Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18

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Gain of function mutations in fibroblast growth factor (FGF) receptors cause chondrodysplasia and craniosynostosis syndromes. The ligands interacting with FGF receptors (FGFRs) in developing bone have remained elusive, and the mechanisms by which FGF signaling regulates endochondral, periosteal, and intramembranous bone growth are not known. Here we show that *Fgf18* is expressed in the perichondrium and that mice homozygous for a targeted disruption of *Fgf18* exhibit a growth plate phenotype similar to that observed in mice lacking *Fgfr3* and an ossification defect at sites that express *Fgfr2*. Mice lacking either *Fgf18* or *Fgfr3* exhibited expanded zones of proliferating and hypertrophic chondrocytes and increased chondrocyte proliferation, differentiation, and Indian hedgehog signaling. These data suggest that FGF18 acts as a physiological ligand for FGFR3. In addition, mice lacking *Fgf18* display delayed ossification and decreased expression of osteogenic markers, phenotypes not seen in mice lacking *Fgfr3*. These data demonstrate that FGF18 signals through another FGFR to regulate osteoblast growth. Signaling to multiple FGFRs positions FGF18 to coordinate chondrogenesis in the growth plate with osteogenesis in cortical and trabecular bone.

[*Key Words*: FGF18; FGFR2; FGFR3; endochondral bone growth; chondrocyte; osteoblast]

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Fibroblast growth factors (FGFs) are a family of polypeptides that have important roles in cell growth, differentiation, survival, and numerous developmental processes. The 22 members of the FGF family can be grouped into subfamilies based on greater sequence similarity (Ornitz and Itoh 2001). FGFs can activate one of four high-affinity FGF receptor (FGFR) tyrosine kinases, and FGF subfamilies tend to share similar receptor specificity toward specific alternatively spliced variants of FGFRs (Johnson and Williams 1993; Ornitz et al. 1996; Naski and Ornitz 1998). The discovery that several human skeletal dysplasia syndromes result from point mutations in *Fgfr1*, *Fgfr2*, and *Fgfr3* suggests that FGFR signaling is an essential component of the regulatory cascades governing skeletal growth and development (Muenke and Schell 1995; Naski and Ornitz 1998). However, the physiologically functional FGF ligand(s) that signal to these receptors have remained elusive.

Skeletal development is highly regulated by a hierarchy of genetic, endocrine, and mechanical regulatory programs (Caplan and Pechak 1987; Hall and Miyake 1992; Erlebacher et al. 1995; Karsenty et al. 2001). In mammals, formation of the skull and the medial part of the clavicles is achieved by intramembranous ossification. The remainder of the skeleton develops through the process of endochondral ossification in which cartilage is converted into bone. Cartilage is formed by condensation of mesenchymal cells, which subsequently differentiate into growth plate chondrocytes localized at the ends of the growing bone (Hall and Miyake 2000). Growth plate chondrocytes are arranged in columns that sequentially develop through proliferative, prehypertrophic, and hypertrophic stages. Distal hypertrophic chondrocytes undergo apoptosis and are replaced by trabecular bone and bone marrow (Gibson 1998; Gerber and Ferrara 2000). In a separate process, cortical bone is generated by osteoblasts derived from osteoprogenitor cells in the perichondrium (Caplan and Pechak 1987). An essential feature of skeletal growth is the synchronous regulation of endochondral and cortical bone formation.

Several signaling pathways have been shown to be important for the regulation of bone growth. Parathyroid hormone-related peptide (PTHrP) signaling regulates the process of chondrocyte maturation. Targeted disruption of *PTHrP*, which is expressed in the periarticular perichondrium, or its receptor, *PTHrPR*, which is expressed in prehypertrophic chondrocytes, results in premature maturation of chondrocytes and a short-limbed dwarfism (Karaplis et al. 1994; Lanske et al. 1996). *Indian*

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hedgehog (*Ihh*), which is expressed in prehypertrophic and hypertrophic chondrocytes, induces the expression of *PTHrP*, and *Ihh*$^{-/-}$ mice show delayed chondrocyte maturation and a short-limbed dwarfism, similar to that of *PTHrP*$^{-/-}$ mice (St-Jacques et al. 1999). *Ihh* also stimulates chondrocyte proliferation in a largely *PTHrP*-independent pathway (Karp et al. 2000). *Fgfr3* is expressed in proliferating and prehypertrophic chondrocytes (Peters et al. 1993; Naski et al. 1998). *Fgfr3*$^{-/-}$ mice show an expanded growth plate, increased cell proliferation, and increased expression of *Ihh* (Colvin et al. 1996; Deng et al. 1996; Naski et al. 1998). Mutations activating *Fgfr3* or overexpression of an activated *Fgfr3* in proliferating chondrocytes results in decreased chondrocyte proliferation and differentiation and decreased *Ihh* expression and consequently signaling (Naski et al. 1998, Ornitz 2001). These studies suggest that signaling through *Fgfr3* negatively regulates chondrocyte proliferation, differentiation, and the activity of *Ihh* and *PTHrP*. *Fgfr1* and *Fgfr2* are expressed in hypertrophic chondrocytes and perichondrium, respectively (Orr-Urtreger et al. 1991; Peters et al. 1992). The precise function of these receptors in bone development is not known.

One interesting but unsolved issue in skeletal development is the identification of the endogenous ligand(s) for FGFRs expressed in the epiphyseal growth plate and perichondrium/periosteum. Some clues may come from the study of limb development in which several FGFs have essential roles (Martin 1998; Naski and Ornitz 1998). For example, *Fgf8* and *Fgf10* are essential for the progressive outgrowth and patterning of the limb bud (Ohuchi et al. 1997; Min et al. 1998; Lewandoski et al. 2000; Moon and Capecchi 2000). *Fgf4*, *Fgf9*, and *Fgf17* are also expressed in the developing limb (Martin 1998; Colvin et al. 1999). *Fgf8* and *Fgf17* are expressed in some skeletal elements (Xu et al. 1999), and *Fgf2* is abundantly expressed in chondrocytes (Hill et al. 1992; Twal et al. 1994; Luan et al. 1996). However, gene targeting experiments have not identified a role for these ligands, individually, in either limb or skeletal development (Dono et al. 1998; Ortega et al. 1998; Zhou et al. 1998; Moon et al. 2000; Sun et al. 2000; Xu et al. 2000; Colvin et al. 2001a,b). Therefore, either these FGFs are redundant or novel FGFs must function in developing bone to regulate FGFRs.

Fgf18 is most closely related to *Fgf8* and *Fgf17* (Ornitz and Itoh 2001). All three of these ligands share similar receptor specificity towards the c splice forms of FGFR1–3 and display overlapping expression patterns in several tissues (Xu et al. 1999, 2000). Here we show that *Fgf18* is expressed in the developing perichondrium, making it an attractive candidate to regulate the developing skeleton. We demonstrate that *Fgf18* null mice develop a growth plate phenotype similar to that of mice lacking *Fgfr3* and that FGF18 negatively regulates the IHH signaling pathway in developing bone. Decreased endochondral and intramembranous ossification suggests that FGF18 positively regulates osteogenesis and/or osteoblast function through another FGFR. These data demonstrate that FGF18 is an important regulator of both chondrogenesis and osteogenesis and may function to coordinate these developmental processes.

