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Constitutive activation of fibroblast growth factor receptors in human developmental syndromes

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Abbreviations: FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; TD, thanatophoric dysplasia

Summary

Fibroblast growth factor receptors (FGFRs) represent specific receptors for the fibroblast growth factors (FGFs), a family of at least 13 polypeptides. Ligand/receptor interactions between FGFs and their receptors are involved in many fundamental biological processes, particularly cell growth and differentiation during chondrogenesis and myogenesis. The four different human FGFR genes encode related glycoproteins with a common structure consisting of an N-terminal signal peptide, three immunoglobulin (Ig)-like domains, a single transmembrane domain, and an intracellular split tyrosine-kinase domain. FGFs, acting in concert with heparan sulfate proteoglycans, bind to FGFRs and result in their activation, involving homo- or hetero-dimerization of receptors, leading to trans-phosphorylation of the kinase domains. The activated receptors can then phosphorylate various intracellular proteins involved in signal transduction, although much remains to be learned concerning these signal transduction pathways downstream of activated FGFRs. Many mutations in different domains of FGFR1, FGFR2 and FGFR3 have recently been identified as causing various human craniosynostosis and dwarfism syndromes, and the molecular consequences of these mutations are beginning to be unraveled. Craniosynostosis syndromes, characterized by premature ossification and fusion of the cranial sutures of the skull, arise primarily from mutations in the extracellular domain FGFR2, although specific mutations in other FGFRs may also underlie related craniosynostosis syndromes. Skeletal dwarfism syndromes, characterized by disproportionate short stature and macrocephaly, arise predominantly from mutations in FGFR3 and include achondroplasia, the most common genetic form of dwarfism, as well as the thanatophoric dysplasias (type I and type II). Recent studies demonstrate that a common mechanism, constitutive activation of receptor signaling, underlies most of these disorders. The mutations responsible for the craniosynostosis and skeletal dwarfism syndromes map variously to either the extracellular domain, the transmembrane domain, or the tyrosine kinase domain of these receptors, suggesting multiple mechanisms of aberrant receptor activation. An overview of the developmental consequences arising from mutations in FGFR family members will be presented, including an examination of the molecular mechanisms underlying these defects.

I. Skeletal Development

The development of the human skeleton is a highly complex and regulated process. Osteogenesis (bone formation) includes both intramembranous ossification, responsible for development and fusion of the flat bones of the skull, and endochondral ossification, which occurs at the growth plates of the vertebrae, the pelvis, and the long bones of the extremities. In the process of intramembranous ossification, primitive mesenchymal cells differentiate into osteoblasts (bone-forming cells) that secrete a collagen-glycosaminoglycan matrix which

subsequently becomes calcified. In contrast, endochondral ossification is a two-step process in which mesenchymal cells first condense and differentiate into chondrocytes that secrete a cartilaginous template. As the chondrocytes proliferate, hypertrophy and die in an ordered sequence, osteoblasts carried by infiltrating blood vessels deposit bone matrix to replace the degrading cartilage template (Gilbert et al., 1994).

Reflecting the complexity of skeletal development, more than 150 different disorders of osteochondrogenesis have been described (Spranger, 1992). For instance, defects may occur in the condensation or differentiation of the mesenchyme, in the structure or regulation of components of the extracellular matrix, and/or in the normal proliferation and maturation of the chondrocytes (Mundlos and Olsen, 1997a,b). Recently, the specific genes defective in some of these human skeletal disorders have been identified (Reardon, 1996), and many encode proteins falling into three categories: structural proteins, including several different collagens (Mundlos and Olsen, 1997a,b); transcription factors, including Sox9 (Wagner et al., 1994; Foster et al., 1994), MSX2 (Jabs et al., 1993) and Twist (El Ghouzzi et al., 1997; Howard et al., 1997); and growth factors and their receptors, including the fibroblast growth factor receptors (FGFRs) (Webster et al., 1996). In this review we will focus on skeletal and cranial malformation syndromes associated with mutations in FGFRs.

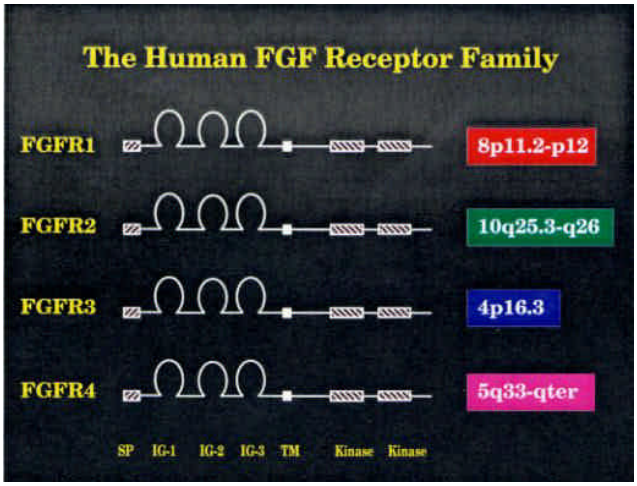


Figure 1. The FGFR family. The overall structure of the four human FGFR family members is shown, together with their chromosomal locations.

II. Fibroblast growth factor receptors: signaling, ligand-binding, and expression

Fibroblast growth factors (FGFs) comprise a family of structurally-related heparin-binding proteins with pleiotropic actions. For instance, they are able to stimulate proliferation in certain cell types, and induce, inhibit or maintain the differentiated phenotype of other cell types. FGFs also exhibit potent neurotrophic and angiogenic activities, and play key roles in embryogenesis (Johnson and Williams, 1993). FGFs, in association with heparan sulfate proteoglycans, bind with high affinity to the extracellular domain of a family of four transmembrane tyrosine kinases, FGFR1-FGFR4, shown schematically in **Figure 1**.

This binding results in receptor homo- or heterodimerization, leading to trans-phosphorylation and activation of the intracellular kinase domains (Spivak-Kroizman et al., 1994a; Ullrich and Schlessinger, 1990). The activated receptors can then phosphorylate substrate molecules which transmit signals into the cell. Some of the effectors involved in FGFR signaling have been identified, and include components of the Ras/MAPK pathway. In contrast to many other receptor tyrosine kinases that directly interact with adaptor proteins following ligand stimulation, it is still unclear exactly how the signal from FGFR is transmitted to Ras (Mohammadi et al., 1996a). Recent studies suggest that tyrosine phosphorylation of both FRS2 and Shc, causing recruitment of the Ras activator complex, Grb2/Sos, to the plasma membrane, links FGFR1 activation to the Ras/MAPK pathway (Kouhara et al., 1997), although a physical association of FGFRs with these proteins has not been demonstrated. Ligand activation of FGFR1 also leads to phosphorylation of and association with PLC-γ (Mohammadi et al., 1991), although PLC-γ–dependent phosphatidylinositol hydrolysis does not appear to be required for either FGF-dependent mitogenesis (Mohammadi et al, 1992) or for differentiation (Spivak-Kroizman et al, 1994b; Muslin et al., 1994). There is significant homology in the catalytic domains of all four FGFRs, suggesting that they regulate many of the same signaling pathways, although differences have been observed in the strength of signals generated by activation of the different receptors (Ornitz and Leder, 1992; Wang et al., 1994).

