Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3

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SUMMARY

Fibroblast growth factor receptor 3 (FGFR3) is a key regulator of skeletal growth and activating mutations in Fgfr3 cause achondroplasia, the most common genetic form of dwarfism in humans. Little is known about the mechanism by which FGFR3 inhibits bone growth and how FGFR3 signaling interacts with other signaling pathways that regulate endochondral ossification. To understand these mechanisms, we targeted the expression of an activated FGFR3 to growth plate cartilage in mice using regulatory elements from the collagen II gene. As with humans carrying the achondroplasia mutation, the resulting transgenic mice are dwarfed, with axial, appendicular and craniofacial skeletal hypoplasia. We found that FGFR3 inhibited endochondral bone growth by markedly inhibiting chondrocyte proliferation and by slowing chondrocyte differentiation. Significantly, FGFR3 downregulated the Indian hedgehog (Ihh) signaling pathway and Bmp4 expression in both growth plate chondrocytes and in the perichondrium. Conversely, Bmp4 expression is upregulated in the perichondrium of Fgfr3−/− mice. These data support a model in which Fgfr3 is an upstream negative regulator of the hedgehog (Hh) signaling pathway. Additionally, Fgfr3 may coordinate the growth and differentiation of chondrocytes with the growth and differentiation of osteoprogenitor cells by simultaneously modulating Bmp4 and patched expression in both growth plate cartilage and in the perichondrium.

Key words: Fibroblast growth factor, FGF, FGF receptor 3, Fgfr3, Bone growth, Achondroplasia, hedgehog, patched, Bmp4

INTRODUCTION

Skeletal growth is regulated by a hierarchy of genetic, endocrine and mechanical regulatory programs. These programs ensure coordinated growth of both the cartilaginous and bony portions of the skeleton. Recently, fibroblast growth factor (FGF) receptor 3 (FGFR3) has been identified as a critical regulator of endochondral bone growth. Autosomal dominant mutations in Fgfr3 cause the dwarfing chondrodysplasias, achondroplasia (Rousseau et al., 1994; Shiang et al., 1994), hypochondroplasia (Bellus et al., 1995), and thanatophoric dysplasia (Tavormina et al., 1995a, b). Additionally, mice homozygous for null alleles of Fgfr3 exhibit skeletal overgrowth (Colvin et al., 1996; Deng et al., 1996). The contrasting phenotypes between the Fgfr3−/− mice and the human dwarfing conditions resulting from mutations in Fgfrs suggest that the mutations causing dwarfism are gain of function alleles (this has recently been proved biochemically; Naski et al., 1996; Webster et al., 1996; Webster and Donoghue, 1996), and that Fgfr3 negatively regulates bone growth. Whether FGFR3 achieves this regulation directly or indirectly through interactions with regulatory signaling pathways is not known.

Endochondral bone growth is a tightly regulated developmental process that occurs in the epiphyseal growth plate, a specialized cartilaginous tissue found at the ends of growing long bones (Caplan and Pechak, 1987). Growth plate chondrocytes are arranged in columns that sequentially and synchronously progress through proliferative, prehypertrophic and hypertrophic stages (Caplan and Pechak, 1987; Caplan, 1988). The hypertrophic chondrocytes die and are replaced by trabecular bone and bone marrow through a process that includes apoptosis of hypertrophic chondrocytes, vascular invasion of the growth plate, resorption of the cartilaginous matrix and recruitment of osteoblasts that deposit the trabecular bone matrix. Fgfr3 is expressed in the epiphyseal growth plate and is most highly expressed in a histomorphological domain that encompasses proliferating and prehypertrophic chondrocytes. This expression pattern suggests a direct role for FGFR3 in regulating chondrocyte proliferation and possibly differentiation.

Trabecular bone is formed by endochondral ossification in the growth plate. In a separate process, osteoblasts derived from osteoprogenitor cells in the perichondrium generate cortical bone. Longitudinal bone growth requires synchronous cortical and endochondral bone formation. This implies that
endochondral bone formation, the process of chondrocyte growth and differentiation, must be coordinated with osteoblast differentiation and the synthesis of cortical bone derived from osteoprogenitor cells in the perichondrium. The mechanisms coordinating these two processes are poorly understood. FGFR3 profoundly regulates longitudinal bone growth but is only expressed in the cartilaginous growth plate (Shiang et al., 1994; Tavormina et al., 1995a,b; Colvin et al., 1996; Deng et al., 1996). This suggests that signals downstream of FGFR3 must regulate bone formation adjacent to the epiphyseal growth plate.

Studies of Fgfr3 null mice show prolonged expression of markers for cell proliferation (Deng et al., 1996) and overexpression of FGFR3 in a chondrocytic cell line results in diminished cell proliferation (J. Henderson, M. C. Naski and D. M. Ornitz, unpublished data). In addition to affecting chondrocyte proliferation, evidence also suggests that FGFR3 may regulate chondrocyte differentiation. Histological studies of biopsies from individuals with achondroplasia show either extensive or focal disorganization of the growth plate (Ponseti, 1970; Rimoin et al., 1970; Briner et al., 1991). Furthermore, Fgfr3−/− mice have an expanded zone of hypertrophy in the epiphyseal growth plate (Colvin et al., 1996; Deng et al., 1996) and in vitro experiments demonstrate that the addition of FGFR3 to cultured chondrocytes inhibits chondrocyte differentiation (Kato and Iwamoto, 1990). These observations suggest that FGFR3 signaling may affect chondrocyte differentiation in vivo.

Along with FGFs, endochondral bone growth is regulated by many signaling molecules including growth hormone, insulin-like growth factor-1 (IGF-1), parathyroid hormone related protein (PTHrP), Indian hedgehog (Ihh) and bone morphogenetic proteins (BMPs) (Reddi, 1994; Erlebacher et al., 1995). Recently, a feedback loop was described in which Ihh and PTHrP interact to coordinate chondrocyte differentiation (Lanske et al., 1996; Vortkamp et al., 1996).

