et al.* (1991) expanded this initial work and came to the conclusion that, on the contrary, cell surface HLGAGs are physiologically significant because they are required for the binding of FGF-2 to its high affinity cell surface receptor. Yayon *et al.* found that Chinese hamster ovary (CHO) cells expressing a high affinity FGF receptor, FGFR-1, and cell surface HLGAGs could bind [125 I]FGF-2 while mutant CHO cells that did not express the cell surface HLGAGs could not, even when they did express FGFR-1. In addition, they found that binding to the HLGAG-deficient mutant CHO cells could be restored by the addition of heparin or heparan sulfate. Lindahl and Hook (1978) had already argued that HLGAGs were capable of inducing a conformational change in proteins to which they bound. Consequently, Yayon *et al.* (1991) proposed that both free and cell surface HLGAGs were capable of imparting a receptor-compatible conformational shift on FGF-2 and thereby promote FGF-2 binding to its receptor. This model explained Moscatelli's observation regarding the competition of FGF-2 off the cell-surface HLGAGs with heparin as not being a competition but rather a substitution of one molecule (cell surface HLGAG) capable of facilitating FGFR binding with another molecule (heparin) capable of doing the same thing.

It has also been demonstrated that cell surface HLGAGs not only facilitate binding to FGFR-1, but also to FGFR-2 (Mansukhani *et al.* 1992), and that this binding facilitates

FGF signaling, triggering mitogenesis and angiogenesis (Rapraeger *et al.* 1991, Aviezer *et al.* 1994). Based on the above observations Klagsbrun and Baird (1991) proposed a model for the role of cell surface HLGAGs in FGF signaling. First, the HLGAGs recruit FGFs to the cell surface, increasing their concentration and making activation of their receptors more thermodynamically favorable. Secondly, the HLGAGs induce a conformational shift in either FGFs or their receptors such that binding between the two is favored.

More recently the thinking has shifted from HLGAGs inducing a conformational shift to their presentation of FGFs in a form more likely to activate their receptors. Ornitz *et al.* (1992) first proposed that heparin facilitates FGF oligomerization and speculated as to a role this may have in triggering receptor dimerization and activation. Ornitz and coworkers showed that FGF-induced mitogenic activity is heparin-dependent and that heparin is required for FGF-2 binding to FGFR-1 in a cell-free assay. The dynamics of this cell-free assay also allowed Ornitz and coworkers to argue that heparin facilitates FGF oligomerization. Spivak-Kroizman *et al.* (1994a) supported this argument by showing through isothermal titration calorimetry that FGF-1 forms a 1:1 complex with the extracellular domain of the FGF receptor. The fact that heparin is capable of binding many molecules of FGF also supports the argument that the FGF-1-heparin complex can bind several receptors, resulting in their dimerization and activation. In addition, a synthetic heparin analog, sucrose octasulfate, which binds only one FGF-1 molecule, is capable of blocking FGF receptor dimerization and activation. Consequently, the current understanding of the ability of FGFs to activate their receptors is that they induce receptor dimerization, and that this dimerization is facilitated by HLGAGs.

FGF receptor dimerization and activation

FGF receptors, like other receptor tyrosine kinases, transmit extracellular signals to various cytoplasmic signal transduction pathways through tyrosine phosphorylation. Following ligand binding and dimerization, the receptors become capable of phosphorylating specific tyrosine residues on their own and each other's cytoplasmic tails (Lemmon &

Schlessinger 1994). The ability of FGFRs in a liganded dimer pair to transphosphorylate each other extends to FGFR heterodimers as well as homodimers (Bellot *et al.* 1991), allowing for additional complexity in FGF signaling. Phosphorylated tyrosine residues, in turn, recruit other signaling molecules to the activated receptors and propagate the signal through many possible transduction pathways (Pawson 1995). Consequently, the key step from the extracellular to the intracellular signaling pathways is receptor dimerization.

Inhibition of receptor dimerization in the absence of FGF

As ligand binding and subsequent dimerization initially determines receptor activation, in order for signals to be appropriately transmitted a mechanism must be present to prevent FGFR dimerization in the absence of FGF. Two mutually consistent theories have been proposed to explain FGFR dimerization only in the presence of FGF. Kan *et al.* (1996) have shown that divalent cations and HLGAGs can cooperate to maintain the FGFR in a conformation that restricts dimerization and prevents receptor activation. Wang *et al.* (1997) identified the sequence that regulates this inhibition as being in the extracellular domain of FGFR between IgII and IgIII from Glu¹⁶⁰ to Lys¹⁷⁶, a region which promotes receptor self-association. In the proposed model, divalent cations and HLGAGs suppress this region's intrinsic tendency to promote receptor dimerization. However, binding of FGF to FGFR releases this suppression and can thereby promote receptor dimerization.

Plotnikov *et al.* (1999) proposed an alternate, though not inconsistent, mechanism to prevent FGFR dimerization in the absence of ligand. There is a region in IgII containing several basic amino acids that is probably important for HLGAG binding to FGFR. Consequently, it may be possible that HLGAGs themselves could cross-link two FGFRs by binding to this region of IgII. However, Plotnikov and coworkers proposed that the acid box, a region between IgI and IgII, is capable of interacting with this basic region of IgII, thereby preventing HLGAG binding. Following FGF binding, this inhibition would be lifted and dimerization could occur. However, as there are some splice variants that lack the acid box yet exhibit no peculiarities in dimerization, this mechanism may not be absolutely necessary.

Solving the HLGAG-FGF-FGFR trimolecular complex

It is important to note that in both of the mechanisms to prevent ligand-independent dimerization described above, different mechanisms are capable of promoting FGFR dimerization. Kan's and Wang's model addresses an intrinsic tendency for FGFR dimerization mediated by a sequence

between IgI and IgII, while Plotnikov's theory addresses a dimerization stabilized by HLGAGs. As we shall see, the trimolecular complex of HLGAG, FGF and FGFR is probably stabilized by numerous interactions between each member.

Early models of the HLGAG-FGF-FGFR complex proposed that the stoichiometry consisted of only one FGF molecule cross-linking two FGFRs with or without the participation of HLGAGs. Pantoliano *et al.* (1994) put forward a model in which single FGF and HLGAG molecules formed a bridge between two FGFR molecules resulting in receptor dimerization. Alternatively, Springer *et al.* (1994) identified high-affinity (Tyr²⁴, Tyr¹⁰³, Leu¹⁴⁰, Met¹⁴²) and low-affinity (Lys¹¹⁰-Trp¹¹⁴) FGFR binding sites on FGF, and, based on the bivalent nature of FGF, proposed that a single FGF molecule could cross-link two FGFRs. However, while both of these theories contained parts of the picture, they did not account for the participation of HLGAG-mediated FGF dimerization and oligomerization in FGFR activation.

An alternative model of Spivak-Kroizman *et al.* (1994a) proposed that HLGAGs promote the formation of FGF dimers that in turn cross-link FGFRs (see Fig. 4). This model relied on the one-to-one interaction between FGF and FGFR shown by isothermal titration calorimetry, and the fact that HLGAGs can bind several FGF monomers. In this model HLGAGs cross-link FGFRs by using FGFs as adaptors. Unfortunately, the Spivak-Kroizman model did not explain the essential HLGAG-FGFR interaction in receptor dimerization (Kan *et al.* 1993).

Within the past year three new, very similar, models have been proposed to explain the HLGAG-FGF-FGFR complex (Fig. 5). Venkataraman *et al.* (1999), Plotnikov *et al.* (1999) and most recently, Stauber *et al.* (2000) have independently proposed models in which a 2 FGF:2 FGFR dimer is stabilized by the addition of an HLGAG. In these models each FGFR in the dimer binds one FGF, and the complex itself is stabilized by HLGAG binding across a canyon formed by the FGF-FGFR pairs. In each of the FGF-FGFR pairs, IgII and IgIII both wrap around a single FGF molecule: IgII binding at Springer's high affinity binding site (see above) and IgIII binding at the low affinity site (Venkataraman *et al.* 1999) or with other amino acids (Plotnikov *et al.* 1999, Stauber *et al.* 2000). Together, these pairs form a highly positive canyon at the IgII-FGF interface into which a single HLGAG can bind, thus placing minimal size restrictions on the HLGAG such that it must span both pairs in order to dimerize receptors. In addition, the extracellular domains of each FGFR stabilize the dimer by direct interactions, probably at the linker region between IgII and IgIII.