The extracellular domains of the FGFRs are also highly homologous, and are comprised of three immunoglobulin-like (Ig-like) domains containing characteristic cysteine residues, with an acidic region between the first and second Ig domains. Alternative splicing is common within the extracellular domain of FGFRs, and forms of the receptors possessing only the second and third Ig domains are recognized (Johnson et al., 1991). FGFs bind to Ig-2 and Ig-3 and the linker region between these two Ig domains, although it is the choice of

exon (IIIb or IIIc) in the 3' half of the third Ig domain of FGFR1-FGFR3 that is critical in determining the ligand-binding specificities of each receptor (Johnson and Williams, 1993). Of the nine well-characterized FGFs, only FGF-1 (aFGF) binds with high affinity to all receptor isoforms, and certain FGF/FGFR interactions, such as FGF-7 (KGF) with FGFR2b, are highly specific (Ornitz et al., 1996).

The biological activities of the four FGFRs are determined not only by their differences in signaling and ligand-binding affinities, but also by their distinct spatial and temporal expression patterns. For instance, FGFR1 is expressed in the primitive ectoderm of the postimplantation embryo, and FGFR1-deficient embryos die prior to gastrulation (Deng et al, 1994). During organogenesis, FGFR1 is widely expressed throughout the mesenchymal tissues, including the limb buds, whereas FGFR2 is expressed in the surface ectoderm and epithelia (Orr-Urtreger et al., 1991; Peters et al., 1992). The two different isoforms of FGFR2, KGFR and bek, are expressed in different layers of the developing skin, with bek also highly expressed in bones of the vertebrae, limbs, skull and ribs (Orr-Urtreger et al., 1993). Unlike FGFR1 or FGFR2, FGFR3 expression is less widespread in early embryogenesis, and is restricted to the glial cells of the brain, the differentiating hair cells of the cochlear duct, and the cartilage of the vertebrae, skull and long bones (Peters et al., 1993). FGFR4 expression is most apparent in the endoderm of the developing gut, liver and lung, in skeletal muscle, and in the endochondral cartilage of the ribs and the olfactory and auditory regions (Stark et al., 1991).

III. Role of FGFs and FGFRs in bone development

As noted above, all four receptors are expressed to some extent in developing bone. Specifically, FGFR1, FGFR2 and FGFR3 have overlapping expression patterns in prebone cartilage rudiments, whereas in later stages of bone formation, FGFR1 is expressed primarily in osteoblasts and hypertrophic cartilage, FGFR2 is expressed in the perichondrium/periostium and in the presumptive bone marrow, and FGFR3 expression is confined to resting cartilage (Peters et al., 1993). These observations suggest that individual FGFRs play distinct and important roles in skeletogenesis.

FGFs are clearly intimately involved in bone and limb development. They act as potent mitogens for chondrocytes, yet they also inhibit chondrocyte terminal differentiation (Kato and Iwamoto, 1990). Additionally, FGFs have been shown to enhance extracellular matrix formation by chondrocytes and to accelerate vascular invasion and ossification of growth plate cartilage (Baron et al., 1994).

Local application of several different FGFs can also induce the formation of ectopic limb buds in the chick, which develop into complete limbs with a normal skeletal structure (Cohn et al., 1995).

Consistent with a key role for FGFs and FGFRs in bone development, transgenic mice overexpressing FGF-2 exhibit a variety of skeletal malformations resembling human chondrodysplasia syndromes, including shortening and flattening of the long bones and macrocephaly (Coffin et al., 1995). On the other hand, targeted disruption of the murine FGFR3 gene results primarily in an expansion of the zones of proliferating and hypertrophic chondrocytes at the bone growth plates, resulting in enhanced growth of the long bones and vertebrae (Deng et al. 1996; Colvin et al. 1996).

IV. Mutations in FGFRs are associated with human skeletal dysplasias

The initial clue that FGFRs are involved in human cranial and skeletal disorders came from genetic linkage studies. The gene for achondroplasia, the most prevelant form of short-limb dwarfism, was mapped by several groups to 4p16.3, a chromosomal region that includes the FGFR3 gene (Le Merrer et al., 1994; Velinov et al., 1994). A recurrent mutation in this gene was rapidly confirmed in virtually all patients with achondroplasia (Shiang et al., 1994; Rousseau et al, 1994). At about the same time, certain autosomal dominant craniosynostosis syndromes were mapped to the FGFR2 locus on 10q25 (Reardon et al., 1994; Jabs et al., 1994; Schell et al., 1995), and to chromosome 8p11.2-p12, a region that included the FGFR1 gene (Muenke et al., 1994). Various mutations were identified in these candidate genes (see below), suggesting that they were directly responsible for the craniofacial and digital anomalies observed in these syndromes.

V. FGFR mutations in craniosynostosis syndromes

The flat bones of the skull of the neonate are normally discrete, enabling molding and overlap to occur during compression in the birth canal as well as allowing the skull to grow in parallel with the growth of the brain. The fibrous sutures between these bones gradually interdigitate and close with bony bridging, although complete fusion of the cranial and facial sutures takes place over the lifetime of the individual (Cohen, 1997). Craniosynostosis, a relatively common birth defect, occurs as a result of premature ossification and fusion of one or more of the cranial sutures. Several related syndromic forms of craniosynostosis have been identified, which all share characteristic craniofacial features including abnormal head

