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FGFs in Endochondral Skeletal Development

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ABSTRACT

The mammalian skeleton developments and grows through two complementary pathways: membranous ossification, which gives rise to the calvarial bones and distal clavicle, and endochondral ossification, which is responsible for the bones of the limbs, girdles, vertebrae, face and base of the skull and the medial clavicle. Fibroblast growth factors (FGFs) and their cognate FGF receptors (FGFRs) play important roles in regulating both pathways. However, the details of how FGF signals are initiated, propagated and modulated within the developing skeleton are only slowly emerging. This prospect will focus on the current understanding of these events during endochondral skeletal development with special attention given to concepts that have emerged in the past few years. J. Cell. Biochem. 110: 1046–1057, 2010.

KEY WORDS: FGF; FGFR; SKELETAL DEVELOPMENT; ENDOCHONDRAL OSSIFICATION; GROWTH PLATE

The nature and regulation of the developmental pathways that generate the mammalian skeleton is extremely complex. They involve two sequential phases—morphogenesis and growth—and two distinct forms of ossification—membraneous and endochondral. Fundamentally, the first phase establishes the embryonic skeleton, while the second is responsible for its slow transition to a final adult form. Most of the skeleton forms and grows through endochondral ossification, which involves the synthesis of a cartilage template that is replaced as bone. By contrast, the calvarial bones and the distal clavicle are produced directly from osteoblastic cells through membraneous ossification.

Despite its complexity, much of the biology that underlies mammalian skeletal development has been unraveled in the past decade largely through a combination of human and mouse genetics. The first has led to the identification of genes whose functions are important for skeletal development by virtue of harboring mutations that cause human skeletal dysplasias. Mouse genetic engineering has uncovered many of the developmental pathways and regulatory circuits through which these genes act. Accumulating evidence from this work has pointed to the Fibroblast growth factors (FGFs) and their cognate receptors (FGFRs) as important players in both phases of membranous and endochondral ossification. This review will focus primarily on FGFs in endochondral skeletal development. For more information on these topics, the reader is referred to a number of comprehensive reviews of FGFs and skeletal development published in the past decade [Martin, 1998; Ornitz and Marie, 2002; Chen and Deng, 2005; Eswarakumar et al., 2005; Ornitz, 2005; Mariani et al., 2008; Su et al., 2008; Horton and Degnin, 2009; Pourquie, 2009].

FGFs

Mammalian FGFs encompass a family of structurally related proteins that share a conserved sequence of 120 amino acids and display high sequence identity [Itoh and Ornitz, 2008]. Twenty-three FGFs are named, but since human FGF19 corresponds to mouse FGF15, the family contains only 22 members.[1] Most *FGFs* have been genetically targeted in mice generating phenotypes that range from lethality to no recognized abnormality. Excluding early embryonic lethal mutants, skeletal phenotypes have been observed in mice lacking *FGF2*, *FGF18*, and *FGF23*. Conditional targeting has been used in some cases to restrict defects primarily to the limbs. These studies have revealed additional skeletal abnormalities, especially when two FGFs are simultaneously targeted, that is, FGF4/FGF8.

FGF ligands can be divided into three subfamilies: canonical FGFs (FGF1-10, 16–18, 20, 22), hormone-like FGFs (FGF15/19, 21, 23) and intracellular FGFs (FGF11-14) [Itoh and Ornitz, 2008]. Canonical FGFs are secreted proteins that bind and activate FGFRs in a paracrine manner. Heparin or heparan sulfate proteoglycans are

[1]By convention, upper case is used to designate human genes, transcripts and proteins, while lower case is employed for the mouse counterparts. However, this can be confusing when combining observations from the two species. For simplicity, we have used upper case for both species.

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required for these FGFs to interact with FGFRs. These proteoglyans may reside in the extracellular matrix, that is, perlecan [Govindraj et al., 2006; Smith et al., 2007] or they may take the form of cell surface receptors, such as syndecans [Kirsch et al., 2002]. Subtle differences in the composition and structure of proteoglycans appear to modulate signaling output from ligand-bound FGFRs [Schlessinger et al., 2000; Allen and Rapraeger, 2003]. In fact, enzymatic modification of the extracellular matrix (ECM) can modulate FGF-induced signaling [Lin et al., 1999; Settembre et al., 2008].

As molecules that bind FGFs, proteoglycans also contribute to local retention of canonical FGFs, explaining their paracrine mode of action. Hormone-like FGFs have low affinity for heparin-binding sites and affect target cells elsewhere in the body in an endocrine fashion. They require the cell surface co-receptors, Klotho or ßKlotho, which are synthesized by target cells, to activate FGFRs. Intracellular FGFs act through FGFR-independent means.

FGFs influence a wide range of biologic activities in the contexts of development, homeostasis, injury repair and regeneration. For example, canonical FGFs typically affect proliferation and differentiation of many cell types during development and tissue repair. The hormone-like FGFs regulate metabolic pathways. During homeostasis, FGF23 produced in bone acts in an endocrine fashion to activate FGFR1c/Klotho in the kidney to regulate phosphate and vitamin D metabolism [Shimada et al., 2004; Sitara et al., 2004; Kurosu et al., 2006; Urakawa et al., 2006; Kurosu and Kuro, 2008]. Knock out studies in mice have revealed considerable redundancy both within and across the different FGF subfamilies.