These three groups differ on a few other possible interactions. For example, Plotnikov *et al.* (1999) also have the dimer stabilized by one FGF binding not only to its own

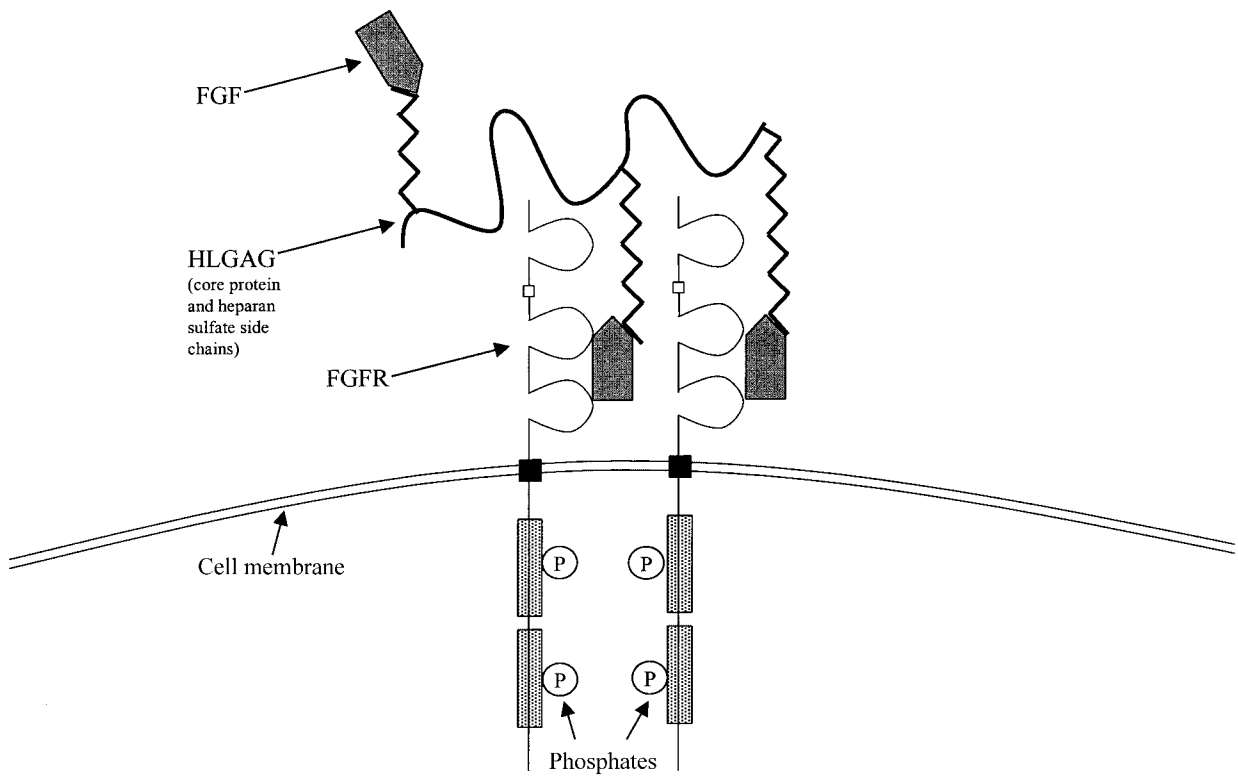


Figure 4 Spivak-Kroizman and coworkers' model for FGFR activation. HLGAGs oligomerize FGFs, which in turn cross-link FGFRs. (Modified from Spivak-Kroizman *et al.* 1994a.)

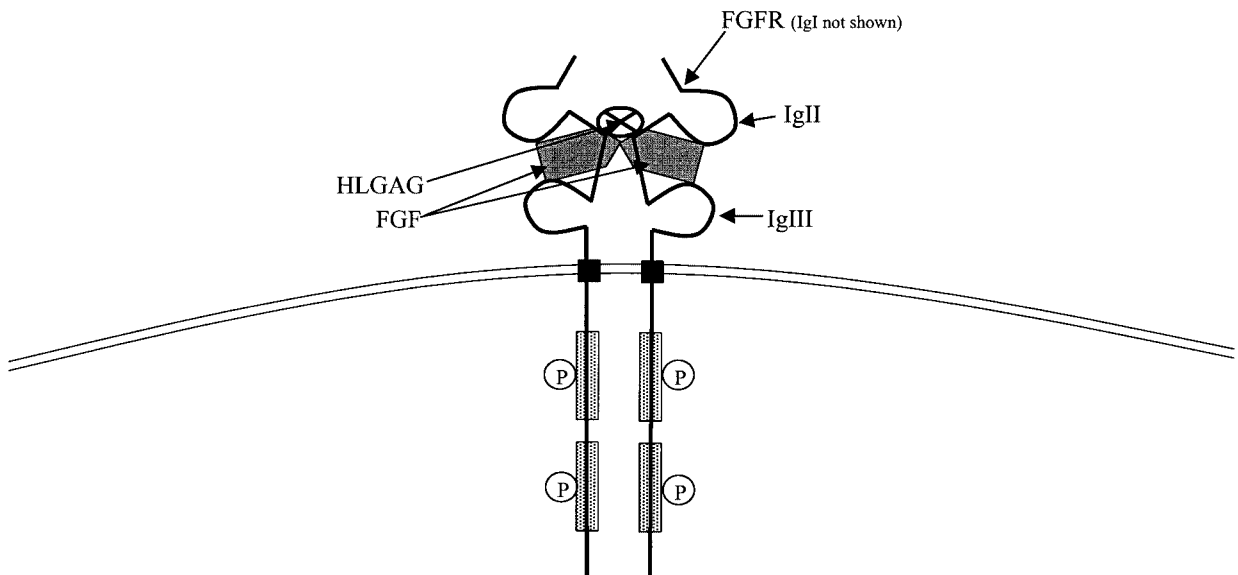


Figure 5 Simplification of the model of Venkataraman *et al.* (1999) and Plotnikov *et al.* (1999) for FGF-FGFR-HLGAG interaction and receptor dimerization. (Modified from Venkataraman *et al.* 1999.)

FGFR's IgII, but also to the IgII in the adjoining pair. However, all three models take into account the interaction between HLGAGs and both FGFs and FGFRs, as well as the numerous interactions between FGFs and FGFRs themselves.

FGFR signal transduction

As we described above, the activated tyrosine kinase receptor recruits target proteins of the signaling cascade to its cytoplasmic tail and modifies them by phosphorylation. One way these recruited target proteins may be localized to the activated receptor is through the interaction between their Src-homology 2 (SH2) domains and specific phosphotyrosine residues on the activated receptor (Pawson 1995). These SH2-containing proteins may be substrates for receptor-mediated phosphorylation themselves, or they may function as adaptor proteins to recruit other target proteins. Tyrosine kinase receptors generally propagate signal transduction by phosphotyrosine-induced conformational changes in their target proteins resulting in activation of various catalytic activities.

Structure of the FGFR cytoplasmic domain

Most studies of FGFR-mediated signal transduction have been carried out using FGFR-1 as the prototypical FGFR. The signaling pathways from different FGFRs are probably

quite similar owing to the high degree of homology at the amino acid level between different receptor types (Johnson & Williams 1993). In addition, Raffioni *et al.* (1999) have shown, by using chimeric receptors comprised of the cytoplasmic domains of FGFR-1, FGFR-3 and FGFR-4 linked to the extracellular domain of the PDGF receptor, that the principle difference between FGFRs in this model is the strength of tyrosine kinase activity, not any differences in target proteins (Raffioni *et al.* 1999).

There are seven tyrosine residues in the cytoplasmic tail of FGFR-1 that can be substrates for phosphorylation: Tyr⁴⁶³, Tyr⁵⁸³, Tyr⁵⁸⁵, Tyr⁶⁵³, Tyr⁶⁵⁴, Tyr⁷³⁰ and Tyr⁷⁶⁶. Tyr⁶⁵³ and Tyr⁶⁵⁴ are important for the catalytic activity of the activated FGFR and are essential for signaling (Mohammadi *et al.* 1996) (see Fig. 6). Tyr⁷⁶⁶ has been shown to bind the SH2 domain of phospholipase C-gamma (PLC γ) and is necessary for FGFR activation of PLC γ (Mohammadi *et al.* 1991). However, the other tyrosines can be mutated to phenylalanine residues, which are not substrates for auto-phosphorylation, without loss of mitogen activated protein (MAP) kinase (MAPK)-activity and mitogenic signaling in rat L-6 fibroblasts (Mohammadi *et al.* 1996), putting their roles in FGFR signal transduction in question. It is interesting to note that a 90-kDa phosphoprotein has been observed associated with the adaptor molecule Grb2 following activation of both the wild-type and phenylalanine-mutated receptors. This suggests that FGFR signaling may