Results

Fgf18 *expression in developing long bone*

The origin of the source of the FGF signal acting on FGFRs in the growth plate is not known. Examination of *Fgf18* mRNA expression in developing long bone at E14.5 detected a prominent signal in the perichondrium and developing joints (Fig. 1). The expression of *Fgf18* in the perichondrium juxtaposed a source of an FGF ligand with *Fgfr3*-expressing proliferating chondrocytes, *Fgfr1*-expressing hypertrophic chondrocytes, and *Fgfr2*-expressing perichondrium and periosteum. This expression pattern suggests a paracrine mechanism of action of FGF18 on chondrocytes and an autocrine or juxtacrine signal to osteoblasts.

Targeting the Fgf18 *gene*

To study the in vivo functions of the *Fgf18* gene, an *Fgf18* null allele was generated through homologous recombination. The *Fgf18* targeting strategy eliminated the first exon in the protein coding region, including the translation initiation site and the signal peptide (Fig. 2a). One correctly targeted embryonic stem (ES) cell clone was used to generate chimeric male mice that passed the targeted allele to offspring (Fig. 2b,c). Mice heterozygous for the targeted allele (*Fgf18*$^{-/+}$) have a normal phenotype. These mice were bred to produce homozygous mice lacking a functional *Fgf18* gene (*Fgf18*$^{-/-}$). Comparison of in situ hybridization patterns in wild-type and *Fgf18*$^{-/-}$ mice showed loss of expression in sites where *Fgf18* is normally expressed, such as developing craniofacial tissue (Fig. 2d).

Because β-galactosidase was introduced into exon 1 of *Fgf18*, the pattern of β-galactosidase activity should indicate sites where *Fgf18* is normally expressed. Staining for β-galactosidase in the cranium showed expression along the endosteal and periosteal surfaces of the calvarial bones (Fig. 2e). In developing limb, expression was restricted to the perichondrium, presumptive joint space, and in interdigital mesenchyme (Fig. 2f–i). This expression pattern was consistent with the pattern of *Fgf18* mRNA expression and demonstrated no obvious changes in expression in heterozygous versus homozygous mice.

Fgf18$^{-/-}$ mice survived embryonic development but died in the early neonatal period. *Fgf18*$^{-/-}$ embryos were about 10%–15% smaller than normal littermates and died of cyanosis within 30 min after birth, probably due to respiratory failure. More than 90% of *Fgf18*$^{-/-}$ embryos developed a complete cleft palate (see below). However, cleft palate is normally not associated with early neonatal death, and mice with cleft palate usually can survive up to 24 h (Peters et al. 1998; Halford et al. 2000).

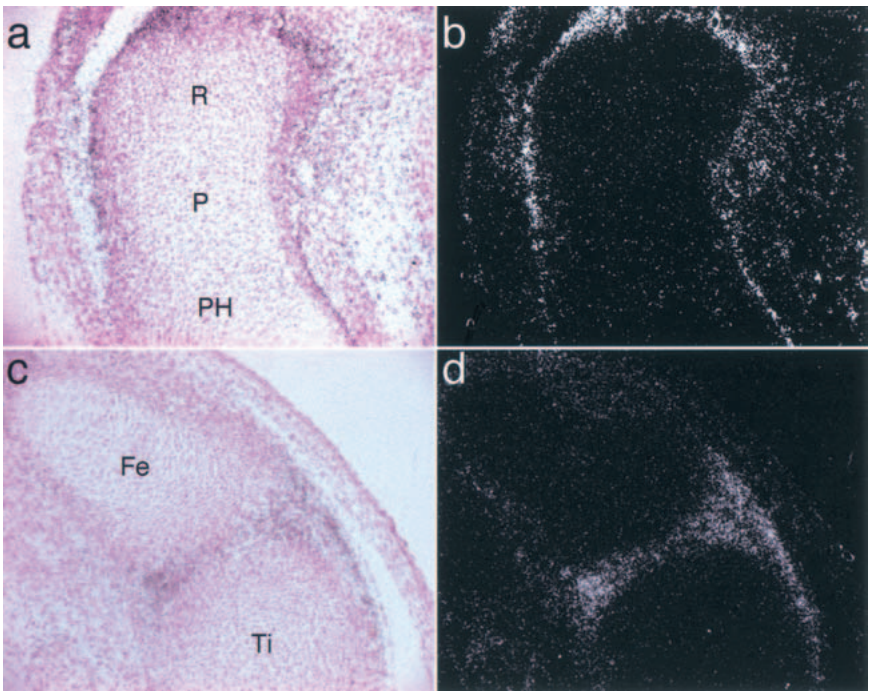

Figure 1. *Fgf18* expression in E14.5 developing limb. (*a*) Proximal humerus growth plate counterstained with hematoxylin and viewed in bright field. (*b*) *Fgf18* in situ hybridization viewed in dark field. Note the expression of *Fgf18* in the perichondrium. (*c*) Knee joint counterstained with hematoxylin and viewed in bright field. (*d*) *Fgf18* in situ hybridization viewed in dark field. Note the expression of *Fgf18* in the joint tissue and perichondrium. (R) Reserve chondrocytes; (P) proliferating chondrocytes; (PH) prehypertrophic chondrocytes; (Fe) femur; (Ti) tibia.

Skeletal pathology of Fgf18*-targeted mice*

All *Fgf18*$^{-/-}$ mice exhibited skeletal abnormalities (Fig. 3a). Skeletal preparations at different stages of embryogenesis showed that ossification in *Fgf18*$^{-/-}$ mice lagged ~2 d behind that of wild-type littermates (Fig. 3b,c,e,f). The radius and tibia often showed increased curvature and the ossified portion was shortened in these bones (Fig. 3a,c,e,f). Incomplete development of the fibula was also observed in four of 11 mice (Fig. 3c; data not shown). The ribs of all *Fgf18*$^{-/-}$ skeletons were deformed, resulting in reduction of thoracic cavity volume. This defect could contribute to mechanical problems with ventilation and lead to the observed cyanosis and neonatal death. In contrast, skeletons from *Fgfr3*$^{-/-}$ mice were more like the wild-type skeletons at this stage of development, but showing some curvature of the tibia and expanded cartilage regions (Fig. 3d; data not shown).

In addition to defects in the appendicular and axial skeletons, specific defects were also observed in the craniofacial bones and palate. A general reduction in cranial ossification was observed in *Fgf18*$^{-/-}$ but not in *Fgfr3*$^{-/-}$ mice (Fig. 3b). The cranial vault in newborn *Fgf18*$^{-/-}$ mice was slightly smaller and more rounded, reflecting changes in the size and shape of the calvarial elements. Mesenchymal regions that preform the cranial sutures were widened, probably reflecting decreased growth of calvarial bones (data not shown). The facial skeleton also showed an underdeveloped maxilla (Fig. 3b) and a cleft palate (Fig. 3g,h). Preliminary analysis indicates that the cleft palate may result from a failure of the palatal shelves to properly elevate. These findings were unique to *Fgf18*$^{-/-}$ mice and were not observed in *Fgfr3*$^{-/-}$ mice.

