[Frontiers in Bioscience 3, d781 -794, August 1, 1998]

FGF SIGNALING IN SKELETAL DEVELOPMENT

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Received12/22/98 Accepted 2/17/98

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1. ABSTRACT

The fibroblast growth factor receptor family consists of four receptor tyrosine kinases which bind with varying affinity and specificity to a family of at least fifteen polypeptide ligands. The receptors and ligands perform many essential functions during growth, development and repair. Recent discoveries show that a growing number of skeletal abnormalities result from mutations in the fibroblast growth factor receptors. These findings have led to a greater understanding of the role of fibroblast growth factor signaling during skeletogenesis and have focused research interests on the effects of fibroblast growth factors on endochondral and intramembranous bone development.

2. INTRODUCTION

The fibroblast growth factors (FGFs) comprise a family of at least fifteen structurally related proteins (reviewed in (1-5). FGFs were first described in the 1970s as an activity that stimulated the proliferation of NIH 3T3 cells. Since that time, FGFs have been shown to support the proliferation of a variety of both mesenchymal and epithelial cells (2). In addition, FGFs are potent regulators of cell migration (6) and differentiation (2). Animal studies have proven that FGFs are required for diverse developmental processes, including inner ear and tail development (7), hair follicle maturation (8, 9), early embryogenesis (10) and skeletal growth and differentiation (11-13).

The FGF receptors are a family of four receptor tyrosine kinases encoded by four distinct genes (14-21). Each receptor recognizes a unique subset of the FGF family of ligands (22). Alternative splicing of the extracellular domain expands the repertoire of ligands that can be recognized by each receptor, while restricting the repertoire of ligands recognized in specific tissues (23-25). The patterns of receptor expression and alternative splicing match ligand binding specificity with proximally expressed ligands (26, 27). During embryonic development, receptor-ligand pairs are often matched across epithelial-mesenchymal boundaries. This permits the inductive effects FGFs to be targeted to neighboring tissues and thereby regionally instruct cells to proliferate, migrate or differentiate. Receptor-ligand interactions are further modified by heparan sulfate proteoglycans (HSPG). These molecules have been shown by a variety of studies to be required for ligand binding in vitro (20, 28, 29) and appear to be required for the formation of an active receptor complex. This hierarchy of regulation indicates that the specific ligand-receptor interactions of a given cell are determined by i) expression of one or multiple FGF receptors, ii) alternative splicing of the receptor mRNA iii) interactions of the receptor and ligand with heparan sulfate proteoglycans and iv) the ligands present in the cellular environment.

2.1 FGF receptor alternative splicing

FGF receptors are members of the receptor tyrosine kinase superfamily (30). These proteins consist of an extracellular ligand binding domain, a single transmembrane domain and an intracellular tyrosine kinase domain. The FGF receptor extracellular region contains

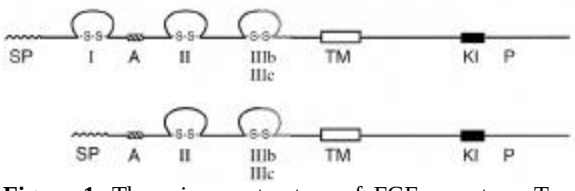

Figure 1. The primary structure of FGF receptors. Top: Full-length FGF receptor with three Ig-like domains. The major alternative splicing pathways will express either Ig-like domain IIIb or IIIc. The stippled region beginning in Ig-like domain III is the sequence subject to alternative splicing. Bottom: Short form of the receptor expressing Ig-like domain II and III. SP, signal peptide; A, acidic region; I, II, III, Ig-like domains; TM, transmembrane domain; KI, kinase insert; P, putative site of autophosphorylation; s-s, disulfide bond.

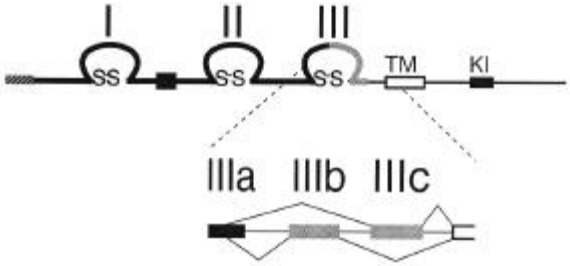

Figure 2. Alternative splicing of FGF receptors in the immunoglobulin-like domain III region. Alternatively spliced exons IIIa, IIIb, IIIc are shown. Abbreviations as in figure 1.

three immunoglobulin-like (Ig-like) domains, a heparin binding domain and a stretch of seven conserved acidic amino acids (figure 1) (14). A hierarchy of alternative mRNA splicing events determine the number of Ig-like folds in the extracellular domain, as well as the ligand binding properties of the receptor. One major splicing event excises the exons encoding the amino-terminal Ig-like domain (domain I) leading to a form of the receptor with two Ig-like domains (figure 1) (31). The ligand binding properties of both the full length (three Ig-like domain) and truncated (two Ig-like domain) receptor appear to be similar (32). Additional RNA splicing events regulate the use of two mutually exclusive exons and result in two alternative versions of Ig-like domain III (IIIb or IIIc) (23, 24, 31). The genomic DNA encompassing the carboxy-terminal half of Ig-like domain III in FGF receptors 1, 2 and 3 is remarkably conserved both in the number of exons and the arrangement of the intron/exon boundaries (23, 33-35). Alternative splicing of these exons results in either IIIb or IIIc isoforms of the FGF receptor (figure 2), dramatically effecting the ligand-receptor binding specificity (23, 25). Alternative splicing is regulated in a tissue-specific manner (36-38). Utilization of either the "b" or "c" exon is dependent upon the identity of the cell which synthesizes the mRNA. The "b" exon appears to be expressed in epithelial lineages while the "c" exon is expressed in mesenchymal lineages (35, 36, 38, 39).

