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REVIEW

FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease

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Over the last decade the identification of mutations in the receptors for fibroblast growth factors (FGFs) has defined essential roles for FGF signaling in both endochondral and intramembranous bone development. FGF signaling pathways are essential for the earliest stages of limb development and throughout skeletal development. In this review, we examine the role of FGF signaling in bone development and in human genetic diseases that affect bone development. We also explore what is presently known about how FGF signaling pathways interact with other major signaling pathways that regulate chondrogenesis and osteogenesis.

Overview of skeletal development

Skeletal elements are formed through two distinct developmental processes. Endochondral ossification gives rise to long bones that comprise the appendicular skeleton, facial bones, vertebrae, and the lateral medial clavicles. Intramembranous ossification gives rise to the flat bones that comprise the cranium and medial clavicles. Both types of ossification involve an initial condensation of mesenchyme and the eventual formation of calcified bone. However, intramembranous bone formation accomplishes this directly, whereas endochondral ossification incorporates an intermediate step in which a cartilaginous template regulates the growth and patterning of the developing skeletal element.

Development of endochondral bones initiates shortly after the formation of the limb bud with the condensation of loose mesenchyme, marked by expression of type II collagen (Fig. 1A; Kosher et al. 1986; Nah et al. 1988). Condensing mesenchyme forms an anlage for the endochondral skeleton and can either branch or segment to form individual skeletal elements (Hall and Miyake 1992, 2000). Differentiation of condensing mesenchyme gives rise to a proliferating population of centrally localized type II collagen-expressing chondrocytes and more peripherally localized type I collagen-expressing perichondrial cells (Kosher et al. 1986). At this stage, chondrocytes begin to elaborate a specialized extracellular matrix containing type II collagen. Midway between the ends of this elongated cartilaginous template, chondrocytes exit the cell cycle, hypertrophy, and begin to synthesize type X collagen in place of type II collagen (Schmid and Linsenmayer 1985).

To synthesize bone, a center of ossification forms in the mid-hypertrophic zone by neovascularization of the initially avascular cartilaginous template. The secretion and mineralization of a type I collagen-containing extracellular matrix is mediated by osteoblasts that are associated with the newly developed vasculature. As bones grow, this center of ossification propagates toward the two epiphyseal plates.

The epiphyseal growth plate consists of well-demarcated zones of cells that follow an elegant developmental program (Caplan and Pechak 1987; Hall and Miyake 1992; Olsen et al. 2000; Wagner and Karsenty 2001; Karsenty and Wagner 2002). Proximally (toward the end of a developing bone), a pool of chondrocytes (called the resting or reserve zone) supplies cells to a population of proliferating chondrocytes. Proliferating chondrocytes in turn differentiate to form a transient pool of prehypertrophic and then a more long-lived pool of hypertrophic chondrocytes. At the distal end of the epiphyseal growth plate, hypertrophic chondrocytes die by apoptosis and are replaced by trabecular bone. In this manner, hypertrophic chondrocytes provide a template for the formation of trabecular bone.

In addition to the cartilaginous and trabecular core, endochondral bone contains a hard outer shell of cortical bone (the bone collar), which surrounds the marrow cavity centrally and is contiguous with the perichondrium proximally (Caplan and Pechak 1987). The perichondrium contains precursor pools of cells that give rise to osteoblasts that line the endosteal (inner) and periosteal (outer) surface of cortical bone. Osteoblasts differentiate into osteocytes, which become embedded within cortical bone. The center of ossification in the perichondrium

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Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.990702.

Zydus EX1011
U.S. Patent No. 12,233,106

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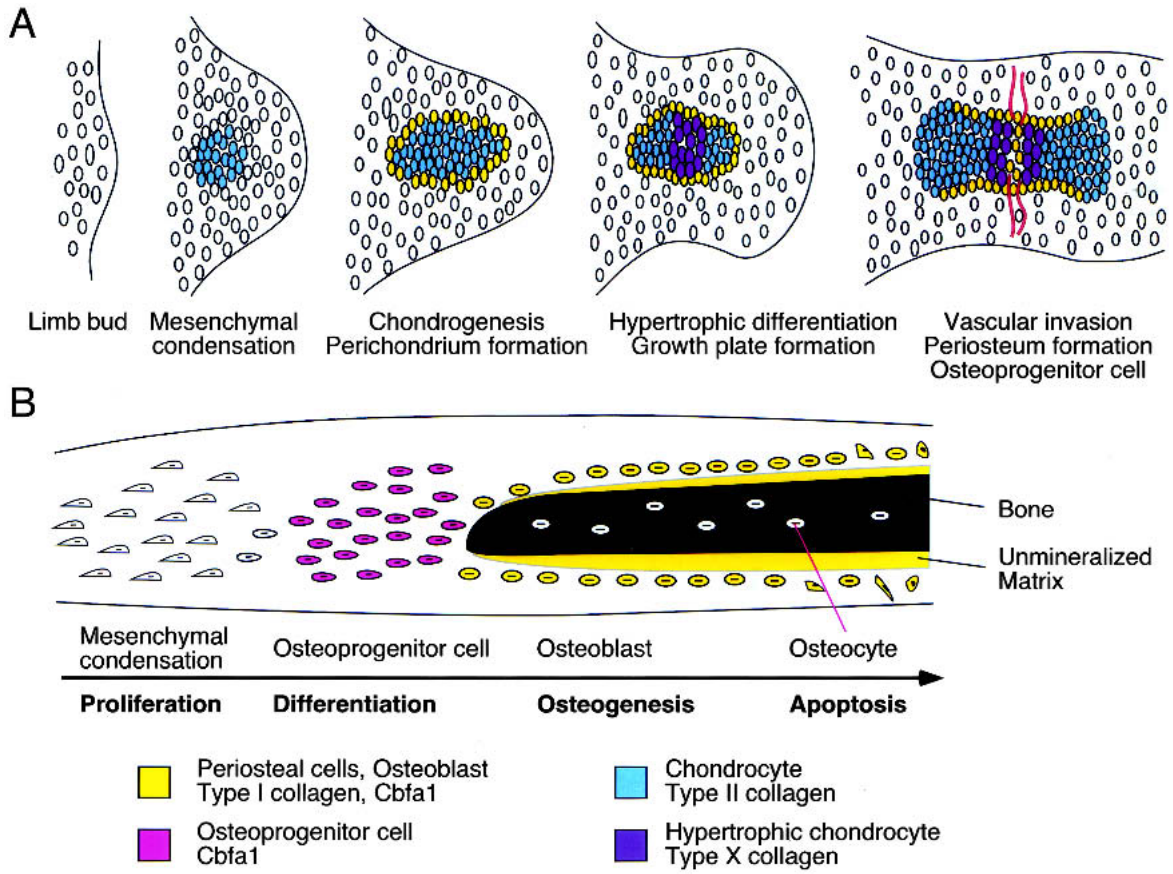

Figure 1. Endochondral and intramembranous bone development. (*A*) Schematic representation of a developing endochondral bone. Endochondral skeletal development begins with the formation of a mesenchymal condensation, expressing type II collagen (blue). Centrally, cells differentiate into chondrocytes, which hypertrophy and express type X collagen (purple). Progression to the mature growth plate accompanies development of the perichondrium (yellow), vascular invasion, and the formation of a center of ossification containing type I collagen-expressing osteoblasts (yellow). (*B*) Schematic representation of a developing intramembranous bone. Undifferentiated mesenchymal cells differentiate into osteoprogenitor cells expressing *Cbfa1* (pink). Osteoprogenitor cells progress to mature osteoblasts that express *Cbfa1* and *type I collagen* (yellow). These cells deposit and mineralize bone matrix. Osteoblasts either die by apoptosis or are embedded in the matrix, becoming osteocytes.

is aligned with the center of ossification at the chondro–osseous junction of the growth plate. A key feature of the growth of long bones is the coordination of chondrogenesis and ossification in the epiphyseal growth plate and osteogenesis in the perichondrium/periosteum (Fig. 1A).

Cranial vault development is a complex process involving cells of neural crest origin (Couly et al. 1993) and paraxial mesoderm that contribute to intramembranous bones of the cranial vault and sutures (Noden 1992; Opperman 2000). Intramembranous bone growth begins with the condensation of mesenchymal cells (Fig. 1B). Ossification centers are formed by direct bone matrix deposition-forming plates, which expand during development but do not fuse at the junction with other cranial bones (Hall and Miyake 2000). The junction between calvarial bones is a functional structure called a suture, which is responsible for the maintenance of a separation between membranous bones and for regulating the expansive growth of the skull. In the vicinity of the suture, a minority of osteogenic mesenchymal cells differentiate into osteoprogenitor cells and then into osteoblasts that express type I collagen, bone sialoprotein, and osteocalcin, and synthesize bone matrix along the bone margins. Growth and differentiation at the suture is regulated by interactions between the mesenchyme, the osteogenic front, and the Dura mater, a tough, fibrous membrane forming the outer envelope of the brain and the inner lining of cranial bones and sutures. (see Fig. 3C below; Most et al. 1998; Opperman 2000; Morriss-Kay et al. 2001). Because sutures are the major sites of intramembranous bone growth during cranial vault development, the events occurring at the suture are essential for the regulation of intramembranous ossification (Cohen 2000a).