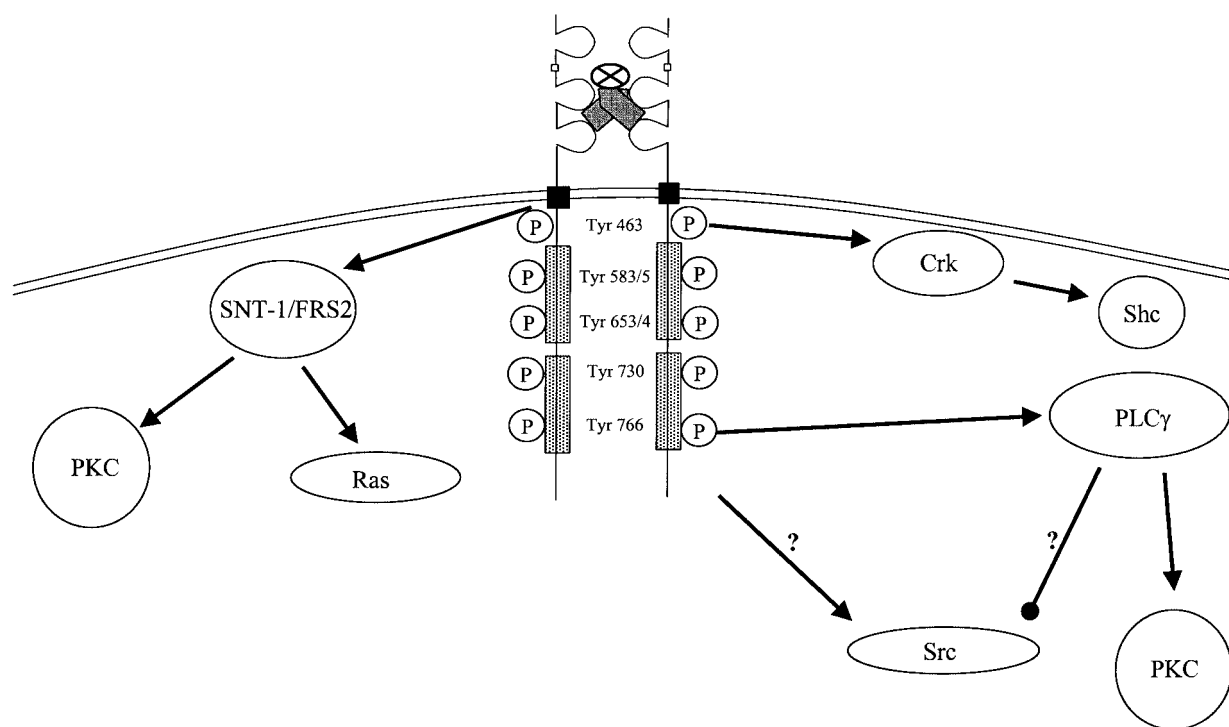


Figure 6 Signaling through the cytoplasmic domain of FGFR. Symbols for HLGAG and FGF as in Fig. 5.

not rely only on the phosphotyrosine-SH2 pathway, an idea we will explore in more detail below.

The PLC γ signaling pathway

Activated PLC γ cleaves phosphatidyl-inositol-4,5-bisphosphate to inositol trisphosphate (IP₃) and diacylglycerol (DAG). In turn, IP₃ facilitates the release of calcium stores from the endoplasmic reticulum while DAG and calcium activate PKC. PLC γ was identified as a 150-kDa phosphoprotein associated with FGFR following ligand-dependent activation (Burgess *et al.* 1990), and this association is due to binding between the SH2 domain of PLC γ and Tyr⁷⁶⁶ of FGFR-1 (Mohammadi *et al.* 1991). Two groups independently mutated this tyrosine to phenylalanine, showing that this residue is essential for phosphatidylinositol hydrolysis (Mohammadi *et al.* 1992, Peters *et al.* 1992). However, mutation of Tyr⁷⁶⁶ did not affect FGFR-mediated mitogenesis, neuronal differentiation (Spivak-Kroizman *et al.* 1994b), or mesoderm-induction in a *Xenopus* animal cap model (Muslin *et al.* 1994). This implies that either PLC γ signaling is redundant with respect to mitogenesis and differentiation, or that the PLC γ pathway is important for some other function of FGFR signaling. Although the PLC γ pathway is not directly involved in cell motility (Langren *et al.* 1998), it may be involved in some other form of cytoskeletal alteration as the actin-binding protein profilin participates in PLC γ signaling (Goldschmidt-Clermont *et al.* 1991).

The Src signaling pathway

Src is a non-receptor tyrosine kinase that may link FGFR signaling to cortactin (Zhan *et al.* 1993), a focal adhesion-associated protein that binds filamentous actin (Wu *et al.* 1991b). This connection would provide an alternate pathway to that of PLC γ for FGFR-mediated cytoskeletal alterations. However, there are conflicting reports concerning the interaction of Src and FGFR. Zhan *et al.* (1994) found a direct interaction by immunoprecipitation with recombinant FGFR-1. On the other hand, Langren *et al.* (1995) saw no direct interaction and instead proposed that, as the Tyr⁷⁶⁶ to Phe⁷⁶⁶ mutant had high levels of phosphorylated Src, the PLC γ pathway inhibits Src activity.

Crk-mediated signaling

Crk is an SH2/SH3-containing adaptor protein which may link FGFR to the downstream signaling molecules Shc, C3G and Cas, which may in turn propagate a mitogenic signal from FGFR. Larsson *et al.* (1999) have shown that Crk binds via its SH2 domain to Tyr⁴⁶³ of the activated FGFR. Signaling through Crk has no effect on cell motility, yet endothelial cells expressing FGFR-1 with a phenylalanine

substitution at Tyr⁴⁶³ failed to proliferate and the activity of both Erk2 and Jun kinase was suppressed. These results are in contradiction to those of Mohammadi *et al.* (1996), who determined that Tyr⁴⁶³ was not important for mitogenesis. Perhaps these differences are reflective of differences in FGFR signaling intrinsic to different cell types, as Mohammadi and coworkers used fibroblasts while Larsson and coworkers' results were seen in endothelial cells.

The SNT-1/FRS2 signaling pathway

As mentioned above with studies of tyrosine-mutated FGF receptors, both the wild-type and the mutant receptor lacking all non-catalytic tyrosine residues have been shown to phosphorylate a novel 90-kDa protein, suggesting the existence of an alternative pathway to that of phosphotyrosine recruitment of SH2-containing proteins. This 90-kDa protein was independently identified as SNT-1 (Wang *et al.* 1996) or FRS2 (Kouhara *et al.* 1997) by two separate groups who both showed that SNT-1/FRS2 linked FGFR activation to the Ras/MAPK signaling pathway important for growth factor-induced cell-cycle progression. Activation of SNT-1/FRS2 recruits the adaptor protein Grb-2/Sos that in turn recruits Ras to the FGFR complex (Kouhara *et al.* 1997). In addition to associating with Grb-2, activated FRS2 also binds the protein tyrosine phosphatase Shp2 (Ong *et al.* 2000). Ong *et al.* (1997) have shown through co-immunoprecipitation that Shp2 associates with both FRS2 and the docker protein Gab-1. SNT-1/FRS2 is localized to the inner leaflet of the cell membrane by myristylation and interacts with FGFR-1 at amino acids 407-433 of the juxtamembrane region (Xu *et al.* 1998). In addition to linking FGFR signaling to the Ras/MAPK pathway, Lim *et al.* (1999) have shown recently that SNT-1/FRS2 can link FGFR activation to atypical protein kinase C isoforms, although the role this may play in mitogenesis or chemotaxis has yet to be characterized.

Interestingly, Ong *et al.* (2000) have shown that FRS2 is constitutively associated with FGFR1, independent of receptor activation. Nerve growth factor (NGF) receptors also utilize FRS2 in their signaling pathways; however association of FRS2 with NGF receptors is dependent on receptor activation. Consequently, FGFR1 may regulate NGF signaling by sequestering FRS2 from liganded NGF receptor.

Thus FGFRs mediate signal transduction by at least two independent pathways. First, FGFRs utilize the traditional SH2-linked pathway joining FGFR directly to PLC γ and Crk, and probably indirectly to Src. Secondly, FGFR is linked to SNT-1/FRS2 through an interaction at the juxtamembrane domain. Regulation of this second pathway has yet to be determined, as it seems to function independently of receptor phosphorylation, although this pathway appears at least superficially analogous to that of the insulin receptor and the insulin receptor substrate (IRS)-1 (Yenush & White 1997).

Through these, and perhaps other, yet to be defined pathways, FGFRs mediate the diverse effects of FGFs.

Biological function of FGFs

Angiogenesis and wound healing

The well-characterized role of FGF-1 and FGF-2 as fibroblast and endothelial cell growth factors (Folkman & Shing 1992) suggests that a significant biological function of FGFs is as positive regulators of angiogenesis. We will discuss FGFs as factors promoting tumor angiogenesis in the later section dealing with FGF signaling in cancer; however, here we will focus on the normal biological role of FGFs. Angiogenesis plays a significant biological role in wound healing and exogenous application of FGF-2 has been found both to promote skin wound healing in healing-impaired db/db mice (Tsuboi & Rifkin 1990) and to promote healing of infarcted myocardium following an ischemic insult in both canine (Yanagisawa-Miwa *et al.* 1992) and porcine (Watanabe *et al.* 1998) models.