FGFRs

There are four high-affinity FGF receptors (FGFR1-4) [Schlessinger, 2000; Eswarakumar et al., 2005; Mohammadi et al., 2005; Ornitz, 2005; Chen et al., 2007; Su et al., 2008; Beenken and Mohammadi, 2009]. These function as transmembrane receptor tyrosine kinases (RTKs) comprised of an extracellular ligand-binding domain consisting of three immunoglobulin-like subdomains designated D1-3, an acid box domain, a transmembrane domain, and an intracellular domain containing a split tyrosine kinase subdomain (Fig. 1). Alternative splicing of D3 generates 'b' and 'c' isoforms of FGFR1-3 that are expressed primarily in epithelial and mesenchymal cells, respectively, where each isoform has distinct ligand specificity. Skeletal tissues express primarily the ''c'' isoforms, although there is evidence for low levels of the related b isoforms [Pandit et al., 2002; Minina et al., 2005]. Because some FGFs are expressed only in epithelial or mesenchymal cells, this lineage-specific pattern of receptor isoform production enables regulatory interplay between the two germ layers during development.

Biologically relevant ligand:receptor interactions are regulated through binding specificity, which is determined by variation in amino acid sequence among the FGFs and the seven FGFR forms. The temporal and spatial expression of FGFs and FGFRs, together with variations in receptor binding specificity and affinity, which are influenced by local proteoglycan environment, contribute considerable diversity and complexity to FGF signaling [Dailey et al., 2005].

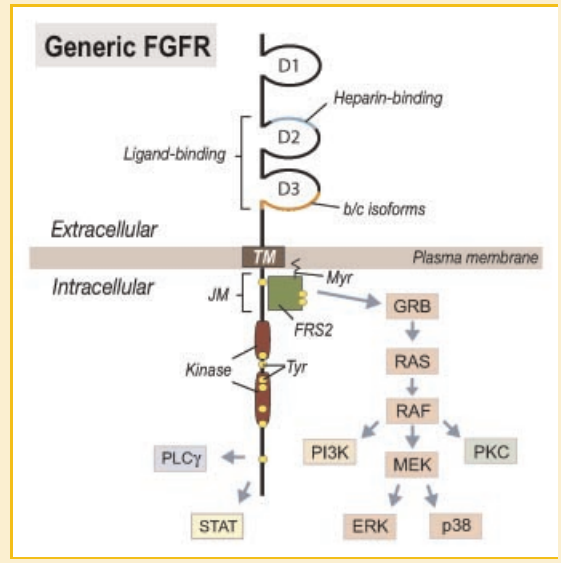

Fig. 1. Domain structure of generic FGFR. Ligand binding occurs in immunoglobulin-like subdomains D2 and D3, while heparin binds to the D2 loop. Tyrosine residues (Tyr) subject to transphosphorylation and initiation of downstream signals map mainly to the split kinase domain. FRS2, which relays signals from activated FGFR through its own tyr residues binds to the receptor near the transmembrane (TM) domain. Targeting of FRS2 to this juxtamembrane (JM) region of the receptor is facilitated its myristoylation (Myr). Receptor activation involves the sequence of ligand binding, dimerization of receptor monomers, activation of tyrosine kinase, phosphorylation of key tyrosine residues on the receptor and on FRS2 and recruitment of molecules to propagate signals downstream. The region labeled b/c isoforms corresponds to the region of the D3 loop that differs between the b and c isoforms of the receptor.

In addition to the four classical FGFRs, another FGFR designated FGFRL1 has been identified in cartilage tissues [Wiedemann and Trueb, 2001]. Its extracellular domain closely resembles that of the classical FGFRs, but its intracellular domain lacks a tyrosine kinase domain. Whereas FGFRL1 is essential for normal skeletal development [Catela et al., 2009; Rieckmann et al., 2009], most evidence to date suggests that it functions as a non-signaling decoy receptor for FGFs [Steinberg et al., 2010].

FGFR ACTIVATION, REGULATION, AND SIGNALING

The current model of ligand-induced FGFR activation suggests that canonical FGFs bind to their cognate FGFRs in the presence of heparin or heparan sulfate proteoglycans to induce dimerization of receptor monomers, which leads to transactivation of the receptor's tyrosine kinase activity [Plotnikov et al., 1999; Eswarakumar et al., 2005; Mohammadi et al., 2005; Chen et al., 2007; Chen et al., 2008a]. Seven tyrosine residues within the intracellular domain of FGFR1, most of which are conserved in FGFR2-4, have been mapped as phosphorylation sites [Hart et al., 2001]. Transphosphorylation of the tyrosine kinase domain of FGFR1 and, by homology, other FGFRs, occurs through a sequential and precisely ordered phosphorylation sequence, which when disturbed can alter the

signaling properties of the receptor [Lew et al., 2009]. Signals are propagated from activated FGFRs by the recruitment of signaling molecules to these phosphorylated residues or to closely linked docking proteins of the Fibroblast growth factor substrate 2 (FRS2) family that bind to the receptor and provide additional tyrosine residues that are phosphorylated in response to receptor activation. The molecular interactions between signaling molecules and the activated FGFR or FRS2 are mediated by phosphotyrosine-binding domains in the recruited signaling molecules and involve both phosphotyrosine-dependent and phosphotyrosine-independent interactions (Fig. 1) [Bae et al., 2009]. These signals are modulated by a growing number of negative regulatory molecules such as Sef (Similar expression to FGF), which binds to FGFRs [Kovalenko et al., 2003; Tsang and Dawid, 2004], Sprouty/Spred family members, which inhibit the Ras-MAPK pathway [Bundschu et al., 2005; Guo et al., 2008], as well as Sulfatase-Modifying Factor 1 (SUMF1), which modifies the ECM to regulate the local retention of FGFs during chondrogenesis [Uchimura et al., 2006; Settembre et al., 2008]. Signals are further modulated by positive regulatory molecules, such as the newly discovered fibronectin leucine-rich transmembrane (FLRT) gene family members [Bottcher et al., 2004; Haines et al., 2006; Gong et al., 2009].