2.2 Glycosaminoglycan interactions

FGFs bind the glycosaminoglycans, heparin and heparan sulfate (1, 40). Heparan sulfate proteoglycans (HSPGs) are located on the cell surface and within the extracellular matrix and serve as "low affinity" (Kd 10-9M), high capacity binding sites for FGF (40). The interaction of FGF with HSPGs has been established by demonstrating decreased binding of FGFs to cells deficient in cell-surface heparan sulfate (20, 41, 42). Additionally, treating cells with heparin degrading enzymes or with inhibitors of glycosaminoglycan sulfation inhibits the binding of and response to FGFs (29, 43). The affinity of FGFs for heparin-like molecules may significantly limit the diffusion and release of growth factor into interstitial spaces (40, 44). FGFs may therefore exert their effects very close to their site of production, making the spatial and temporal patterns of expression of FGFs and FGF receptors an important biological regulatory mechanism.

Heparin and heparan sulfate proteoglycans bind directly to FGFs and FGF receptors and thereby modulate the activation of the FGF receptor. Cells that express FGF receptor 1 and are deficient in HSPGs, require heparin in the binding media for high affinity FGF binding (Kd 2-20x10-11M) (20, 29). Furthermore, heparin is required for FGF to bind to a soluble FGF receptor in a cell-free system and for FGF to activate its receptor when expressed in growth factor (interleukin 3) dependent lymphoid cell lines (22, 23, 28, 45). The mechanism by which FGF interacts with its receptor may involve the formation of a low affinity complex between FGF and the FGF receptor which can then be stabilized by heparin. The increase in affinity between FGF and the FGF receptor in the presence of heparin is estimated to be approximately 4-10 fold (46, 47).

2.3 FGF receptor Mutations in Human Disease

The essential role for FGF receptor signaling in the regulation of skeletal development has been accentuated by studies of human genetic diseases. Recently, several human skeletal dysplasias have been linked to point mutations in the genes encoding FGF receptors 1, 2 and 3 (figure 3). These disorders can be broadly classified into two groups: 1) the dwarfing chondrodysplasias, including hypochondroplasia (HCH) (48), achondroplasia (ACH) (49-52), thanatophoric dysplasia (TD) (53-56), and 2) the craniosynostoses, including Crouzon syndrome (CS) (57-67), Pfeiffer syndrome (PS) (58, 60, 64, 68, 69), Jackson-Weiss syndrome (JWS) (59, 60, 63), Beare-Stevenson cutis gyrata (70), Apert syndrome (AS) (71) and a nonsyndromic craniosynostosis (72). All of the mutations are autosomal dominant and frequently arise sporadically. The great majority of these disorders result from point mutations in the coding sequence of the receptor that result in a single amino acid substitution (figure 3).

The dwarfing conditions, HCH, ACH and TD result from dominant mutations in the FGF receptor 3 gene. HCH is a mild and relatively common skeletal disorder with clinical features similar to that of ACH. ACH is the most common form of genetic dwarfism. ACH is characterized by shortening of the proximal and, to a lesser extent, distal long bones. The cranium of ACH patients is characterized by frontal bossing, and the face is characterized by a depressed nasal bridge. Rare