FGFs and FGF receptors—a brief introduction

FGFs comprise a family of 22 genes encoding structurally related proteins (Ornitz and Itoh 2001). Six subfamilies of FGFs, grouped by sequence similarities, tend to

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share biochemical and functional properties and are expressed in specific spatial and developmental patterns. Four distinct FGF receptor tyrosine kinase molecules bind and are activated by most members of the FGF family. Alternative mRNA splicing produces FGF receptors with unique ligand binding properties (Johnson and Williams 1993; Ornitz et al. 1996). Alternative splicing is mostly tissue-specific, producing epithelial variants (b splice forms) and mesenchymal variants (c splice forms; Miki et al. 1992; Orr-Urtreger et al. 1993; Chellaiah et al. 1994; Naski and Ornitz 1998). FGF activity and specificity are further regulated by heparan sulfate oligosaccharides, in the form of heparan sulfate proteoglycans. Heparin/Heparan sulfate, FGF, and an FGF receptor (FGFR) associate to form a trimolecular complex (Rapraeger et al. 1991; Yayon et al. 1991; Ornitz et al. 1992). Heparan chains, themselves, have unique tissue-specific modifications that are required for, and may actually regulate, functional ligand–receptor interactions (Rapraeger 1995; Guimond and Turnbull 1999; Ornitz 2000; Allen et al. 2001).

Mutations in FGF receptors in chondrodysplasia and craniosynostosis syndromes

The importance of FGF signaling in skeletal development was first revealed with the discovery that a point mutation in the transmembrane domain of FGFR3 is the etiology of Achondroplasia, the most common genetic form of dwarfism in humans (Fig. 2; Rousseau et al. 1994; Shiang et al. 1994). Since this discovery, the etiology of many other human skeletal dysplasias have been attributed to specific mutations in the genes encoding *FGF receptors 1*, *2*, and *3* (Muenke and Schell 1995; Wilkie

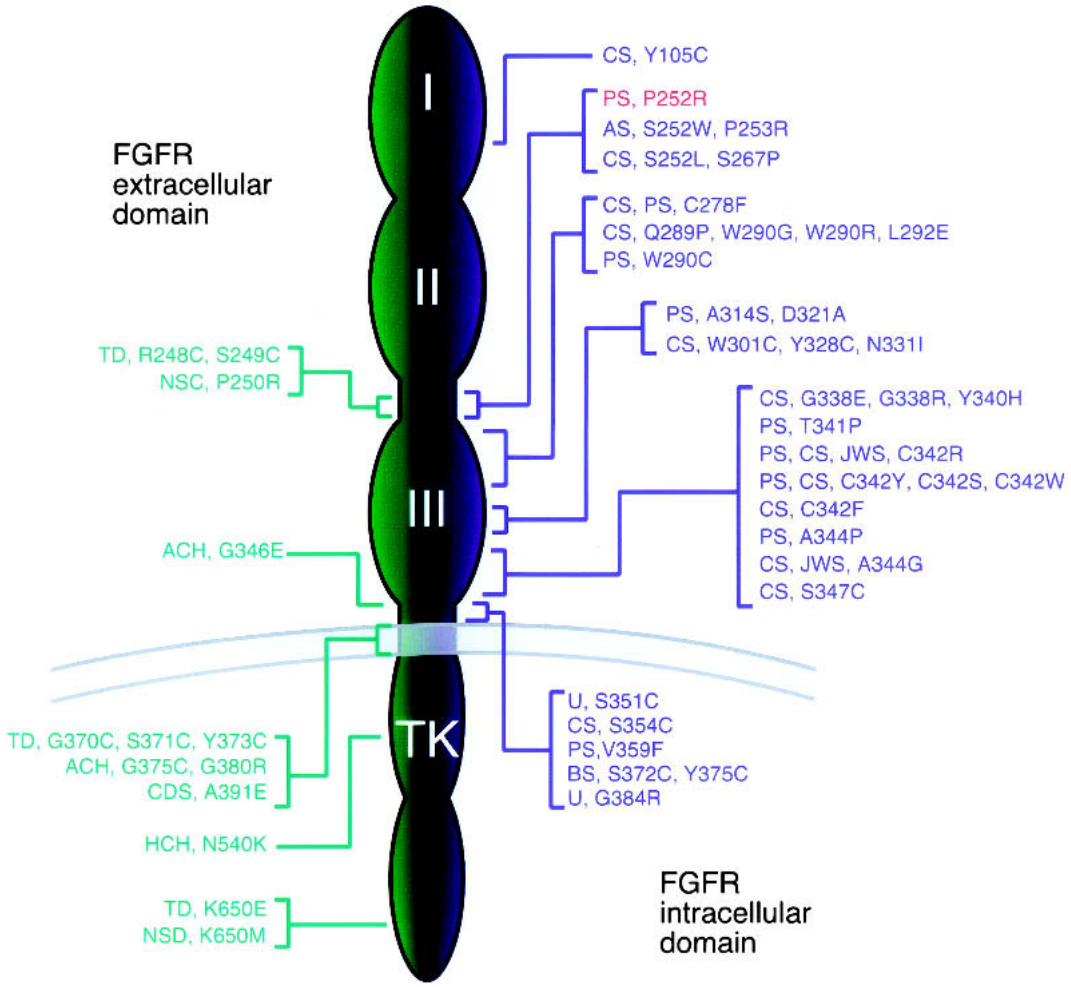

Figure 2. FGF receptor mutations in human chondrodysplasia and craniosynostosis syndromes. (*Left*, green) Mutations in FGF receptor 3. (ACH) Achondroplasia, (TD) thanatophoric dysplasia, (HCH) hypochondroplasia, (CDS) Crouzonodermoskeletal syndrome (Crouzon syndrome and acanthosis nigricans), (NSC) non-syndromic craniosynostosis. (*Right*, blue) Mutations in FGF receptor 2. (CS) Crouzon syndrome, (JWS) Jackson-Weiss syndrome, (PS) Pfeiffer syndrome, (AS) Apert syndrome, (BS) Beare-Stevenson cutis gyrata, (U) unclassified. (*Right*, red) (PS) A single mutation in FGF receptor 1 that causes Pfeiffer syndrome. The numbers represent the position of the mutant amino acid in the human coding sequence. Amino acids are abbreviated using standard single-letter abbreviations. Data source: Wilkie (1997), Naski and Ornitz (1998), and included references.

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1997; Naski and Ornitz 1998; Cohen 2000c; Britto et al. 2001a; Ornitz 2001). These disorders can be broadly classified into two groups: (1) the dwarfing chondrodysplasia syndromes, which include hypochondroplasia (HCH) (Bellus et al. 1995), achondroplasia (ACH) (Rousseau et al. 1994; Shiang et al. 1994; Ikegawa et al. 1995; Superti-Furga et al. 1995), thanatophoric dysplasia (TD) (Rousseau et al. 1995, 1996; Tavormina et al. 1995a,b); and (2) the craniosynostosis syndromes, which include Apert syndrome (AS) (Wilkie et al. 1995b), Beare-Stevenson cutis gyrata (Przylepa et al. 1996), Crouzon syndrome (CS) (Jabs et al. 1994; Reardon et al. 1994; Gorry et al. 1995; Meyers et al. 1995, 1996; Oldridge et al. 1995; Park et al. 1995; Rutland et al. 1995; Schell et al. 1995; Steinberger et al. 1995; Wilkie et al. 1995a), Pfeiffer syndrome (PS) (Muenke et al. 1994; Lajeunie et al. 1995; Rutland et al. 1995; Schell et al. 1995; Meyers et al. 1996), Jackson-Weiss syndrome (JWS) (Jabs et al. 1994; Park et al. 1995; Meyers et al. 1996), and a non-syndromic craniosynostosis (NSC) (Bellus et al. 1996). All of these mutations are autosomal dominant and frequently arise sporadically.

Chondrodysplasia syndromes and mutations in FGFR3

ACH is characterized by reduced growth of long bones with proximal elements more severely affected than distal elements (referred to as rhizomelia). Additional phenotypic features include frontal bossing of the cranium, cranio-somatic disproportion, and frequently, compression of the foramen magnum associated with neurological sequela. HCH is characterized by mild short stature and shares some clinical features with ACH (Bellus et al. 1995). TD is characterized by a very severe skeletal dysplasia and is clinically similar to homozygous cases of ACH (Stanescu et al. 1990). Both TD and homozygous ACH are generally lethal during the first several months of life. In histologic sections, the growth plates of patients with ACH display narrowed zones of proliferating and hypertrophic chondrocytes with disorganization of the chondrocyte columns (Briner et al. 1991). The pathophysiology and genetics of these diseases have been extensively reviewed (Muenke and Schell 1995; Horton 1997; Vajo et al. 2000).