Wound repair progresses in four phases: inflammation, contraction, repair and regeneration. During the initial inflammatory response, a fibrin- and fibronectin-rich exudate containing numerous inflammatory cells and platelets invades the site of injury. Next, myofibroblasts, that have probably differentiated from pericytes or mesenchymal stromal cells, act to contract the wound, reducing the area to be repaired. The chief hallmark of the repair phase is the formation of granulation tissue, a richly vascularized connective tissue. Granulation tissue is characterized by the migration of endothelial cells and fibroblasts through a network of noncollagenous extracellular matrix resulting in the formation of new capillaries. Late repair is characterized by the deposition of collagen and the organization of the newly formed blood vessels. During the last phase of wound healing, regeneration, the lost epithelial cells are replaced as appropriate.

There is evidence to suggest that FGFs may play a role in at least three of the four phases of wound repair: inflammation, repair and regeneration. FGFs are stored not only in the extracellular matrix itself, but also in endothelial cells (McNeil *et al.* 1989) and fibroblasts (Werner *et al.* 1991). As mentioned above, the release of FGFs from these cells during wound repair may in fact be stimulated by creation of the wound itself. McNeil *et al.* (1989) have shown that various growth factors are released by mechanically wounded endothelial cells and have proposed that mechanical force is both a stimulus and a mechanism for FGF-2 release from endothelium. Of course, there is probably also some other signal for FGF release from endothelium and the stroma, as angiogenesis is not always due to a mechanical injury.

FGFs in inflammation

The role of FGFs in inflammation is supported by the localization of FGF-1 to the synovium of inflammatory arthritic joints (Sano *et al.* 1990) and to allografts showing the histological morphology of chronic allograft rejection (Zhao *et al.* 1993, 1995). The inflammation of arthritis and chronic rejection is characterized by a proliferation of lymphocytes. Production of interleukin-2, a powerful lymphocyte growth factor, has been shown to be stimulated by FGF-1 (Byrd *et al.* 1999), suggesting that FGFs, along with other factors, can induce the migration of inflammatory cells. FGFs may also play a role in the initial phase of wound repair by regulating platelet production, as platelets are also important constituents of the inflammatory response. FGF-4 has been shown to stimulate the proliferation of megakaryocyte progenitor cells (Konishi *et al.* 1996) and FGF-2 knockout mice have abnormalities in their serum platelet levels (Zhou *et al.* 1998).

FGFs in repair

The potential role of FGFs in the repair phase is obvious considering the powerful proliferative effects they have on endothelial cells and fibroblasts. Another important role of FGFs in the repair phase may be the facilitation of endothelial cell migration by regulation of proteolysis and adhesion molecules. FGF-2 has been shown to induce the urokinase-type plasminogen activator (uPA) gene in both endothelial cells (Gualandris & Presta 1995) and fibroblasts (Besser *et al.* 1995). uPA is a serine protease that converts the zymogen plasminogen to plasmin, a trypsin-like protease which cleaves, among other substrates, the fibrin deposited by initial clot formation. uPA can be localized to the cell surface by means of the uPA receptor, resulting in a pericellular zone of fibrinolysis (Werb 1997), and facilitating the migration of endothelial cells through the fibrin clot.

FGF-2 may also facilitate endothelial cell migration during the repair phase of wound healing through regulation of cell-surface adhesion molecules, most notably the $\alpha V\beta 3$ integrin complex. Expression of the $\alpha V\beta 3$ integrin complex on the surface of human microvascular endothelial cells is increased by treatment with FGF-2 (Sepp *et al.* 1994). The $\alpha V\beta 3$ integrin complex, also known as the vitronectin receptor, mediates endothelial cell binding to extracellular components such as vitronectin and fibrinogen. In addition, expression of the $\alpha V\beta 3$ integrin complex can also localize matrix metalloproteinases to the surface of endothelial cells providing another mechanism for the creation of a pericellular zone of fibrinolysis in addition to that mediated by uPA (Brooks *et al.* 1996).

FGFs in regeneration

Evidence for a potential role of FGFs in the final phase of wound healing, regeneration, comes from studies of renal

tubule repair following chemically induced proximal tubule damage. By means of *in situ* hybridization, Ichimura *et al.* (1996) have localized expression of FGF-7 to interstitial cells and FGFR-2(IIIb), a specific FGF-7 receptor isoform, to the tubular epithelium. This segregation of receptor and ligand expression suggests that FGFs, most notably FGF-7, may facilitate epithelial regeneration by means of a paracrine loop, with stromal cells releasing FGF-7 and the target epithelial cells expressing the appropriate receptor. Interestingly, Ichimura *et al.* (1996) also noted that the regeneration of renal tubules closely resembles the late stages of kidney development, in particular the differentiation of epithelium.

FGFs in development

FGFs play a role in development even prior to implantation, as FGF-signaling induces cell division of embryonic and extraembryonic cells of the mouse embryo starting as early as the fifth cell division (Chai *et al.* 1998). FGFs also seem to play a significant role in gastrulation, the formation of the three germ layers. FGFs have been shown to be mesoderm-inducing factors in *Xenopus*, as FGF-3 and FGF-4 are capable of inducing mesoderm derived from *Xenopus* animal pole cells in animal cap experiments (Paterno *et al.* 1989). The temporal and spatial localization of FGF-5 mRNA in pluripotent embryonic ectoderm and cells forming the three primary germ layers during gastrulation implicates FGF-5 as a regulatory factor of gastrulation (Hebert *et al.* 1991). In mouse embryos, FGF-8 is required for cells that have undergone an epithelial-mesenchymal transition to move away from the primitive streak (Sun *et al.* 1999). In FGF-8 knockout mice this failure in migration results in the absence of embryonic mesoderm- and endoderm-derived tissues, as well as a disturbance in the patterning of the prospective neuroectoderm.

FGFs have also been shown to be relevant in organogenesis, particularly in that of the nervous system, the lung and limbs. FGF-8 is important in midbrain development (Crossley *et al.* 1996a) and cell patterning of the neural plate (Ye *et al.* 1998), while FGF-3 plays an important role in induction of the inner ear (Represa *et al.* 1991). FGF-10 plays a key role in lung development (Sekine *et al.* 1999) by triggering branching and differentiation of lung epithelium (Warburton *et al.* 1999). However, the most fully characterized role of FGFs is in limb development.

FGFs in limb development

FGFs have been shown to play a significant role in limb development in chick and mouse model systems. Normal limb development begins as the protrusion of a limb bud composed of lateral plate mesoderm and its covering ectoderm (Martin 1998). The mesenchymal cells in the limb bud will eventually differentiate to form the skeletal elements

and connective tissues of the limb, while muscles are formed by cells that migrate into the developing limb from the somites. As the limb bud elongates, it forms recognizable skeletal elements which form from proximal to distal. Outgrowth and patterning of the developing limb has been found to depend on three distinct signaling centers. One of these is the apical ectodermal ridge (AER), a thickening of the ectoderm that runs anterior to posterior on the tip of the limb bud. The AER is chiefly responsible for proximal to distal development through signaling with FGF family members. A second, the zone of polarizing activity (ZPA), is in the mesenchyme at the posterior margin of the limb bud and is responsible for anterior–posterior axis determination through signaling with the sonic hedgehog (*Shh*) gene. And the third, the ectoderm at the limb tip which is not part of the AER, the so-called non-ridge ectoderm, is responsible for dorsal–ventral patterning through signaling with the *Wnt7a* gene.

The signals from the AER, ZPA and non-ridge ectoderm act on undifferentiated mesenchyme within the limb bud, the so-called ‘progress zone’. The progress zone remains at the tip of the developing limb under the ectoderm and the cells in the progress zone proliferate as a result of signals from the AER resulting in the elongation of the limb bud. As the limb bud elongates, the progress zone advances as well, leaving cells behind which terminally differentiate as skeletal elements of the limb. The time of exit from the progress zone is the key determinant of whether the mesenchymal cells will form proximal or more distal structures (Summerbell *et al.* 1973). Thus, cells in the progress zone are exposed to signaling from three distinct sources resulting in proximal–distal, anterior–posterior and dorsal–ventral patterning.

FGFs in induction of limb buds

FGFs play an important role in limb bud induction. Cohn *et al.* (1995) showed that beads soaked in FGF-1, -2 or -4 and placed in the flanks of chick embryos induce the formation of ectopic limb buds which can develop into almost normal limbs. It is remarkable that a single growth factor is sufficient to induce the development of a limb and this is indicative of the key role FGFs play in limb bud induction as well as in maintenance of limb development. The normal source of the limb induction signal has been identified as the intermediate mesoderm (IM) which lies between the lateral plate mesoderm and the somites and is composed of nephrogenic mesoderm (NM) and the Wolffian duct (WD) (Geduspan & Solursh 1992). Crossley and coworkers have shown that FGF-8 is expressed in the developing limb bud and in the NM, and that its expression in the NM may be due to an uncharacterized signal from the WD (Crossley & Martin 1995, Crossley *et al.* 1996b). Thus, limb bud induction seems to occur as a result of a signal from the WD, which in turn triggers FGF-8 expression in the NM. FGF-8 is then capable

of acting on the lateral plate mesoderm and inducing limb bud formation.