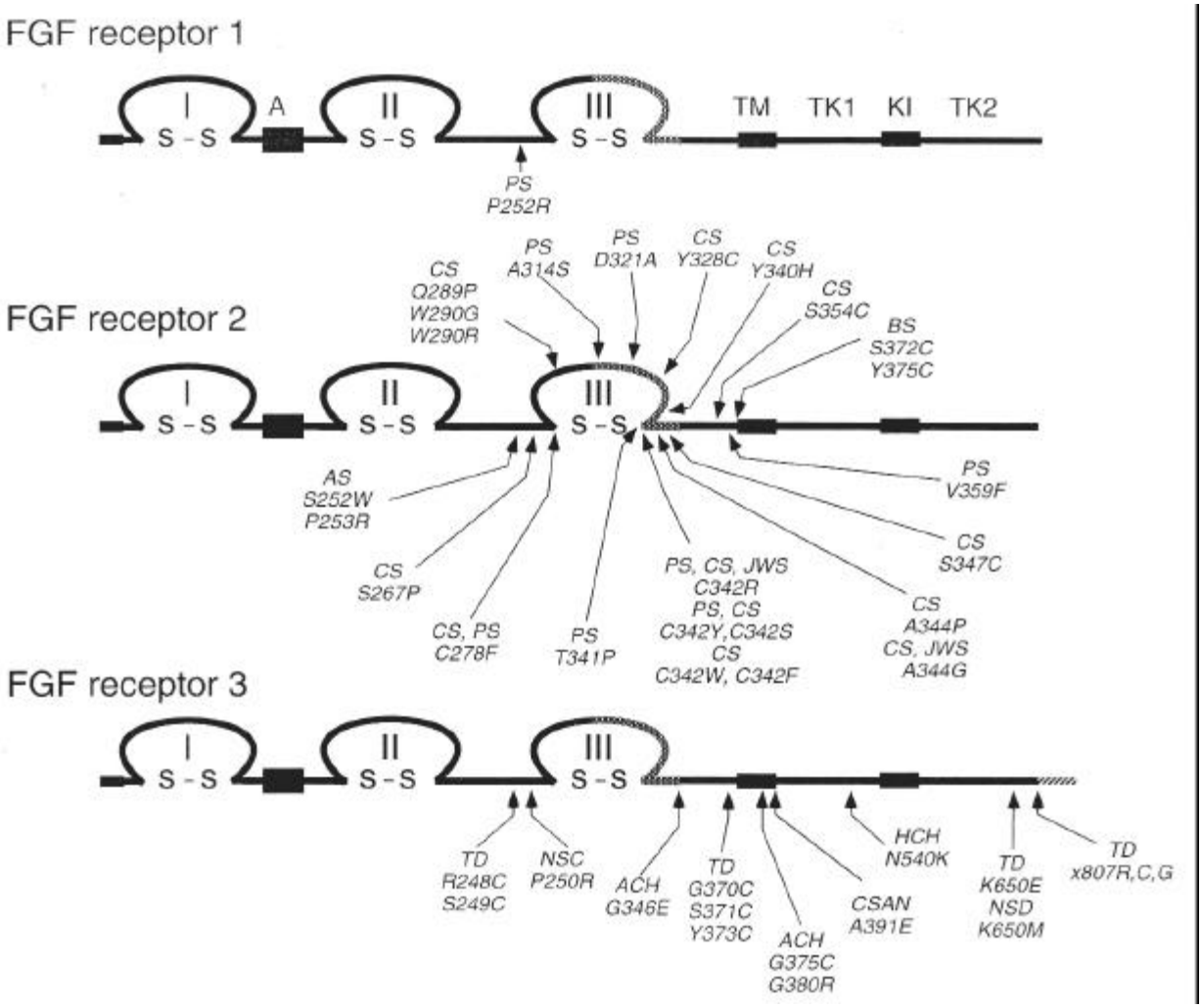

Figure 3. Mutations in the FGF receptor genes in human skeletal diseases. Top: FGF receptor 1 showing a single point mutation causing Pfeiffer syndrome (PS). Abbreviations as in Figure 1. Middle: FGF receptor 2 showing the mutations responsible for Crouzon syndrome (CS), Jackson-Weiss syndrome (JWS), Pfeiffer syndrome (PS), Apert syndrome (AS) and Beare-Stevenson cutis gyrata (BS). Bottom: FGF receptor 3 showing the mutations responsible for achondroplasia (ACH), thanatophoric dysplasia (TD), hypochondroplasia (HCH), Crouzon syndrome & acanthosis nigricans (CSAN) and a non-syndromic craniosynostosis (NSC). The stippled line attached to the end of FGF receptor 3 represents an extension of the protein resulting from mutations in the stop codon of the receptor. The numbers represent the position of the amino acid in the coding sequence for the human receptor. Amino acids are abbreviated using standard single letter abbreviations.

homozygous cases of ACH usually result in neonatal lethality (73). These individuals have features similar to that of TD. TD results from several dominant mutations in the FGF receptor 3 gene. TD is the most common lethal-neonatal skeletal disorder and is clinically similar to homozygous ACH (73).

PS, CS, JWS, BS and AS are clinically distinct syndromes characterized by craniosynostosis (premature closure of the cranial sutures) and distinct facial features. In addition, PS, JWS, BS and AS have variable phenotypes in the distal extremities consisting of syndactyly of the hands and feet or broad thumbs and big toes. In general, the mutations affecting craniofacial development have resulted from mutations in FGF receptors 1 or 2. Recently however, mutations in FGF receptor 3 have also been shown to cause CS and a non-syndromic craniosynostosis (66, 72) (for review of the FGF receptor mutations and the corresponding clinical abnormalities see (74, 75)).

3. LIMB DEVELOPMENT

3.1 FGF signaling and limb initiation

The first step in the development of the vertebrate limb is the initiation of a site in the flank of the embryo where the presumptive limb will grow. Foil barrier and tissue transplant studies of early chick limb development have suggested the existence of a diffusible substance that regulates the initiation of limb outgrowth (76). Subsequent studies, placing an FGF soaked bead on the flank of the