The phenotypes of HCH, ACH, and TD display progressively increasing clinical severity. Early goals of the analysis of mutations that cause these diseases were to establish a relationship between disease severity and the biochemical consequence of the mutations. Present work aims to understand the developmental function of FGFR3 and how FGFR3 signaling interacts with other signaling pathways. A long-term goal is to devise ways to ameliorate the phenotype in people carrying the ACH mutation.

The etiology of most cases of ACH is a glycine-to-arginine substitution in the transmembrane domain of FGFR3 (Fig. 2). This mutation activates FGFR3 in the absence of ligand. However, the mutant receptor is further activated in the presence of ligand (Naski et al. 1996; Webster and Donoghue 1996; Li et al. 1997). Two different substitution mutations account for most cases of TD. An arginine-to-cysteine substitution in the extracellular domain (ECD) of FGFR3 (TD type 1) results in ligand-insensitive constitutive activation and a lysine-to-glutamic acid substitution in the tyrosine kinase domain of FGFR3 (TD type II) results in ligand-sensitive hyperactivation of the receptor (Naski et al. 1996). Although the phenotypes of ACH and TD are related, the mechanisms by which these mutations activate FGFR3 are diverse. The transmembrane domain mutation may act by uncoupling ligand-mediated receptor activation from receptor internalization and degradation, leading to increased levels and consequently increased signaling of the receptor (Monsonego-Ornan et al. 2000). The extracellular arginine-to-cysteine substitution in TD leads to the formation of disulfide-linked dimers, which result in constitutive activation of FGFR3 (Naski et al. 1996). In contrast, the intracellular lysine-to-glutamic acid substitution is thought to stabilize a noninhibitory conformation of the kinase regulatory loop (Mohammadi et al. 1996). In summary, the degree of activation of FGFR3 correlates well with the severity of the chondrodysplasia. This relationship provides the first evidence for negative regulation of chondrogenesis by FGFR3. This genotype–phenotype correlation is supported by several mouse models harboring activating mutations in FGFR3 (Table 1; Naski et al. 1998; Chen et al. 1999, 2001; Li et al. 1999; Wang et al. 1999; Segev et al. 2000; Iwata et al. 2001).

FGF receptor mutations induce craniosynostosis syndromes

Craniosynostosis is characterized by premature fusion of the cranial sutures. Associated phenotypes in some of the craniosynostosis syndromes also include malformations in the appendicular skeleton and nonskeletal phenotypes such as mental retardation. The histological analysis of fused cranial sutures in human craniosynostosis revealed normal mesenchymal cell proliferation but increased bone formation at the sites of primary ossification. This results from increased osteoblast maturation rather than an alteration in osteoblast number (De Pollak et al. 1996; Lomri et al. 1998). Mutations in *Fgfr1*, *Fgfr2*, and *Fgfr3* have been associated with craniosynostosis syndromes. However, the majority of craniosynostosis syndromes are associated with mutations in *Fgfr2* (Fig. 2; Muenke and Schell 1995; Park et al. 1995; Malcolm and Reardon 1996; Webster and Donoghue 1997; Wilkie 1997; Burke et al. 1998; Naski and Ornitz 1998; Lajeunie et al. 1999; Ornitz 2001). The pathophysiology and genetics of craniosynostosis syndromes have been recently reviewed (Wilkie 1997, 2000; Cohen 2000b; Britto et al. 2001a; Wilkie and Morriss-Kay 2001). Most of the mutations are missense, or small in-frame insertions or deletions that affect the highly conserved extracellular FGFR ligand-binding domain. However, some recently described mutations involve the intracellular tyrosine kinase domain of FGFR2 and presumably function to activate downstream signaling pathways (Kan et al. 2002).

Most mutations in the *Fgfr* gene family are dominant

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Table 1. *Mouse models for FGF signaling in skeletal development*

Mouse model	Method/mechanism	Phenotype	Reference
Ectopic FGF2 expression	PGK promoter transgenic expression	Enlarged occipital bones, skeletal dwarfism	Coffin et al. 1995
Ectopic FGF2 expression	Adenoviral expression in suture	Coronal suture synostosis	Greenwald et al. 2001
Ectopic FGF9 expression	Type II collagen promoter transgenic expression	Achondroplasia-like dwarfism	Garofalo et al. 1999
Increased FGF3/FGF4 expression	Retrovirus insertion	Crouzon-like dysmorphology	Carlton et al. 1998
FGF2-deficient mice	Knockout mutation	Inhibition of bone formation/bone mass	Montero et al. 2000
FGF18-deficient mice	Knockout mutation	Delayed suture closure, expanded growth plate	Liu et al. 2002; Ohbayashi et al. 2002
Dominant-negative FGFR1	Adenoviral expression in suture	Inhibition of calvarial suture fusion	Greenwald et al. 2001
FGFR3-deficient mice	Knockout mutation	Skeletal overgrowth	Colvin et al. 1996; Deng et al. 1996
Gain of function, FGFR2c	Knockout of exon 9(IIIc), abberant alternative splicing	Coronal synostosis	Hajihosseini et al. 2001; Yu and Ornitz 2001
P250R mutation, FGFR1	Knockin mutation	Craniosynostosis	Zhou et al. 2000
K644M mutation, FGFR3	Knockin mutation	Severe dwarfism	Iwata et al. 2001
S365C mutation, FGFR3	Knockin mutation	Severe dwarfism	Chen et al. 2001
K644E mutation, FGFR3	Knockin mutation	Achondroplasia-like dwarfism	Li et al. 1999
K644E mutation, FGFR3	Knockin mutation	Thanatophoric dysplasia-like dwarfism	Iwata et al. 2000
G380R mutation, FGFR3	Type II collagen or *Fgfr3* promoter transgenic expression, Knockin mutation	Achondroplasia-like dwarfism	Naski et al. 1998; Wang et al. 1999; Segev et al. 2000
G369C mutation, FGFR3	Knockin mutation	Achondroplasia-like dwarfism	Chen et al. 1999

and are thought to be gain-of-function mutations (Naski and Ornitz 1998; Cohen 2000c; Ornitz 2001; Wilkie and Morriss-Kay 2001). Several of the mutations in *Fgfr1* and *Fgfr2* in CS, PS, and JWS constitutively activate the receptor by stabilizing intermolecular disulfide bonds, causing ligand-independent dimerization and signaling (Neilson and Friesel 1995; Wilkie et al. 1995a; Galvin et al. 1996; Robertson et al. 1998). Other mutations are thought to prolong the duration of receptor signaling or alter ligand-binding specificity (Anderson et al. 1998; Mansukhani et al. 2000; Plotnikov et al. 2000; Yu et al. 2000; Ibrahimi et al. 2001). For example, one of the CS mutations in *Fgfr2* results in ligand-independent activation and dramatically decreased ligand binding (Mangasarian et al. 1997). However, the mutant receptor in AS has lost ligand-binding specificity and thus can be activated by inappropriate ligands (Plotnikov et al. 2000; Yu et al. 2000; Ibrahimi et al. 2001; Yu and Ornitz 2001). A mutation in FGFR3 has also been associated with a Crouzon-like phenotype and associated dermal thickening and hyperpigmentation (Crouzon syndrome with acanthosis nigricans). This syndrome is now referred to as Crouzonodermoskeletal syndrome (CDS) (Cohen 2000c).

The importance of FGF signaling in cranial bone development is further supported by mice harboring activating mutations in FGF signaling pathways (Table 1). Mice overexpressing FGF2 develop enlarged occipital bones, which are formed in part by intramembranous ossification (Coffin et al. 1995). Increased expression of FGF3 and FGF4, induced by retroviral insertion, results in a phenotype that resembles CS (Carlton et al. 1998). Additionally, gain-of-function mutations in FGFR1 and FGFR2 in mice affect calvarial development. The P250R mutation in FGFR1 (orthologous to the P252R substitution associated with PS in humans) causes premature fusion of calvarial sutures (Zhou et al. 2000). In addition, a dominant mutation that affects alternative splicing in *Fgfr2* causes coronal synostosis in mice (Hajihosseini et al. 2001; Yu and Ornitz 2001). In contrast, loss-of-function mutations in *Fgfr*s have been less informative. Embryos lacking FGFR1 or FGFR2 die prior to skeletal development (Deng et al. 1994; Arman et al. 1999), and mice lacking FGFR3 do not have obvious defects in calvarial bones (Colvin et al. 1996; Deng et al. 1996). However, a secreted soluble FGFR2 ECD can inhibit FGF signaling and causes skull abnormalities in mice that resemble those induced by *Fgfr* mutations in humans (Celli et al. 1998). Of the FGFs thus far targeted in mice, only mice lacking FGF18 have revealed a phenotype in calvarial development. *Fgf18* null mice have delayed calvarial ossification, indicating a requirement for FGF18 in intramembranous bone formation (Liu et al. 2002; Ohbayashi et al. 2002).