Abnormal chondrogenesis in the growth plate of Fgf18$^{-/-}$ *mice*

In the developing long bone, cells in the growth plate progress through stages of proliferation, hypertrophy, and apoptosis. The distal hypertrophic zone is eventually invaded by vascular elements and replaced with trabecular bone. The defined histomorphological zones of the growth plate outline the various stages of chondrocyte differentiation. Histological analysis showed an overall intact cellular architecture in the growth plate of *Fgf18*$^{-/-}$ embryos. However, the zones of proliferating and hypertrophic chondrocytes were significantly elongated (Fig. 4a,b; Table 1). The height of the distal femoral hypertrophic zone at E16.5 and E18.5 was increased by 60% ($P < 0.005$) and 37% ($P < 0.02$) in *Fgf18*$^{-/-}$ mice relative to littermate controls, respectively. The height of the distal femoral proliferating zone was also increased at E16.5 by 14% ($P < 0.02$) in *Fgf18*$^{-/-}$ mice relative to littermate controls (Fig. 4a,b; Table 1). These data are similar to that observed in *Fgfr3*$^{-/-}$ mice (Colvin et al. 1996).

The expanded proliferating and hypertrophic chondrocyte regions resulted in an enlarged growth plate in *Fgf18*$^{-/-}$ mice compared to normal littermates (Figs. 3, 4a,b; data not shown). However, the overall length of the long bones was nearly normal. Examination of skeletal preparations demonstrated a delay in the formation of ossification centers in *Fgf18*$^{-/-}$ mice, which could account for the shortened mineralized region (Fig. 3).

Fgf18 *inhibits chondrocyte proliferation and differentiation*

Long bone growth requires the continuous proliferation and differentiation of chondrocytes in the epiphyseal

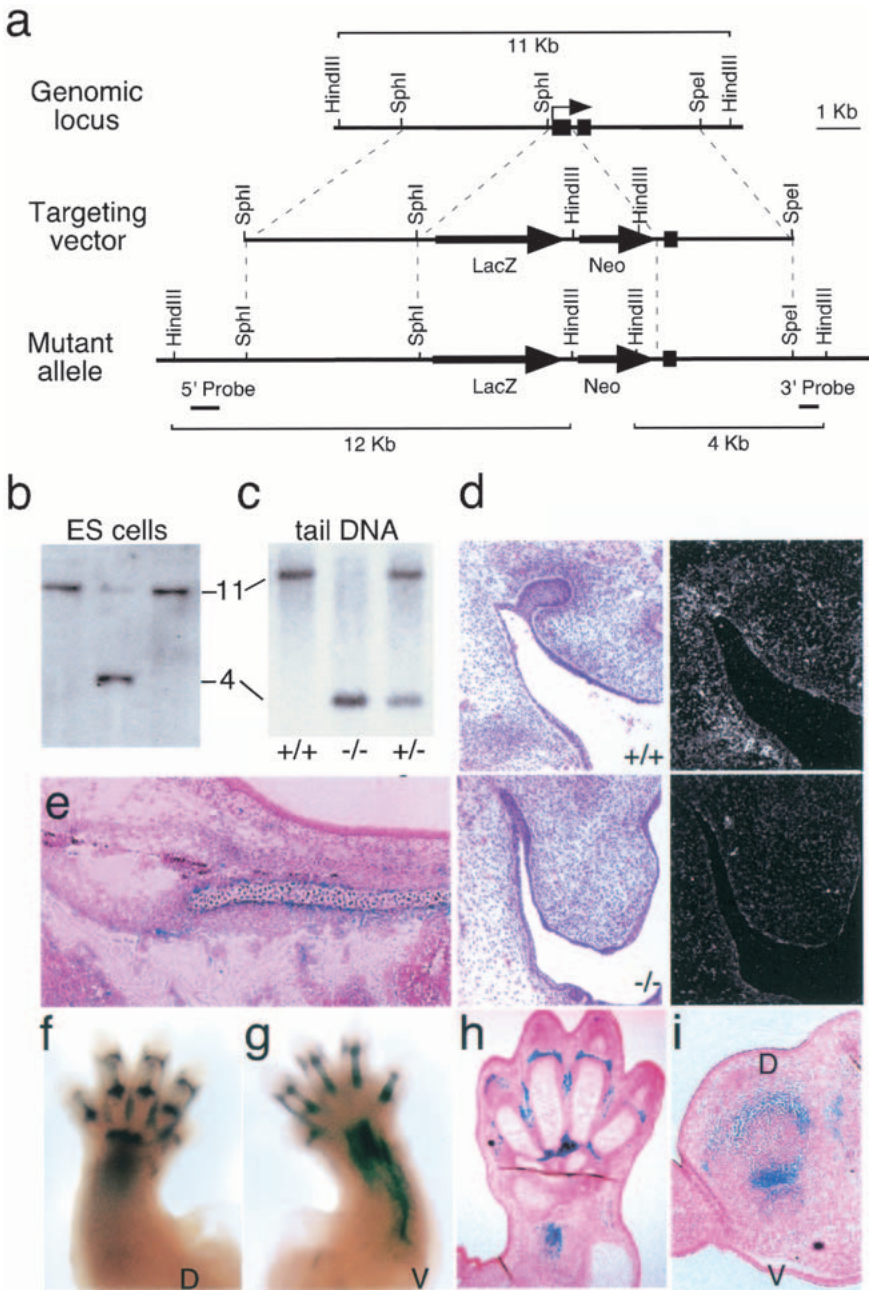

Figure 2. Generation of an *Fgf18-LacZ* targeted allele. (*a*) A schematic representation of the *Fgf18* genomic locus, the targeting vector and the mutant allele generated following homologous recombination. (*b*) Southern blot identification of the *Fgf18* targeted allele in embryonic stem cells using a 3′ probe and a *Hin*dIII digest. (*c*) Southern blot identification of the *Fgf18* targeted allele in tail DNA using a 3′ probe and a *Hin*dIII digest. The 3′ probe (as indicated in *a*) identifies the wild-type allele as an 11-kb *Hin*dIII fragment and the mutant allele as a 4-kb *Hin*dIII fragment. (*d*) In situ hybridization detection of *Fgf18* mRNA expression in the developing palatal shelf in wild-type mice (*upper* panels) and in $Fgf18^{-/-}$ tissue (*lower* panels). Brightfield and darkfield images are shown. Note the absence of expression in $Fgf18^{-/-}$ tissue (*lower* panels). (*e–i*) Staining for β-galactosidase enzyme activity in $Fgf18/^{-/+}$ cranium and limbs of E14.5 mice. (*e*) A sagittal section through the parietal bone showing β-galactosidase enzyme activity in the periosteum and endosteum. (*f*,*g*) Dorsal (*f*) and ventral (*g*) views of a whole mount-stained limb showing β-galactosidase enzyme activity in skeletal elements. (*h*, *i*) Histological sections through the distal limb stained for β-galactosidase enzyme activity. A planar section is shown in (*h*) and a cross section is shown in (*i*). (D) Dorsal; (V) ventral.