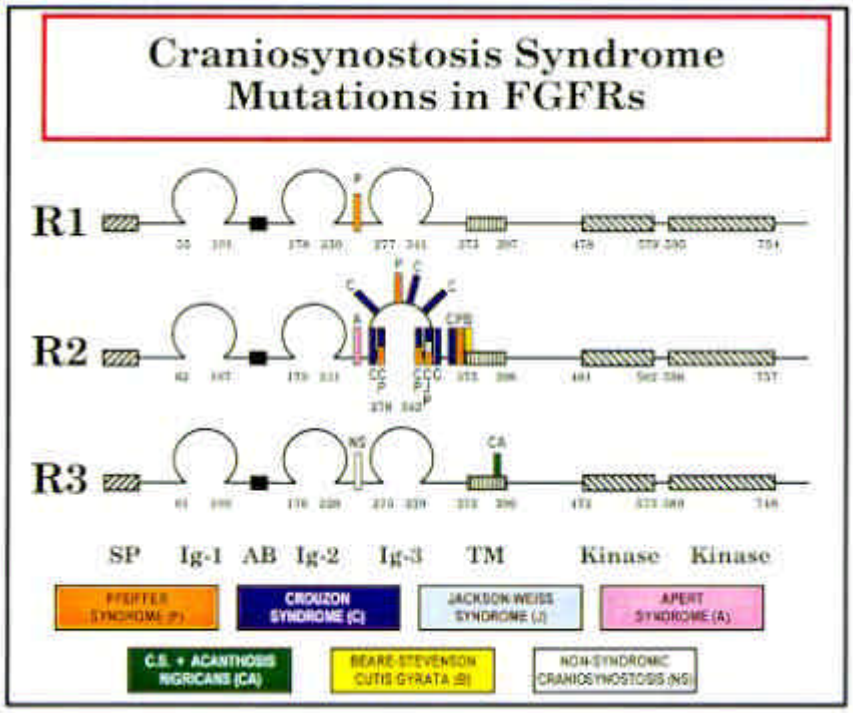


Figure 2. The locations of point mutations in FGFR1, FGFR2 and FGFR3 giving rise to craniosynostosis syndromes.

Abbreviations: AB, acid box; Ig, immunoglobulin-like domain; Kinase, split tyrosine kinase domain; SP, signal peptide; TM, transmembrane domain. The numbers indicate the amino acid residue number at the approximate boundaries of each domain.

shape, protruding eyes, and midface underdevelopment. These syndromes can be distinguished by the pattern of associated limb involvement. For instance, in Crouzon syndrome there is no apparent malformation of the hands or feet, whereas in Pfeiffer syndrome the thumbs and great toes are broad and medially deviated, and in Apert syndrome there is severe and symmetric fusion of the bones of the hands and feet. Jackson-Weiss syndrome has a high degree of phenotypic variability, but generally is associated with anomalies of the feet (Cohen, 1986).

Craniosynostosis syndromes are occasionally associated with skin disorders. For instance, Crouzon syndrome with acanthosis nigricans is a distinct syndrome involving hypertrophy of the skin and hyperpigmentation (Meyers et al., 1995). Beare-Stevenson cutis gyrata is an often-lethal craniosynostosis disorder characterized by furrowed skin, hyperpigmentation, and abnormalities of the digits, the umbilical cord and the anogenital region (Hall et al., 1992).

Surprisingly, given the distinct expression patterns of the different FGFRs discussed previously, related craniosynostosis syndromes have been mapped to point mutations in FGFR1, FGFR2 and FGFR3. The mutations found in FGFR-related craniosynostosis syndromes are shown diagrammatically in **Figure 2**. For instance, a Pro-Arg substitution in the linker region between Ig-like domains 2 and 3 occurs in each of the three receptors, causing Pfeiffer syndrome when expressed in FGFR1 (Muenke et al. 1994), Apert syndrome when expressed in FGFR2 (Wilkie et al. 1995b), and a non-syndromic craniosynostosis when expressed in FGFR3 (Bellus et al. 1996; Muenke et al., 1997). Often, the identical mutation results in clinically distinct disorders in different individuals; for instance, substitutions at Cys342 in the Ig3 domain of FGFR2 lead to Pfeiffer, Crouzon or Jackson-Weiss syndromes (Wilkie et al., 1995a; Meyers et al., 1996; Park et al., 1995; Reardon et al., 1994; Rutland et al., 1995; Steinberger et al., 1995), although these phenotypes usually breed true within families. It is also of interest that substitutions leading to craniosynostosis are found both in FGFR2 exon IIIa, in which case they are expressed in both bek and KGFR forms of the receptor, and also in exon IIIc, in which case they are exclusive to the bek isoform, without apparent phenotypic differences.

VI. FGFR mutations in dwarfism syndromes

Several related forms of short-limb dwarfism, including achondroplasia, hypochondroplasia, and thanatophoric dysplasia, have recently been linked to mutations in different structural and functional domains of FGFR3 (Rousseau et al., 1994; Shiang et al., 1994; Superti-Furga

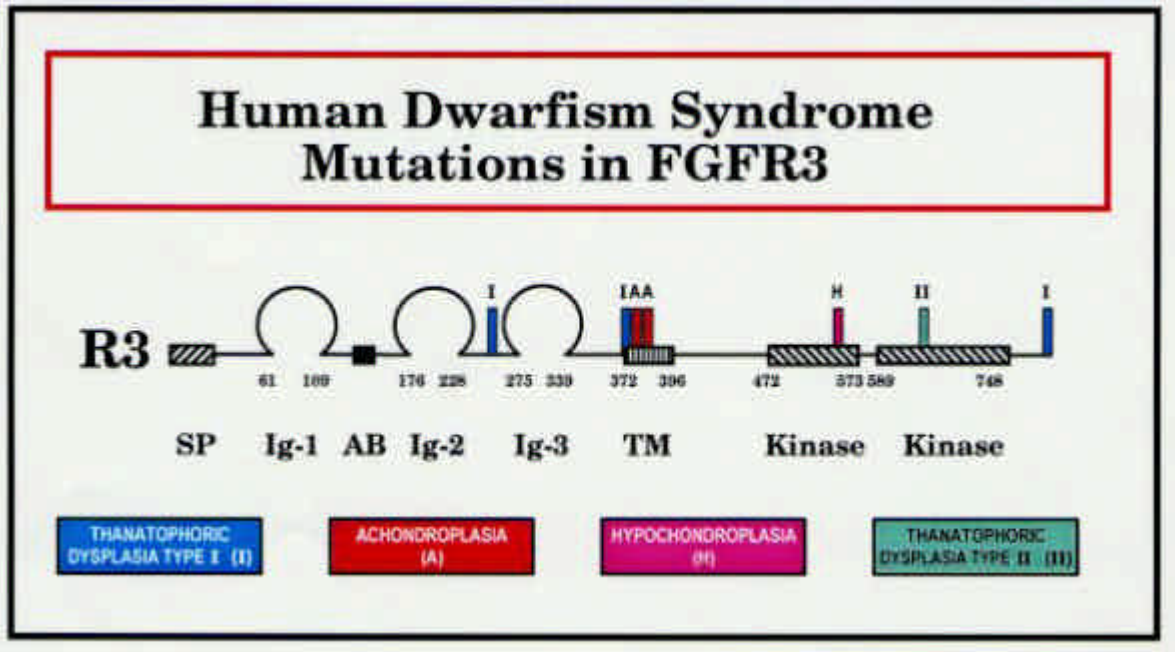


Figure 3. The locations of point mutations in FGFR3 giving rise to dwarfism syndromes.
Numbers and symbols are as described in the legend to Fig. 2.

et al., 1995; Ikegawa et al., 1995; Tavormina et al., 1995a,b; Rousseau et al., 1996; Rousseau et al., 1995). The mutations found in FGFR3 in these dwarfism syndromes are shown in **Figure 3**.