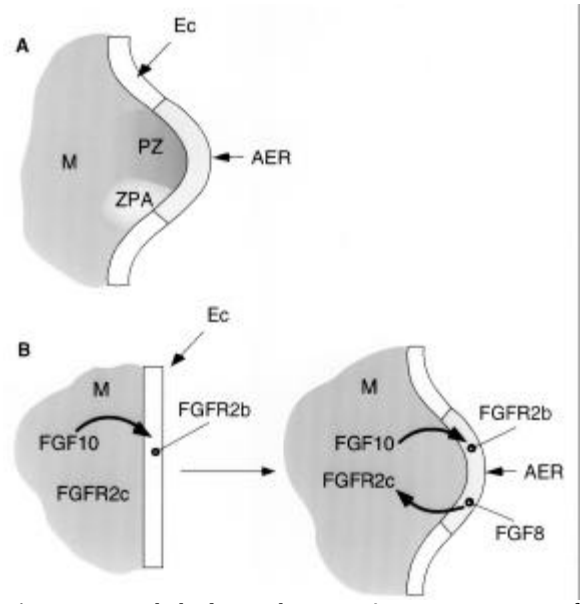

Figure 4. Limb bud *Development* A, Representation of early limb development following the formation of the apical ectodermal ridge (AER), zone of polarizing activity (ZPA), and progress zone (PZ). M, limb bud mesenchyme and Ec, surface ectoderm. B, Model for the initiation of the limb bud. Ec, surface ectoderm where FGF receptor 2 IIIb (FGFR 2b) and FGF 8 are expressed. M, mesenchyme wherein FGF 10 and FGF receptor 2 IIIc (FGFR 2c) are expressed.

embryo, demonstrated that FGFs could induce the formation of a limb, suggesting that FGFs may be the diffusible element responsible for initiating the limb field (77-79). Several different FGFs, including FGF 2, 4, 8 and 10, applied to the flank of the embryo can initiate the pathway(s) leading to limb development (77-80). This raises the question as to which FGF(s) functions in vivo to initiate the site of limb formation. Recent studies of chick limb development showed that FGF 8 and FGF 10 are expressed temporally and spatially in a manner consistent with their function as the endogenous initiators of limb development (79). FGF 10 is localized to the lateral plate mesoderm at the site of the presumptive limb bud in chick (79) and mouse (26). The expression of FGF 10 precedes that of FGF 8 suggesting that FGF 10 may be the primary initiator of the limb bud.

3.2 FGF signaling and limb growth

FGFs also perform essential functions during the progressive outgrowth and patterning of the limb bud. The limb bud is a specialized structure consisting of three functionally and spatially defined domains (figure 4). The apical ectodermal ridge (AER) is a specialized thickening of epithelium at the tip of the growing limb. It functions to stimulate elongation of the limb and maintain signals required for patterning the limb. FGFs 2, 4, and 8 are expressed in the AER (81-84). The progress zone (PZ) is a domain of undifferentiated mesenchymal cells, expressing FGF 10, that lie beneath the AER. The proliferative cues elaborated by the AER stimulate the PZ and maintain the cells in an undifferentiated state. The zone of polarizing activity (ZPA) is a region of posterior mesenchyme, adjacent to the AER that functions as a molecular compass; providing spatial cues to orient the growing limb with respect to the anterior-posterior axis, resulting in the stereotyped anatomy of the limb. These three functional domains operate coordinately and interdependently. Epithelial-mesenchymal signaling, mediated by FGFs and their receptors, is essential for establishing and maintaining these specialized domains.

FGF 10 is expressed in the lateral plate mesoderm and later in the limb mesenchyme and acts to initiate and maintain FGF 8 expression in the AER. Reciprocally, FGF 8 maintains expression of FGF 10 in the underlying mesenchyme (79). The proliferation of cells within the PZ and the subsequent outgrowth of the limb results from the actions of FGFs produced in the AER. This has been demonstrated by experiments showing that when the AER is excised from the growing limb, the limb becomes truncated in proportion to the developmental stage that the AER is removed (85-87) and that growth can be restored by replacing the AER with a source of FGF placed at the tip of the growth arrested limb (78, 88, 89). Establishment of the ZPA and the interdependence of the ZPA and AER also requires an FGF signal. Sonic hedgehog (SHH) is the molecular determinant of the ZPA that induces signaling cascades that control the anterior-posterior spatial orientation of the limb (90 , 91). Initiation of SHH expression is thought to require FGF. Removal of the posterior AER, and thus FGF 4 and FGF 8 in sites overlying the ZPA, results in a loss of SHH expression (92). Conversely, ectopic expression of SHH expands the expression of FGF 4 (92, 93). Thus, feedback signaling pathways exist between FGF4 in the AER and SHH in the ZPA. These signals maintain the AER and ZPA and are thus essential for both limb elongation and patterning.

3.3 FGF receptors and limb development

FGF signals in the growing limb are mediated by high affinity FGF receptors. The specific receptors and their splice variants expressed in the developing limb match the ligand binding specificity of the receptor with the ligand expected to act at that site. For example, FGF 10 is expressed in the mesenchyme underlying the AER and is proposed to maintain and initiate formation of the AER (79) (figure 4B). In support of this, the AER expresses the IIIb splice form of FGF receptor 2 (37), a receptor that can be efficiently activated by FGF 10 (94). FGF receptor 2 signaling is absolutely required for the initiation of the limb as evidenced by studies in which a presumptive null allele was introduced into the mouse Fgfr 2 gene by homologous recombination (26). In mice lacking normal FGF receptor 2 activity, development of the limb bud is completely abolished. This leads to a model for initiation of the limb, whereby FGF 10 induces expression of FGF 8 in the AER through its interaction with FGF receptor 2 IIIb. FGF 8, in turn, maintains FGF 10 expression in the mesenchyme through its interaction with FGF receptor 2 IIIc (figure 4B). Limb bud mesenchyme also expresses FGF receptor 1 (95). However, in vitro FGF 10 activates FGF receptor 1 IIIb poorly and FGF receptor 1 IIIc not at all (22). Therefore,