FGF signaling in the developing limb bud

Fibroblast growth factors are involved in the earliest stages of limb development and in the formation of skel-

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etal elements within the developing limb. The expression patterns and function of FGFs in early limb development have been extensively reviewed (Martin 1998). Briefly, in the developing limb bud, the epithelial splice form of FGF receptor 2 (FGFR2b) is expressed in ectoderm, and the mesenchymal splice form of FGF receptor 1 (FGFR1c) is expressed in limb mesenchyme (Fig. 3A). An early step in the initiation of the limb bud involves signaling from mesenchymally expressed FGF10 to FGFR2b to form the apical ectodermal ridge. FGF8 is subsequently expressed in the apical ectodermal ridge and is thought to signal back to FGFR1c in limb mesoderm (Fig. 3A,B). This pattern of reciprocal signaling is one of several essential events required for outgrowth and patterning of the limb.

The mesenchymal condensation

FGF receptor expression

The mesenchymal condensation is the first morphologic event leading to bone formation (Hall 1987; Hall and Miyake 1992). *Fgfr2* expression is first observed in mesenchyme as mesenchymal cells begin to coalesce in the central core of the developing limb (Fig. 3A). At the condensation stage of limb development, *Fgfr1* expression persists in limb mesenchyme and in mesenchymal cells in the periphery of the condensation, whereas *Fgfr2* expression can be observed in the morphologically distinct mesenchymal condensations but not in the surrounding loose connective tissue (Fig. 3A). These patterns of expression have been observed in chicken, mouse, and hu-

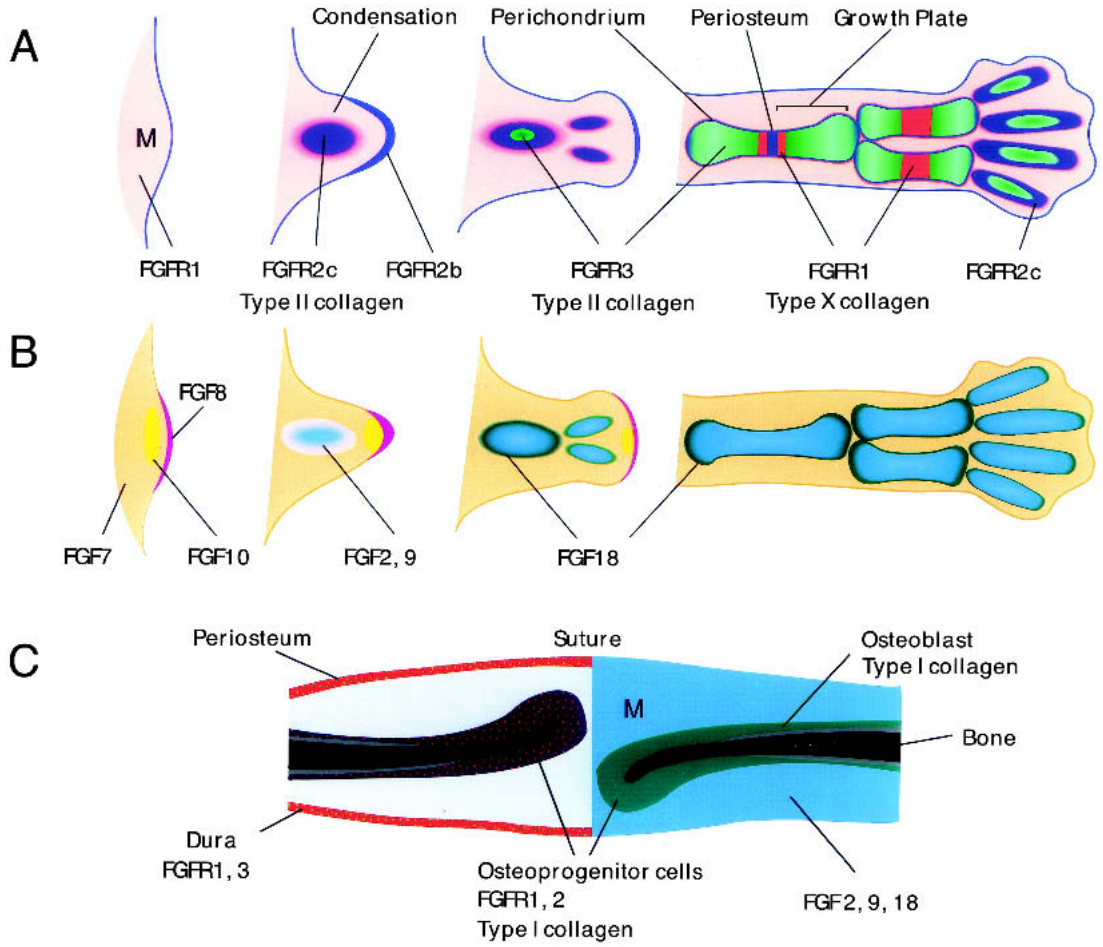

Figure 3. *Fgf* and *Fgf receptor* gene expression in developing endochondral and intramembranous bone. (*A*) *Fgf receptor* gene expression. *Fgfr1* is expressed in limb mesenchyme (M) and in the periphery of the mesenchymal condensation (pink). *Fgfr2* is first expressed in condensing mesenchyme (purple). *Fgfr3* expression is initiated as chondrocytes differentiate and proliferate (green). *Fgfr1* expression is present as chondrocytes hypertrophy (red), and *Fgfr2* expression is prominent in osteoblasts in the ossification center (purple). Both *Fgfr1* and *Fgfr2* appear to be coexpressed in the perichondrium (pink). (*B*) *Fgf* gene expression. At the limb bud stage, *Fgf10* is expressed in distal mesenchyme (M) (yellow) and *Fgf8* is expressed in the overlying apical ectodermal ridge (AER) (pink). *Fgf2* and *Fgf9* expression is observed in the developing condensation (blue), and *Fgf18* expression is observed in the perichondrium and presumptive joint positions (green). *Fgf7* is expressed in loose mesenchyme and perichondrium surrounding the mesenchymal condensation (tan). (*C*) (*Right*) *Fgf* expression. In developing cranial bones, *Fgf2*, *Fgf9*, and *Fgf18* (blue) are expressed in mesenchymal cells in the suture separating the two osteogenic fronts. *Fgf18* also is expressed in cells lining the endosteal and periosteal surface of calvarial bones. (*Left*) *Fgf receptor* expression. *Fgfr1* (red) and *Fgfr3* (green dots) are found in the dura mater and periosteum, whereas *Fgfr1* (red dots) and *Fgfr2* (purple) are expressed in osteoprogenitor cells at the osteogenic front separating the suture.

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man limb development (Orr-Urtreger et al. 1991; Peters et al. 1992; Szebenyi et al. 1995; Delezoide et al. 1998). Later in skeletal development, *Fgfr1* and *Fgfr2* expression persists in the perichondrium and periosteum in patterns that suggest expression in the osteoblast lineage (Delezoide et al. 1998). As chondrogenesis begins in the center of the condensation, *Fgfr3* expression is first observed (Peters et al. 1993).

FGF signaling in condensing mesenchyme

The physiologic ligands that activate FGFRs in the mesenchymal condensation have been difficult to identify. At the condensation stage, *Fgf9* is expressed within condensing mesenchyme (Fig. 3B; Colvin et al. 1999). However, in the absence of FGF9 there are no apparent defects in skeletal development (Colvin et al. 2001a,b). Similarly, *Fgf2*, *Fgf5*, *Fgf6*, and *Fgf7* are expressed in loose mesenchyme outside the condensation (Haub and Goldfarb 1991; deLapeyriere et al. 1993; Mason et al. 1994; Finch et al. 1995; Savage and Fallon 1995). However, mice lacking these FGFs have no apparent defects in skeletal development (Hebert et al. 1994; Guo et al. 1996; Fiore et al. 1997). It is possible that a combination of these and other FGFs may constitute the complete FGF signal to the developing condensation.