Achondroplasia is characterized by disproportionate shortening of the long bones, macrocephaly, spinal curvature, and midface underdevelopment. Although individuals with achondroplasia are of normal intelligence, they often have delayed motor development and an increased incidence of respiratory problems (Wynne-Davies et al., 1985). This disorder, affecting about 1 in 26,000 individuals (Oberklaid et al., 1979), is usually sporadic, but when familial is transmitted as an autosomal dominant trait with complete penetrance. Homozygous achondroplasia is usually lethal shortly after birth, due to impaired development of the ribs resulting in early respiratory failure. Thanatophoric dysplasia (TD) is a severe disorder resembling homozygous achondroplasia, and can generally be divided into two subclasses. TDI is distinguished by the presence of femoral bowing, whereas the femurs are straight in TDII. Furthermore, TDII is invariably associated with cloverleaf skull (a form of multiple suture craniosynostosis), whereas cloverleaf skull occurs only occasionally in TDI individuals (Langer et al., 1987). On the milder end of the spectrum of short-limb dwarfisms, hypochondroplasia is quite similar to achondroplasia except the skeletal abnormalities observed are considerably less severe (Wynne-Davies et al., 1985). The underlying defect in each of these dwarfisms appears to be varying degrees of disruption of the normal proliferation and differentiation of chondrocytes at the epiphyseal plates of the long bones.

The mutations responsible for all cases of achondroplasia identified to date reside in the transmembrane domain of FGFR3, and result in either a Gly380Arg substitution (Rousseau et al., 1994; Shiang et al., 1994), or, much more rarely, a Gly375Cys substitution (Superti-Furga et al., 1995; Ikegawa et al., 1995). In contrast, three distinct types of point mutations give rise to TDI: mutations to Cys at the extracellular/transmembrane domain junction (Tavormina et al., 1995b; Rousseau et al., 1996); mutations to Cys in the linker region between Ig-like domains 2 and 3 (Tavormina et al., 1995a,b); and mutations that allow read-through of the stop codon (Rousseau et al. 1995). A Gln540Lys substitution in the proximal portion of the split tyrosine kinase domain is found in many patients with hypochondroplasia (Bellus et al., 1995), and a Lys650Glu mutation in the kinase activation loop is responsible for all cases of thanatophoric dysplasia type II identified to date (Tavormina et al., 1995b).

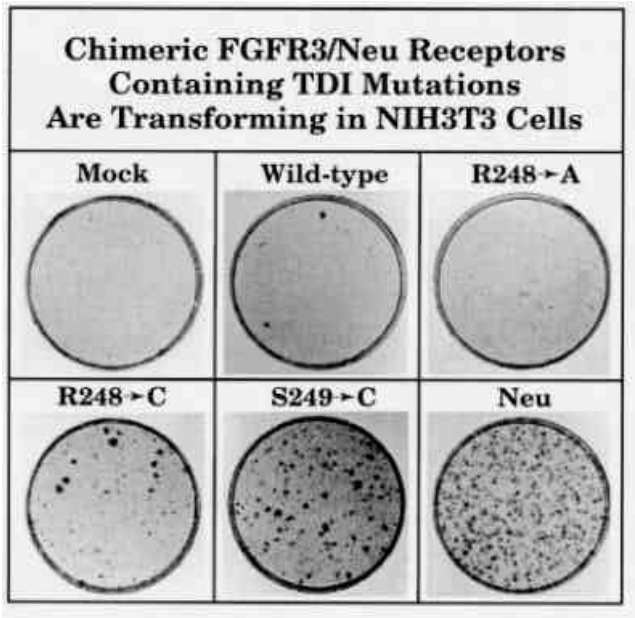


Figure 4. Focus formation assay of chimeric FGFR3/Neu receptors containing TDI mutations.

Chimeric FGFR3/Neu constructs containing interlinker region mutations were transiently transfected into NIH3T3 cells, which were subsequently scored for focus formation. (A) mock-transfected cells (nontransformed); (B) wild type FGFR3; (C) R248A; (D) R248C; (E) S249C; (F) activated Neu containing the mutation V664E as a positive control.

VII. Constitutive FGFR activation underlies both craniosynostosis and dwarfism syndromes

Many different mutations in FGFR1, FGFR2 and FGFR3 have been identified in craniosynostosis and dwarfism syndromes, and it was initially unclear whether these mutations resulted in loss of receptor function (either through dominant negative effects or through loss of ligand binding capacity), alteration of receptor localization (perhaps due to misfolding), or enhanced signaling capacity. Evidence has recently been accumulating that suggests that each of these mutations leads to constitutive activation of receptor signaling, although by different mechanisms, and that this may explain, at least in part, the similar biological consequences of such different FGFR mutations. The effects of mutations in the extracellular, transmembrane and kinase domains of FGFRs will be discussed separately in this review.

VIII. Extracellular domain mutations

Several of the mutations that arise in the extracellular domain of FGFR2 and FGFR3 in bone development disorders are caused by mutations that either destroy or create Cys residues. For instance, in Crouzon and Pfeiffer syndromes, mutations at each of the conserved Cys residues at the base of the third Ig domain of FGFR2 have been observed (Wilkie et al., 1995a; Meyers et al., 1996; Oldridge et al., 1995; Reardon et al., 1994; Rutland et al., 1995; Steinberger et al., 1995). Normally, these residues are presumed to be involved in a disulfide bond, which stabilizes the Ig domain structure, and disruption of this bond appears to underlie constitutive activation of FGFR2 observed in Crouzon mutants (Galvin et al., 1996). In Xenopus FGFR2, mutation of either of these Cys residues results in FGF-independent signaling, as measured by induction of mesoderm in animal pole explants (Neilson and Friesel 1995; Neilson and Friesel 1996). Furthermore, the mutant receptors form covalent homodimers, exhibit increased tyrosine autophosphorylation, and are unable to bind ligand. These data suggest that destruction of one of the paired Cys residues not only disrupts normal folding of the third Ig domain, but also mimics ligand binding by constitutively dimerizing the receptor.