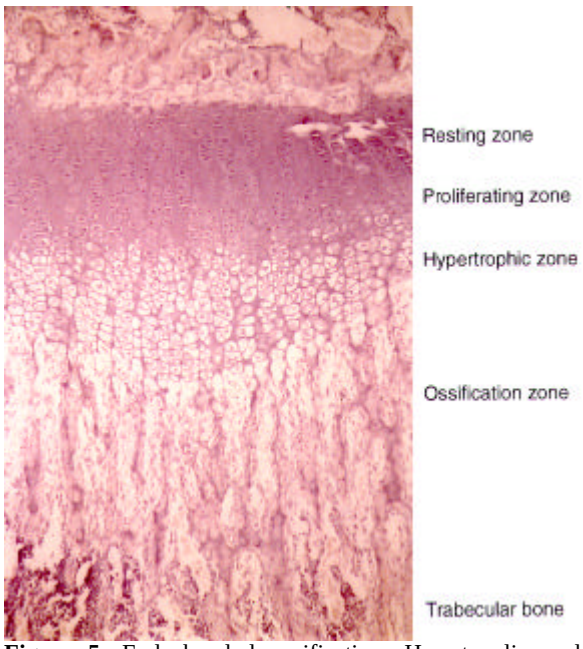

Figure 5. Endochondral ossification. Hematoxylin and eosin stained section of the epiphyseal growth plate from the proximal tibia of a two week old mouse.

FGF receptor 1 does not appear to be the target of FGF 10. Targeted inactivation the IIIc isoform of FGF receptor 1 is lethal during early embryogenesis (96). In contrast, using the same targeting strategy to delete the IIIb splice form yields a mouse that is viable and fertile (96), indicating that the IIIc isoform is required for most of the functions of FGF receptor 1 and is presumably the splice form present in the limb mesenchyme. Using this guideline, FGF 4 and 8 expressed in the AER act on the underlying mesenchyme to activate FGF receptor 1 IIIc and FGF receptor 2 IIIc, thereby promoting the elongation of the limb.

The significance of FGF receptor signaling in the developing limb is accentuated by the finding that certain human skeletal disorders with abnormalities of the digits result from mutations in FGF receptors. Apert syndrome and Jackson-Weiss syndrome result from mutations in FGF receptor 2 (58, 71). These syndromes are characterized by premature fusion of the cranial sutures and syndactyly of the hand and feet. Pfeiffer syndrome, which is characterized by broad great toes and thumbs in addition to premature fusion of cranial sutures, results from a single mutation in FGF receptor 1 or one of several mutations in FGF receptor 2 (60, 68, 69). Jackson-Weiss and Pfeiffer syndrome can result from several different mutations in FGF receptor 2 (74), including mutations of Cys342, a conserved residue in the third Ig-like fold of the receptor (figure 3). This residue is believed to normally be bound in a disulfide linkage that stabilizes the Ig-like domain. Substitution of other amino acids at this site (as in Jackson-Weiss and Pfeiffer syndromes) is then presumed to leave the opposing cysteine residue available for the formation of inter- rather than intramolecular disulfide bonds. Indeed, studies in Xenopus showed that mutation of this cysteine residue results in disulfide linked receptor homodimers and ligand-independent receptor signaling (97). Thus, these results indicate that some, and perhaps all, of the mutations causing Jackson-Weiss and Pfeiffer syndrome are the result of activating mutations in an FGF receptor.

Interestingly, the limb phenotypes of JWS and PS are restricted to the distal most portion of the limb. This implies that the outgrowth and patterning of the more proximal portions of the limb are insensitive to constitutive FGF receptor signaling and suggests that these portions of the limb are constructed under conditions of excess ligand. If so, a constitutively active receptor would be functionally equivalent to the normal conditions of proximal limb development where the receptor is fully activated by a saturating concentration of ligand. In contrast, the distal most limb appears to be more dependent on the degree of FGF receptor signaling. Here, the receptor may be functioning under conditions where the available ligand is limiting. Additional FGF receptor signaling supplied by an activated receptor would be expected to disrupt the normal growth and patterning of the digits. Recent fate mapping studies in chick limb development show that the digits are formed by cell populations originating in the mesenchyme arising just below the AER (98). A portion of these cells migrate to positions anterior to their site of origin, suggesting that altered FGF receptor signaling may perturb the migration of cells destined to form the digits. Interestingly, we and others have found that the S252W mutation of the Apert syndrome alters the ligand binding affinity of the receptor (75, 99). The increased affinity for ligand may result in enhanced activation of the receptor in mesenchymal cells destined to form the digits. This may lead to abnormal migration of cells toward a source of ligand which may result in aberrant mesenchymal condensations and, consequently, abnormal digits.