The role of FGF signaling in condensing mesenchyme is poorly understood. In primary chondrocytes and in undifferentiated mesenchymal cells, FGF signaling pathways induce the expression of SOX9, an essential transcription factor for chondrocyte differentiation (Murakami et al. 2000). Additionally, FGFR3 signaling may enhance chondrocyte proliferation in the mesenchymal condensation, even though it is well established that this receptor negatively regulates chondrogenesis during late embryonic and postnatal development (Iwata et al. 2000, 2001). Defects in signaling in the mesenchymal condensation could lead to skeletal abnormalities such as the bony syndactyly resulting from mutations in FGFR2 in Apert syndrome. The Apert mutations result in loss of ligand binding specificity, allowing inappropriate activation of FGFR2 in the mesenchymal condensation by mesenchymally expressed ligands such as FGF7 and inappropriate activation of FGFR2b by ligands such as FGF2, FGF6, and FGF9 (Yu et al. 2000; Yu and Ornitz 2001).

Endochondral ossification

Expression of FGFs and FGF receptors in endochondral bone

Shortly after formation of a mesenchymal condensation, chondrogenesis ensues, and *Fgfr3* expression is initiated in chondrocytes in the differentiated core of the mesenchymal condensation (Fig. 3A). At this stage, overlap in expression may exist with *Fgfr2*. As the epiphyseal growth plate is formed, *Fgfr1* expression is initiated as chondrocytes further differentiate and hypertrophy. Interestingly, *Fgfr1* and *Fgfr3* have very distinct domains of expression with little overlap; *Fgfr3* is expressed in proliferating chondrocytes, whereas *Fgfr1* is expressed in prehypertrophic and hypertrophic chondrocytes (Peters et al. 1992, 1993; Deng et al. 1996). This juxtaposition of FGFR1 and FGFR3 expression domains suggests unique functions. Expression of *Fgfr3* in the reserve and proliferating zone suggests a direct role for FGFR3 in regulating chondrocyte proliferation and possibly differentiation (Peters et al. 1993; Delezoide et al. 1998; Naski et al. 1998). In contrast, the expression of *Fgfr1* in hypertrophic chondrocytes (Peters et al. 1992; Delezoide et al. 1998) suggests a role for FGFR1 in survival of the hypertrophic chondrocyte, in regulating a feedback signal to control the rate of differentiation, in regulating the production of the unique extracellular matrix products of these cells, or in signaling their eventual apoptotic death. Interestingly, examination of FGFR3 protein in rib cartilage shows the presence of large amounts of cleaved FGFR3 ECD within the extracellular matrix of hypertrophic chondrocytes, suggesting that proteolytic processing could regulate the activity of FGFR3 and that the FGFR3 ECD could bind ligand and thus modify FGFR1 signaling in hypertrophic chondrocytes (Pandit et al. 2002).

Several FGFs are expressed in developing endochondral bone (Fig. 3B). Historically, FGF2 was the first FGF ligand to be isolated from growth plate chondrocytes (Sullivan and Klagsbrun 1985). Subsequently, *Fgf2* expression has also been observed in periosteal cells and in osteoblasts (Hurley et al. 1994, 1999; Sabbieti et al. 1999). Targeted deletion of FGF2 causes a relatively subtle defect in osteoblastogenesis, leading to decreased bone growth and bone density. However, no defects in chondrogenesis were observed (Montero et al. 2000). *Fgf9* is also expressed in chondrocytes (Colvin et al. 1999; Garofalo et al. 1999). However, the skeleton of *Fgf9*$^{-/-}$ mice is apparently normal at birth when these mice die of other causes (Colvin et al. 2001a,b).

In the perichondrium, expression of *Fgf7*, *Fgf8*, *Fgf17*, and *Fgf18* has been observed (Mason et al. 1994; Finch et al. 1995; Xu et al. 1999; Liu et al. 2002; Ohbayashi et al. 2002), suggesting a possible paracrine signal to the growth plate. Recent genetic studies have identified a defect in chondrogenesis and osteogenesis in mice lacking FGF18 (Liu et al. 2002; Ohbayashi et al. 2002). Mice lacking FGF7, FGF8, and FGF17 have apparently normal chondrogenesis, or in the case of FGF8 die prior to skeletal development (Guo et al. 1996; Meyers et al. 1998; Xu et al. 2000). Issues of functional redundancy among these and other FGFs will need to be addressed in the future.

Signaling pathways that regulate endochondral bone development

The growth plate is a developmental center that integrates many diverse signals to coordinate the complex patterning and growth of the skeleton. Each growth plate must appropriately respond to positional cues and endo-

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crine and local signals to coordinate the formation of each unique skeletal element. Local signaling pathways that are known to coordinate endochondral bone growth include (but are not limited to) the parathyroid hormone related peptide (PTHrP), Indian hedgehog (IHH), bone morphogenic proteins (BMPs), and FGFs. Current research aims to understand the function of these and other signaling pathways and how they interact with each other to coordinate bone growth.

PTHrP and IHH form a feedback loop that regulates the rate of chondrocyte differentiation (Fig. 4A). *Ihh* is expressed in distal prehypertrophic and proximal hypertrophic chondrocytes (Bitgood and McMahon 1995), whereas *Pthrp* is expressed in perichondrial and periarticular chondrocytes (Amizuka et al. 1994; Karaplis et al. 1994; Lee et al. 1995). The PTH/PTHrP receptor (PPR) is expressed in distal proliferating and proximal prehypertrophic chondrocytes proximal to *Ihh*, positioned to regulate the rate of chondrocyte differentiation. The IHH receptor, Patched (PTC), and associated signaling molecules (Smoothened, Gli1, Gli3), are located throughout the growth plate and perichondrium (Iwasaki et al. 1997). IHH induces the expression of PTHrP (Vortkamp et al. 1996; Chung et al. 2001; Minina et al. 2001), and in mice lacking IHH, chondrocytes prematurely differentiate, resulting in a short-limbed dwarfism (St-Jacques et al. 1999). A similar phenotype is observed in mice with a targeted disruption of *Pthrp* or the *Ppr* (Karaplis et al. 1994; Lanske et al. 1996). The net effect of this feedback loop is to regulate the number of chondrocytes undergoing hypertrophic differentiation (Kronenberg and Chung 2001). IHH also has been shown to stimulate chondrocyte proliferation independently of PTHrP signaling (St-Jacques et al. 1999; Karp et al. 2000; Chung et al. 2001).

The IHH/PTHrP signaling loop also interacts with members of the bone morphogenic protein (BMP) family.