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In this study we have created a mouse model for the human genetic disease, achondroplasia. We show that expressing an activated Fgfr3 in the growth plate downregulates the expression of Ihh, the Ihh receptor, patched and Bmp4, whereas patched and Bmp4 expression are upregulated in Fgfr3−/− mice. Significantly, Bmp4 expression is modulated in both growth plate chondrocytes and in the perichondrium. These data suggest that Fgfr3 is genetically upstream of the Ihh signaling pathway and is a global coordinator of endochondral ossification. We further demonstrate that Fgfr3 regulates endochondral ossification by inhibiting chondrocyte proliferation and differentiation.

MATERIALS AND METHODS

Transgene expression vector

To target FGFR3 to proliferating chondrocytes, the Fgfr3 cDNA was cloned into a transgenic expression vector (pI757) containing the promoter and enhancer sequences from the rat type II collagen gene (Yamada et al., 1990; Weir et al., 1996). The murine Fgfr3 cDNA (either wild type or containing the G380R mutation) was excised from the MIRB expression vector (Chellasai et al., 1994; Naski et al., 1996) with HindIII and Asp700. The hGH gene (containing a deletion of the BglII in exon V, which prevents synthesis of a functional protein) was excised from G4e/hGH (Ornitz et al., 1991) with EcoRV and HindIII. These fragments were ligated into the HindIII site of pBS SK in which the Clal site was replaced with a BamHl linker so that the resulting Fgfr3ΔhGH fusion transcript was flanked by BamHI sites. The insert was excised with BamHI and cloned into the BamHI site of pI757 (generously provided by Y. Yamada, NIH, USA) (Yamada et al., 1990; Bruggeman et al., 1991). The Agel site of pI757 was replaced with a Nol linker, and the targeting construct was excised with AflIII and NorI and injected into oocytes. Transgenic mice were generated in inbred FVB/N mice using established methods (Hogan et al., 1986).

Genotyping of mice

Transgenic animals were identified by PCR using the primers 5′AGTTGACCATGACCTTACAG3′ and 5′TCTTGTGTGTTTCTCCTCAGTG3′, which amplify 360 bp of human growth hormone (hGH) sequence present in the transgene. PCR amplification included 27 cycles of 94°C for 60 seconds, 55°C for 60 seconds and 72°C for 90 seconds. Homozygous transgenic animals were detected by Southern blotting using genomic DNA digested with BglII and a probe consisting of a 0.81 kb PstI fragment from the Fgfr3 cDNA. The homozygous animals were identified by determining the ratios of the signal intensity of the Fgfr3ΔhGH transgene to that of the endogenous Fgfr3 using a phosphorimager (Molecular Dynamics). Homozygous animals yielded a ratio approximately twice that of their heterozygous littermates. Homozygosity was then verified by mating to wild-type FVB/N mice and assessing the genotype of the F1 progeny. Fgfr3−/− mice were generated by mating C57BL/6 Fgfr3+/+ mice. The genotype of the offspring was determined by Southern blotting as described previously (Colvin et al., 1996).

RT-PCR analysis of transgene expression

Epiphyseal cartilage was isolated from the distal femur and proximal tibia of 2-week-old animals. The tissues were snap-frozen in liquid nitrogen, and mRNA was isolated (QuickPrep® mRNA purification kit, Pharmacia). First strand cDNA was synthesized with MMLV reverse transcriptase (Gibco-BRL) for 50 minutes at 42°C, and PCR (25 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes) was performed with primers 5′TCAGGAGGTGTC- TCCGCAAAC3′ and 5′TGATGTCTAGTGGCGTCA3′, which recognize a sequence within exons IV and V of the hGH gene. In situ hybridization

Digoxigenin-labeled riboprobes were synthesized according to the manufacturer’s instructions (DIG RNA™ labeling kit, Boehringer-Mannheim). Radiolaabeled riboprobes were transcribed in the transcription buffer supplied by the manufacturer (Boehringer-Mannheim) at 37°C for 60 minutes with 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 7.5 μM UTP and 60 μM [35S]UTP (specific activity >1000 mCi/mmol). Formalin-fixed tissue sections were deparaffinized in xylene and rehydrated through a graded series of alcohols. The sections were digested with Pro tease K (5 μg/ml) at 37°C in phosphate-buffered saline (PBS) for 5 minutes and acetylated (0.1 M triethanolamine, pH 8.0, 0.25% acetic anhydride) for 15 minutes at room temperature. Tissue sections were hybridized overnight at 55°C (12,000 cpm/ml riboprobe, 50% formamide, 4× SSC, 1× Denhardt’s solution, 10% dextran sulfate, 50 mM DTT, 500 μg/ml yeast t-RNA and 300 μg/ml denatured herring sperm DNA). Following hybridization, the sections were washed for 10
minutes at room temperature in 2x SSC (2x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 5.5) and digested in 10 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 1 mM EDTA with 20 µg/ml RNase A (Boehringer-Mannheim) for 30 minutes at 37°C. Following RNase digestion, slides were sequentially washed at 55°C in 2x SSC, 0.2x SSC and 0.1x SSC for 15 minutes/wash. The sections were dehydrated through a graded series of alcohols, air-dried and autoradiographed using a 1:1 ratio of NTB emulsion (Kodak) to water. Sections were developed in D-19 developer (Kodak) for 4 minutes at 14°C, stopped in water for 1 minute and fixed with Kodak rapid fixer for 5 minutes. After washing, the slides were stained with Harris Hematoxylin and Eosin, coverslips placed on top and bright- and dark-field images obtained. The sections probed with digoxigenin-labeled riboprobe were hybridized and washed in a similar fashion. The signal was detected according to the manufacturer’s (Boehringer-Mannheim) instructions using the Nitroblue Tetrazolium, 5-bromo-4-chloro-3-indoyl-phosphate substrate solution.