growth plate. These two processes are tightly regulated by several signaling pathways and must be coordinated to keep long bone growth in balance. The elongated proliferating and hypertrophic zones in $Fgf18^{-/-}$ growth plates suggested that *Fgf18* negatively regulates chondrocyte proliferation and/or differentiation, similar to that observed in mice lacking *Fgfr3*. To assess the effect of FGF18 on chondrocyte proliferation, pregnant females were injected with BrdU at E16.5. After two hours, embryos were collected and BrdU incorporation into chondrocytes in the proximal tibia and distal humerus was detected by immunohistochemistry. BrdU incorporation in proliferating chondrocytes of these two growth plates was increased by 14% (P = 0.001) and 24% ($P < 0.02$), respectively. BrdU incorporation in the reserve zone of the proximal tibia was also increased by 36% ($P < 0.05$) (Fig. 4c,d; Table 2). These data suggested that FGF18 either directly or indirectly inhibits chondrocyte proliferation during normal long bone growth and is consistent with FGF18 signaling to FGFR3 in proliferating chondrocytes.

The size of the hypertrophic zone is regulated by the rate of chondrocyte differentiation and chondrocyte apoptosis. Apoptotic chondrocytes are localized to a narrow band of cells in the hypertrophic chondrocyte-trabecular bone interface. No significant differences in TUNEL labeling were observed in $Fgf18^{-/-}$ and wild-type control mice (data not shown). This suggested that chondrocyte apoptosis was unlikely to cause hypertrophic

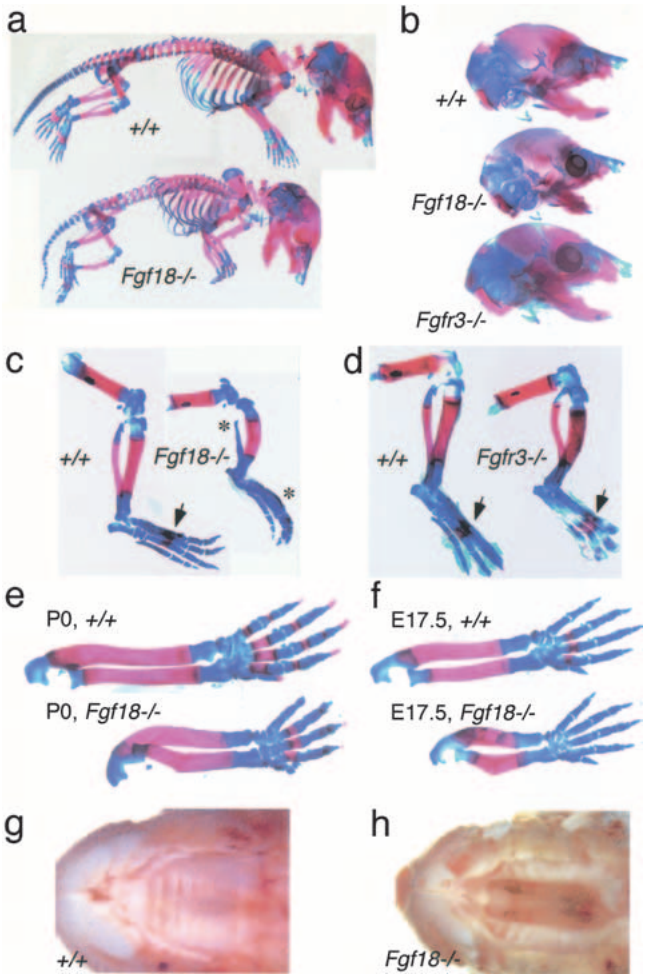

Figure 3. Morphological analysis of the skeletal phenotype in *Fgf18*$^{-/-}$ mice. (*a*) Alizarin red- and alcian blue-stained skeletal preparations of neonatal (P0) mice. The *Fgf18*$^{-/-}$ skeleton is shown below and a wild-type littermate is shown above. Note the deformed ribs and smaller thoracic cavity in *Fgf18*$^{-/-}$ mice. (*b*) Alizarin red- and alcian blue-stained skull from an E17.5 wild-type littermate (*upper*), an *Fgf18*$^{-/-}$ embryo (*middle*), and an *Fgfr3*$^{-/-}$ embryo (*lower*), showing decreased growth of cranial bones in the *Fgf18*$^{-/-}$ but not in the *Fgfr3*$^{-/-}$ skull. (*c*,*d*) Higher-magnification view of the hind limbs from an E17.5 *Fgf18*$^{-/-}$ embryo (*c*) and *Fgfr3*$^{-/-}$ embryo (*d*). Each panel also shows a wild-type littermate control. (*) Indicates a noticeable defect in *Fgf18*$^{-/-}$ mice. The arrow indicates the ossification zone in the metatarsal bones. (*e*,*f*) Forelimbs from P0 (*e*) and E17.5 (*f*) embryos showing delayed ossification in *Fgf18*$^{-/-}$ mice. Note that the P0 *Fgf18*$^{-/-}$ limb looks similar to the E17.5 wild-type limb. (*g*,*h*) Palate morphology from a wild-type littermate (*g*) and a neonatal *Fgf18*$^{-/-}$ mouse (*h*). Note the complete cleft palate in the *Fgf18*$^{-/-}$ mouse.

zone elongation in *Fgf18*$^{-/-}$ mice. Hypertrophic chondrocytes were examined for type X collagen expression, a specific differentiation marker for all hypertrophic chondrocytes. In situ hybridization showed an expanded domain of type X collagen expression corresponding to the expanded hypertrophic zone of the *Fgf18*$^{-/-}$ growth plate (Fig. 4e,f). Furthermore, the expression level of type X collagen appeared to be increased. This suggested that more extracellular matrix was produced by *Fgf18*$^{-/-}$ hypertrophic chondrocytes and may reflect increased chondrocyte differentiation and increased metabolic activity.