A number of signaling pathways have been identified downstream of FGFR activation, including the STAT, MAP kinase, PLCγ, PI3 kinase, PKC and AKT pathways.[2] The pathways most relevant to skeletal development involving FGFR3 in the growth plate are discussed below. The specific pathways activated by FGFRs and the strength of downstream signals depend on the particular receptor, ligand, and their cellular context, which reflects numerous factors including the developmental and metabolic state of the cells and input from other signaling pathways that modulate FGF signals, such as BMP pathways [Yoon et al., 2006]. FGF signals also influence other signaling pathways, such as WNT and TGF-β pathways [Dailey et al., 2005; Mukherjee et al., 2005].

Although not well defined in the context of skeletal development, there is also evidence that FGFs and FGFRs interact directly with other integral membrane proteins and receptors typically involved in cell:cell and cell:matrix adhesion, such as NCAM/L1 [Kiselyov et al., 2003; Christensen et al., 2006; Francavilla et al., 2009], cadherins [Suyama et al., 2002; Bryant et al., 2005], Eph receptors [Yokote et al., 2005; Fukai et al., 2008] and integrins [Suyama et al., 2002; Aszodi et al., 2003; Kiselyov et al., 2003; Mori et al., 2008; Francavilla et al., 2009]. Several models have been proposed including those in which FGFR activation is induced through its interaction with other integral membrane proteins in the absence of FGF ligands. This latter phenomenon has been referred to as non-canonical FGFR signaling.

In addition to the phosphotyrosine-dependent and -independent interactions between the activated FGFR or its FRS2 proxy and recruited signaling molecules, other factors modulate the strength of FGFR signal output. One factor is survival of the activated receptor, that is, the signal strength associated with an activated receptor is increased as its turnover rate is reduced and vice versa. Both FGFR1 and FGFR3 are targeted to lysosomes for degradation by c-Cbl (Casitas B-lineage lymphoma)-mediated ubiquitination, a mechanism that also terminates signaling of a number of other RTKs [Cho et al., 2004; Haugsten et al., 2008; Komada, 2008]. In response to distinct canonical and non-canonical ligands, c-Cbl is displaced from the activated FGFR complex diverting it from a lysosomal destination to a recycling pathway at the plasma membrane [Belleudi et al., 2009; Francavilla et al., 2009]. This diversion prolongs the receptor's signaling lifetime and thereby alters its overall signal output [Steinberg et al., 2010]. As FGF and non-canonical FGFR ligands are expressed in a spatially and temporally regulated fashion during chondrogenesis, ligand-induced changes in receptor trafficking further increases the complexity of FGFR signaling in the growth plate.

ENDOCHONDRAL SKELETAL DEVELOPMENT

As noted earlier, endochondral skeletal development can be divided conceptually into two phases. The first phase involves morphogenesis or formation of the embryonic skeleton, which ends at mid gestation as the functional growth plate is established (Fig. 2A–H). The second phase consisting of mostly linear growth, plays out within the growth plates (Fig. 2H,I) and continues through skeletal maturation. FGFs are involved in both phases.

MORPHOGENESIS: FORMATION OF THE ANLAGEN

Limb development provides a good model to examine skeletal formation. In early limb outgrowth, expression of FGFs 2, 4, 8, and 9 can be detected in the apical ectodermal ridge (AER) [Martin, 1998]. Skeletal morphogenesis per se begins with the formation of condensations of mesenchymal cells in areas of the embryo destined to become bone (Fig. 2A). These structures give rise to both membranous and endochondral skeletal elements. FGF 2, 5, 6, and 7 expression has been observed in the mesenchyme surrounding them [Haub and Goldfarb, 1991; deLapeyriere et al., 1993; Mason et al., 1994; Finch et al., 1995; Savage and Fallon, 1995]. The initial condensation is most likely mediated by cell–fibronectin interactions [Gehris et al., 1997]. At high cell density these cell–matrix interactions are replaced by cell–cell interactions mediated through the transient expression of NCAM and N-cadherin [Oberlender and Tuan, 1994; Tavella et al., 1994]. The first evidence of endochondral development is the appearance of chondrocyte differentiation markers (Fig. 2B), most notably expression of *SOX9* (SRY (sex determining region Y)-box 9) the master gene for chondrocyte differentiation. *SOX9* activates its target genes in chondrogenic cells, resulting in secretion of extracellular matrix rich in cartilage matrix proteins, such as types II, IX and XI collagen, aggrecan and cartilage oligomeric matrix protein [Morris et al., 2002].

FGFR2 expression coincides with the appearance of chondrocyte markers in the mesenchymal condensations. The condensations consist of two distinct, highly proliferative cell populations: the

[2] Abbreviations used for signaling pathways: AKT = family of serine/threonine kinases, also known as protein kinases B (PKB); BMP = bone morphogenetic protein; ERK = extracellular signal-related protein kinase; MAP kinase = mitogen activated protein kinase; PI3 kinase = phosphoinositol 3 kinase; PKC = protein kinase C; PLCγ = phospholipase Cγ; STAT = signal transducer and activator of transcription; TGF-β = transforming growth factor β.