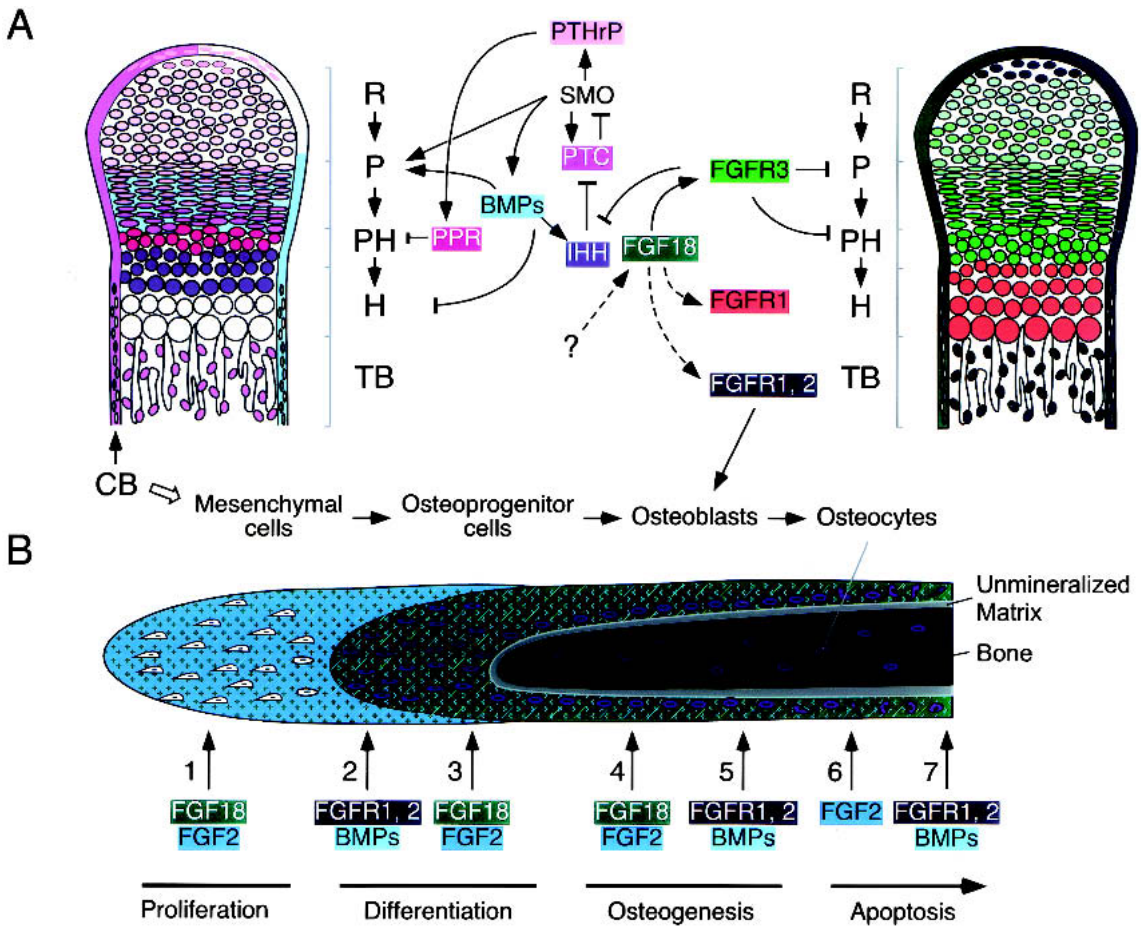

Figure 4. Regulatory pathways in endochondral and intramembranous bone. (*A*) Chondrocytes progress through reserve (R), proliferating (P), prehypertrophic (PH), and hypertrophic (H) stages. Expression patterns and interactions of molecules that regulate these events are color coded and diagramed. (IHH) Indian hedgehog; (PTC) patched; (SMO) smoothened; (BMPs) bone morphogenic proteins; (PTHrP) parathyroid hormone related peptide; (PPR) PTHrP receptor. (*B*) During intramembranous bone development, the proliferation of mesenchymal cells is followed by their commitment to become osteoprogenitor cells. Osteoprogenitor cells differentiate into pre-osteoblasts and then into bone matrix-forming mature osteoblasts. The stimulatory effects (arrow) of FGFs, FGFRs, and BMPs on cell proliferation (1), differentiation (2,3), osteogenesis (4,5) and apoptosis (6,7) during this progressive differentiation pathway are shown.

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Unlike PTHrP, BMP signaling promotes the proliferation of chondrocytes and delays maturation of differentiated hypertrophic chondrocytes, resulting in an enlarged skeleton (Fig. 4A; Duprez et al. 1996; Zou et al. 1997; Minina et al. 2001). This effect of BMP occurs independently of IHH and PTHrP (Minina et al. 2001). A further consequence of BMP signaling is to increase the domain of *Ihh* expression (Kawakami et al. 1996; Pathi et al. 1999; Capdevila and Belmonte 2001; Minina et al. 2001). IHH signaling also interacts with the BMP pathway by up-regulating expression of *Bmp2* and *Bmp4* in the growth plate (Pathi et al. 1999; Minina et al. 2001). The net effect of this signaling loop is to promote chondrogenesis.

FGF signaling pathways in the growth plate

Analysis of both gain-of-function and loss-of-function mutations in FGFR3 at late gestational and postnatal stages of mouse development shows that the net consequence of signaling through this receptor is to limit chondrocyte proliferation and differentiation (Fig. 4A; Naski and Ornitz 1998; Ornitz 2001). This effect is mediated in part by direct signaling in chondrocytes (Henderson et al. 2000; Rozenblatt-Rosen et al. 2002) and in part indirectly, by regulating the expression of the IHH/PTHrP/BMP signaling pathways. Mice harboring an activating mutation in FGFR3 have decreased expression of *Ihh*, *Ptc*, and *Bmp4* (Naski et al. 1998; Li et al. 1999; Chen et al. 2001), whereas in mice lacking FGFR3, *Ihh*, *Ptc*, and *Bmp4* expression are up-regulated (Naski et al. 1998; data not shown). The overall function of FGFR3 is consistent with a direct action of FGFR3 on proliferating chondrocytes (see below) and an indirect consequence of modulating Hedgehog and BMP signaling (Fig. 4A).

The ligands that signal to FGFR3 during endochondral ossification have been elusive. However, recent data show that growth plate histology of mice lacking FGF18 is similar to that of mice lacking FGFR3. This includes the up-regulation of *Ihh* and *Ptc* expression and increased chondrocyte proliferation. These similarities provide strong evidence for a ligand–receptor relationship between FGF18 and FGFR3 (Liu et al. 2002; Ohbayashi et al. 2002). Furthermore, in vitro, FGF18 can activate FGFR3c (Xu et al. 2000) and stimulate the proliferation of cultured articular chondrocytes (Ellsworth et al. 2002). FGF18 is most closely related to FGF8 and FGF17 (Ornitz and Itoh 2001) and may not be the only FGF ligand functioning in developing bone. There is some evidence for expression of FGF8 and FGF17 in developing bone (Xu et al. 1999), suggesting that there may be additional redundancy among these three ligands.

Chondrogenesis and osteogenesis must be tightly coordinated to correctly form and generate a biomechanically robust structure. Regulation of endochondral ossification by FGFs, IHH, PTHrP, and BMPs involves signaling between different populations of chondrocytes and between chondrocytes and the perichondrium. Several studies have shown that the perichondrium elaborates a signal that negatively regulates both chondrocyte proliferation and differentiation (Long and Linsenmayer 1998; Haaijman et al. 1999; Alvarez et al. 2001). The expression pattern of *Fgf18* and the phenotype of *Fgf18* null mice make FGF18 a good candidate for this signal. However, PTHrP and BMPs are also expressed in the perichondrium and may also contribute to this signal. In the reverse direction, IHH is the only factor identified thus far that signals from chondrocytes to the perichondrium (Karaplis and Goltzman 2000; Chung et al. 2001).

Mice lacking *Fgf18* have a more severe phenotype than mice lacking *Fgfr3* (Liu et al. 2002). Unlike in *Fgfr3*$^{-/-}$ mice, *Fgf18*$^{-/-}$ mice show delayed ossification in the long bone and decreased growth of cranial bones. These observations suggest that FGF18 may signal to FGFR1 in hypertrophic chondrocytes and to FGFR1 or FGFR2 in the perichondrium and periosteum. Like IHH and BMPs, FGF18 may also serve to coordinate the development of these closely associated compartments. A prediction of this model is that the expression of *Fgf18* in the perichondrium must be tightly regulated by other factors. Interestingly, TGF-β requires the presence of the perichondrium to inhibit chondrocyte proliferation and differentiation (Alvarez et al. 2001). Therefore, TGF-β, as well as IHH and BMPs, could act by regulating *Fgf18* expression in the perichondrium.

Response of the chondrocyte to FGF signaling

Inhibition of chondrocyte proliferation could result from either unique signaling properties of FGFR3 or a unique response of the proliferating chondrocyte to an FGFR signal. FGFR1 and FGFR3 have different signaling properties in some cell types in vitro (Wang et al. 1994; Lin et al. 1996; Naski et al. 1996). These differences may be attributable to differences in the strength of the tyrosine kinase signal but not to the specific signaling pathway activated (Raffioni et al. 1999). Consistent with this, both receptor kinase domains appear to have similar activities when expressed in proliferating chondrocytes in vivo (Wang et al. 2001). This observation supports the hypothesis that the proliferating chondrocyte itself is uniquely responsive to an FGFR signal. Interestingly, during embryonic development, constitutive FGFR3 activation enhances chondrocyte proliferation (Iwata et al. 2000, 2001). Taken together, these data suggest that the proliferating growth plate chondrocyte is a uniquely differentiated type of chondrocyte that acquires specific responsiveness to FGF signals during late embryonic and postnatal skeletal development.

This unique response of the chondrocyte may result from both indirect signals mediated by IHH and direct signals from both IHH and FGFR3. Expression of constitutively activating mutations in FGFR3 or treatment of cells with FGF can induce nuclear translocation of STAT1 and STAT3 and induce the expression of the cell cycle inhibitor p21(WAF1/CIP1) (Su et al. 1997; Sahni et al. 1999; Hart et al. 2000). Furthermore, chondrocytes isolated from patients with Thanatophoric dysplasia, showed nuclear STAT1, increased *Bax* expression, decreased *Bcl2* expression, and an increase in the number of apoptotic chondrocytes, suggesting that signaling

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through STAT1 may also regulate cell death (Legeai-Mallet et al. 1998).

In vivo, the dwarfism phenotype observed in mice expressing activating mutations in FGFR3 correlated with the activation of STAT proteins and up-regulation of cell cycle inhibitors (p16, p18, and p19; Chen et al. 1999; Li et al. 1999). Interestingly, in primary chondrocytes derived from mice lacking STAT1, FGF signaling failed to induce chondrocyte growth inhibition (Sahni et al. 1999). Additionally, mating FGF2-expressing transgenic mice into a *Stat1* null background corrected the chondrodysplasia phenotype characteristic of this transgenic line (Sahni et al. 2001). These data support a model in which STAT1 mediates the growth inhibition by FGFR3.