Detection of bromodeoxyuridine-labeled and apoptotic cells

Mice received an intraperitoneal injection of bromodeoxyuridine (BrdU; 100 µg/g body mass), and were killed 1 or 36 hours later. Control experiments determined that 36 hours allowed sufficient time for chondrocyte differentiation, as indicated by the appearance of labeled nuclei in late hypertrophic chondrocytes. Similar results were obtained in studies of rat growth plate chondrocytes (Farnum and Wilsman, 1993). Additionally, these authors showed that the BrdU labeling index achieved steady state in less than 8 hours post-injection. Labeled chondrocytes were detected as described (Morgenbesser et al., 1993). Briefly, tissue sections were deparaffinized in xylenes and rehydrated through a graded alcohol series. Endogenous peroxidases were inactivated in 10% methanol, 3% H2O2 in PBS for 30 minutes at room temperature. The tissue was digested for 20-40 minutes with 200 µg/ml pepsin in 0.01 N HCl, denatured in 2 N HCl for 45 minutes at room temperature, neutralized in 0.1 M sodium borate, pH 8.5, for 10 minutes and then washed in PBS for 10 minutes at room temperature. The samples were blocked for 30 minutes at room temperature with 1% horse serum (Vector Laboratories ABC kit) in PBS, incubated overnight at 4°C with a 1:20 dilution of mouse anti-BrdU (Becton-Dickinson) in 1% horse serum, then washed with PBS at room temperature. Secondary antibody (Vector ABC kit) was applied at a 1:200 dilution in blocking solution, incubated at room temperature for 1 hour then washed with PBS. The DAB colorimetric reaction was done according to the manufacturer’s instructions (Vector Laboratories, Inc.). Proliferation indices were calculated as the number of BrdU-labeled cells per grid divided by the total number of cells per grid. The grid circumscribed a portion of the proliferative zone of the growth plate as viewed through a 40x objective and generally contained a total of 50 to 100 cells. For each growth plate, the fraction of labeled cells in three distinct grid locations was calculated and averaged. The growth plates examined included the proximal tibia, distal femur and proximal humerus. The BrdU-labeled cells of the growth plates were counted in the perichondrium adjacent to the growth plates of the proximal tibia and distal femur. 12 growth plates of FGFR3<sup>ach</sup> or wild-type littermates, 18-20 days of age, were analyzed. Endothelial cells and tendon were excluded from the count. Apoptotic chondrocytes were detected in formalin-fixed paraffin-embedded tissues using the ApopTag<sup>®</sup> Plus (Oncor, Gaithersburg MD) TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) kit.

Skeletal preparations

Skeletons were prepared as described previously (Williams, 1941; Fig. 1. Generation of transgenic mice that express FGFR3<sup>ach</sup> in growth plate chondrocytes. (A) Bright-field image of 18.5-day embryonic mouse proximal tibia showing resting (r), proliferating (p), prehypertrophic (ph) and hypertrophic (h) chondrocytes, perichondrium (pc) and the primary spongiosa of bone (b). Chondrogenesis proceeds sequentially from resting to hypertrophic chondrocytes. (B) Dark-field view of the tissue section shown in A probed with an antisense FGFR3 riboprobe. (C) Type II collagen expression in 18.5-day embryonic tibia detected with a digoxigenin-labeled antisense riboprobe. (D) Transgene expression vector containing the type II collagen promoter (Col II-pr), and a β-globin splice donor and acceptor, placed 5' to the FGFR3 cDNA containing the G380R mutation found in the human disease, achondroplasia. The 3' end of FGFR3 is fused to the hGH gene, which provides additional splice as well as polyadenylation sequences, and the enhancer for the type II collagen gene (Col II-en). (E) FGFR3<sup>ach</sup> expression determined by rt-PCR using PCR primers that recognize spliced hGH sequences. A-E represent separate transgenic lines and + is a positive control for hGH transcripts. (F,G) Bright- and dark-field images demonstrating expression of the FGFR3<sup>ach</sup> transgene in phalanges of an 18.5-day embryonic limb probed with a 200-bp antisense hGH riboprobe. (H) Growth curve of homozygous FGFR3<sup>ach</sup> line A females (squares, n=4,5) and wild-type females (circles, n=3,4). Each curve shows average weights for a separate litter of animals. Error bars represent ± s.e.m. A similar curve was seen for line B mice. Growth curves of transgenic mice overexpressing wild-type FGFR3 were not significantly different from wild-type mice. A,B,C,F,G, ×20.
Bone length was determined using Foster-Findley image analysis software. For long bones, the length was calculated along a computer-generated line that followed the midline between epiphyseal growth plates.

**RESULTS**

**Generation of a mouse model for achondroplasia**

To investigate the functions of FGFR3 during endochondral ossification and during the development of skeletal dysplasias, transgenic mice were constructed that express an activated FGFR3 (containing the G380R mutation responsible for the human disease achondroplasia, hereafter referred to as FGFR3<sup>ach</sup>) in the growth plate. Expression of the transgene was targeted to cartilage using type II collagen promoter and enhancer sequences, which have been shown to confer specific, high level transgene expression in chondrocytes (Horton et al., 1987; Yamada et al., 1990; Bruggeman et al., 1991; Weir et al., 1996; Schipani et al., 1997). The collagen II promoter was chosen because the pattern of collagen II expression (resting and proliferating chondrocytes) overlaps that of FGFR3 and, significantly, neither gene is expressed in hypertrophic chondrocytes or in the perichondrium (Fig. 1A-D).

Because the G380R mutation of FGFR3 causes a dominant skeletal dysplasia, we expected to observe a phenotype in transgenic founder animals. In fact, three transgenic founders died during the first several weeks of postnatal life as a consequence of severe dwarfism. Of five established transgenic lines, three expressed the transgene (Fig. 1E). Although the RT-PCR assay is not fully quantitative, lines A and B consistently showed the highest levels of expression, and were further characterized.

The pattern of expression of the transgene was assessed by in situ hybridization. An RNA probe specific to the hGH portion of the transgenic mRNA demonstrated expression in the growth plate cartilage of an 18.5-day transgenic embryo (Fig. 1F,G). Expression of the transgene was detected in all growth plates examined as well as in the developing vertebrae. Expression was not observed in wild-type littermates. Transgene expression overlapped that of type II collagen and endogenous Fgfr3 (compare the expression to that of Fgfr3 in Fig. 1B and collagen II in Fig. 1C), and like endogenous Fgfr3, the transgene was excluded from hypertrophic chondrocytes and the perichondrium.