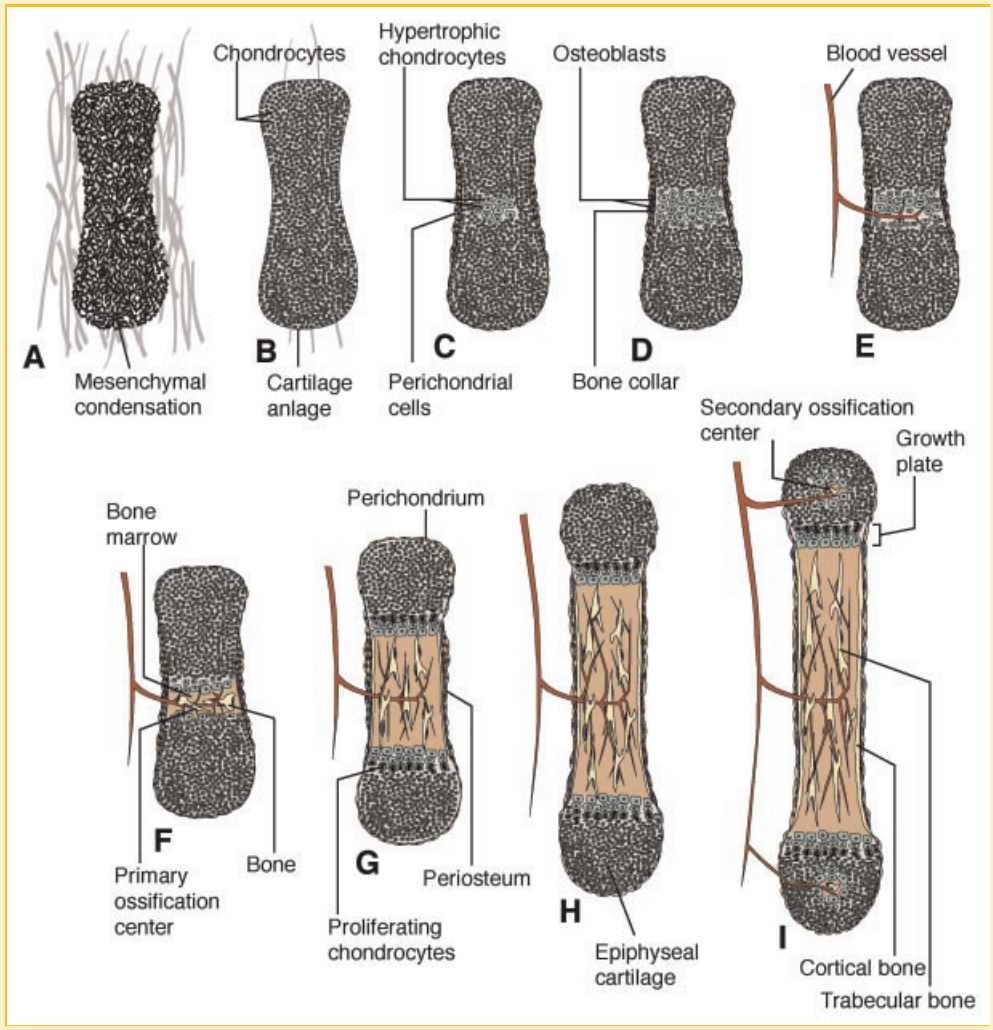

Fig. 2. Endochondral bone development from mesenchymal condensation stage to formation of mature growth plate. Chondrocytes differentiate within mesenchymal condensations to form cartilage anlagen of future bones (A,B). Coincident with the appearance of the perichondrial bone collar (C,D), the chondrocytes in the central anlague hypertrophy followed by invasion of vascular and osteoblastic cells from the collar (E) and formation of the primary ossification center (F). This process expands toward the bone ends eventually forming mature growth plates (H). Secondary ossification centers later form in the epiphyseal cartilage (I). This figure was modified from reference [Horton, 2006] with permission.

central cells that express type II collagen and give rise to chondrocytes, and the peripheral cells that transiently express type II collagen before adopting an osteoblast cell fate expressing alkaline phosphatase and type I collagen. *FGFR1* is detected in the surrounding loose mesenchyme and overlaps with expression of *FGFR2* around the periphery of the condensations [Ornitz, 2005]. *FGFs 2*, *5*, *6*, and *7* have been detected within the central condensing mesenchyme [Ornitz, 2005], whereas *FGF9* has been shown in the surrounding mesenchyme destined to become the perichondrium [Hung et al., 2007]. As the condensations take on the appearance of cartilaginous structures, that is, anlagen of the future skeleton, *FGFR3* expression can be found in the (central) chondrocytic cells. It has been difficult to assign specific functions to the different FGF ligands and receptors at these early stages of skeletal development and models describing these early events continue to evolve [Yu and Ornitz, 2008].

Concurrent with the onset of type II collagen expression, NCAM and N-Cadherin expression is lost, FGFR3 is expressed and adhesion is driven by integrin–ECM interactions. Integrins are essential for chondrocytic maturation and differentiation and may act coordinately with FGFRs [Aszodi et al., 2003; Grashoff et al., 2003]. For example, knockout of β1-integrin or integrin-linked kinase (ILK) targeted to cartilage mimics many phenotypic features of mouse models for FGFR3 gain of function in achondroplasia.

MORPHOGENESIS: DIFFERENTIATION AND VASCULARIZATION

Soon after the cartilage anlagen are formed, chondrocytes in the centers of these structures begin to exit the cell cycle and undergo terminal differentiation, which is often referred to as hypertrophy (Fig. 2C) [Morris et al., 2002; Kronenberg, 2003; Horton, 2006]. This process involves down regulation of *SOX9* and other cartilage-specific genes and up regulation of Runt-related transcription factor

2 (*RUNX2)* and genes characteristic of the prehypertrophic and hypertrophic chondrocyte phenotypes. The former is characterized by expression of Indian hedgehog (*IHH*) [St-Jacques et al., 1999; Mariani et al., 2008], and the latter by expression of a number of genes including those encoding type X collagen, osteopontin, matrix metalloproteinase 13 (MMP-13) and vascular endothelial growth factor (VEGF). These biosynthetic changes are accompanied by substantial enlargement of cells and mineralization of the matrix surrounding the hypertrophic chondrocytes [Morris et al., 2002; Chung, 2004]. The precise role of FGFs at this early stage of ossification is not well defined. FGFR3 is believed to regulate chondrocyte proliferation and differentiation, while FGFR1 mediates cell survival, terminal differentiation, ECM modification, as well as cell death.