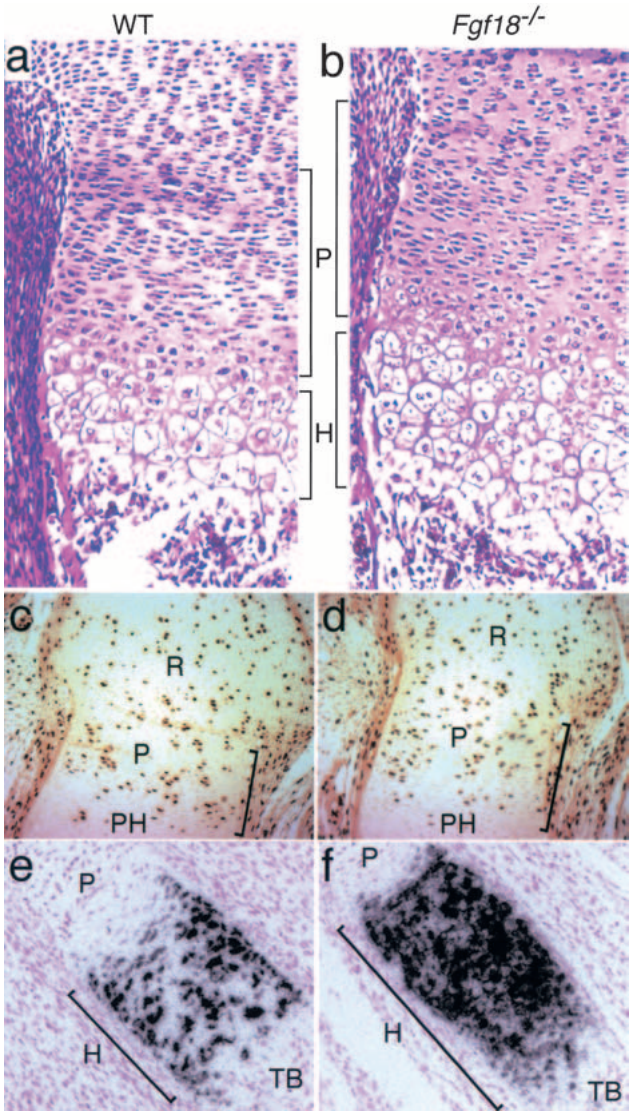

Figure 4. Histological analysis and identification of proliferating and hypertrophic chondrocytes using BrdU immunohistochemistry and type X collagen in situ hybridization. (*a*,*b*) Hematoxylin and eosin-stained section of the distal femur from an E16.5 wild-type embryo (*a*) and an *Fgf18*$^{-/-}$ embryo (*b*). Note the increased height of the *Fgf18*$^{-/-}$ proliferating and hypertrophic zones relative to that of the control. (*c*,*d*) Immunohistochemical detection of BrdU-labeled chondrocytes in the epiphyseal growth plate of the distal femur from an E16.5 wild-type embryo (*c*) and an *Fgf18*$^{-/-}$ embryo (*d*). (*e*,*f*) Type X collagen expression in the distal fibula growth plate of an E18.5 wild-type littermate (*e*) and *Fgf18*$^{-/-}$ mouse (*f*). (R) Reserve chondrocytes; (P) proliferating chondrocytes; (PH) prehypertrophic chondrocytes; (H) hypertrophic chondrocytes; (TB) trabecular bone.

Table 1. *Bone morphometric data*

Age	Growth plate[a]	Genotype	N[b]	Mean length (mm)[c]	Length % control	*P* value
E16.5	Distal femur HC	+/+	4	0.30 ± 0.01		
		–/–	4	0.48 ± 0.08	160	<0.005
	Distal femur PC	+/+	4	0.47 ± 0.02		
		–/–	4	0.54 ± 0.04	114	<0.02
E18.5	Distal femur HC	+/+	4	0.24 ± 0.02		
		–/–	4	0.33 ± 0.04	137	<0.01
	Distal femur PC	+/+	4	0.43 ± 0.04		
		–/–	4	0.46 ± 0.03	108	0.4

[a](PC) Proliferating chondrocyte zone; (HC) hypertrophic chondrocyte zone.
[b]Number of animals examined.
[c]Mean length was determined with Zeiss Axio Vision 3.0 software.

Fgf18 *inhibits* Ihh *signaling*

Chondrogenesis requires a well-coordinated transition from proliferating to hypertrophic chondrocytes. *Ihh* stimulates chondrocyte proliferation and delays the transition from proliferation to hypertrophy. In *Ihh*$^{-/-}$ mice, hypertrophic chondrocytes predominate in the growth plate (St-Jacques et al. 1999; Karp et al. 2000). The defective chondrogenesis in *Fgf18*$^{-/-}$ mice suggested that *Fgf18* might interact with *Ihh* signaling. To assess the consequence of loss of *Fgf18* on *Ihh* expression and signaling, the expression of *Ihh* and its receptor, *patched*, was examined in the growth plates of both wild-type and *Fgf18*$^{-/-}$ mice.

Consistent with previous reports, expression of *Ihh* was restricted to prehypertrophic and proximal hypertrophic chondrocytes in both wild-type and *Fgf18*$^{-/-}$ mice (Fig. 5a–d). However, the intensity of the *Ihh* signal was significantly stronger in *Fgf18*$^{-/-}$ growth plates compared to wild-type littermates. *patched* is expressed in proliferating chondrocytes, perichondrium, and the cartilage-bone interface (St-Jacques et al. 1999). The expression of *patched* is induced by Hedgehog signaling, and therefore the level of *patched* expression is a measure of the strength of the Hedgehog signal (Chen and Struhl 1996; Ingham 1998). Expression of *patched* was detected in the proliferating chondrocytes, perichondrium, and the cartilage-bone interface in both wild-type and *Fgf18*$^{-/-}$ mice (Fig. 5e–h). The expression of *patched* was significantly increased in the proliferating chondrocyte region of *Fgf18*$^{-/-}$ growth plates. However, the expression level of *patched* in the perichondrium and the cartilage-bone interface showed no significant differences between wild-type and the *Fgf18*$^{-/-}$ growth plates. These data support a model in which *Fgf18* regulates chondrogenesis in part by inhibiting IHH signaling in prehypertrophic and proximal hypertrophic chondrocytes.

Fgf18 *promotes osteogenesis*

The phenotypic similarities between *Fgf18*$^{-/-}$ and *Fgfr3*$^{-/-}$ mice strongly suggest that FGF18 acts through FGFR3 to inhibit chondrocyte proliferation, differentiation, and IHH signaling. However, there are also significant differences. Close comparison of skeletal preparations from *Fgf18*$^{-/-}$ mice and *Fgfr3*$^{-/-}$ mice clearly demonstrated a delayed ossification in *Fgf18*$^{-/-}$ mice, which was not observed in *Fgfr3*$^{-/-}$ mice (Fig. 3b–d; data not shown). This raises the possibility that FGF18 may also signal through other FGFRs to regulate osteogenesis and/or osteoblast function.

To study the detailed molecular mechanism by which FGF18 normally regulates osteogenesis, we examined the expression of preosteogenic and osteogenic markers. *Osteopontin* (*Op*) and *osteocalcin* (*Oc*) are expressed in mature osteoblasts (Rodan and Noda 1991). At E15.5, expression of *Op* was significantly decreased in the humerus of *Fgf18*$^{-/-}$ mice compared to littermate control

Table 2. *Proliferation index for growth plate chondrocytes*

Age	Growth plate[a]	Genotype	N[b]	BrdU+/0.01 mm^2	Proliferation % control	*P* value
E16.5	Distal humerus PC	+/+	2	1.82 ± 0.04		
		–/–	2	2.25 ± 0.07	124	<0.02
	Proximal tibia PC	+/+	4	2.38 ± 0.05		
		–/–	3	2.71 ± 0.08	114	0.001
	Proximal tibia RC	+/+	3	2.38 ± 0.3		
		–/–	3	3.64 ± 0.5	136	<0.05

[a](PC) Proliferating chondrocyte zone; (RC) reserve chondrocyte zone.
[b]Number of animals examined.