FGF functions in intramembranous bone formation

FGF and FGFR signaling in intramembranous bone formation

The expression of FGFs and FGFRs is temporally and spatially regulated during craniofacial development. During intramembranous bone formation, *Fgf2*, *Fgf4*, and *Fgf9* are expressed in sutural mesenchyme in early craniofacial skeletogenesis, suggesting that they may be involved in regulating calvarial osteogenesis (Kim et al. 1998; Mehrara et al. 1998; Rice et al. 2000; Britto et al. 2001b). *Fgf18* (Liu et al. 2002; Ohbayashi et al. 2002) and *Fgf20* (Hajihosseini and Heath 2002) are also expressed in developing calvarial bones, and mice lacking *Fgf18* have defects in calvarial development (Liu et al. 2002; Ohbayashi et al. 2002). *Fgf18* is first detected in calvarial mesenchymal cells and later in development is expressed in osteogenic mesenchyme and in differentiated osteoblasts on the endosteal and periosteal surface of cranial bones (Fig. 3C; Liu et al. 2002; Ohbayashi et al. 2002).

Fgfr1 is expressed in the calvarial mesenchyme and later in osteoblasts, whereas *Fgfr2* is expressed at sites of ossification in differentiating osteoblasts (Hughes 1997; Britto et al. 1998, 2001b; Delezoide et al. 1998; Marie et al. 2000; Ohbayashi et al. 2002). *Fgfr3* is not found in calvarial mesenchymal cells or periosteal cells but is detected at low levels in sutural osteogenic fronts at late stages of development (Delezoide et al. 1998; Molteni et al. 1999; Marie et al. 2000; Rice et al. 2000). However, mice lacking *Fgfr3* do not have defects in calvarial development (Colvin et al. 1996; Deng et al. 1996). It is therefore likely that intramembranous bone formation is controlled primarily by FGFR1 and FGFR2.

Transcriptional mechanisms regulating calvarial bone formation and suture development

Several transcription factors control osteogenic differentiation at the level of the suture. The early commitment of mesenchymal stem cells into osteoblasts requires expression of CBFA1/RUNX2, a transcription factor that regulates several osteoblast genes including type I collagen, bone sialoprotein, osteopontin, and osteocalcin (Ducy et al. 1997). CBFA1 is essential for intramembranous bone formation, and haploinsufficiency at the *Cbfa1* locus leads to delayed membranous ossification in mice and humans (Komori et al. 1997; Lee et al. 1997; Mundlos et al. 1997). Other transcription factors are also important for intramembranous bone formation. MSX1 and MSX2 are homeobox-containing transcription factors that are associated with the differentiation of neural crest-derived intramembranous calvarial bones. *Msx2* is expressed in the mesenchyme and acts by inhibiting calvarial osteoblast differentiation (Towler et al. 1994; Dodig et al. 1999). MSX2 overexpression in mice and mutations in *Msx2* in humans induce precocious bone formation and craniosynostosis by increasing the number of osteoprogenitor cells (Jabs et al. 1993; Liu et al. 1995, 1999). In contrast, haploinsufficiency of *Msx2* decreases cell proliferation, delays suture closure, and causes defects in skull ossification in both humans and mice (Dodig et al. 1999; Satokata et al. 2000; Wilkie et al. 2000). Inactivation of DLX5, a transcription factor that regulates osteocalcin expression (Newberry et al. 1998), induces delayed ossification of membranous bone in mice (Acampora et al. 1999). Haploinsufficiency of *Twist*, a basic helix–loop–helix transcription factor, results in premature cranial ossification (el Ghouzzi et al. 1997; Howard et al. 1997). *Twist* is expressed in sutural mesenchyme and affects osteoblast gene expression and apoptosis in calvarial osteoblasts (Yousfi et al. 2001, 2002). Conversely, delayed suture closure is associated with trisomy at the human *Twist* locus (Stankiewicz et al. 2001). It is likely that these factors interact to control cranial suture ossification.

FGF signaling affects the expression and activity of several transcription factors that are required for calvarial osteogenesis. In rat or mouse calvarial cells, FGF2 activates osteocalcin transcription. This activity is inhibited by the transcription factor MSX2 and is activated by DLX5 (Newberry et al. 1996, 1998, 1999). Similarly, FGF4 can stimulate *Msx1* gene expression and cell proliferation (Kim et al. 1998), and FGF2 can up-regulate *Twist* expression in mouse calvarial mesenchyme (Rice et al. 2000). *Twist* heterozygous mice show altered FGFR protein expression (Rice et al. 2000), suggesting that TWIST acts upstream of FGF signaling pathways. Additionally, Twist could be a potential transcriptional regulator that mediates the negative effect of FGF2 on type I collagen expression in calvarial cells (Fang et al. 2001). Thus, FGF/FGFR, MSX, and TWIST interact to control cranial suture development in a coordinated manner.

Biological functions of FGFs in cranial bone formation

The biological effects of FGFs on osteogenic cells depend on their stage of differentiation. In vitro studies show that FGF1, FGF2, FGF4, or FGF18 can potentiate growth of fetal or neonatal calvarial osteoblasts (Canalis et al. 1988; Tang et al. 1996; Hurley et al. 2001; Shimoaka et al. 2001) but not mature osteoblasts (Debiais et al. 1998; Mansukhani et al. 2000). In vivo, FGF2 increases the

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number of osteogenic cells and promotes calvarial osteogenesis (Mundy et al. 1999). In contrast, calvarial osteogenesis is decreased in mice lacking FGF2, or following inhibition of endogenous FGF2 activity (Montero et al. 2000; Moore et al. 2002). These data support a role for endogenous FGF2 as an autocrine mitogenic factor in calvaria.

FGF2 production by calvarial osteoblasts is up-regulated by FGF2 itself, and by parathyroid hormone, PGE2, and TGF-β (Hurley et al. 2001). Thus, the balance between high and low levels of endogenous FGF2 may constitute a mechanism to control proliferation and ensure normal cranial vault morphogenesis (Moore et al. 2002). In addition to FGF2, FGF18 appears to be important for calvarial development. Recent analysis of *Fgf18* null embryos revealed a transient decrease in the proliferation of osteogenic mesenchymal cells associated with delayed suture closure (Ohbayashi et al. 2002). Therefore, at least two FGFs control osteogenic cell proliferation during calvarial development.

FGFs also regulate calvarial cell differentiation. In vitro, FGFs inhibit type I collagen expression and bone nodule formation of rat calvarial cells (Tang et al. 1996; Hurley et al. 2001). However, prolonged treatment with FGF2 increases calvarial osteoblast differentiation (Debiais et al. 1998). FGF2 promotes osteocalcin transcription in rodent and human calvarial osteoblasts (Schedlich et al. 1994; Boudreaux and Towler 1996; Newberry et al. 1996, 1998; Debiais et al. 1998). This effect, together with the reported increase in sodium-dependent phosphate transport (Suzuki et al. 2000), may contribute to the regulation of intramembranous calcification. Recent in vitro data indicate that FGF18 can act like FGF2 to regulate calvarial osteoblast differentiation in vitro (Shimoaka et al. 2001). Consistent with this, decreased terminal differentiation of osteoblasts was observed in *Fgf18* null mice, which may contribute to delayed suture ossification (Ohbayashi et al. 2002). Thus, FGF18 is required for both the promotion of osteogenic mesenchymal cell proliferation and osteoblast differentiation in developing calvarial bones (Fig. 4B).

In addition to regulating rates of proliferation and differentiation, FGF signaling may also regulate cell death in the cranial suture, a critical factor for determining suture patency. Perturbations in the number of apoptotic cells can lead to either premature or delayed suture closure (Rice et al. 1999). The effects of FGF signaling on osteoblast apoptosis are dependent on the developmental stage. FGF2 reduces apoptosis in cultured calvarial cells (Hill et al. 1997) but stimulates apoptosis in the developing coronal suture (Mathijssen et al. 2001). In transgenic mice overexpressing FGF2, apoptosis is restricted to more differentiated calvarial osteoblasts at the osteogenic front (Mansukhani et al. 2000). These observations and studies on proliferation and differentiation show that FGFs can control the balance between undifferentiated and differentiated osteogenic cells by increasing the proliferation of immature cells and by promoting the differentiation and apoptosis of more mature osteoblasts in developing calvaria (Fig. 4B).

FGFs may also affect calvarial osteogenic cells by regulating endogenous factors such as insulin-like growth factors (IGFs) and their regulatory binding protein BP5 (Canalis and Gabbitas 1995; Hurley et al. 1995), as well as vascular endothelial growth factor (Saadeh et al. 2000), hepatocyte growth factor (Blanquaert et al. 1999), and TGF-β (Noda and Vogel 1989; Debiais et al. 1998). TGF-β2 and TGF-β3 are also secreted by the dura mater and regulate osteogenic suture cell proliferation and apoptosis (Cohen 1997; Opperman 2000). Both TGF-β1 and FGF2 expression are up-regulated as cranial sutures fuse, suggesting a paracrine signal from dura to regulate suture closure (Most et al. 1998). Some BMPs are also expressed in the dura, sutural mesenchyme, and the osteogenic front (Opperman 2000). BMP signaling promotes the differentiation and apoptosis of calvarial osteoblasts (Hay et al. 1999, 2001; Yamaguchi et al. 2000) and may thus act in concert with FGFs to control calvarial growth and differentiation during intramembranous bone development (Fig. 4B; Opperman 2000; Marie et al. 2002).