Concurrent with these events, mesenchymal cells that surround the cartilage anlagen differentiate into osteoblasts forming a perichondrial collar of membranous bone around the center of the anlagen (Fig. 2C,D) [Kronenberg, 2003; Long et al., 2004; Horton, 2006]. This collar appears to serve as a staging area for the subsequent invasion of the cartilage anlagen. Although FGFs 7, 8, 9, 17, and 18 have been observed in the perichondrium [Mason et al., 1994; Finch et al., 1995; Xu et al., 1999; Liu et al., 2002; Ohbayashi et al., 2002], FGF9 and 18 appear to be the primary ligands involved in chondrogenesis and the promotion of angiogenesis [Hung et al., 2007; Liu et al., 2007]. VEGF and possibly other angiogenic factors secreted from hypertrophic chondrocytes in the central anlagen induce sprouting angiogenesis from the perichondrium (Fig. 2E) [Colnot et al., 2005]. This invasion brings osteoclasts, osteoblasts and haematopoetic cells along with blood vessels into the anlagen as the most terminally differentiated chondrocytes die by apoptosis. The osteoclasts degrade most of the matrix surrounding dying hypertrophic chondrocytes leaving fragments that act as scaffolding for deposition of bone matrix by the osteoblasts. Coordination of osteoblasts and osteoclasts during bone remodeling involves bidirectional signaling between ephrins in osteoclasts and EphB4 in osteoblasts [Zhao et al., 2006]. Whether or not this interaction also involves cross talk with FGFRs expressed in osteoblasts has not been explored. Bone marrow becomes established in spaces between the bony trabeculae. The net result of these events is formation of primary ossification centers (Fig. 2F).

Once the centers are established, ossification spreads toward the ends of the would-be bones as a front within the anlagen (Fig. 2G) [Morris et al., 2002; Horton, 2006]. Chondrocytes adjacent to the ossification front terminally differentiate to prehypertrophic and hypertrophic chondrocytes, which facilitates the hypertrophy, degradation, and replacement of the cartilage. Much of the cartilage anlage is converted to bone except near the ends where chondrocytes proliferate as stacks of flattened cells that synthesize abundant cartilage matrix before proceeding through the prehypertrophic and hypertrophic phases of terminal differentiation. These events become structurally organized, synchronized and compacted into a thin band of tissue—the growth plate or physis—located between the expanding front of bone and epiphyseal cartilages at the ends of the bones (Fig. 2G–I). Secondary ossification centers subsequently form in the epiphyseal cartilages (Fig. 2I). These centers expand mostly at the expense of epiphyseal cartilages and are eventually converted to bone except for a thin layer of articular cartilage at the joint surface.

FGFR1, 2, and 3 can be detected at this stage of endochondral bone development (Fig. 3). *FGFR3* is expressed by differentiated chondrocytes with highest levels in proliferating chondrocytes, while *FGFR1* is expressed in prehypertrophic and hypertrophic chondrocytes [Ornitz, 2005; Jacob et al., 2006]. Expression of both *FGFR1* and *2* can be detected in the osteoblastic cells of the primary ossification centers; osteoclasts express FGFR1 [Chikazu et al., 2001].

LINEAR GROWTH

Growth plates serve as engines for linear growth for endochondral bones from the time they are established in mid gestation through skeletal maturity. They do so through de novo generation of cartilage template followed by the sequential turnover and replacement of this template with new bone at the ossification front. Growth plates are highly ordered, dynamic structures with leading edges where new cells are 'born' as a result of proliferation and trailing edges where chondrocytes die and bone matrix is deposited on remnants of the degraded cartilage template (Fig. 4). Because cells at the same stage of the growth plate chondrocyte life cycle tend to be the same distance from the ossification front, the growth plate appears as zones that correspond to the stages of endochondral ossification. Growth plates vary in appearance by age, anatomic location and species.

Many growth factors including BMPs, other members of the TGF-β superfamily, insulin-like growth factors (IGFs), WNTs, hedgehog proteins, retinoids and FGFs, have been detected within or nearby the growth plate and are implicated in regulating different aspects of growth plate functions [Fujimori et al., 2009; Kronenberg et al., 2009]. Most relevant here and in contrast to early development, expression of *FGFs 1*, *2*, *6*, *7*, *9*, *18*, *21*, and *22* has been detected in the perichondrium of the postnatal rat proximal tibia [Lazarus et al., 2007]. *FGFs 2*, *7*, *18*, and *22* were identified in the growth plate proper, but at lower levels than in the perichondrium. A compilation of FGF and FGFR expression patterns is illustrated in Figure 3. Krejci et al. [2007] reported evidence for 15 different FGF ligands in human fetal growth plate cartilages, but based on functional assays concluded that only FGFs 1, 2, and 17 were likely to activate FGFR3. It is difficult to determine which of these reported FGF ligands is physiologically significant. However, as discussed below, *in vivo* experiments suggest that FGF18 and possibly FGF9 are significant at least for FGFR3.

Most, if not all, of these growth factors likely function as elements of regulatory circuits; however, only a few of these circuits have been delineated. The best defined regulatory circuit to date, which is in fact relevant to FGF signaling, is the IHH-PTHrP (parathyroid hormone-related protein) feedback loop that regulates the pool sizes of proliferating and hypertrophic chondrocytes and the rate of chondrocyte terminal differentiation in embryonic bones and in postnatal bones [Kronenberg, 2003; Kronenberg et al., 2009]. IHH secreted from prehypertrophic chondrocytes induces *PTHrP* expression in cells distal to the growth plate, that is, periarticular cells and articular chondrocytes in embryos, and later in growth plate proliferating chondrocytes [Chen et al., 2008b]. PTHrP then signals through its receptor, PTHR1, to inhibit chondrocyte