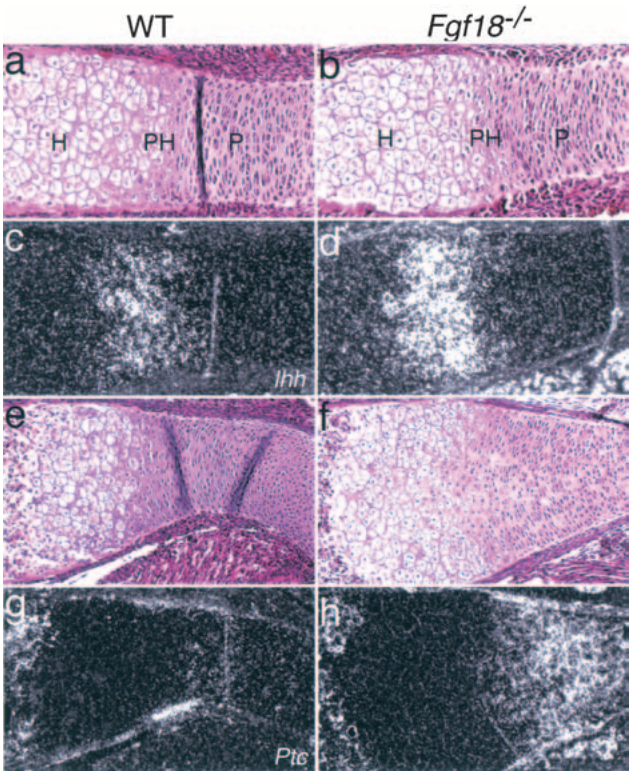

Figure 5. In situ detection of *Ihh* and *patched* in the developing growth plate. (*a–d*) *Ihh* expression in the distal fibula growth plate of an E16.5 wild-type (*a*,*c*) and *Fgf18*$^{-/-}$ (*b*,*d*) mouse. (*e–h*) *patched* expression in the distal humerus growth plate of an E16.5 wild-type (*e*,*g*) and *Fgf18*$^{-/-}$ (*f*,*h*) mouse. Brightfield (*a*,*b*,*e*,*f*) and darkfield (*c*,*d*,*g*,*h*) images are shown. (P) Proliferating chondrocytes; (PH) prehypertrophic chondrocytes; (H) hypertrophic chondrocytes.

mice (Fig. 6c,d). *Oc* expression was similarly down-regulated in *Fgf18*$^{-/-}$ mice at this stage (data not shown). *Cbfa1* is one of the earliest osteogenic markers and is expressed in perichondral/periosteal mesenchymal cells committed to become osteoblasts. CBFA1 is required for the specification of the osteogenic lineage (Ducy et al. 1997; Karsenty et al. 1999). *Cbfa1* expression was similar in the perichondrium/periosteum and endosteum of both wild-type and *Fgf18*$^{-/-}$ mice. However, the expression level of *Cbfa1* in trabecular bone was greatly decreased (Fig. 6e,f). These data demonstrated that osteoprogenitor cells were present in the perichondrium/periosteum but functional osteoblasts were deficient in the trabecular region, suggesting that FGF18 function is required in the process of osteoblast maturation/proliferation. Alternatively, FGF18 could regulate the influx of osteoblasts into the trabecular region by regulating the expression of angiogenic factors or molecules required for the remodeling of the extracellular matrix in the hypertrophic chondrocyte zone. To test these possibilities, we examined the expression of vascular endothelial growth factor (*Vegf*) and matrix metalloproteinase (*Mmp9*). No significant differences in *Vegf* and *Mmp9* expression were observed in wild-type and *Fgf18*$^{-/-}$ mice (Fig. 6g,h; data not shown). These data suggested that FGF18 may have a more direct effect on osteoblast development in trabecular bone or mediate the influx of osteoblasts by other means.

Discussion

Mutations in FGF receptors demonstrate that FGF signaling is intimately involved in the regulation of bone development and growth. However, the physiologic FGF ligand(s) that must signal to FGFRs in developing bone remain elusive. Here we show a skeletal phenotype in mice lacking *Fgf18*, demonstrating that FGF18 is an important mediator of skeletal development. Comparison of the growth plate of *Fgf18*$^{-/-}$ mice with that of *Fgfr3*$^{-/-}$ mice shows several similar features, which strongly suggests that FGF18 is a physiologic ligand for FGFR3. How-

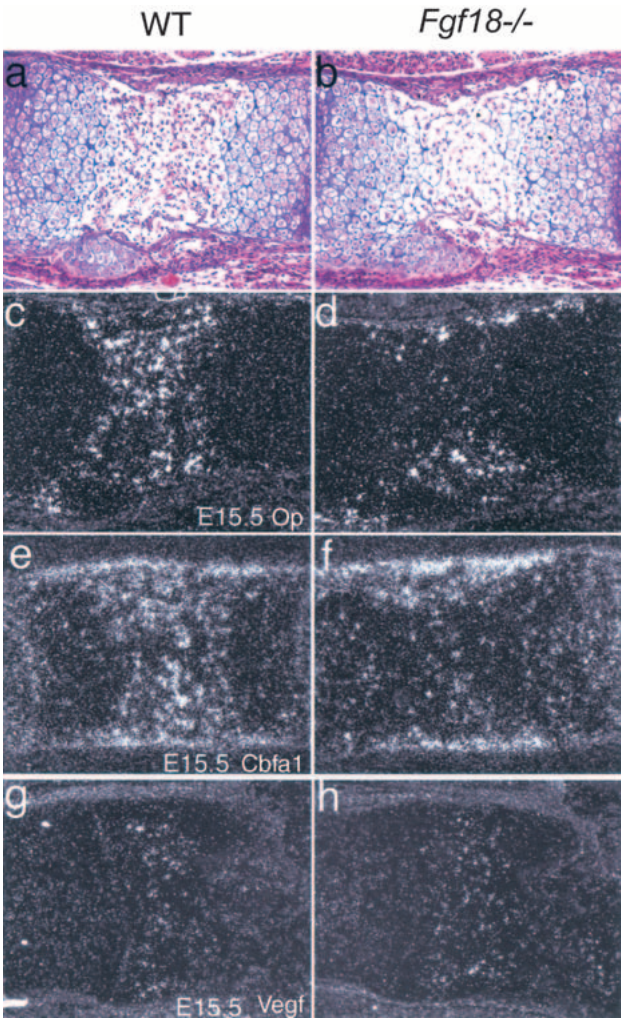

Figure 6. In situ detection of *osteopontin*, *Cbfa1*, and *Vegf* in the developing growth plate of the humerus at E15.5. (*a*,*b*) Brightfield images. (*c–h*) Darkfield images. (*c*,*d*) *Op* expression. (*e*,*f*) *Cbfa1* expression. (*g*,*h*) *Vegf* expression. (*a*,*c*,*e*,*g*) Sections from wild-type littermates. (*b*,*d*,*f*,*h*) Sections from *Fgf18*$^{-/-}$ mice.

ever, phenotypic differences demonstrate that FGF18 must also signal to other FGFRs in developing bone.