FGFs in proliferation of developing limb

FGFs also play a key role in the established limb bud as the proliferative signal from the AER. The dependence of limb development on an intact apical ectodermal ridge has been known since 1948, when Saunders showed that removal of the AER terminated any further limb development (Saunders 1948). The application of FGF-4-soaked beads to the exposed mesenchyme following apical ectodermal ridge removal led to essentially normal limb development (Niswander *et al.* 1993), showing that FGF-4 is sufficient to replace the AER. However, Fallon *et al.* (1994) showed that FGF-2-soaked beads are also sufficient to replace the AER and, in addition, FGF-2 is the only detectable FGF in chick limb bud extracts, suggesting that FGF-2 is the prime candidate for the chick limb bud AER signal. These observations have led developmental biologists to two key functions of the FGF produced by the AER. First, FGF stimulates proliferation of cells in the progress zone, leading to limb bud elongation and a pool of cells from which skeletal elements can differentiate. Secondly, FGF maintains sonic hedgehog (Shh) expression in the zone of polarizing activity (Vogel & Tickle 1993, Crossley *et al.* 1996b). In addition, Shh can act on cells in the AER to maintain FGF-4 expression, resulting in a positive-feedback loop between the FGFs and Shh (Laufer *et al.* 1994). The interaction between the apical ectodermal ridge and the zone of polarizing activity through FGFs and sonic hedgehog confirms a molecular link between proximal-distal and anterior-posterior patterning (see Fig. 7).

The actions of FGFs in limb bud induction and in the established limb have led to a model for the role of FGFs in induction, initiation and maintenance in limb development (Cohn *et al.* 1995, Crossley *et al.* 1996b, Martin 1998). Limb bud induction is triggered by FGF-8 inducing the expression of *fgf-10*. FGF-10 then induces *fgf-8* in ectodermal cells resulting in the formation of the AER. FGFs from the AER maintain cell proliferation in the progress zone, while FGF-2 (Fallon *et al.* 1994), FGF-4 (Laufer *et al.* 1994) and FGF-8 (Crossley *et al.* 1996b) induce *Shh* expression in the ZPA. Outgrowth and patterning of the limb then results from the combined effects of FGF and Shh and their regulation of many genes in their target cells, including the HoxD family of genes.

Knockout studies of FGFs

The key role FGFs seem to play in angiogenesis and development might lead one to conclude that animals lacking particular FGFs would manifest serious abnormalities. This is true with the *fgf-4*^{-/-} (Feldman *et al.* 1995) and *fgf-8*^{-/-} (Sun *et al.* 1999) mutations which are embryonic lethal, while *fgf-10*^{-/-} mice die at birth due to insufficient lung development (Sekine *et al.* 1999). On the other hand, *fgf-2*^{-/-} and *fgf-6*^{-/-} mice are not only viable, but are phenotypically indistinguishable from wild-type animals by gross examination (Fiore *et al.* 1997, Ortega *et al.* 1998). This observation is probably due to regulatory redundancies in processes controlled by *fgf-2* and *fgf-6*: thus while certain FGFs may contribute to various processes, this does not mean that they are necessary for proper regulation, as other family members may be able to substitute.

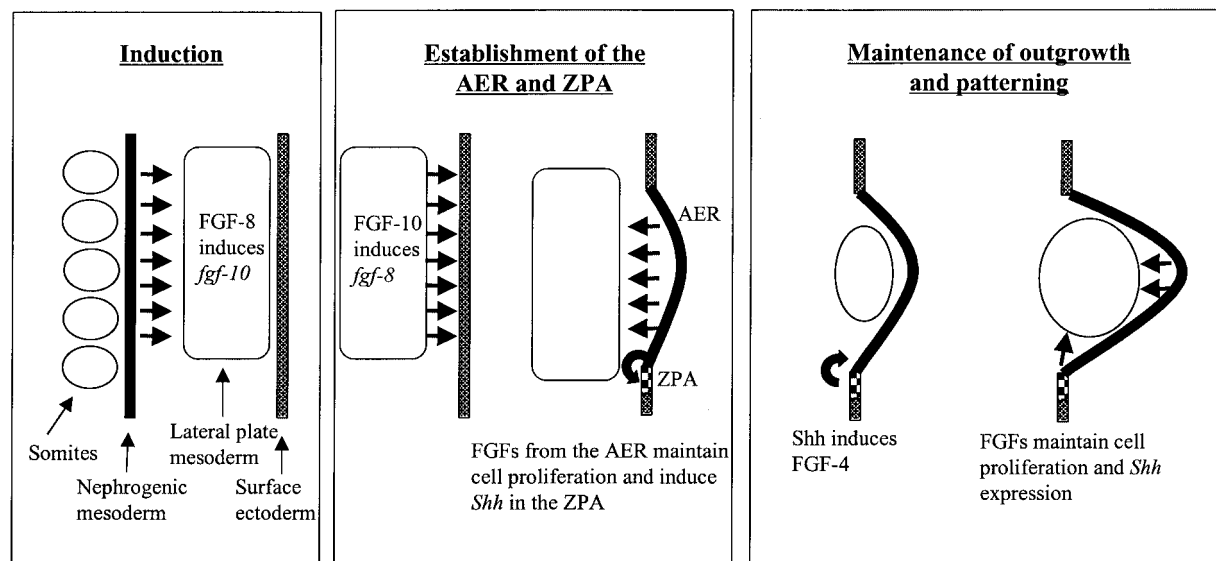


Figure 7 Possible roles of FGFs in induction, initiation and maintenance of limb development. (Modified from Martin 1998.)

This is almost certainly the case with angiogenesis, as *fgf-2^{-/-}* mice demonstrate only a three-day lag over wild-type animals in excisional skin wound repair (Ortega *et al.* 1998), no alteration in the dynamics of vessel repair following mechanical injury (Zhou *et al.* 1998) and a comparable level of retinal neovascularization in a murine model of oxygen-induced ischemic retinopathy (Ozaki *et al.* 1998). On the other hand, control of vascular tone does seem to be affected in the *fgf-2^{-/-}* mice. They display decreased vascular smooth muscle contractility (Zhou *et al.* 1998) and an impaired baroreceptor reflex as elicited by isoproterenol, a β -adrenergic agonist (Dono *et al.* 1998). As a result of these two defects, *fgf-2^{-/-}* mice have a lower mean arterial pressure than wild-type animals. This suggests that whatever role FGF-2 may play in angiogenesis, some other factor can replace it, while with regulation of vascular tone, other factors cannot compensate for the loss of FGF-2.

One other area where FGF-2 seems to play an indispensable role is in cortical development. *fgf-2^{-/-}* mice display a reduction in neuronal density in the motor cortex, neuronal deficiencies in the cervical spinal cord and ectopic neurons in the hippocampal commissure (Ortega *et al.* 1998). The neuronal density deficit is probably due to a loss of an FGF-2-induced increase in the number of rounds of division of cortical progenitors (Vaccarino *et al.* 1999). Micro-injection of FGF-2 at embryonic day 15.5 into the cerebral ventricles of *fgf-2^{-/-}* mice results in an 18% increase in cortical volume and an 87% increase in the number of neurons in the adult cortex.

While it is possible that the lack of profound phenotypic defects in *fgf-2^{-/-}* mice is due to compensation by other FGF family members, FGF-1 does not seem to be the active factor in this regard. Miller *et al.* (2000) have recently shown that *fgf-1^{-/-}* and *fgf-2^{-/-}* double knockout mice displayed similar mild phenotypic defects as *fgf-2^{-/-}* single knockout mice, suggesting that FGF-1 is not the factor that compensates for a lack of FGF-2.

Thalidomide: limb defects and cancer

The discussion of the role of FGFs in limb development is particularly relevant in light of the newly found use of thalidomide as an antiangiogenic drug in the treatment of cancer. Thalidomide was first introduced as a sedative in the 1950s, but fell out of use when, in 1961, McBride and Lenz described a link between limb defects in babies and maternal thalidomide use (McBride 1961, Lenz 1962). The most prominent congenital defect following fetal exposure to thalidomide metabolites (the parent drug is itself harmless) is phocomelia, or shortening of the limbs, suggesting that thalidomide metabolites interfere with proximal–distal patterning in the developing limb bud. The mechanism for this interference seems to be the action of thalidomide metabolites in blocking proliferation of limb bud

mesenchyme in the progress zone (Stephens 1988). The result is that cells in the progress zone do not leave to form more proximal structures and only distal structures are formed from the limb bud.