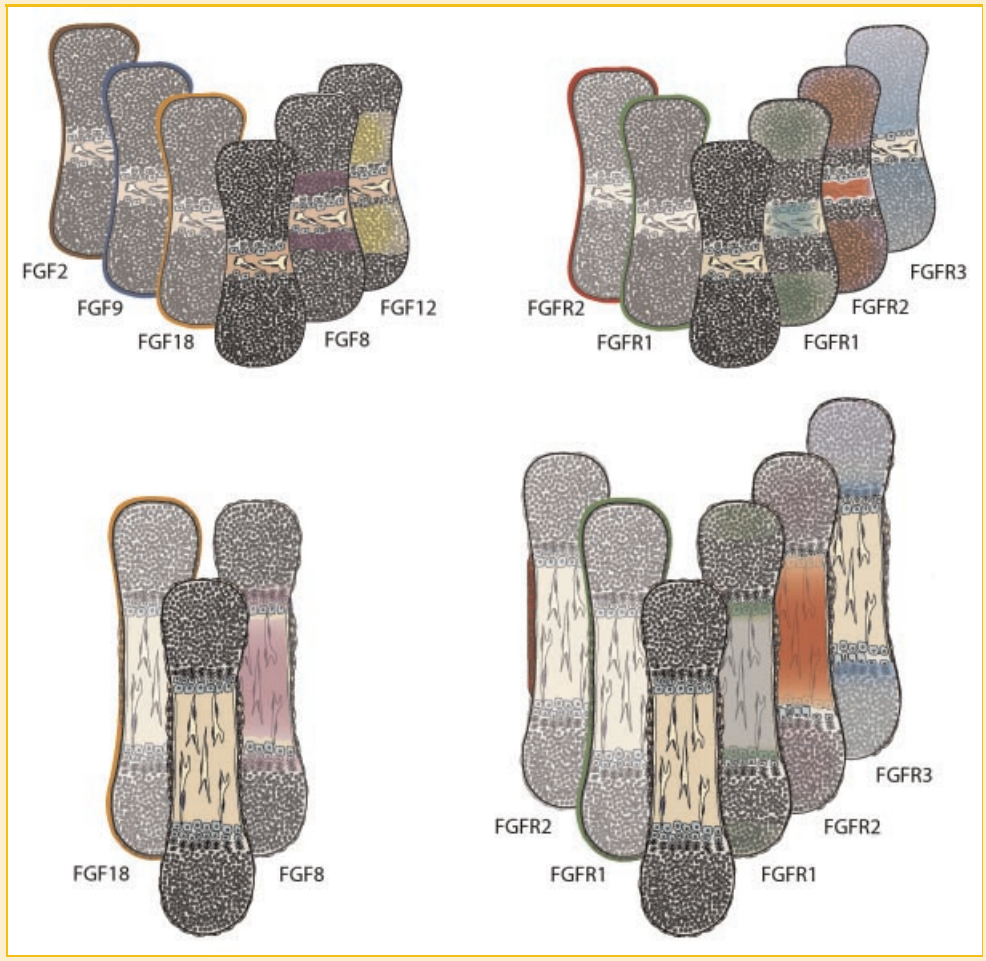

Fig. 3. Approximation of FGF/FGFR expression in an endochondral bone following formation of the primary ossification center (above) and after the growth plate has matured (below). Expression patterns of FGFs is shown on left and FGFRs on right. Within each panel perichondrium/periosteum expression is depicted on the left; cartilage and bone expression is on the right. This illustration was constructed from long bone in situ hybridization results compiled from several reports [Liu et al., 2002; Ohbayashi et al., 2002; Minina et al., 2005; Hung et al., 2007; Yu and Ornitz, 2007].

hypertrophy and thereby prevent further production of IHH. This action keeps the chondrocytes in a proliferative state and sustains the zone of proliferating chondrocytes. IHH produced by prehypertrophic chondrocytes also acts independent of PTHrP [Mak et al., 2008].

FGFR3 has been established as an important negative regulator of the mature post-embryonic growth plate. Indeed, heterozyous gain-of-function mutations of FGFR3 are known to cause the most common forms of dwarfism in humans— achondroplasia, thanatophoric dysplasia, and hypochondroplasia [Horton et al., 2007]. FGFR3 signaling reduces the pace of cartilage template formation and turnover. Most evidence suggests that FGFR3 inhibits both the proliferation and terminal differentiation of growth plate chondrocytes and synthesis of matrix by these cells [Horton and Lunstrum, 2002; Kronenberg, 2003; Ornitz, 2005]. This inhibitory function is compatible with its expression in cells exiting the cell cycle. However, it is proposed alternatively that FGFR3 induces premature terminal differentiation reducing the number of cells that contribute to template synthesis [Minina et al., 2002; Dailey et al., 2005].

It has been suggested that FGFR3 promotes growth plate chondrocyte proliferation in the early to mid stages of skeletal development, switching to its inhibitory function as the growth plate matures later in gestation [Chen and Deng, 2005; Ornitz, 2005]. The evidence comes from mouse embryos examined at days 14–15 (proliferation) and at day 18 (inhibition) of the mouse 18-day gestation [Iwata et al., 2000]. The apparent switch in function most likely reflects differences in so-called cellular context of cells at the different developmental stages. The specific factors that mediate this switch have yet to be identified.