The level of transgene expression does not exactly match that of endogenous Fgfr3 in that the highest levels of transgene expression are near the articular surface, whereas endogenous Fgfr3 is most abundant in proliferating chondrocytes. All transgenic lines examined showed similar patterns of expression.

**Skeletal pathology in FGFR3<sup>ach</sup> mice**

Both of the transgenic lines characterized were dwarfed. The phenotype of line A was less severe than that of line B but was readily apparent when bred to homozygosity, indicating a dose-dependent effect of the transgene. Mice homozygous for the transgene (line A) were 30-40% smaller than wild-type animals at 4 weeks of age (Fig. 1H); heterozygous line B mice were similarly dwarfed. Skeletal preparations showed shortening of both the axial and appendicular skeleton (Fig. 2). Long bones and craniofacial skeletal elements formed by endochondral ossification were also shortened (Table 1). The smaller craniofacial bones lead to the appearance of a dome-shaped cranium similar to the frontal bossing seen in achondroplasia (Fig. 2C). Unlike achondroplasia, the proximal and distal bones of the limbs of the transgenic mice were proportionally shortened. Both the proximal and distal skeletal elements were 15-21% shorter than in wild-type mice (Table 1). Differences between achondroplasia and the phenotypes observed in FGFR3<sup>ach</sup> transgenic mice may be attributable to differences in transgene expression along the proximal-distal axis.

In addition to shortening of the appendicular skeleton, specific defects were also observed in the vertebrae. Approximately 15% of the mice developed a severe kyphosis (Fig. 2B), and all mice had specific patterning defects in the vertebrae.
dorsal axis of the vertebrae. Examination of the lumbar vertebrae showed that the caudal aspects of the vertebrae were blunted, particularly at the articular surfaces. More rostrally, vertebrae showed abnormalities in the dorsal midline, including absence of the spinous processes and in some instances a non-ossified gap in the dorsal midline of both cervical and thoracic vertebrae (Fig. 2C,D).

**FGFR3 inhibits chondrocyte proliferation and differentiation**

The growth of long bones requires the continuous proliferation and differentiation of chondrocytes in the epiphyseal growth plate. The defined histomorphological zones of the growth plate outline the various stages of chondrocyte differentiation. The stages of growth and differentiation can be most simply envisaged as a linear differentiation pathway in which chondrocytes proceed sequentially through resting, proliferative, prehypertrophic and hypertrophic phases. Late hypertrophic chondrocytes die and are replaced by trabecular bone and marrow elements (Fig. 1A). The observation that activating mutations in Fgfr3 cause dwarfing conditions suggests that FGFR3 negatively regulates chondrocyte proliferation and/or differentiation. Consistent with this, Fgfr3 is highly expressed in both proliferating and pre-hypertrophic chondrocytes.

Histological examination of the growth plate from FGFR3\textsuperscript{ach} mice showed an overall intact histomorphologic architecture; however, both the hypertrophic and proliferative zones were significantly smaller than in littermate controls (Fig. 3A,B). Additionally, although the time of initiation of the primary ossification centers during embryonic development was unchanged (data not shown), postnatal formation of the secondary ossification centers in the epiphyses of the proximal tibia and distal femur of FGFR3\textsuperscript{ach} mice was delayed by 2-3 days (Fig. 3A,B).

Histologically, the linkage of cortical bone growth with epiphyseal chondrocyte growth and differentiation was maintained in FGFR3\textsuperscript{ach} mice. This is indicated by the regular appearance of osteoblasts depositing osteoid adjacent to the hypertrophic chondrocytes of the proximal tibia of 8- to 20-day-old mice (Fig. 3C,D, arrowheads).

The effect of FGFR3\textsuperscript{ach} on the proliferation of epiphyseal chondrocytes was assessed by BrdU labeling for 1 hour prior to killing. At 18.5 days of embryonic development (E18.5) no significant difference was observed in the labeling index of the transgenic animals compared to their wild-type littermates (Fig. 4A,B). In contrast, a profound effect of the FGFR3\textsuperscript{ach} transgene on chondrocyte proliferation was observed postnatally (Fig. 4C,D). Incorporation of BrdU into chondrocytes in the proximal tibia of 20-day-old mice was reduced 60% relative to that of wild-type mice. The inhibition of cell proliferation was similar in other growth plates including the distal femur and proximal humerus of 18- to 20-day-old mice (data not shown). The fraction of chondrocytes in the zone of proliferation that incorporate BrdU was 0.098±0.04 in FGFR3\textsuperscript{ach} animals, compared to 0.25±0.05 in wild-type animals (P<0.005). These data demonstrate that FGFR3 either directly or indirectly suppresses chondrocyte proliferation during postnatal bone growth; alternatively, FGFR3 could suppress the transit of resting chondrocytes into the proliferative zone.

In addition to the small proliferative zone, the hypertrophic zone in FGFR3\textsuperscript{ach} mice was significantly smaller than in wild-type littermates. The size of the hypertrophic zone can be regulated by modulators of chondrocyte proliferation and chondrocyte death. An example of a modulator of chondrocyte differentiation is the PTHrP receptor (PTHrP-R). In PTHrP\textsuperscript{R−/−} mice the size of the proliferating zone is markedly shortened but the size of the hypertrophic zone is near normal (Lanske et al., 1996). In contrast, MMP-9 promotes chondrocyte cell death. In MMP-9\textsuperscript{−/−} mice the hypertrophic

![Fig. 3. Histological comparison of the epiphyseal growth plate from the proximal tibia of a 10-day-old wild-type (A,C) and FGFR3\textsuperscript{ach} line B (B,D) mice. A and B show Haemotoxylin and Eosin stained sections of growth plate. p, proliferating chondrocytes; h, hypertrophic chondrocytes; pc, perichondrium; 2°, secondary ossification center. C and D show enlarged views (boxed in A and B) of osteoblasts depositing osteoid (arrowheads) adjacent to hypertrophic chondrocytes.](image)
Table 1. Bone morphometric data