There are numerous examples of mutations within the third Ig domain of FGFR2 that cause craniosynostosis syndromes yet do not directly alter Cys residues. Some of these mutations, including Trp290Gly and Thr341Pro, involve residues that are predicted to play an important structural role in the correct folding of the Ig domain . These mutations were also demonstrated to result in ligand-independent FGFR2 activation and receptor dimerization (S. C. Robertson and D. J. Donoghue, unpublished data), presumably by destabilizing the disulfide-bond that normally forms within the third Ig domain, instead allowing disulfide bond formation to occur between receptor monomers.

Mutations in the conserved Arg-Ser-Pro tripeptide in the Ig2-Ig3 linker region of FGFR1, FGFR2 and FGFR3 have also been observed in craniosynostosis and dwarfism syndromes (see **Figures 2** and **3**). Some of these mutations have been examined in the context of chimeric receptors, where the extracellular domain is derived from FGFR and the intracellular domain is derived from the proto-oncogene Neu. In these constructs, signaling through the Neu tyrosine kinase is used as reporter for receptor activation, and results in the formation of transformed foci in NIH 3T3 fibroblasts, which can be easily scored. TDI mutations that create unpaired Cys residues in the Ig2-Ig3 linker region of FGFR3, including Arg248Cys and Ser249Cys, have been engineered in FGFR3-Neu chimeric receptors and shown to cause ligand-independent signaling through the Neu tyrosine kinase

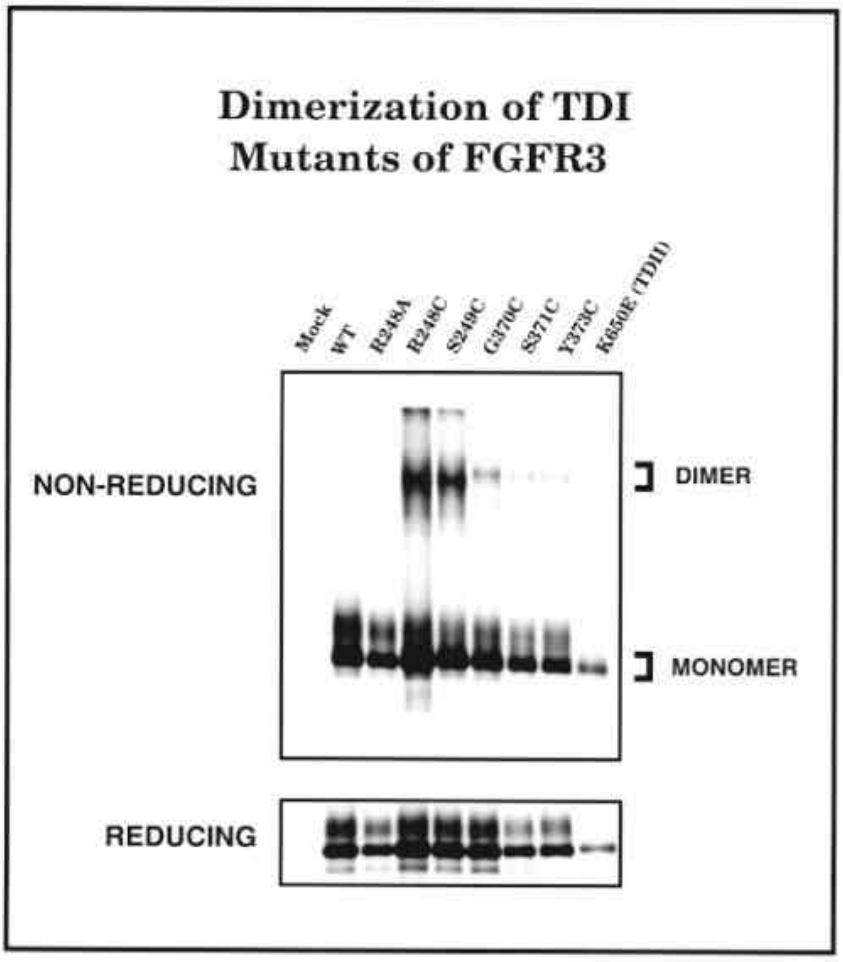


Figure 5. Ligand independent dimerization of TDI mutants of FGFR3.

Lysates from [35S]-labeled cells were immunoprecipitated and electrophoresed under nonreducing and reducing conditions through SDS-PAGE gradient gels. Proteins were transferred to nitrocellulose and immunoblotted using FGFR3 antiserum. (Top) Non-reducing gel. The positions of dimeric and monomeric forms of FGFR3 are indicated. (Bottom) Reducing gel. Monomeric FGFR3 is shown, indicating equivalent expression of all constructs.

(d'Avis et al., 1997). **Figure 4** presents a typical transformation assay of chimeric FGFR3/Neu receptors carrying TDI mutations. When examined in the context of full-length FGFR3, mutations such as the TDI mutations Arg248Cys and Ser249Cys also lead to constitutive receptor dimerization, which can be readily observed by SDS-PAGE under non-reducing gel conditions, as shown in **Figure 5**. A control mutation, Arg248Ala, does not cause receptor activation or dimerization, implying that it is the introduction of a novel disulfide bond, as opposed to an alteration of the structure in this region, that is responsible for TDI. The consequences of other interlinker mutations to non-Cys residues in craniosynostosis disorders are still unknown. However, in contrast to Ig3 domain mutations, Ig2-Ig3 linker domain mutations apparently do not abolish FGF binding (Neilson and Friesel, 1996; Naski et al., 1996). Perhaps these mutations affect the sensitivity or specificity of the receptor to small amounts of ligand, which could also play a role in their abnormal regulation in vivo.

IX. Transmembrane and extracellular juxtamembrane domain mutations

The introduction of an Arg residue into the normally hydrophobic transmembrane domain of FGFR3 has been shown to be responsible for the vast majority of cases of achondroplasia (Rousseau et al., 1994; Shiang et al., 1994). Interestingly, this Gly380Arg mutation is in an analogous position to the Val664Glu mutation that activates the proto-oncogene Neu (Bargmann et al., 1986). **Figure 6** presents an alignment of the transmembrane domain sequences of FGFR3 and Neu, showing the location of activating mutations. Activation of Neu has been proposed to involve stabilization of the receptor in a dimeric conformation due to hydrogen bond formation, leading to elevated receptor tyrosine kinase activity and cellular transformation (Sternberg and Gullick 1989). It was demonstrated that the achondroplasia mutation has similar functional consequences, as evidenced by the fact that substitution of the transmembrane domain of Neu with the transmembrane domain of mutant human FGFR3 causes ligand-independent signaling through Neu (Webster and Donoghue, 1996). Consistent with this model, resi-