Identification of the physiologic ligand(s) for FGFR3 has been elusive, mostly because of the proximity to the growth plate of multiple FGF ligands and their apparent redundancy. A vital clue came from targeting *FGF18* in mice, which produced a skeletal phenotype very similar to that observed in mice lacking FGFR3 [Liu et al., 2002; Ohbayashi et al., 2002]. Further studies on FGF18 found its expression up regulated in perichondrial cells by RUNX2 [Hinoi et al., 2006]. Because IHH upregulates *RUNX2* [St-Jacques et al., 1999; Komori, 2005; Shimoyama et al., 2007], these observations raise the possibility of a second feedback loop in

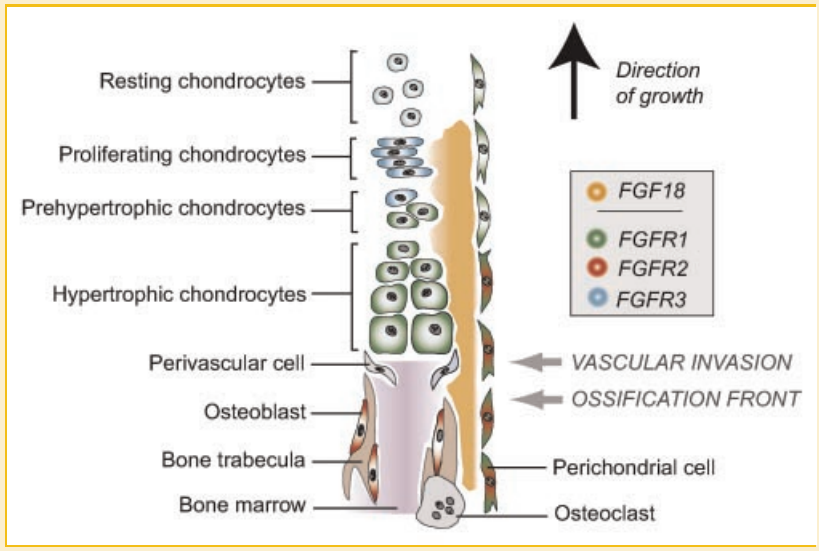

Fig. 4. Structure of mature growth plate with relevant cell types and landmarks. Cells are color coded for FGFRs and the predominant FGF (FGF18). See text for further discussion.

which IHH stimulates *RUNX2* expression by differentiating osteoblastic cells in the perichondrium adjacent to the growth plate, which then promotes expression of *FGF18*. FGF18 could in turn diffuse back to nearby proliferating chondrocytes to bind and activate FGFR3, antagonizing further chondrocyte proliferation and terminal differentiation. The existence of this feedback loop is speculative at this time.

Liu and Ornitz have also suggested that FGF18 promotes vascular invasion of the growth plate independent of FGFR3 by inducing VEGF expression [Liu et al., 2007]. If so, it might coordinate neovascularization and recruitment of osteoblasts and osteoclasts to the ossification front. Thus, FGF18 might play a central role in orchestrating different components of endochondral ossification, but more studies are needed to confirm this function. There is also evidence that FGF9 contributes to regulation of the growth plate, especially proximally. Early in chondrogenesis the FGF9 null mouse best phenocopies mice with the conditional knockout of FGFR1 suggesting that FGF9 may signal through FGFR1 at these stages. Loss of FGF9 contributes to a delayed expression of IHH and PTHr1 early in chondrogenesis, suggesting that FGF9 may work in concert with FGF18 to regulate the IHH-PTHrP feedback loop [Hung et al., 2007].

The functions of FGFR1 and FGFR2 in the growing endochondral skeleton are less well characterized compared to FGFR3. Yu et al. [2003] conditionally inactivated FGFR2 in condensing mesenchyme, eliminating its expression in both chondrocytic and osteoblastic lineages. The resulting skeletal phenotype was dominated by postnatal dwarfism and osteopenia, which was attributed to defective proliferation of osteoprogenitor cells. The dwarf mice exhibited a reduction of hypertrophic chondrocytes in the growth plate, but the mechanism has not been determined. *FGFR1* has also been conditionally ablated [Jacob et al., 2006]. Using a *Col2-Cre* driver to ablate *FGFR1* in both chondrocytic and osteoblastic progenitor cells present in mesenchymal condensations, Jacob et al. detected an expanded hypertrophic zone in late gestation (E16-5-18.5) mouse embryos. The findings were attributed to disturbed vascular invasion and delayed maturation of the hypertrophic chondrocytes. When *FGFR1* ablation was restricted to the osteoblastic lineage by using a *Col1-Cre* driver, the observations suggested that FGFR1 suppresses proliferation and stimulates differentiation of osteoprogenitor cells. Ostensibly, these findings imply that FGFR1 signals have opposite effects on differentiation of growth plate chondrocytes and osteoblasts. However, given the complex feedback between these two lineages, it is difficult to draw any firm conclusions.

PATHWAYS THAT PROPAGATE AND MODULATE FGFR SIGNALS IN THE GROWTH PLATE

Many signaling pathways have been proposed to transmit FGFR signals in the developing endochondral skeleton [Hart et al., 2000; Eswarakumar et al., 2005]. Those downstream of FGFR3 have received the most attention with the strongest evidence pointing to STAT and MAP kinase pathways [Li et al., 1999; Sahni et al., 1999; Murakami et al., 2004; Zhang et al., 2006]. Indeed, STAT signals induce expression of cell cycle inhibitors such as $p21^{Waf1/Cip1}$ (cyclin-dependent kinase inhibitor 1A) and down regulate expression of promitotic factors [Su et al., 1997; Dailey et al., 2003]. Both p38 and ERK arms of the MAP kinase pathway propagate FGFR3 signals that antagonize chondrocyte proliferation and terminal differentiation [Murakami et al., 2004; Zhang et al., 2006]. In fact, targeting expression of transgenes that constitutively activate these pathways in cartilage leads to skeletal phenotypes characteristic of achondroplasia in mice.

Pathways have been identified that modulate the strength of FGFR3 signals. For example, SOCS (Suppressors of cytokine-inducible stimulation of STAT) proteins induced in response to FGFs

modulate FGFR-STAT signals [Ben-Zvi et al., 2006]. *Snail 1*, a transcription factor normally expressed in prehypertrophic and hypertrophic chondrocytes and induced by FGFR3 signaling, inhibits both chondrocyte proliferation and terminal differentiation and it may be necessary for FGFR3 signaling [de Frutos et al., 2007]. Snail 1 regulates both STAT and MAP kinase pathways and is proposed to act as a coordinator of FGFR3 signaling in the growth plate.