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<th>Bone</th>
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<td>79</td>
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aMice from transgenic line B (FGFR3ach/+/*+) were compared to wild-type (+/+/*+) controls.

bBone length was determined with Foster-Findley PC Image software using limbs from animals 16 to 18 days old. For long bones the length represented the distance, calculated along the midline, between epiphyseal growth plates. The mandible length was measured from the mandibular notch to origin of the incisor.

cTwo-tailed t-test for independent samples comparing bone lengths of transgenic animals (FGFR3ach/+/*+) to wild type (+/+/*+).

zone is dramatically elongated relative to the proliferating zone (Vu et al., 1998). To assess the contribution of FGFR3 to these processes, cells in the pre-hypertrophic zone (cells that are committing to differentiate) were identified by examining the domain of PTHrP-R expression in FGFR3ach mice. Cells committed to die were identified by TUNEL.

Apoptotic chondrocytes are localized to a narrow band of cells in the distal hypertrophic zone where ossification begins (Vu et al., 1998). No significant differences in TUNEL-labeled cells were observed in FGFR3ach and control animals (data not shown), suggesting that FGFR3 affects cell differentiation rather than cell death. The significantly decreased size of the hypertrophic zone in FGFR3ach mice (5-6 cells wide versus 10-15 cells wide in wild-type mice; Fig. 3) suggests that, in addition to a consequence of a decreased pool of proliferating chondrocytes, FGFR3 may also slow differentiation.

The PTHrP receptor is most highly expressed in a narrow band of post-proliferative, pre-hypertrophic chondrocytes (Lee et al., 1995, 1996). In PTHrP-R−/− mice, the differentiation of chondrocytes is greatly accelerated, indicating that PTHrP signaling limits the rate of chondrocyte differentiation (Lanske et al., 1996). The PTHrP receptor was used as a marker of cells committed to pre-hypertrophic differentiation (Lee et al., 1996). In 18-day-old FGFR3ach mice the band of expression of the PTHrP-R is narrowed compared to wild-type littermates (Fig. 5A-D and data not shown), suggesting that the pool of cells committed to differentiate is decreased. Additionally, E18.5 Fgr3−/− mice show an expanded region of PTHrP-R expression compared to wild-type littermates (Fig. 5E,F). These data are therefore consistent with FGFR3 acting to decrease the pool of cells committed to differentiation.

To further examine the effect of FGFR3ach on the differentiation of chondrocytes, the fate of BrdU-labeled cells in the growth plate was examined. We hypothesized that slowed differentiation of chondrocytes may, over time, lead to an accumulation of BrdU-labeled cells in the growth plate of FGFR3ach mice. To test this hypothesis, 16-day-old mice were pulsed with BrdU and killed 36 hours later. Interestingly, and consistent with a role for FGFR3 as an inhibitor of chondrocyte differentiation, the labeling index increased 2.5-fold, from 0.098±0.04 (1 hour post-injection) to 0.26±0.04 (36 hours post-injection) in FGFR3ach mice, compared to from 0.25±0.05 (1 hour post-injection) to 0.3±0.03 (36 hours post-injection) in the wild-type littermates. The increase in the labeling index at 36 hours does not solely reflect increased labeling as the index approaches steady state, since others have shown that steady state is achieved less than 8 hours post-injection (Farnum and Wilsman, 1993). Thus, the accumulation of BrdU-labeled cells over time supports the notion that chondrocyte differentiation is slowed in FGFR3ach mice. As noted above, formation of the secondary ossification centers are delayed in FGFR3ach mice (Fig. 3A,B), a finding consistent with an inhibitory role for FGFR3 on chondrocyte differentiation.

**Signaling between the growth plate and perichondrium**

The coupling of cortical bone growth to cartilage growth is supported by the observation of a dramatic decrease in cell proliferation in the perichondrium of FGFR3ach mice compared to the littermate controls (Fig. 4C,D, asterisks). The number of BrdU-labeled cells in the perichondrium of the long bones was 1.7±1.5 in FGFR3ach mice (n=12) compared to 6.8±3 in wild-type littermates (n=12). To investigate possible pathways of communication between cartilage and the perichondrium, and to investigate potential modulators of chondrocyte and osteoprogenitor cell differentiation, expression of Bmps 2, 4 and 7 was examined by in situ hybridization. BMP family members are potent regulators of mesenchymal cell differentiation and are expressed in both the
growth plate and the perichondrium (Kingsley, 1994; Zou et al., 1997a,b; Vortkamp et al., 1998). Analysis of Bmp 2 and 7 showed similar expression in FGFR3\(^{-/}\) and wild-type mice (data not shown), whereas Bmp4 expression was greatly reduced in 8-, 14- and 20-day-old FGFR3\(^{-/}\) animals in both the perichondrium and in the growth plate (Fig. 6A,B and data not shown). Consistent with these observations, in Fgfr3\(^{-/-}\) mice, Bmp4 expression was increased in the perichondrium at E18.5 (Fig. 6C,D). The observations of decreased proliferation in the perichondrium and decreased Bmp4 expression in FGFR3\(^{-/}\) mice suggests that osteoprogenitor cell differentiation is inhibited. Furthermore, these findings suggest that the differentiation of chondrocytes and osteoprogenitor cells in the perichondrium is coordinated and that BMP4 may act as one of the links between cortical bone growth and chondrocyte growth.

Osteoprogenitor cells expressing the PTHrP-R are found in the perichondrium tightly opposed to cartilage (Lee et al., 1995). Additional evidence for decreased osteoblast differentiation was the reduced expression of the PTHrP-R in the perichondrium of FGFR3\(^{-/}\) mice (Fig. 5C,D, arrows). These findings further support the existence of signaling pathways that coordinate chondrocyte and osteoblast differentiation and again suggest that FGFR3 can regulate these pathways and thus indirectly regulate perichondrial bone growth.