Although FGFs are generally considered to be mitogens for a variety of cell types, several studies have shown that signaling through FGFR3 inhibits chondrocyte proliferation in vivo (Colvin et al. 1996; Naski et al. 1998; Sahni et al. 1999). Mice lacking *Fgfr3* exhibit an increase in the number of proliferating chondrocytes and an enlarged growth plate. *Fgf18*$^{-/-}$ mice also exhibit similar features. Recent data demonstrate that this inhibition may be due to a direct effect on the chondrocyte (Sahni et al. 1999; Henderson et al. 2000) and may be independent of the specific FGFR tyrosine kinase domain expressed (Wang et al. 2001). The expression of *Fgf18* in the perichondrium, adjacent to *Fgfr3*-expressing proliferating chondrocytes, is consistent with our hypothesis that FGF18 acts as a paracrine factor signaling through FGFR3 to inhibit chondrocyte proliferation (Fig. 7).

In *Fgf18*$^{-/-}$ mice and *Fgfr3*$^{-/-}$ mice, both the proliferating zone and hypertrophic zone were expanded. Gain- and loss-of-function experiments with FGFR3 showed that in addition to inhibiting chondrocyte proliferation, FGFR3 also inhibited chondrocyte differentiation (Colvin et al. 1996; Deng et al. 1996; Naski et al. 1998). In *Fgf18*$^{-/-}$ mice, chondrocyte differentiation was assessed by examining the expression of the hypertrophic chondrocyte differentiation marker, type X collagen, and the extent of apoptosis in hypertrophic chondrocytes. The increased expression of type X collagen and the similar amount of apoptosis in distal hypertrophic chondrocytes was again consistent with the phenotype of *Fgfr3*$^{-/-}$ mice and suggests that the most likely mechanism for hypertrophic zone elongation is increased chondrocyte differentiation. Increased differentiation could be a direct effect of decreased signaling through FGFR3 in proliferating and prehypertrophic chondrocytes, it could be due to changes in signaling through other pathways (see below), or it could be a consequence of an increased pool of proliferating chondrocytes.

The perichondrium that surrounds developing cartilage has been shown to transmit signals that negatively regulate both chondrocyte proliferation and differentiation in in vitro perichondrium-free cultures of either chick or mouse bones (Long and Linsenmayer 1998; Haaijman et al. 1999; Alvarez et al. 2001). However, the molecular identity of this signal has not been identified. The expression of *Fgf18* in the perichondrium, the observed increased BrdU incorporation in the proliferating chondrocyte zone, and the increased type X collagen expression in *Fgf18*$^{-/-}$ mice suggests that FGF18 is a good candidate for this signal.

In addition to a direct effect on proliferation and differentiation mediated through FGFRs, FGF signaling

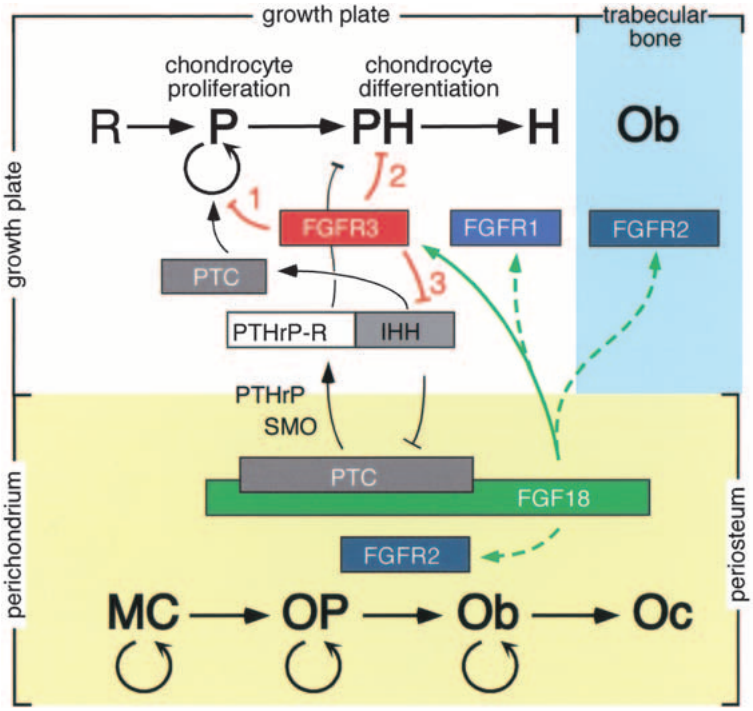

Figure 7. Model for *Fgf18* regulation of long bone growth. In a linear model of chondrogenesis, chondrocytes sequentially develop through reserve (R), proliferating (P), prehypertrophic (PH), and hypertrophic (H) stages. The rectangles indicate the relative expression domains of signaling molecules in the growth plate. Previous studies show that FGFR3 inhibits (1) chondrocyte proliferation, (2) chondrocyte differentiation, and (3) *Ihh* expression in prehypertrophic chondrocytes (Naski et al. 1998). *Fgf18* is expressed in the perichondrium and is proposed to activate FGFR3 signaling in proliferating and prehypertrophic chondrocytes (green arrow). FGF18 may also signal to FGFR1 in hypertrophic chondrocytes and FGFR2 in the perichondrium and in trabecular bone. Decreased IHH signaling through Patched (PTC) and Smoothened (SMO) results in decreased *PTHrP* expression in the periarticular perichondrium and signaling to the PTHrP receptor (PTHrP-R) in prehypertrophic chondrocytes. PTHrP-R signaling delays chondrocyte maturation. FGF18 could inhibit chondrocyte proliferation either directly through the action of FGFR3 in proliferating chondrocytes or indirectly by repressing the *Ihh–Patched–PTHrP* signaling pathway. Contrary to its function in chondrogenesis, FGF18 positively regulates osteogenesis. In a linear model of osteogenesis, mesenchymal cells (MC) develop into osteogenic progenitor cells (OP), which express *Cbfa1*. OP cells then differentiate into mature osteoblasts (OB) which eventually become entrapped in bone as osteocytes (OC). In cortical bone, FGF18 promotes osteoblast maturation/proliferation, but has no effect on the formation of the osteoprogenitor cell population. This suggests that FGF signaling affects differentiation of the committed osteoprogenitor cell. The delayed formation of trabecular bone suggests that FGF18 signals directly through FGFR1 or indirectly through other factors to regulate ossification of hypertrophic chondrocytes. FGF18 signaling to FGFR3 in proliferating chondrocytes and potentially to FGFR2 in the perichondrium/periosteum or FGFR1 in hypertrophic chondrocytes places FGF18 in a position to coordinate rates of growth and differentiation in developing bone.

may have an indirect effect mediated by other signaling molecules expressed in developing bone. In both *Fgf18* and *Fgfr3* knockout mice, *Ihh* expression is increased, and in mouse models for achondroplasia, *Ihh* expression is suppressed (Naski et al. 1998; Chen et al. 2001). Thus FGF18 and FGFR3 are negative regulators of IHH signaling. $Ihh^{-/-}$ embryos show a smaller zone of proliferating chondrocytes with reduced BrdU incorporation (St-Jacques et al. 1999). These data suggest that one function of IHH signaling is to stimulate chondrocyte proliferation. It is possible that the suppression of chondrocyte proliferation by FGF18 is mediated indirectly by suppressing IHH signaling. Conversely, IHH could stimulate chondrocyte proliferation by inhibiting FGF18. The increased expression of *Ihh* and its receptor, *patched*, in the growth plate of $Fgf18^{-/-}$ mice is most consistent with the former possibility.