The teratogenic metabolites of thalidomide also seem to have antiangiogenic properties. D'Amato *et al.* (1994) showed that orally administered thalidomide is an inhibitor of angiogenesis induced by FGF-2 in a rabbit cornea micropocket assay, and given intraperitoneally, thalidomide significantly inhibits FGF-2- and vascular endothelial growth factor (VEGF)-induced corneal neovascularization in a mouse model (Kenyon *et al.* 1997). In addition, thalidomide has recently been shown to inhibit endothelial cell proliferation itself *in vitro* (Moreira *et al.* 1999). Taken together, these results suggest that thalidomide metabolites may interfere with FGF signaling; however, the precise nature of this interference remains unknown.

FGF signaling in cancer

Following release into the extracellular environment, polypeptide growth factors such as the FGFs bind cell surface receptors that, in turn, can activate many signal transduction cascades. These signal transduction pathways will then activate various genetic programs through the concerted regulation of transcription factors, stimulating cell growth by promoting cell cycle progression and inhibiting pathways of cell death. All components of this pathway, from the polypeptide growth factors to the transcription factors, are potential oncoproteins. That is, loss of regulation at any step can result in the driving of those downstream components to promote cell growth beyond control, thus resulting in neoplastic growth. Here we will discuss alterations in both FGFs themselves as well as the FGF receptors and the potential roles such alterations may have in cancer. We will also briefly examine the genetic programs activated by FGF signaling. Finally, we will discuss the evidence for a role of FGF signaling in steroid hormone-dependent cancers.

FGFs in cancer

Overexpression of secreted FGFs

As there are no documented activating mutations in FGFs themselves, the clearest mechanism by which FGFs may contribute to unregulated cell proliferation is by overexpression; thus potential sources for secreted FGFs are the most important target of investigation. Within epithelial tumors there are, in the simplest terms, only two possible sources of FGFs: the tumor cells themselves or the surrounding stromal cells. FGFs may be released from either of these sources and they may also act on either of these sources; consequently FGFs may act in an autocrine or a

paracrine manner or both. There are at least three possibilities: first, FGFs may simply be overexpressed and secreted by the tumor cells themselves; secondly, FGFs may be secreted by the stromal cells in response to a signal from the tumor cells; and thirdly, FGFs may be secreted by the tumor in response to a signal from non-transformed cells.

An example of the first possibility, that of FGF secreted by the tumor cells as an autocrine growth factor and a paracrine angiogenic factor, has been shown to occur in the case of human gliomas (Takahashi *et al.* 1992). *fgf-2* mRNA has been shown to be expressed in over 94% of human gliomas (Takahashi *et al.* 1990); however, the FGF-2 protein has not been detected in normal brain by immunohistochemistry (Takahashi *et al.* 1992). The expression of FGF-2 has also been shown positively to correlate with the degree of malignancy and vascularity in human gliomas (Takahashi *et al.* 1992). Because FGF receptors are expressed on both the tumor cells and non-tumor cells (Takahashi *et al.* 1991), it is not surprising that FGF-2 can act as an autocrine growth factor on the tumor itself, as well as act to promote angiogenesis in the surrounding stroma.

The second possibility, that of FGFs secreted by non-transformed stromal cells in response to a signal from the tumor, is illustrated with FGF-5 in pancreatic cancer. *fgf-5* mRNA has been localized by *in situ* hybridization to cancer-associated macrophages and fibroblasts, yet it is not detected in fibroblasts in normal pancreatic tissue (Kornmann *et al.* 1997). Fibroblasts can be induced to express FGF-5 by epidermal growth factor (EGF), PDGF and transforming growth factor- α (TGF α) (Werner *et al.* 1991) and all of these factors are overexpressed in human pancreatic cancer tissues (Korc *et al.* 1992, Ebert *et al.* 1995). This suggests that FGF-5 is secreted as a paracrine growth factor by the non-transformed cells in the tumor stroma in response to

signaling factors present in the tumor. Such a paracrine signaling pathway would need to be completed by signaling through FGFRs in response to FGFs. By overexpressing a truncated dominant-negative FGFR-1, Wagner *et al.* (1998) showed that two human pancreatic cell lines, Panc-1 and MIA PaCa-2, are dependent on signaling through FGFR-1 for activation of the MAP kinase cascade, increased proliferation, and, most importantly, increased tumor formation *in vivo* in response to FGFs.

In a converse mechanism to that illustrated with FGF-5 in pancreatic cancer, the production of FGF-2 by the transformed cells in Kaposi's sarcoma seems to be a result of signals from the non-transformed stromal cells. The transformed cells in Kaposi's sarcoma, so-called 'spindle cells', have been shown to produce and release FGF-2 in response to tumor necrosis factor- α (TNF α), IL-1 and interferon- γ (INF γ) (Samaniego *et al.* 1998). Kaposi's sarcoma is characterized by an inflammatory cell infiltrate and TNF α , IL-1 and INF γ are all released by these activated T-lymphocytes (Samaniego *et al.* 1998). In this situation the inflammatory cells probably release mediators which cause the tumor cells to oversecrete FGF-2.

Release of sequestered FGFs from the extracellular matrix

Another possibility for dysregulation of FGF signaling in cancer as a result of increased availability of FGFs may be due to the mobilization of FGFs from the ECM. There are currently two models for the release of FGFs from this ECM reservoir: enzymatic cleavage of ECM components, by proteases or heparanases, resulting in the release of FGFs, or binding to a carrier protein, which can then deliver FGFs to their receptors (see Fig. 8). Numerous studies have investigated the release of soluble FGFs from the ECM by

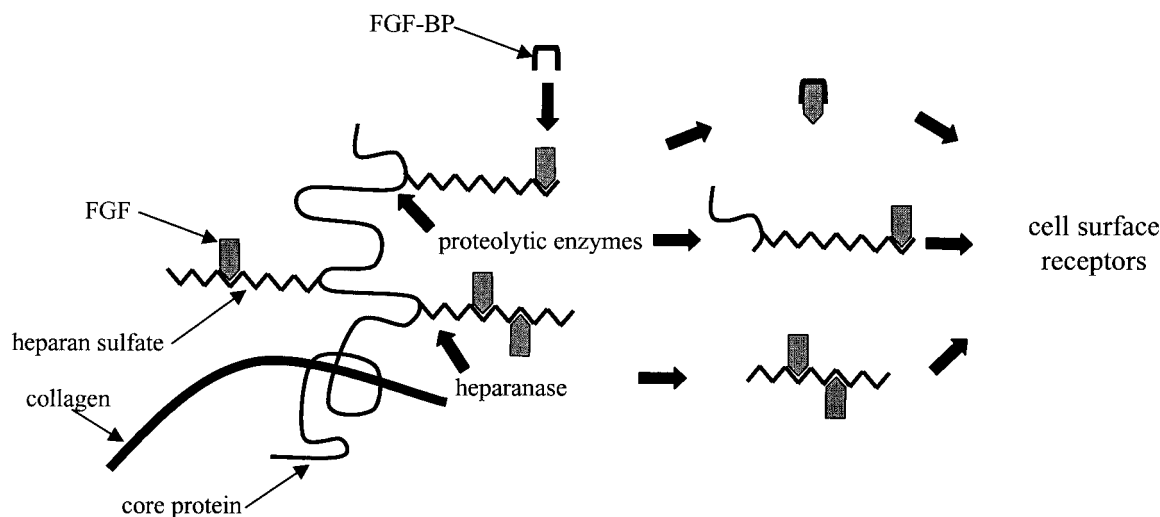


Figure 8 Three possible mechanisms for the mobilization of stored FGFs from the ECM. (Modified from Rak & Kerbel 1997.)

the digestion of the glycosaminoglycan portion of HLGAGs through the activity of heparanases (Vlodavsky *et al.* 1988, Bashkin *et al.* 1989, Moscatelli 1992), and recently the mammalian gene for heparanase has been isolated independently by Vlodavsky *et al.* (1999) and Hulett *et al.* (1999). They have shown expression of this gene at the mRNA and protein levels in metastatic human and rat cell lines as well as in samples of human breast, colon and liver carcinomas. Interestingly, expression of the heparanase gene seems to correlate with invasive phenotype of several human breast cancer cell lines: the non-metastatic cell line MCF-7 does not express the heparanase gene, the moderately metastatic cell line MDA-MB 231 showed moderate levels of heparanase activity and gene expression, while the highly metastatic cell line MDA-MB 435 had high levels of heparanase activity and gene expression. In addition, low metastatic murine T-lymphoma and melanoma cells transfected with the heparanase cDNA developed the ability to metastasize to lung and liver, while the parent cells did not display this phenotype. The ability of heparanases to release bound stores of FGFs, which can then trigger angiogenesis, is consistent with the observed role of heparanases in promoting metastasis. Thus, by regulating expression of heparanases some tumors may be able to mobilize FGFs from the ECM.