In Drosophila melanogaster, the BMP homologue, decapentaplegic is induced by hedgehog (Hh) (Basler and Struhl, 1994). Similarly, in vertebrates, signaling pathways that induce Bmp expression, are often regulated by members of the Hh family (Laue et al., 1994; Bitgood and McMahon, 1995; Zou et al., 1997a,b). Because Ihh is expressed in cartilage and is thought to regulate cartilage differentiation (Lanske et al., 1996; Vortkamp et al., 1996), Ihh expression was examined in FGFR3\(^{-/}\) mice to determine whether it could function to regulate Bmp4 expression. Consistent with previous reports (Lanske et al., 1996; Iwasaki et al., 1997; Vortkamp et al., 1998), expression of Ihh was restricted to hypertrophic and pre-hypertrophic chondrocytes in both wild-type and FGFR3\(^{-/}\) mice (Fig. 7A and B). However, the area of Ihh expression was smaller in FGFR3\(^{-/}\) mice because of the smaller hypertrophic zone. Additionally, the intensity of the Ihh signal was significantly weaker in 8-day (Fig. 7A,B), 14-day and 20-day (data not shown)-old FGFR3\(^{-/}\) mice than in littermate controls.

To assess the consequences of decreased Ihh expression, the expression of the Hh receptor, patched, was examined in growth plates from 8- to 20-day-old mice. The expression of patched is induced by Hh (Bitgood et al., 1996; Goodrich et al., 1996), and therefore the level of patched expression is a measure of the strength of the Hh signal. In wild-type animals patched expression was observed in prehypertrophic and proliferating chondrocytes and was weakly expressed in the perichondrium (Fig. 7C). In contrast, in FGFR3\(^{-/}\) mice, the expression of patched was dramatically reduced in proliferating chondrocytes, minimally detectable in prehypertrophic chondrocytes, and not detectable in the perichondrium (Fig. 7D). The intensity of collagen X expression was similar in FGFR3\(^{-/}\) mice and wild-type littermates (Fig. 7E,F). Additional evidence that FGFR3 inhibits Hh signaling is the finding that patched expression is upregulated in Fgfr3\(^{-/-}\) mice compared to wild-type littermates (Fig. 7G,H). These data are consistent with significantly decreased Ihh signaling throughout the growth plate and perichondrium of FGFR3\(^{-/}\) mice and support a model in which FGFR3 negatively regulates Ihh and indirectly negatively regulates Bmp4 expression.

![Fig. 5. PTHrP receptor expression in the limbs of FGFR3\(^{-/}\) and Fgfr3\(^{-/-}\) mice.](image-url) (A,B) Bright-field image showing proliferating (p) and hypertrophic (h) chondrocytes and perichondrium (pc) in the proximal humerus of an 18-day-old line B FGFR3\(^{-/}\) mouse (B) and wild-type littermate (A). (C,D) Dark-field image of tissue sections from FGFR3\(^{+/}\) (D) and wild-type (C) animals hybridized with an antisense riboprobe specific for PTHrP-R. Arrows indicate osteoprogenitor cells in the perichondrium that express the PTHrP-R. (E,F) Dark-field image of PTHrP-R expression in the proximal femur of 18.5 day Fgfr3\(^{+/}\) (E) and Fgfr3\(^{-/-}\) (F) embryos. Similar results were observed in the proximal tibia, distal femur and proximal humerus of FGFR3\(^{-/}\) or Fgfr3\(^{-/-}\) mice compared to their respective controls.
DISCUSSION

Multiple signaling pathways converge on the growth plate to regulate the growth and differentiation of the skeleton. Recently, the etiology of several human genetic diseases affecting skeletal development has been attributed to over 24 distinct dominant mutations in FGF receptors 1, 2 and 3 (Webster and Donoghue, 1997; Naski and Ornitz, 1998). Mutations in FGFR3 cause hypochondroplasia, achondroplasia and thanatophoric dysplasia, diseases that directly affect the function of the growth plate. To investigate genetic pathways utilized by FGFR3 to regulate chondrocyte growth and differentiation, transgenic mice were engineered to express the activating FGFR3 mutation that causes achondroplasia (FGFR3\textsuperscript{ach}) in chondrocytes. The effects of FGFR3\textsuperscript{ach} signaling on the proliferation and differentiation of epiphyseal chondrocytes showed that FGFR3\textsuperscript{ach} dramatically inhibits both chondrocyte proliferation (either directly or by slowing the entry of resting chondrocytes into the proliferating zone) and differentiation. The consequence of this effect on chondrogenesis is a histologically shortened growth plate and a gross phenotype resembling the human skeletal disorder, achondroplasia. Examination of signaling pathways that regulate chondrocyte differentiation showed that FGFR3\textsuperscript{ach} inhibits Ihh signaling and \textit{Bmp4} expression in cartilage and perichondrium. These data suggest that \textit{Fgfr3} is genetically upstream of Ihh and that FGFR3 may globally coordinate chondrogenesis and osteogenesis during skeletal growth.

Regulation of cell proliferation by FGFR3

It is surprising and provocative that FGFR3 signaling inhibits chondrocyte proliferation because, classically, FGFs are considered powerful mitogens for many cell types including primary chondrocytes (Gospodarowicz and Mescher, 1977; Klagsbrun et al., 1977; Basilico and Moscatelli, 1992). Several possibilities can account for the seemingly paradoxical mitogenic activities of FGFs. In vivo, in chondrocytes, FGFR tyrosine kinase activity may act through unique signaling pathways that inhibit proliferation. Alternatively, because the FGFR family consists of four high-affinity receptor tyrosine kinases, FGFR3 may have signaling properties that are distinct from that of other FGFRs. For example, FGFR3 may activate signals that inhibit cell proliferation, whereas the other FGFRs may stimulate cell proliferation. In support of this, FGFR3 is a poor mitogenic receptor compared to FGFR1 in BaF3 and PC12 cells (Lin et al., 1996, 1998; Naski et al., 1996) and recent in vitro studies, in which a constitutively active FGFR3 (containing the K650E mutation) was overexpressed in a non-chondrocytic cell line, showed decreased cell proliferation and a coincident increase in STAT1 activity (Su et al., 1997). STAT1 can upregulate the expression of certain cell cycle inhibitors, such as p21 (Chin et al., 1996). The effect of FGFR3 on cell cycle mediators in vivo and the differential signaling properties of the FGFRs will require further investigation. Another important possibility is that the suppression of chondrocyte proliferation may be mediated indirectly by other signaling molecules. BMP4 and Patched signaling pathways, which are significantly downregulated in proliferating zone chondrocytes in response to activated FGFR3, must also be considered as potential regulators of chondrocyte proliferation in vivo.