Ihh, produced by prehypertrophic chondrocytes, induces the expression of *PTHrP* in the periarticular perichondrium (Lanske et al. 1996; Vortkamp et al. 1996). *PTHrP* acts through *PTHrP-R* in proliferating chondrocytes to delay their transition from proliferation to hypertrophy and thus reduces the number of cells expressing *Ihh*. In this way, the rate of chondrocytes leaving the proliferation cycle is precisely controlled (Lanske et al. 1996; Vortkamp et al. 1996). The data presented here provide evidence that *Ihh* is also regulated by a second mechanism in which FGF18 signals from the perichondrium through FGFR3 in the proliferating/prehypertrophic chondrocytes to suppress *Ihh* expression (Fig. 7). These data also identify FGF18 as a perichondrial regulator of endochondral bone growth. A similar role has been identified for PTHrP (Karaplis et al. 1994; Lanske et al. 1996). The next important step will be to determine how feedback mechanisms regulate the expression of *Fgf18*.

Thus far we have considered phenotypic similarities between $Fgf18^{-/-}$ mice and $Fgfr3^{-/-}$ mice. However, the formation and extent of ossification is delayed in $Fgf18^{-/-}$ mice but not in $Fgfr3^{-/-}$ mice. This raises the possibility that FGF18 may also signal through FGFR1 in hypertrophic chondrocytes and FGFR2 in the perichondrium/periosteum to regulate osteogenesis (Fig. 7). The normal level of *Cbfa1* expression in the perichondrium/periosteum of $Fgf18^{-/-}$ mice suggests the presence of normal numbers of osteoprogenitor cells. These data demonstrate that FGF18 most likely functions to promote osteoblast maturation/proliferation (observed delayed ossification of cortical bone), but is not required for the early specification of the osteogenic cell lineage (Fig. 7). However, the expression of *Cbfa1*, *Op*, and *Oc* is greatly reduced in the ossification zone giving rise to trabecular bone, which demonstrates a deficiency of functional osteoblasts in this region. This deficiency may be due to a defect in the ability of osteoblast progenitors to populate the distal hypertrophic zone or a defect or delay in vascular invasion of the distal hypertrophic zone. The normal expression of VEGF and MMP9 suggests that at least some of the signals required for the ossification process are intact in $Fgf18^{-/-}$ mice. Our data are therefore most consistent with FGF18 directly regulating osteogenesis or vascular invasion of the distal hypertrophic zone.

Experiments in which *Fgfr2* has been conditionally knocked out in developing bone demonstrates that FGFR2 positively regulates bone growth (K. Yu and D. Ornitz, unpubl.). FGFR2 could thus be an autocrine/juxtocrine receptor to transduce an FGF18 signal in the perichondrium/periosteum. FGF18 expression is thus localized, both spatially and developmentally, in a position where it could coordinate both chondrogenesis in the growth plate and osteogenesis in cortical and trabecular bone (Fig. 7). The calvarial phenotype in $Fgf18^{-/-}$ mice also supports a model in which FGF18 signals through FGFR1 and/or FGFR2, because these are the predominant FGFRs expressed in developing calvarial bones (Iseki et al. 1999; Rice et al. 2000). Further experiments will be required to determine the extent of FGF18 signaling through other FGFRs.

Materials and methods

Generation of Fgf18*-targeted mice*

A 7.1-kb *Sph*I–*Spe*I genomic clone containing exons 1 and 2 was used to construct the targeting vector. Exon 1 was deleted and replaced by a 7-kb DNA fragment containing a β-galactosidase cassette followed by a neoselection cassette. The targeting construct was linearized with *Not*I prior to electroporation into RW-4 embryonic stem cells. Selected G418-resistant clones were screened by Southern blot analysis of *Hin*dIII-digested genomic DNA using both 5′ and 3′-probes (Fig. 2a). Cells from one positive clone were injected into blastocysts of C57BL6/J mice and transferred into the uteri of pseudopregnant females. Chimeric males were mated with C57BL6/J females, and offspring were screened by Southern blot analysis of tail genomic DNA. Heterozygous littermates were mated to obtain homozygous animals (Fig. 2a–c).

Skeletal preparations

Skeletons were prepared as described previously (Colvin et al. 1996). For postnatal day 0 (P0) skeletal preparations, carcasses were skinned and eviscerated, and then soaked in acetone for 12–24 h, cleared in 2% KOH (12–24 h), stained with alizarin red S and alcian blue (12–24 h), cleared in 1% KOH/20% glycerol, and stored in glycerol. For embryo skeleton preparations, fetuses were skinned and eviscerated, stained with alizarin red S and alcian blue (12–24 h), cleared in 1%KOH/20% glycerol, and stored in glycerol.

Histological analysis

Tissues were fixed in 4% paraformaldehyde/PBS or 10% formalin, decalcified if necessary in EDTA or Decalcifying Solution (Stephens Scientific), and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E). Computer imaging using AxioVision 3.0 software (Zeiss) was used to calculate the length of proliferating and hypertrophic chondrocyte zones. Each length was measured three times along the midline of the corresponding growth plate in H&E stained sections. Proliferating and hypertrophic zones were demarcated as shown in Figure 4. The top of the brackets around the proliferating chondro-

cyte zone were defined based on bone morphology; that is, the narrowest part of the growth plate.

Analysis of cell proliferation

Anti-BrdU immunohistochemistry was carried out as described (Naski et al. 1998) with minor modifications. Pregnant mice were injected intraperitoneally with BrdU (100 mg/kg) 1 h before sacrifice. Embryos were fixed in 4% paraformaldehyde at 4°C for 2 h. BrdU was detected with an anti-BrdU antibody (Becton Dickinson Immunocytometry Systems) using the ABC kit (Vector Lab) according to the manufacturer's instructions. All of the BrdU-positive nuclei of reserve and columnar proliferating chondrocytes were counted and the area of the proliferating chondrocyte zone was measured using AxioVision 3.0 image software (Zeiss). The number of BrdU-positive nuclei per 0.01 mm^2 area was calculated for *Fgf18*$^{-/-}$ and wild-type littermate embryos. At least three sections were counted for each embryo examined.

In situ hybridization

In situ hybridization was performed as described (Xu et al. 1999). The plasmids used for generating P^{33}-labeled riboprobes were generously provided by B. Olsen (*Type X collagen*), M. Scott (*patched*), A. McMahon (*Ihh*), K. Lee (*Osteopontin*), K. Nakashima (*Cbfa1*), and G. Karsenty (*VEGF*).

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