4. ENDOCHONDRAL OSSIFICATION

4.1 FGFs and cartilage

The pre- and postnatal development of long bones and vertebrae occurs via endochondral ossification. In this process a cartilaginous template is formed by mesenchymal cells which, under the control of regional morphogenetic and proliferative cues, coalesce and secrete an extracellular matrix that is essential for their differentiation. After the cartilaginous templates have been established, bone formation ensues from ossification centers that form in the center of long bones and proceed as a wave extending toward the two ends. This process begins in the primary ossification centers during embryonic development and is recapitulated during post-natal skeletal growth at the epiphyseal growth plates. During endochondral ossification, chondrocytes differentiate through a series of well-defined morphological zones within the epiphyseal growth plate (figure 5). A zone of proliferation provides a renewable source of chondrocytes for longitudinal bone growth. After exiting the cell cycle these maturing chondrocytes secrete a matrix composed of chondroitin-sulfate proteoglycans and type II collagen, as well as other matrix components. Encapsulated in this matrix, the chondrocytes undergo hypertrophy and

subsequently express type X collagen and alkaline phosphatase. Hypertrophic chondrocytes undergo an apoptotic death as their surrounding matrix is mineralized and replaced by trabecular bone (100).

The effects of FGFs on cartilage physiology have been studied both in vitro and in vivo. The addition of FGF 2 to chondrocytes cultured in soft agar, as well as monolayer culture, resulted in a dramatic increase in cell proliferation (101-103). In fact, when compared to other known mitogens for chondrocytes, FGF-2 was a more potent mitogen than insulin-like growth factor-1, transforming growth factor-beta (TGF-beta) and epidermal growth factor (101, 104). Cultured rabbit growth plate chondrocytes grown as a pelleted mass synthesize an abundant extracellular matrix, undergo cellular hypertrophy and initiate mineralization of the surrounding matrix. In certain respects, the pellet cultures recapitulate events of chondrocyte maturation in the epiphysis during endochondral ossification. The addition of FGF 2 to these cultures only modestly effects cell proliferation, but profoundly effects chondrocyte differentiation (11, 105). FGF 2 inhibited the terminal phase of chondrocyte differentiation as evidenced by a dramatic inhibition of both the rise in alkaline phosphatase activity and the deposition of calcium (11). Interestingly, the cultures became less sensitive to the effects of FGF 2 once the cells differentiated to hypertrophic chondrocytes. This was shown to correlate with the loss of FGF receptor expression in the terminally differentiated cells, as evidenced by the loss of binding of radiolabeled FGF 2 to the cells (105).

4.2 FGF receptors and endochondral ossification

FGF receptors 1 and 3 are expressed in the epiphyseal growth plate. FGF receptor 3 is expressed in proliferating chondrocytes, whereas FGF receptor 1 is expressed in hypertrophic chondrocytes (106, 107). FGF receptor 3 is also expressed in the cartilage of the developing embryo, prior to formation of ossification centers. The results of Iwamoto *et al*. (105) using a pellet culture system show that terminally differentiated chondrocytes do not bind FGF-2. These data suggest that FGF-2 has a low affinity for FGF receptor 1 or that matrix cofactors, required for receptor binding, are not present in hypertrophic chondrocytes (108). *In vitro* data indicate that FGF-2 binds avidly to FGF receptor 1 in the presence of heparin (22). Thus, the absence of binding of FGF 2 may reflect differences in FGF receptor expression in the pellet culture model compared to the growth plate, a lack of correlation between receptor expression determined by in-situ hybridization and synthesis of the protein, or modulation of ligand-receptor affinity during chondrocyte hypertrophy by extracellular matrix components. Expression of syndecan-3, a transmembrane glycosaminoglycan capable of binding and presenting FGFs to their receptor, is restricted to proliferating chondrocytes (108). The absence of this co-receptor in hypertrophic chondrocytes could explain the lack of a detectable interaction between FGF 2 and FGF receptor 1.

Data showing that FGF 2 inhibits the terminal differentiation of chondrocytes (105) in conjunction with the finding that FGF receptor 3 is expressed in proliferating but not hypertrophic chondrocytes suggests that FGF receptor 3 mediates the inhibitory effects of FGF 2 on chondrocyte differentiation. This model predicts that in the absence of FGF receptor 3 signaling, accelerated chondrocyte differentiation would occur. However, in FGF receptor 3 null mice the primary ossification centers form normally (109) and skeletal overgrowth ensues. These data suggest that the primary role for FGF receptor 3 may be to inhibit chondrocyte proliferation (106, 109). This is supported by recent data showing that FGF receptor 3 can induce the expression of cell-cycle inhibitors (110). Additionally, transgenic mice overexpressing FGF-2 are dwarfed (111), consistent with a role for FGF receptor 3 as an inhibitor of chondrocyte proliferation. The growth plate in FGF receptor 3 null mice showed an expansion of the hypertrophic zone, while in transgenic mice overexpressing FGF 2, a reduction of the hypertrophic zone was observed. This implies that FGF receptor 3 may also directly or indirectly regulate chondrocyte differentiation by altering the rate at which cells enter the hypertrophic phase. An interesting and as of yet unresolved issue is the identification of the endogenous ligand(s) for FGF receptor 3 in the epiphyseal growth plate. FGF 2 is a likely candidate given that it is present in the growth plate (112) and is a known ligand for FGF receptor 3 (22). Surprisingly, however, targeted disruption of the mouse Fgf 2 gene produces no gross or histological skeletal phenotype (113). Therefore, other FGFs in addition to FGF 2 must be present within the growth plate and may be functionally redundant with FGF 2.