Expression of an FGF ligand may be limiting during postnatal bone development

In postnatal FGFR3\textsuperscript{ach} mice the BrdU-labeling index was decreased by 60% relative to that of wild-type animals. In contrast, during embryonic development the FGFR3\textsuperscript{ach} transgene had no effect on the proliferation of chondrocytes. This suggests that during embryonic growth, chondrocyte proliferation is insensitive to the effect of an activated FGFR, either because the concentration of FGF is in excess, saturating FGFR signaling pathways, or because other mitogens such as IGF-1 function dominantly during embryonic life and mask the growth inhibitory effects of FGFR3. Interestingly, the effects of IGF-1 on skeletal growth are greater during embryonic development than postnatally (Baker et al., 1993; Liu et al., 1993); thus, IGF-1 is a mitogen that may suppress the effects of FGFR3. Biochemical analyses of FGFR3\textsuperscript{ach} demonstrate that low ligand concentrations FGFR3\textsuperscript{ach} is weakly activated compared to the wild-type receptor. However, at saturating ligand concentrations both wild-type FGFR3 and FGFR3\textsuperscript{ach} have comparable activity (Naski et al., 1996). Thus, under conditions of excess ligand, the G380R mutation would have little or no effect because the cell is already receiving and responding to a maximal FGFR3 signal. In support of this we observe no difference in the expression of the downstream targets of FGFR3, \textit{patched} and \textit{Ihh}, in 18.5-day FGFR3\textsuperscript{ach}...
Regulation of bone growth by Fgfr3

4985

Regulation of bone growth by Fgfr3

Regulation of bone growth by Fgfr3

Regulation of bone growth by Fgfr3

Embryos compared to wild-type littermates (data not shown). Postnatally, either ligand or receptor could be limiting. However, transgenic mice that overexpress wild-type FGFR3 in the growth plate do not have abnormal chondrocyte proliferation (unpublished data), and transgenic mice that overexpress FGF2 develop a dwarfing condition similar to that of achondroplasia (Coffin et al., 1995). These observations suggest that postnatally, in the growth plate, the concentration of FGF ligand is limiting relative to that of FGFR3.

Regulation of chondrocyte differentiation

The effect of FGFR3 on chondrocyte differentiation was assessed by examining the size of the hypertrophic zone, the flux of BrdU-labeled cells through the growth plate over a 36-hour period, the band width of PTHrP-R and Ihh-expressing cells (cells committed to differentiate) and cell death (exit from the hypertrophic zone). The absence of any significant difference in the numbers of apoptotic cells in the distal hypertrophic zone of FGFR3ach mice compared to controls suggested that decreased chondrocyte differentiation and not increased cell death may be primarily responsible for the observed reduction in the size of the hypertrophic zone.

Short-term BrdU labeling showed that the labeling index was 2.5-fold lower in FGFR3ach mice compared to controls, whereas 36 hours after labeling only a 1.15-fold difference was observed. These data suggest that labeled cells accumulate in the FGFR3ach growth plate, because the flux of cells from the proliferating zone into the hypertrophic zone may be slowed. This conclusion is further supported by the observation of a narrowed band of both PTHrP-R and Ihh expression in FGFR3ach mice and a wider domain of PTHrP-R expression in Fgfr3−/− mice. The PTHrP-R is most highly expressed in post-proliferative chondrocytes in a band of cells transitional between proliferating and hypertrophic cells, and Ihh is expressed in cells committed to hypertrophy (Lee et al., 1995, 1996; Vortkamp et al., 1998). Thus, the quantity of cells expressing the PTHrP-R and Ihh should reflect early events in cell fate determination within the growth plate. The diminished expression of these markers suggests that the rate at which cells exit the proliferative phase and commit to hypertrophy is slowed. Alternatively, FGFR3 may directly inhibit the expression of the PTHrP-R and/or Ihh. However, if PTHrP-R expression was inhibited, the expected phenotype would be opposite to that which is observed in FGFR3ach mice because the loss of PTHrP or the PTHrP-R results in accelerated chondrocyte differentiation and premature ossification (Fig. 8) (Karaplis et al., 1994; Lanske et al., 1996; Lee et al., 1996).

Further evidence for a direct inhibition of chondrocyte...
growth of both bone and cartilage. FGFR3 can globally coordinate skeletal growth by controlling both the growth plate and perichondrium (step 5). In this manner, and patched in turn inhibits differentiation. Our data showing that diminished expression of Bmp4 in FGFR3ach mice correlates with an inhibition of chondrocyte proliferation or the entry of cells into the proliferative zone (step 1) and differentiation (step 2). The inhibition of differentiation may occur near the prehypertrophic region. This may be the result of a direct action of FGFR3 (solid lines) or indirectly as a result of inhibiting Ihh expression (red dashed line, step 3). Ihh is normally upregulated during chondrocyte hypertrophy and can bind to and inactivate its receptor, Patched, within the growth plate and perichondrium (step 4). The interaction of Ihh with Patched releases the inhibitory actions of Patched on Smoothened (Smo), thereby activating downstream signaling events, which result in the stimulation of PThrP-R and patched expression. The PThrP-R in turn stimulates chondrocyte proliferation and slows differentiation. In the proposed signaling pathway, FGFR3 inhibits Ihh expression in the growth plate, which in turn inhibits patched and Bmp4 expression (Zou et al., 1997a,b) in both the growth plate and perichondrium (step 5). In this manner, FGFR3 can globally coordinate skeletal growth by controlling the growth of both bone and cartilage.

differentiation by FGFR3 is that the formation of the secondary ossification center is delayed in the FGFR3ach mice.