The studies of Saksela & Rifkin (1990) provide a good *in vitro* example of the regulation of FGF release by proteolysis of the protein backbone of HLGAGs. They have shown that plasmin releases [¹²⁵I]FGF-2 bound to the ECM secreted by bovine capillary endothelial (BCE) cells in culture. This mechanism may allow for a positive-feedback loop, as FGF-2 is capable of increasing plasminogen-activator activity in BCE cells (Saksela *et al.* 1987). Plasminogen added to a culture of BCE cells with the addition of FGF-2 results in an increased release of labeled FGF-2 from the ECM (Saksela & Rifkin 1990). Thus, FGF-2 release can be increased by the stimulation of proteolytic activity in the pericellular environment through the activity of FGF-2 itself.

A second mechanism for the regulation of FGF release from the ECM may be through the activity of a carrier protein that shuttles FGFs from their site of storage to FGF receptors. The FGF-binding protein (FGF-BP) is a 17-kDa protein originally isolated from the human epidermoid carcinoma cell line A431 which binds FGF-1 and FGF-2 in a non-covalent, reversible manner (Wu *et al.* 1991a). Transfection of the human adrenal adenocarcinoma cell line SW-13, which expresses FGF-2, with an expression vector for FGF-BP results in a malignant phenotype as determined by colony formation in soft agar and the growth of tumors in nude mice (Czubayko *et al.* 1994). FGF-BP is known to be tightly regulated during development: high levels have been detected in skin and intestine perinatally, yet it is downregulated in adult tissues (Kurtz *et al.* 1997). However,

FGF-BP is upregulated in adult skin during early stages of carcinogenesis as well as in squamous cell carcinoma (SCC) and some colon carcinoma cell lines (Czubayko *et al.* 1997, Kurtz *et al.* 1997). Depletion of FGF-BP in human SCC (ME-180) and colon carcinoma (LS174T) cell lines through the use of FGF-BP targeting ribozymes decreases growth and angiogenesis in a xenograft tumor model (Czubayko *et al.* 1997). These observations have led to the possibility that regulation of FGF-BP may be just as important as direct regulation of FGF production because of the ability of FGF-BP to mobilize the FGF reservoir. In fact, retinoids may act to inhibit growth of SCC cell lines *in vitro* through the down-regulation of FGF-BP levels (Liaudet-Coopman *et al.* 1997) and phorbol ester promotion of skin cancer may be through a stimulatory effect at the FGF-BP promoter (Harris *et al.* 1998).

FGF receptors in cancer

Disregulation of FGF signaling as a result of alterations at the level of the receptor has been shown to occur in four possible forms: inappropriate expression, point mutations, splice variations and genomic alterations. While not all of these alterations are associated with human cancer, we will, nevertheless, briefly consider each one of these possibilities in turn. FGFRs have also been shown to be overexpressed in comparison to normal tissues by immunohistochemistry of brain (Morrison *et al.* 1994), breast (Yoshimura *et al.* 1998), prostate (Giri *et al.* 1999), thyroid (Shingu *et al.* 1998), melanoma (Ahmed *et al.* 1997) and salivary gland (Myoken *et al.* 1996) tumor samples. With most of the examples of overexpression of FGFRs in cell lines and tumor samples, one or more FGF is often also expressed, creating the possibility for autocrine FGF signaling. Causes for such overexpression are largely uncharacterized; however the role of chromosomal translocation described below is certainly one such mechanism.

There is a wealth of data supporting the existence of activating point mutations of FGFRs, but such mutations have only been found in developmental defects such as skeletal dysplasias (Webster *et al.* 1996) and craniosynostotic syndromes (Mangasarian *et al.* 1997, Chun *et al.* 1998, Gripp *et al.* 1998). In these cases, point mutations may occur in the extracellular, transmembrane or kinase domains and all such mutations result in ligand-independent activation of the FGFRs (Neilson & Friesel 1996). While such mutations would seem to be possible mechanisms leading to cancer development, none has been shown to be involved in human cancer.

An alteration in post-transcriptional processing has also been shown to occur with FGFR-3, but as is the case with point mutations, this alteration has an unknown role in human cancers. A splice variant of FGFR-3 has been reported in breast epithelial cells that is missing exons 7 and

8, which code for the transmembrane domain, yet has an intact kinase domain and is located in the nucleus (Johnson *et al.* 1995). While the nuclear localization of some members of the FGF family themselves raises the possibility of a completely novel autocrine signaling pathway in the nucleus, the role of nuclear localization of this FGFR-3 splice variant is unclear.

Finally, gene rearrangements have also been shown to lead to ligand-independent activation of FGFRs. A constitutively active form of FGFR-2 has been found in a rat osteosarcoma cell line (Lorenzi *et al.* 1996). In this cell line, chromosomal rearrangement leads to the alteration of the C-terminus of the FGFR-2 protein as a result of fusion to a novel gene designated FGFR activating gene 1 (FRAG1). The FGFR-2-FRAG1 fusion protein seems to form constitutive dimers resulting in autophosphorylation of the FGFR-2 kinase domains and activation of the FGF signaling pathway. Another chromosomal translocation in human myeloid cells has also been shown to result in activation of the FGF signaling pathway. Human myeloid cells containing the t(4;14)(q16.3;q32.3) translocation have been shown to have increased expression of FGFR-3 resulting in the activation of an FGF autocrine loop, as determined by blocking antibodies to FGF-4, a principle ligand of FGFR-3 (Otsuki *et al.* 1999).

Genetic programs of tumor growth

FGFs may activate genetic programs which promote cell growth by at least one of three general mechanisms: first, as mitogens for the tumor cells themselves, secondly, by promoting angiogenesis to supply a growing tumor, and thirdly, by inhibiting apoptosis and allowing tumor cells to continue to grow beyond normal constraints.

FGFs as mitogenic factors

FGF-1 and FGF-2 were initially isolated based on their ability to stimulate incorporation of [³H]thymidine in 3T3 fibroblasts, suggesting that they are powerful mitogenic factors. However, it is important to separate the concept of adding an exogenously produced protein to that of overexpressing the gene itself. While FGF-1 and FGF-2 are potent mitogens in their own right, overexpressed *fgf-1* and *fgf-2* cDNAs are only powerful transformants for fibroblasts if a signal sequence is inserted in the 5' region of the cDNA; otherwise they are only weakly transforming (Basilico & Moscatelli 1992). This suggests that inefficient secretion may limit the potential of FGF-1 and FGF-2 to transform cells efficiently. This also suggests that a mutation which allowed the efficient secretion of FGF-1 or FGF-2 might be oncogenic; however, as mentioned above, such a mutation has never been observed.

fgf-3 was initially identified as a gene which is

overexpressed as a result of MMTV insertion upstream of its promoter (Dickson *et al.* 1984). However, it is uncertain how important this overexpression actually is in mammary tumor development, as overexpression of FGF-3 mRNA is not powerfully transforming by itself (Basilico & Moscatelli 1992), probably, like FGF-1 and FGF-2, because of inefficient secretion (Kiefer *et al.* 1993). However, *fgf-3* is consistently expressed in tumorigenic, but not non-tumorigenic, clones of human colon cancer cell lines (Galdemard *et al.* 1995), suggesting that it may be necessary, although not sufficient, for some tumors.

fgf-4 was isolated by screening genes from human cancers themselves for a transforming effect on 3T3 fibroblasts (Sakamoto *et al.* 1986, Delli Bovi & Basilico 1987), attesting to its mitogenic capability. In addition, perhaps because FGF-4 is efficiently secreted, it has been shown to transform fibroblasts by establishing an autocrine loop (Delli Bovi *et al.* 1987, Talarico & Basilico 1991). Interestingly, an amino-terminal truncated FGF-4 mutant has been shown to bind its receptor more tightly than the full length protein (Bellosta *et al.* 1993); however, this form has not been shown to be more oncogenic.

fgf-5 was identified by screening tumor cell lines for sequences capable of transforming 3T3 cells (Zhan *et al.* 1988). In fact, the *fgf-5* gene was identified because the juxtaposition of a retroviral transcriptional enhancer element resulted in FGF-5 overexpression (Zhan *et al.* 1988). Like FGF-4, FGF-5 is also efficiently secreted, suggesting that FGF-5-induced transformation is due to overexpression.

FGFs as angiogenic factors

FGF-1 and FGF-2 are well-defined pro-angiogenic molecules (Folkman & Shing 1992). In addition, FGF-3 and FGF-4 have been shown to induce angiogenesis *in vivo* in chick chorioallantoic membrane assays (Wellstein *et al.* 1992, Costa *et al.* 1994, Yoshida *et al.* 1994). Two other FGFs, FGF-5 and FGF-7, also seem to possess pro-angiogenic properties. FGF-5 co-localizes with VEGF in epiretinal membranes (Schneeberger *et al.* 1997), is expressed by bovine epithelial cells *in vitro* (Keithahn *et al.* 1997), and its expression in choroidal neovascular membranes is associated with age-related macular degeneration (Kitaoka *et al.* 1997); however it remains to be definitively identified as an angiogenic factor. FGF-7, although widely thought of as an epithelial cell mitogen, has been shown to induce *in vivo* neovascularization in the rat cornea (Gillis *et al.* 1999). To date, however, the role of all the other FGFs as angiogenic factors remains undefined.