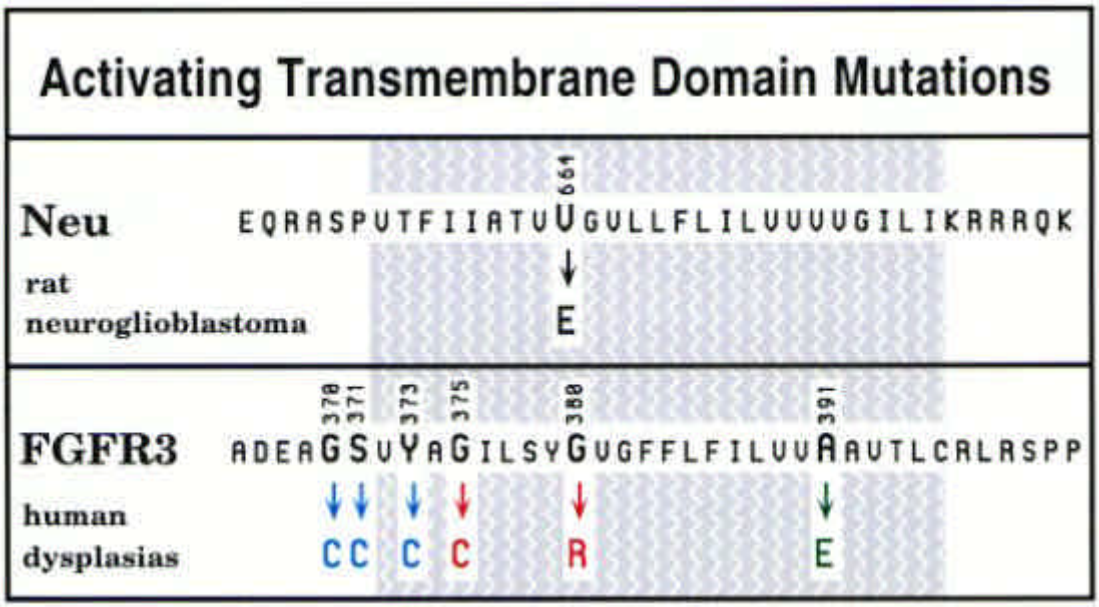
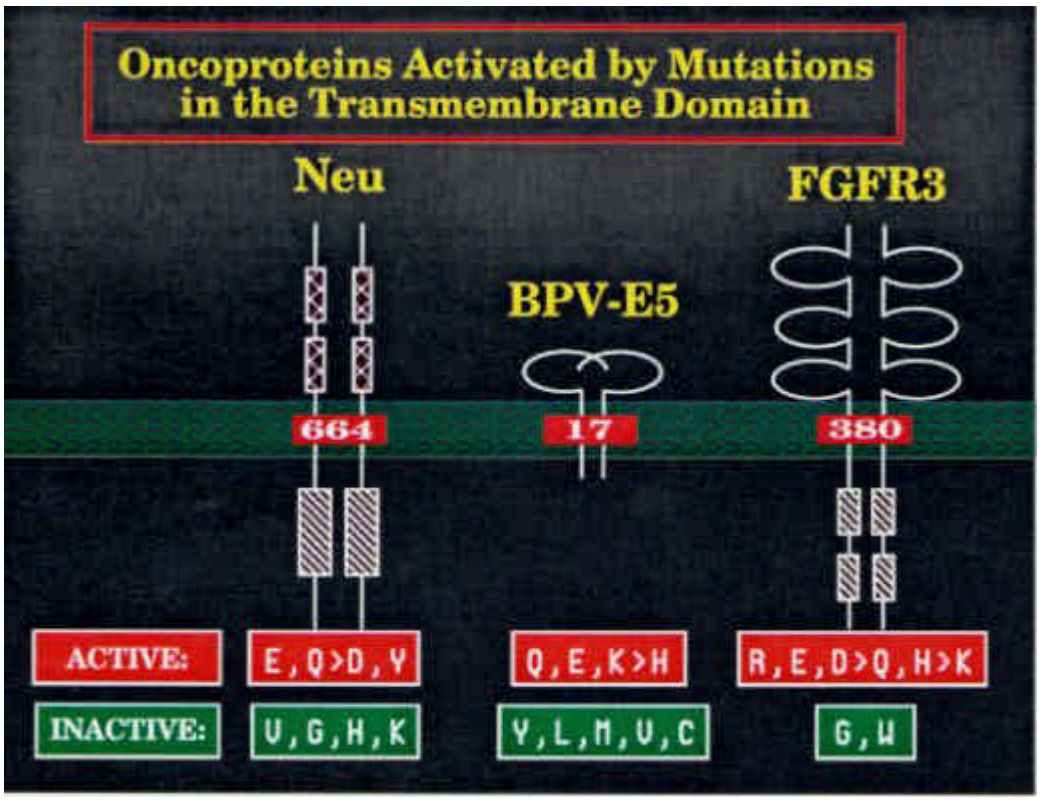


Figure 6. Activating mutations in the FGFR3 transmembrane domain. An alignment of the transmembrane domains (gray) of rat Neu and human FGFR3 is presented, showing the location of activating mutations. The mutation in the Neu oncogene (associated with rat neuroglioblastoma) is shown in black. The mutations in FGFR3 giving rise to thanatophoric dysplasia type I are shown in blue, achondroplasia in red, and Crouzon syndrome with acanthosis nigricans in green.

Figure 7. Oncoproteins and receptors activated by mutations in the transmembrane domain. Amino acids are shown that allow activation of p185c-neu when substituted at residue 664, activation of BPV-E5 when substituted at residue 17, and activation of FGFR3 when substituted at residue 380. Those substitutions that allow activation in these three different systems share the property that they are strongly polar, in an otherwise hydrophobic membrane environment, and thus share the ability to participate in hydrogen bond formation that may stabilize dimer formation.



dues with side chains capable of participating in hydrogen bond formation, but not hydrophobic residues, also activated the chimeric receptor at this position. Additionally, the Gly380Arg mutation activated ligand-independent kinase activity of full-length FGFR3 (Naski et al., 1996; Webster and Donoghue, 1996). **Figure 7** presents the mutations that occur within the transmembrane domain of three different oncoproteins or receptors which can result in activation (Chen et al., 1997). The activating mutations in these cases are all polar residues capable of participating in hydrogen bond formation to stabilize receptor dimers.

A dramatically different phenotype, Crouzon syndrome with the associated skin disorder, acanthosis nigricans, results from a Ala391Glu substitution just 11 residues away from the site of the principal achondroplasia mutation in the transmembrane domain of FGFR3 (Meyers et al., 1995) We have observed that this mutation is also activating in the context of the chimeric system described above (Chen et al., 1997), presumably by stabilization of dimers due to hydrogen bonding. It will be of interest, however, to determine whether this mutant FGFR3 in some way affects signaling through FGFR2, as the phenotype of this disorder is much more characteristic of craniosynostosis syndromes resulting from FGFR2 mutations than of dwarfism syndromes typically arising from FGFR3 mutations.