In addition to direct effects, FGFR3 may also regulate differentiation indirectly by inhibiting Bmp4 expression. Studies of chick limb development have shown that constitutive expression of an activated or dominant negative BMP receptor can dramatically alter chondrogenesis (Kawakami et al., 1996; Zou et al., 1997a,b). These studies show that the activated BMP receptor promotes, whereas a dominant negative receptor inhibits early stages of chondrocyte differentiation. Our data showing that diminished expression of Bmp4 in FGFR3ach mice correlates with an inhibition of chondrocyte differentiation is also consistent with BMP-induced signals promoting early stages of chondrocyte differentiation in the growth plate. Alternatively, FGFR3 may regulate differentiation indirectly through its effects on chondrocyte proliferation. The inhibition of chondrocyte proliferation by FGFR3ach may diminish the pool of cells available for further differentiation. This predicts that the growth plate would effectively close or senesce more rapidly in FGFR3ach mice. However, after up to 1.5 years, the growth plate did not close more rapidly in FGFR3ach mice compared to wild-type littermates.

**Signaling between chondrocytes and the perichondrium**

Recent findings have shown that the perichondrium can elaborate undefined signals that negatively regulate chondrocyte proliferation and differentiation (Long and Linsenmayer, 1998). These authors showed that the effect of the perichondrium on the growth plate is to inhibit chondrogenesis. This similarity to FGFR3 signaling suggests that one mechanism by which FGFR3 may effect chondrocyte development is by indirectly regulating the expression of a factor produced by the perichondrium that can signal in the growth plate. Increased FGFR3 signaling in the growth plate suppressed expression of Bmp4 in both the perichondrium and the growth plate (Fig. 6). Similarly, patched expression is suppressed by FGFR3 in both of these tissues. These observations and the work of Zou et al. (1997a,b) showing that BMP receptors are expressed in the growth plate whereas BMPs are predominantly expressed in the perichondrium, support the existence of signaling pathways between the FGFR3-expressing growth plate chondrocytes and the surrounding perichondrium.

Interactions between FGF and BMP signaling have been observed in several developmental paradigms. Recently, examination of the early events that determine sites of tooth formation, revealed antagonistic interactions between FGF and BMP signaling (Neubuser et al., 1997). Others have found antagonistic interactions between FGFs and BMPs during chondrogenesis (Buckland et al., 1998). These studies of chick limb development show that BMP4-soaked beads promote chondrogenesis when implanted in the limb, and that FGF4 inhibits BMP-induced chondrogenesis. The interactions between FGF and BMP signaling may provide a mechanism to coordinate skeletal growth, which requires that the proliferation and differentiation of chondrocytes be synchronized with the differentiation of osteoblasts and deposition of osteoid. The simultaneous regulation of Bmp4 expression by FGFR3 in the growth plate and perichondrium suggests that BMP4 may be a signal that coordinates the development of these two tissues.

Antagonistic interactions between FGF and BMP signaling may also contribute to the defects observed in the dorsal axis of the vertebrae of FGFR3ach mice. The formation of this domain requires BMP4 signaling, and the loss of Bmp4 expression at this site results in the failure of spinous processes development (Monsoro-Burq et al., 1994, 1996; Liem et al., 1995). Regional expression of FGFR3ach may inhibit the expression or activity of BMP4, similar to that which occurs during the growth of long bones, and result in the observed defects of the dorsal vertebrae.

To investigate the signaling pathways that may couple FGFR3 to Bmp4 expression and to signals that may regulate the differentiation of chondrocytes, we examined the expression of Ihh and its receptor, patched (Marigo et al., 1996; Stone et al., 1996). Ihh activates signaling pathways in both the perichondrium and growth plate (Lanske et al., 1996; Vortkamp et al., 1996). Furthermore, Hh family members are...
known to be potent regulators of Bmp expression (Lauffer et al., 1994). The positive feedback pathway, whereby Hh binds to patched and upregulates patched expression via smoothened, sequesters Hh at the sites where patched is expressed (Chen and Struhl, 1996; Goodrich et al., 1996). The region of patched expression therefore defines the functional limits of Hh signaling (Chen and Struhl, 1996).

The expression of Ihh in the growth plate was significantly decreased in FGFR3<sup>ach</sup> mice. The hedgehog receptor, patched, was also dramatically suppressed in FGFR3<sup>3ch</sup> mice, providing further evidence that Ihh signaling is suppressed. These data are consistent with a model for the regulation of skeletal growth in which Bmp4 and patched are negatively regulated by FGFR3 (Fig. 8). It is possible that Ihh, because of its reduced area and intensity of expression, mediates the effects of FGFR3. Alternatively, FGFR3 may directly inhibit the expression of patched and Bmp4 in chondrocytes or may function through an intermediate other than Ihh, such as the PTHrP receptor. The effects of FGFR3 on Bmp4 and patched expression are observed not only in tissues where FGFR3 is expressed (cartilage), but also in regions where FGFR3 is not expressed (perichondrium). Thus, these data are more consistent with a model whereby FGFR3 acts indirectly through an intermediate such as Ihh. Interestingly, Zou et al. (1997) and Vortkamp et al. (1996) demonstrate that increased Ihh expression results in increased PTHrP expression and consequently decreased chondrocyte differentiation and increased proliferation. Our findings of decreased Ihh expression and decreased chondrocyte proliferation and differentiation in FGFR3<sup>ach</sup> mice suggest that FGFR3 may have a direct dominant effect on the chondrocyte differentiation independent of Ihh.

We thank M. Wuerffel, E. Spinaio, X. Hua and D. O’Donnell for their technical assistance and R. Kopan, J. Gordon, R. Cagan and D. Towler for critically reading this manuscript. This work was supported by NIH grant HD35692, funds from the Lopez Hidalgo Foundation and a Physician Postdoctoral Fellowship from the HHMI (M. C. N.).

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