FGFs as antiapoptotic factors

Bcl-2 is an antiapoptotic protein initially found to be overexpressed in human follicular lymphomas (Tsujimoto *et al.* 1985). The possibility that FGF-2 may participate in the regulation of apoptosis through bcl-2 was first suggested by

Murai *et al.* (1996) because neutralizing antibodies to FGF-2 were found to induce apoptosis in human glioma cell lines overexpressing FGF-2, and this apoptosis could be inhibited by the overexpression of bcl-2. FGF-2 was shown to upregulate expression of bcl-2 in B cell chronic lymphocytic leukemic cell lines resulting in a delay in fludarabine-induced apoptosis (Konig *et al.* 1997). In addition, FGF-1 has been shown to upregulate bcl-2 expression in the human SH-SY5Y neuroblastoma cell line (Raguenez *et al.* 1999). There appears to be a different story in other cancer cell lines, as FGF-2 has been shown to downregulate expression of bcl-2 and actually promote apoptosis in MCF-7 cells (Wang *et al.* 1998). Clearly, the exact role of FGFs in apoptosis remains to be fully elucidated.

FGFs and steroid hormones in cancer

Epithelium whose growth is regulated by endogenous steroid hormones can give rise to tumors that, like the parent tissue, are also dependent on steroid hormones for growth. However, as these tumors progress, they may become independent of steroid hormones for growth, limiting the effectiveness of anti-hormonal therapies for their treatment. This is particularly important with breast or prostate cancer, in which the tumor may progress from a steroid-dependent to a steroid-independent phenotype, rendering it unresponsive to hormonal therapies. An attractive hypothesis to explain the progression to steroid independence is that the tumor acquires the ability to constitutively express autocrine growth factors previously induced by the steroid hormone itself. There is evidence in some cancer models that particular FGFs may function as autocrine growth factors capable of conferring steroid independence.

Such a function has been demonstrated in SC-3 cells, a murine cell line derived from the mouse mammary carcinoma, Shionogi 115, whose growth is markedly increased by treatment with androgens. Koga *et al.* (1995) demonstrated that this androgen-dependent growth is mediated by the simultaneous induction of FGF-8 and FGFR-1. Sato *et al.* (1993) showed that blockade of FGF-8 activity by antisense oligonucleotides blocks androgen-induced growth of SC-3 cells (Sato *et al.* 1993), suggesting that FGF-8 plays a key role in mediating the effect of androgens on this cell line. In addition, expression of the FGF-8 cDNA in androgen-dependent cells facilitates their conversion to an androgen-independent phenotype, yet is not in itself sufficient to induce it (Koga *et al.* 1995), suggesting the important role of simultaneous expression of the receptor in order to complete the autocrine loop.

An FGF-mediated autocrine loop has also been demonstrated in human breast cancer. Either FGF-1 or FGF-4 overexpression in the estrogen-dependent human breast cancer cell line MCF-7 induces an estrogen-independent phenotype as determined by tumor growth and

metastasis in nude mice (McLeskey *et al.* 1993, Kern *et al.* 1994). As this alteration is not due to changes in estrogen receptor levels (McLeskey *et al.* 1998), it is likely that the FGF autocrine loop acts downstream from an estrogen signal. In order to determine if FGF functions as an autocrine growth factor in mediating the estrogen-independent growth of FGF-1 overexpressing cells in nude mice, these cells were transfected with an FGFR-1 vector encoding a truncated dominant negative FGF receptor (Zhang *et al.* 1998). The expression of the dominant negative receptor inhibited the ability of the FGF-1 overexpressing cells to form tumors in the absence of estrogen in ovariectomized nude mice. This suggests that FGF-1 acts as an autocrine growth factor in order to promote estrogen-independent tumor formation in this model. Importantly, disruption of the FGF autocrine loop did not abolish the formation of tumors in mice supplemented with estrogen or tamoxifen, perhaps because of a synergy between estrogen-promoted mitogenicity and a paracrine FGF-1 effect.

The situation in prostate appears to be slightly more complicated. Isolated epithelial and stromal cells from normal rat prostate and androgen-responsive tumor models are themselves androgen-independent for growth (McKeehan *et al.* 1984). Instead, their growth is dependent on various polypeptide growth factors, suggesting that the function of androgen on prostate epithelium may be indirectly mediated by other growth factors (McKeehan *et al.* 1984). Co-culture of prostate epithelial and stromal cells revealed androgen-sensitive growth by the epithelial cells, while the stromal cells were unresponsive (Yan *et al.* 1992). These observations can be explained by the finding that FGF-7 is expressed by prostate stromal cells in response to androgen treatment, but not by prostate epithelial cells (Yan *et al.* 1992). Because prostate epithelial cells express FGFR-2(IIIb) (Miki *et al.* 1992), the receptor specific for FGF-7, this suggests that in the normal prostate, androgens regulate epithelial cell growth by inducing FGF-7 expression in the stromal cells. FGF-7 may then act as a paracrine growth factor on the epithelial component owing to its expression of FGFR-2(IIIb). Recently, FGF-10 has also been identified as a potential paracrine mediator of the androgen signal in the prostate (Lu *et al.* 1999).

The progression of androgen-dependent to androgen-independent prostate cancer may be due to yet another additional FGF signaling pathway. Injection of a mixture of stromal and epithelial cells from a rat prostate tumor model into rats resulted in the formation of non-malignant, differentiated, slowly growing tumors (Yan *et al.* 1993). However, in the absence of stromal cells, the tumors were malignant, poorly differentiated and grew rapidly (Yan *et al.* 1993). This independent and aggressive growth was accompanied by two important changes in gene expression which appear to establish an autocrine signaling loop. First, the cells switched their expression of FGFR-2 from the IIIb

isoform to the IIIc isoform, which binds FGF-2 but not FGF-7, and secondly, they began to express FGF-2 itself (Yan *et al.* 1993). This suggests that in prostate cancer, androgen-dependent growth relies on FGF-7, and perhaps FGF-10 also, as a paracrine growth factor, yet the switch to androgen independence may result from the activity of FGF-2 as an autocrine growth factor.

Conclusion

With all the information of the FGF family and its receptors described above, we can see a basic mechanism for FGF action. FGF is produced by cells, enters the extracellular milieu and eventually binds to and activates cell surface receptors on target cells. Receptor binding triggers a signal transduction cascade mediated by protein phosphorylation, culminating in alterations in gene expression. However, one aspect of this mechanism is still unclear, namely what happens between FGF secretion and receptor activation. We know that FGFs are secreted through either the classical secretory pathway, or, in the case of FGF-1, -2 and -9, by some as yet uncharacterized ER-Golgi-independent pathway. Upon release, FGF quickly becomes associated with the HLGAGs in the ECM. This association may afford FGF protection from proteolysis, as well as creating a local reservoir of growth factors. However, the association of FGF with ECM HLGAGs is a sticky point, as it is unclear how FGF can then localize to the cell surface and activate the FGFR. There are two mutually compatible mechanisms for how FGF may activate the FGFR from its association with the ECM. First, FGF bound to the ECM may not actually be sequestered and may be available to cell surface receptors. If a cell comes in contact with this FGF-primed ECM, the signaling pathway can be activated, thus allowing for a strict spatial regulation of FGF signaling. A second mechanism is that this store of FGFs can be rapidly mobilized through proteolysis, the activity of heparanases, or the activity of a secreted binding protein, FGF-BP. Proteolysis and heparanolytic are attractive mechanisms because they allow the possibility that a large amount of FGF can be released in conjunction with HLGAGs. This is important because HLGAGs are required components of the activated receptor complex along with FGFs. The FGF-BP mechanism is also attractive in the light of our unpublished observation that FGF-2 binds well to FGF-BP or to HLGAGs but not to both at the same time. Thus FGF-BP may pick up FGF from the ECM and carry it to the cell surface where it is dropped off to cell surface HLGAGs. In turn, the cell surface HLGAGs could present FGF to the FGFR and participate in the complex as well. It is possible that each of these mechanisms is operating at different times and in different situations. With the first mechanism, FGF may signal from the ECM to promote chemotaxis and cell migration during development. The second model may explain FGF signaling during wound

repair and tumor angiogenesis, as numerous proteolytic enzymes and heparanases are activated during these processes. And with the FGF-BP model, we have a mechanism for FGF signaling that may be appropriate whenever finely tuned regulation of FGF signaling is required. Clearly, tumor development may be facilitated by the dysregulation of these mechanisms for FGF release through the inappropriate expression of proteases, heparanases or FGF-BP. Future studies of the regulation of these mechanisms will be required to elucidate further the role of FGF signaling in cancer.

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