Mutations in FGF receptor 3 cause the human dwarfing conditions achondroplasia, thanatophoric dysplasia and hypochondroplasia (figure 3). Point mutations in the coding sequence for the receptor cause amino acid substitutions in the extracellular, transmembrane, and kinase domain of the receptor. Additional mutations in the stop codon of FGF receptor 3, presumably resulting in a protein of extended length, also have been identified. These observations emphasize the requirement of tightly regulated FGF receptor 3 activity to maintain normal skeletal growth. We and others have found that the mutations causing achondroplasia and thanatophoric dysplasia are gain of function mutations resulting in increased receptor tyrosine kinase activity (114-117). The G380R mutation in the transmembrane domain of FGF receptor 3, that is responsible for most cases of achondroplasia partially activates FGF receptor 3 (114). By measuring the mitogenic activity of a chimeric receptor consisting of the extracellular domain of FGF receptor 3 fused to the tyrosine kinase domain of receptor 1, we found that the G380R receptor increased the basal activity to approximately 18 percent of its maximal activity. The basal activity of this receptor could be augmented by the addition of ligand and the dose response curve suggested that this receptor has a similar ligand binding affinity to that of the wild type receptor. Studies of receptor tyrosine phosphorylation showed ligand independent receptor autophosphorylation. The K650E and R248C mutations of thanatophoric dysplasia are also activating mutations. These mutations result in ligand

independent receptor activation as evidenced by ligand independent cell proliferation and receptor tyrosine phosphorylation. Significantly, these mutations were more strongly activating than the mutation causing achondroplasia. This implies a correlation between the degree of receptor activation and the severity of the dwarfing condition. The mutations causing thanatophoric dysplasia strongly activate the receptor and lead to a severe phenotype. The R248C mutation constitutively activates FGF receptor 3 by forming a disulfide linked receptor homodimer (114). This mutation introduces an unpaired cysteine residue into the extracellular domain of the receptor that forms an intermolecular disulfide linkage. The K650E mutation occurs in a highly conserved lysine residue of the activation loop of the receptor (118, 119). This mutation results in a constitutively active tyrosine kinase, presumably by altering the structure of this loop to that of an active conformation. Unlike the R248C mutation which shows constitutive activation matching that of maximally stimulated wild-type receptor, the K650E mutant receptor can be further activated by ligand to a level greater that that of the wild-type receptor. These observations are consistent with the role that the R248C mutation regulates dimerization whereas the K650E mutation affects the regulation of the kinase activity.

5. INTRAMEMBRANOUS BONE FORMATION

5.1 FGFs and intramembranous bone formation

Calvarial bone forms directly from mesenchymal cells derived from neural crest (120). These cells migrate to predetermined sites of the embryo where they condense into a multilayered membrane (121). Under the appropriate morphogenetic signals, osteogenesis begins within the core of this membrane. The instructed cells differentiate into osteoblasts which in turn secrete and initiate the mineralization of a matrix rich in type I collagen. Ossification begins at predefined sites of the membrane and radiates outward. In the calvarium, separate osteogenic fronts meet at the cranial sutures. During early post-natal life these sutures remain patent, allowing the cranial vault to grow and expand to accommodate the enlarging brain. Growth of the calvarial bones occurs through the proliferation and differentiation of osteoblasts and the deposition of bone matrix at the margins of the suture. Altered signaling within the regulatory pathways controlling osteoblast growth and differentiation in the calvarium results in dysmorphic facial features. For example, premature closure of the sutures (craniosynostosis) results in characteristic craniofacial features, such as those of the hereditary craniosynostosis.

FGFs have potent effects on the survival, proliferation and differentiation of osteoblasts and their precursors. FGFs have been shown to stimulate the proliferation of cells derived from fetal rat calvarium (122, 123). Cultured rat osteosarcoma cells and osteoblastic MC3T3 cells also proliferate in response to FGFs (124, 125). Additionally, apoptosis of osteoblasts is inhibited by FGF (126). Several studies have shown that FGF inhibits the differentiation of cultured osteoblasts, as evidenced by the inhibition of matrix mineralization, alkaline phosphatase activity and osteocalcin gene expression (124, 127). Concomitantly, an increase in the expression of interstitial collagenase and tissue inhibitor of metalloprotease was observed (128). Interestingly, others have observed that the expression of osteocalcin can be activated by FGF (129-132). These differences in the profiles of gene activation may reflect the duration of the treatment with FGF, the responsiveness of the cells to FGF, or the specific developmental stage of the cells. Prolonged *in vitro* stimulation with FGF may result in the synthesis of collagenases that degrade the surrounding matrix, a feature essential to the fate of many cell lineages. FGF receptors are subject to degradation by matrix proteases (133). Thus, proteolysis could alter osteoblast differentiation by directly affecting FGF receptor signaling. Additionally, the response of cells to FGF may be dependent on the stage of differentiation of the cells; stimulation of mesenchymal precursors may recruit additional cells to the osteoblast lineage, while prolonged or late treatment may cause reversion to an undifferentiated phenotype. Intravenous administration of FGF 1 or 2 in rats results in enhanced endosteal bone formation (12, 13). Thus, prolonged *in vivo* treatment with FGF does not irreversibly inhibit programs for osteoblast differentiation. Local factors such as the availability of glycosaminoglycans that regulate the activity of FGF (44) or the presence of other regulators of bone growth, such as TGF-beta (13) or BMPs may also modulate the *in vivo* activities of FGF.