Another pathway that influences FGFR3 signals transmitted through MAP kinase effectors involves C-type natriuretic peptide (CNP) [Schulz, 2005]. Following binding to its G-protein coupled receptor, natriuretic peptide receptor B (NPR-B), CNP induces accumulation of intracellular cGMP. The genes coding for both CNP and NPR-B are expressed in the proliferative and prehypertrophic zones of the growth plate, respectively, setting up a potential autocrine or paracrine feedback loop. Signals downstream of FGFR3 and NPR-B intersect at the level of *raf-1* such that the CNP-NPR-B signals antagonize MAP kinase signaling [Krejci et al., 2005; Horton et al., 2007].

Modulation of FGFR3 signals by CNP was validated in mouse experiments in which targeting CNP expression to growth plate cartilage corrected the bone growth deficiency that had resulted from expression of an achondroplasia FGFR3 transgene in growth plate cartilage [Yasoda et al., 2004]. The "therapeutic" effect involved reducing MAP kinase-ERK signals downstream of FGFR3. These findings are encouraging for potential treatment of achondroplasia with the caveat that the *Col2a1* promoter/enhancer used to drive *CNP* expression in these mice is quite strong and most likely generated a high local concentration of CNP in the growth plates of these mice. However, in a follow up study, Yasoda et al. corrected the growth deficiency in the achondroplastic mice by continuous infusion of CNP for an extended period of time [Yasoda et al., 2009].

EFFECTS OF FGF23 ON SKELETAL DEVELOPMENT

The hormone-like FGF23, which is produced primarily by mineralized tissues, indirectly influences skeletal development through its regulation of inorganic phosphate homeostasis [Yoshiko et al., 2007; Kurosu and Kuro, 2008; Razzaque, 2009]. There is some debate about where FGF23 signals, FGF23 signals to a FGFR/Klotho complex in the kidney to reduce phosphate resorption and thereby increase urinary phosphate excretion [Liu et al., 2008]. FGF23 suppresses expression of 1-α-hydroxylase to decrease production of the active vitamin D metabolite, $1,25(OH)_2D$, which reduces intestinal phosphate adsorption, further reducing serum phosphate [Shimada et al., 2004]. FGF23 also influences skeletal mineralization and chondrocyte differentiation through a mechanism independent of its effect on phosphate homeostasis [Sitara et al., 2008]. The clinical manifestation of hypophosphatemia is rickets in the growth plate and osteomalacia in bone. FGF23 mutations in humans have been identified in autosomal dominant hypophosphatemic rickets. They alter the structure of FGF23 making it resistant to normal degradation, thereby exaggerating its phosphate-lowering actions. FGF23 is also a direct negative regulator of parathyroid hormone (PTH) synthesis and secretion [Ben-Dov et al., 2007; Krajisnik et al., 2007]. PTH deletion in a hypophosphatemic rickets (Hyp) mouse reverses the phenotype, leading to hyperphosphatemia and hypocalcemia [Bai et al., 2007] and supporting a role for PTH downstream of FGF23 signaling. Interestingly, a second site of Klotho expression is the parathyroid gland [Kuro-o, 2008].

CONCLUSIONS

FGFs play essential roles in both the formation and subsequent growth of the endochondral skeleton. Defining specific receptor interactions and functional responses during the genesis of the skeleton has been difficult because of overlap in expression patterns of both ligands and receptors and apparent redundancy of ligand functions. However, as the growth plate becomes established, FGFRs become segregated such that *FGFR1* is expressed primarily by terminally differentiating chondrocytes and osteoblasts, *FGFR2* by bone cells and *FGFR3* by proliferating chondrocytes. The function of FGFR3 is best defined in this context; it acts as an inhibitor of chondrocyte proliferation and terminal differentiation. In fact, the most common forms of dwarfism in humans result from mutations that enhance FGFR3 inhibition of bone growth. Evidence to date points to FGF18 derived from perichondrial osteoblastic cells and possibly FGF9 as ligands that activate FGFR3. FGF18 might also be part of a negative feedback loop involving IHH secreted from prehypertrophic chondrocytes and mediated by RUNX2 in the perichondrial cells. FGF23, acting as an endocrine hormone, indirectly influences mineralization associated with endochondral ossification by regulating phosphate homeostasis.

Despite progress in understanding the influence of FGFs on endochondral bone development, many fundamental questions remain unanswered. For example, what other local regulatory circuits do FGFs participate in, and how do these circuits interface with non-FGF circuits that regulate cellular events in the growth plate? Similarly, do systemic hormones influence FGF paracrine regulation of skeletal development and if so, how? MAP kinase pathways have been implicated as the primary propagators of FGFR3 inhibitory signals, but the link between these pathways and the cellular machinery that controls proliferation and differentiation is largely unknown.

There is a consensus that canonical FGFs secreted by perichondrial cells, such as FGF18, activate FGFRs in the growth plate. Perichondrial-derived FGFs bind to heparin and heparan-sulfate proteoglycans, which are very abundant in cartilage matrix and would be expected to impede diffusion of ligands beyond a few cell diameters from the perichondrium. How then is the signal transmitted across the growth plate so that chondrocytes in the center respond to the same extent as cells in the lateral edges? Could interactions between FGFRs and integral membrane proteins that mediate cell:matrix contacts contribute to this phenomenon? A number of human diseases, mostly genetic disorders, have now been identified as caused by abnormalities FGFs or FGFRs. A few have been mentioned in this review. It is likely that developing definitive therapies for these conditions will require answering these and related questions.

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