A number of mutations resulting in the creation of Cys residues at the junction of the extracellular and transmembrane domains of FGFR3 warrant mention. Substitution by Cys at residues 370, 371 and 373 of FGFR3 has been observed in the lethal dysplasia, TDI, whereas a Gly375Cys mutation, just two residues away, is found in rare instances of achondroplasia. It has recently been demonstrated that these juxtamembrane TDI mutations result in the formation of stable, disulfide-linked receptor dimers and induce high levels of expression of a c-fos-luciferase reporter construct (d'Avis et al., 1997). In contrast, it is possible that the milder achondroplasia mutation, occurring more deeply within the lipid bilayer of the cell, might result in the formation of weaker dimers and thus less pronounced signaling through FGFR3. Indeed, in a chimeric system involving the extracellular domain of the platelet-derived growth factor receptor, and the transmembrane and intracellular domains from the Gly375Cys mutant FGFR3, these receptors did not signal in a ligand-independent fashion, although they were responded more rapidly and robustly to ligand (Thompson et al., 1997).

X. Kinase domain mutations

As opposed to TDI, where a variety of mutations occur in the extracellular domain of FGFR3, all cases of TDII involve the point mutation, Lys650Glu, in the activation loop of the FGFR3 kinase domain. Several groups have demonstrated that expression of this mutant receptor in mammalian cells leads to strong, constitutive activation of the tyrosine kinase activity of the receptor (Naski et al., 1996; Webster et al., 1996), to a much greater extent than seen for the achondroplasia mutation, suggesting that there may be a correlation between the degree of receptor activation in vitro and the clinical severity of the phenotype. **Figure 8** presents an in vitro kinase assay of immunoprecipitated FGFR3 receptors, showing modest activation for the achondroplasia mutant compared with profound activation for the TDII mutant receptors.

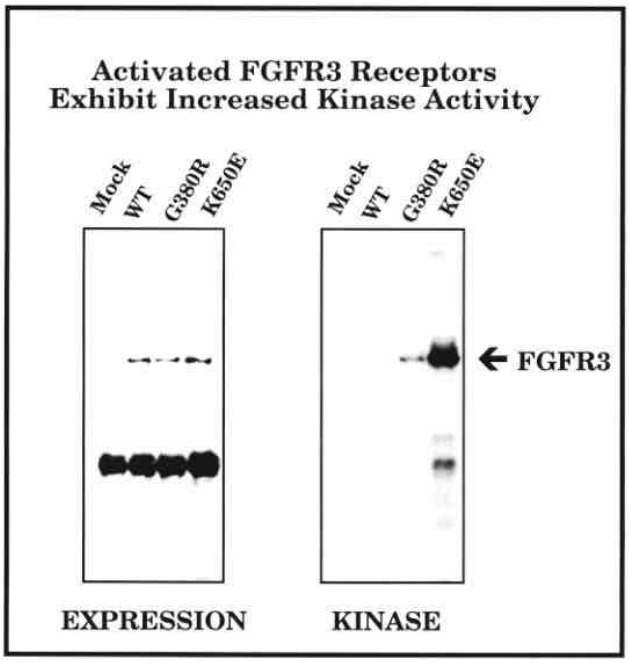


Figure 8. In vitro kinase activity of FGFR3 mutants causing achondroplasia and TDII.

The TDII mutant FGFR3 is constitutively active as a tyrosine kinase. NIH3T3 cells transiently expressing either a vector control (Mock), wild-type FGFR3, the Gly380Arg mutant causing achondroplasia, or the Lys650Glu mutant causing were lysed and immunoprecipitated with FGFR3 antiserum. (Left) Immunoblot of immunoprecipitates with FGFR3 antiserum followed by horseradish peroxidase-conjugated secondary antiserum and ECL development, showing comparable levels of receptor expression. (Right) Autophosphorylation assay. Immunoprecipitates were subjected to in vitro kinase reactions in the presence of $\gamma[^{32}P]$-ATP, and analyzed by SDS-PAGE and autoradiography. Cells expressing the TDII mutant receptor construct exhibited significantly increased autophosphorylation relative to the achondroplasia mutant.

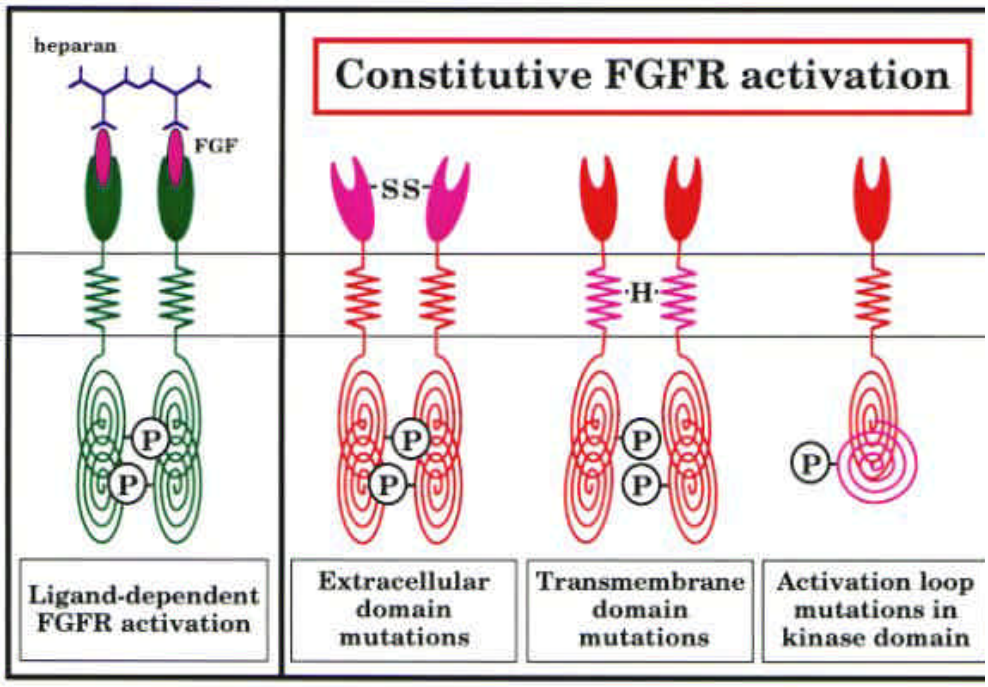


Figure 9. Model for the effect of point mutations on FGFR function.