5.2 FGF receptors and intramembranous bone formation

The important role for FGF signaling in osteoblast differentiation, and in particular the control of the development of cranial sutures, is highlighted by the human craniosynostoses that result from mutations in FGF receptors. Several mutations in FGF receptor 2 that cause Crouzon syndrome are known to be gain of function mutations (97, 134), and it is likely that other mutations causing craniosynostoses are activating as well. This observation suggests that FGF receptor activity within the cranial suture directs an anabolic signal for osteoblast differentiation. In fact, recent experiments studying the relationship of FGF receptor 2 expression and osteoblast differentiation suggest that the two events are linked (135). These studies showed that within the cranial sutures FGF receptor 2 expression is localized to pre-osteoblastic mesenchyme. FGF 2, a potent ligand for FGF receptor 2, is found at highest levels at sites of osteoblast differentiation. The authors speculate that the high levels of FGF 2 strongly activate FGF receptor 2 resulting in accelerated osteoblast differentiation and the down regulation of receptor expression. Consistent with this, studies using calvarial tissues derived from fetuses with Apert syndrome demonstrated enhanced mesenchyme condensation and bone formation (136). It is interesting to note once again that as with the distal extremities, the phenotypic effects of the FGF receptor mutations are localized. During the complex developmental program that creates the calvarial bones, the effects of FGF receptor mutations are localized to the suture. Its is likely that a similar developmental program to that which initiates the differentiation and ossification of the calvarial bones is also responsible for

the growth and differentiation of these bones at the suture margin. Several of the FGF receptor mutations responsible for premature ossification of the sutures have been demonstrated to activate the FGF receptor. The similar phenotype seen with other mutations suggests that they may be gain of function mutations as well. This suggests that the early, very rapid events that lay-down the calvarium are insensitive to an activated FGF receptor. Perhaps because these early developmental steps, which occur very rapidly, operate under conditions of excess ligand. Therefore, an activating mutation in the receptor would have no consequence. However, later developmental steps, such as growth at the suture margin, occur more slowly and may function under conditions of limiting ligand, matching the amount of ligand to the desired degree of growth. In this case, an activated receptor would have profound consequences.

Mutations in FGF receptors 1, 2, and 3 cause numerous human craniosynostoses (figure 3). This observation suggests some overlap in the expression and function of the FGF receptors. Indeed, the same clinical syndrome (Pfeiffer syndrome) can result from mutations in FGF receptor 1 or FGF receptor 2. The receptors are clearly not completely redundant, however, because the same proline to arginine substitution occurring in an absolutely conserved region of FGF receptors 1, 2 and 3 results in distinct clinical syndromes, Pfieffer syndrome, Apert syndrome and a nonsyndromic craniosynostosis, respectively. It is particularly interesting that a few of the mutations in FGF receptor 3 have been found to cause craniosynostoses. While all the mutations described in FGF receptor 1 and 2 affect craniofacial development, most of the mutations in FGF receptor 3 cause dwarfing conditions, implying a primary role for FGF receptor 3 in endochondral rather that intramembranous bone growth. The two mutations in FGF receptor 3 that affect craniofacial development result from amino acid substitutions in the transmembrane domain or linker region between Ig-like folds II and III. Both of these domains are thought to be important for receptor dimerization (74, 137), raising the interesting possibility that these mutant receptors may exert their effects by forming a heterodimer between wild type FGF receptor 2 and the mutant FGF receptor 3, thereby activating FGF receptor 2 signaling pathways. Precedence exists for the formation of receptor heterodimers in that a truncated, dominant negative FGF receptor 1 can inhibit signaling through multiple FGF receptors (137).

6. CONCLUSION

Many recent developments in the biology of FGF receptor signaling have furthered our understanding of the role of these molecules during skeletogenesis. These include the identification of the various skeletal dysplasias that result from mutations in the FGF receptors, the biochemical characterization of the effect of these mutations on receptor function, and the additional characterization of the pleotropic effects of FGFs during limb development, suture formation and endochondral ossification.

Notwithstanding this progress, many questions remain to be answered. While several receptor mutations are known to be gain of function mutations, the effect of many others on receptor activity are unknown. Some of these mutations may alter ligand binding affinity or receptor dimerization. A greater understanding of the biochemical properties of the mutant receptors will help to decipher the specific ligand-receptor pairs that function during *Development* The possibility of direct or indirect interactions between different receptors harboring mutations must also be investigated. Animal models for both the craniosynostosis and dwarfing conditions will be essential to fully understand the effects of FGFs during the complicated processes of osteoblast and chondrocyte differentiation. The compilation of these studies will hopefully link receptor biochemistry and skeletogenesis and guide future efforts to modulate FGF receptor activity for clinical benefit.

7. ACKNOWLEDGMENTS

We would like to thank our colleagues for making manuscripts available to us prior to publication. This work was supported by NIH grant HD35692. M.C.N. is a Howard Hughes Physician Postdoctoral Fellow.

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Key words: FGF Signaling, Receptor Tyrosine Kinase, Intramembranous Ossification, Limb Development, Endochondral Ossification, Skeletal Dysplasia

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