Bone resorption by osteoclasts is required to maintain the shape of craniofacial bones during development. It is therefore significant that FGF2 can increase the formation of osteoclast-like cells (Hurley et al. 1998; Nakagawa et al. 1999) and activate mature osteoclasts (Kawaguchi et al. 2000) through FGFR1 (Chikazu et al. 2000). FGF18 also can promote osteoclast formation through RANKL (receptor activator of NFκB ligand) signaling (Shimoaka et al. 2001). In addition, FGF2 increases the expression of metalloproteinases, collagenases 1 and 3 (Varghese et al. 1995, 2000; Tang et al. 1996; Newberry et al. 1997), tissue inhibitors of metalloproteinases (TIMP) 1 and 3 (Varghese et al. 1995), and stromelysin-3 (which regulates collagenase activity in calvarial cells; Delaney and Canalis 1998). These mechanisms may control bone matrix degradation and remodeling by FGFs during calvarial expansion.

FGF signaling pathways in intramembranous bone formation

Multiple FGF-induced signaling pathways are involved in the control of the calvarial cell phenotype during intramembranous bone formation. FGFs activate ERK and p38 MAPK signaling pathways in osteoblasts (Hurley et al. 1996; Chaudhary and Avioli 1997, 2000; Kozawa et al. 1999; Tokuda et al. 2000; Shimoaka et al. 2001). FGF2 and FGF18 stimulate ERK2 phosphorylation, which promotes mitogenesis (Hurley et al. 1996; Chaudhary and Avioli 1997, 2000; Shimoaka et al. 2001) and down-regulates procollagen gene expression in calvarial osteoblasts (Chaudhary and Avioli 1997, 2000). The protein kinase C (PKC) pathway is involved in the control of sodium-dependent phosphate transport (Suzuki et al. 2000) and expression of N-cadherin in calvarial osteoblasts (Debiais et al. 2001). Mutations in *Fgfr2* that cause Apert syndrome induce constitutive activation of PKC in human calvarial osteoblasts (Fragale et al. 1999; Lomri et al. 2001). This signaling pathway is responsible for the increased differentiation and apoptosis in mutant osteo-

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blasts (Lemonnier et al. 2000, 2001a). Whether *Fgfr* mutations induce alterations in other pathways in calvarial osteoblasts is at present unknown.

Control of cranial suture closure by FGF signaling

Several experiments indicate that FGF signaling enhances suture closure by regulating the progression of undifferentiated cells toward mature osteoblasts. Implantation of FGF beads over a suture accelerates suture closure at late developmental stages (Iseki et al. 1997; Kim et al. 1998; Greenwald et al. 2001; Mathijssen et al. 2001). However, at early developmental stages, FGF4 increases proliferation of undifferentiated mesenchymal cells and delays suture closure (Kim et al. 1998). FGF signaling was suggested to shift the cell proliferation/differentiation balance toward differentiation by down-regulating *Fgfr2* expression (Iseki et al. 1997; Kim et al. 1998; Mehrara et al. 1998; Most et al. 1998; Johnson et al. 2000). Accordingly, *Fgfr2* expression is down-regulated in osteoblast progenitor cells and osteoblasts at the osteogenic front in fused sutures in Apert (Bresnick and Schendel 1998; Lemonnier et al. 2000) and Crouzon syndrome patients (Bresnick and Schendel 1995). Negative autoregulation of *Fgfr2* also characterizes cranial development in Apert and Pfeiffer syndromes (Britto et al. 2001b). In mice, the down-regulation of *Fgfr2* induced by FGF2 application at the osteogenic front is associated with increased *Fgfr1* expression, which may enhance osteoblast differentiation (Iseki et al. 1999). Consistent with this hypothesis, expression of a dominant-negative FGFR1 gene inhibits suture fusion in rat calvaria (Greenwald et al. 2001). These data suggest that gradients of FGFR1 and FGFR2 may regulate the balance between proliferation and differentiation of osteogenic cells in the cranial suture.

Several cellular mechanisms may contribute to the premature suture closure induced by gain-of-function mutations in FGFRs. In nonosteoblastic cells, the expression of FGFR2 harboring a Crouzon mutation results in increased cell proliferation (Galvin et al. 1996). In murine calvarial cells, transfection of the *Fgfr2* S252W (Apert mutation) gene inhibits cell differentiation and increases proliferation (Mansukhani et al. 2000). In human osteoblasts, Apert syndrome mutations do not increase cell proliferation (Lomri et al. 1998; Fragale et al. 1999; Lemonnier et al. 2000) but alternatively, increase the expression of type 1 collagen, osteocalcin, and osteopontin, and enhance osteogenesis (Lomri et al. 1998; Lemonnier et al. 2000). This premature osteogenic cell differentiation induced by Apert *Fgfr2* mutations is associated with increased N-cadherin expression and cell–cell adhesion (Lemonnier et al. 2001b), which is reproduced by application of FGF2 (Debiais et al. 2001). The increased osteoblast differentiation and bone formation induced by activating mutations in *Fgfr2* is consistent with the phenotype of stenotic sutures in human nonsyndromic craniosynostosis (De Pollak et al. 1996; Shevde et al. 2001). The murine FGFR1 P250R mutation also increases expression of osteoblast differentiation genes such as *Cbfa1*, bone sialoprotein, and osteocalcin (Zhou et al. 2000). Similarly, activation of FGFR3 by a G369C mutation also enhances osteoblast differentiation during endochondral bone formation (Chen et al. 1999). Therefore, most activating mutations in FGFRs appear to result in premature differentiation of osteogenic cells in the suture (Marie et al. 2000, 2002; Opperman 2000).

Increased apoptosis is another feature of craniosynostoses that is enhanced by mutations in *Fgfr2*. In murine or human osteoblasts, activating FGFR2 C342Y (Crouzon) or S252W (Apert) mutations promote apoptosis (Mansukhani et al. 2000; Lemonnier et al. 2001a). In human mutant osteoblasts, this is mediated by increased expression of *interleukin-1* and the pro-apoptotic proteins FAS and BAX (Lemonnier et al. 2001a). Thus, FGFR2 activation enhances apoptosis in calvarial osteoblasts following acceleration of terminal osteoblast maturation (Fig. 4B).

Conclusions and unresolved questions

Experimental and genetic evidence shows that FGFs and FGFRs are critical for the control of endochondral and intramembranous bone formation during development. FGF signals control the balance among skeletal cell growth, differentiation, and apoptosis. The identification of signaling molecules that act both upstream and downstream of FGFRs has led to a more comprehensive view of the mechanisms that control skeletal development and has provided a molecular basis for understanding the pathogenesis of chondrodysplasia and craniosynostosis syndromes.

Important unresolved issues include the identification of the pathways that are activated by FGF/FGFR signaling at different stages of skeletal development and how these pathways and molecules feed back to regulate FGF signaling. Thus far only 2 of the 22 known FGF ligands have been shown to be essential for skeletal development. The factors that regulate these FGFs and the identity and function of additional members of the FGF family that function in skeletogenesis remain to be discovered. Additional factors that regulate FGF activity, such as heparan sulfate proteoglycans that act as cofactors for FGF–FGFR interactions, may also have important roles in regulating bone growth. Diseases such as Simpson-Golabi-Behmel syndrome, in which increased skeletal growth occurs as a consequence of loss-of-function mutations in the *glypican-3* gene (DeBaun et al. 2001), could act by modulating the activity of either stimulatory or inhibitory FGF signaling pathways.

Several mouse models for chondrodysplasia and craniosynostosis syndromes have been made. These mice appear to phenocopy many aspects of their human disease counterparts. It will be important to exploit these mouse models to assess not only the developmental biology of the phenotype, but also genetic interactions and novel genes affected by altered FGFR activity in bone tissue. Similarly, loss-of-function mutations in FGFR3 have been informative, but analysis of loss-of-function mutations in FGFR1 and FGFR2 in skeletal development

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will require conditional gene targeting or possibly splice form-specific mutations.

Acknowledgments

This work was supported by NIH grant HD39952, a gift from the Virginia Friedhofer Charitable Trust (D.M.O.), and by INSERM and the Ministère de la Recherche et de la Technologie (P.J.M.). We also thank D. Redmond for help with artwork.

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Genes & Development

FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease

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Genes Dev. 2002, **16:**
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