(**a**) Normal ligand-dependent activation leads to regulated signals for proliferation and differentiation of bones. (**b, c, d**) Certain extracellular domain mutations activate the receptors through the formation of aberrant disulfide bonds, indicated by S-S, leading to constitutive dimerization. Transmembrane domain mutations result in hydrogen (H)-bonded FGFR dimers. Mutations in the activation loop of the kinase domain result in conformational changes that activate receptor tyrosine kinase activity. Constitutive signaling through inappropriately activated FGFRs results in premature maturation of the bones of the skeleton and cranium.

Substitutions at position 650 and at neighboring positions indicate that the Lys650Glu mutation mimics the activating conformational changes that normally accompany autophosphorylation at conserved Tyr residues within the activation loop (Webster et al., 1996). Recently, a second mutation at this residue, Lys650Met, has been identified in Skeletal-Skin-Brain Dysplasia (SSBD) (Tavormina et al., 1997), a severe disturbance in endochondral bone growth and neural and skin development. Despite the involvement of the same residue as that mutated in TDII, and similarly high levels of in vitro receptor activation (Tavormina et al., 1997), there is no observed cloverleaf skull in SSBD, and usually this disorder is not lethal.

A mutation that results in the substitution Asn540Lys in the kinase domain of FGFR3 causes a mild form of dwarfism, hypochondroplasia (Bellus et al., 1995). The prediction would be that this mutation is also able to activate the receptor in a ligand-independent fashion, but probably to a lesser degree than other activating mutations, based on the relative severities of the associated skeletal disorders. The crystal structure of the tyrosine kinase domain of FGFR1 in the inactive conformation was recently solved, which suggested that Asn540 is normally hydrogen bonded to His535 (Mohammadi et al., 1996b). Disruption of this bond would thus be predicted to stabilize the active conformation of the receptor.

XI. Relevance of FGFR mutations in skeletal disorders to human cancers

Although patients with skeletal dysplasias caused by activating FGFR germ-line mutations do not have an apparent increase in tumor frequency, enhanced signaling through FGFRs has been implicated in tumor progression. For instance, amplification or ectopic expression of genes encoding FGFs and FGFRs has been found in neoplastic cells (Adnane et al., 1991; Hattori et al., 1990; Kobrin et al., 1993; Yamanaka et al., 1993; MacArthur et al., 1995; Delli Bovi et al., 1987; Goldfarb et al., 1991; Marics et al., 1989), and overexpression of FGFs results in morphological transformation of cells co-expressing FGFRs in vitro. (MacArthur et al., 1995; Delli Bovi et al., 1987; Goldfarb et al., 1991; Marics et al., 1989). Interestingly, some of the identical activating mutations as those found in the severe skeletal dysplasias TDI, TDII and SSBD have recently been identified in human multiple myeloma (Chesi et al., 1997). In these tumors and cell lines, a translocation leading to the juxtaposition of FGFR3 near the IgH switch region was observed, resulting in the selective overexpression of the mutant FGFR3 allele. We have recently confirmed that a highly activated kinase-domain derivative of FGFR3 (Lys650Glu) can transform NIH3T3 fibroblasts, suggesting a causative role for activated FGFR3 in the development of certain cancers (Webster and Donoghue, 1997).

Why, then, do activating germ-line mutations in FGFR3 cause defects in skeletal, cranial and skin development, rather than cancers? Perhaps in these affected tissues, FGFR3 activation is coupled to signaling pathways leading to differentiation or growth arrest, rather than proliferation. For instance, constitutive activation of STAT1 and elevated expression of the cell cycle inhibitor p21 WAF1/CIP1 has been observed in cartilage cells from a TDII fetus but not a normal fetus (Su et al., 1997). In other cells from TDII patients, such as fibroblast and lymphoid cells where FGFR3 signaling may be coupled to mitogenesis, the level of expression of the receptor may not be sufficient to stimulate the unregulated proliferation necessary for tumor development, consistent with our observation that only greatly enhanced levels of signaling result in morphological transformation of cells (Webster and Donoghue, 1997).

XII. Perspectives and future directions

The recognition that constitutive FGFR activation appears to underlie many human dwarfism and craniosynostosis disorders is an important first step in understanding the role of FGFRs in normal human development. As described above, this activation may occur by a number of distinct mechanisms, depending on which structural domain of the receptor is involved. These different mechanisms are shown schematically in **Figure 9**.

A number of important questions remain, however, and addressing these questions will provide exciting challenges to molecular and developmental biologists for many years to come. For instance, it is unclear how phenotypically similar craniosynostosis syndromes can arise from mutations encoding three different FGFRs (and splice variants thereof), with different spatial and temporal patterns of expression. This observation implies a certain overlap in function of FGFR1, FGFR2 and FGFR3 during development, and might additionally suggest an ability of one receptor to affect signaling through a heterologous receptor. In fact, one study suggests that mutant FGFR alleles may also function in a dosage-dependent dominant-negative fashion to inactivate ligand-dependent signaling from wild-type FGFR alleles (Nguyen et al., 1997).

Constitutive activation as a result of FGFR mutations can also not fully explain why substitutions at neighboring or identical residues within the same receptor result in clinically distinct syndromes. The heterogeneity of phenotypes observed in different individuals with similar mutations probably reflects, to some extent, the absolute degree of receptor signaling, but additionally suggests that other genes are involved that modulate the effects of mutated FGFRs. In particular, subtle differences between individuals in expression of ligands, other FGFR heterodimerization partners, and downstream effectors might ultimately determine the severity of the phenotype and the precise tissues affected.

Finally, studies described here imply a normal role for FGFRs in restraining premature maturation at the growth plates of long bones and at the sutures of the skull. It is as yet unknown at which of the highly regulated steps of proliferation and differentiation constitutive FGFR activation acts to disrupt normal bone maturation. It will be very useful to develop transgenic mice expressing mutant FGFR proteins to help define the normal roles of FGFRs in skeletal and cranial development. Such in vivo systems will allow the examination of developmental changes specifically due to mutant receptors, in the context of the normal complement of heterologous FGFRs, ligands, and effectors, which is a limitation in the interpretation of data from current in vitro assay systems.

As opposed to certain genetic skeletal disorders which appear to be due to loss-of-function mutations, such as Saethre-Chotzen syndrome (El Ghouzzi et al., 1997; Howard et al., 1997) and campomelic dysplasia (Wagner et al., 1994; Foster et al., 1994), the craniosynostosis and dwarfism syndromes discussed in this review are due, at least in part, to gain-of-function mutations in FGFRs. As such, gene replacement therapy is not expected to be of consequence in the treatment of these disorders in the foreseeable future. Nonetheless, an ability to correlate specific FGFR mutations with particular phenotypic consequences, arising from research described in this review, is already proving useful for diagnostic and genetic counseling purposes.

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