Parathyroid Hormone Receptor Type 1/Indian Hedgehog Expression Is Preserved in the Growth Plate of Human Fetuses Affected with Fibroblast Growth Factor Receptor Type 3 Activating Mutations

Sarah Cormier,* Anne-Lise Delezoide,[†]
Catherine Benoist-Lasselin,[‡]
Laurence Legeai-Mallet,[‡] Jacky Bonaventure,[‡] and
Caroline Silve*

From Institut National de la Santé et de la Recherche Médicale U426* and the Service de Biologie du Développement,[†] Assistance Publique-Hôpitaux de Paris, Hôpital Robert Debré, Paris and Institut Fédératif de Recherche 02, Faculté de Médecine Xavier Bichat, Paris; and Institut National de la Santé et de la Recherche Médicale U393[‡] and Département de Génétique, Assistance Publique-Hôpitaux de Paris, Groupe Hospitalier Necker Enfants Malades, Paris, France

The fibroblast growth factor receptor type 3 (FGFR3) and Indian hedgehog (IHH)/parathyroid hormone (PTH)/PTH-related peptide receptor type 1 (PTHrP) systems are both essential regulators of endochondral ossification. Based on mouse models, activation of the FGFR3 system is suggested to regulate the IHH/PTHrP pathway. To challenge this possible interaction in humans, we analyzed the femoral growth plates from fetuses carrying activating FGFR3 mutations (9 achondroplasia, 21 and 8 thanatophoric dysplasia types 1 and 2, respectively) and 14 age-matched controls by histological techniques and *in situ* hybridization using riboprobes for human IHH, PTHrP, type 10 and type 1 collagen transcripts. We show that bone-perichondrial ring enlargement and growth plate increased vascularization in FGFR3-mutated fetuses correlate with the phenotypic severity of the disease. PTHrP and IHH expression in growth plates, bone-perichondrial rings and vascular canals is not affected by FGFR3 mutations, irrespective of the mutant genotype and age, and is in keeping with cell phenotypes. These results indicate that in humans, FGFR3 signaling does not down-regulate the main players of the IHH/PTHrP pathway. Furthermore, we show that cells within the bone-perichondrial ring in controls and patients express IHH, PTHrP, and type 10 and type 1 collagen transcripts, suggesting that bone-perichondrial ring formation involves cells of both chondrocytic and osteoblastic phenotypes. (Am J Pathol 2002, 161:1325–1335)

Endochondral bone ossification is a highly regulated process involved in the formation and growth of cartilaginous templates of long bones, vertebrae, and some craniofacial bones. It requires the linear phenotypic progression of undifferentiated mesenchymal cells into hypertrophic chondrocytes and the subsequent replacement of mineralized cartilage matrix by bone.^{1–4} As part of this process, resting chondrocytes at the growth plate proliferate, then mature and differentiate into hypertrophic chondrocytes and finally undergo apoptosis after mineralization of the surrounding extracellular matrix. Lacunae formed in this way in the mineralized matrix are invaded by blood vessels and bone cells. At each stage, chondrocytes express specific markers including type 2 collagen in the resting, proliferative, and prehypertrophic zones, and type 10 collagen when hypertrophic.

In addition to endochondral ossification, development and growth of bones formed via a cartilage model involve a distinct pattern of bone formation, namely perichondrial bone formation,^{2,3,5} a process whereby bone is formed outside and around the cartilage anlage. When ossification starts, perichondrial ossification generates the primitive cortical bone (or bone collar) and its associated periosteum, and the bone-perichondrial ring that develops around the growth plate at the cartilage/bone junction. Bone-perichondrial ring (also called ossification groove of Ranvier)^{5,6} comprises the bony ring (also called ring of Lacroix)^{5,6} covered on the periosteal side by a loose connective tissue that acts as a germinative (cambium) layer, assuring growth in width. Its growth in length is insured by a germinative zone localized at the epiphyseal tip of the ring.

Two main systems, namely the fibroblast growth factor receptor type 3 (FGFR3) and the Indian hedgehog (IHH)/

Supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM), INSERM/FRM "Réseaux Maladies Rares," Délégation à la Recherche Clinique Assistance Publique-Hôpitaux de Paris (CRC 99-302), and the Ministère de l'Éducation Nationale de la Recherche et de la Technologie and Fondation pour la Recherche Médicale (to S. C.).

Accepted for publication June 28, 2002.

Address reprint requests to Caroline Silve, M.D., Ph.D., INSERM U426 et Institut Fédératif de Recherche 02, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, 75018, Paris, France. E-mail: silve@bichat.inserm.fr.

parathyroid hormone (PTH)/PTH-related peptide (PTHrP) receptor type 1 (PTHR1) signaling pathways, are known to play a key role in regulating endochondral ossification. Recurrent, sporadic, or dominant gain-of-function mutations in the gene encoding FGFR3 cause hypochondroplasia, achondroplasia (ACH), and thanatophoric dysplasia (TD), an allelic series of chondrodysplasias of increasing severity.^{7–10} ACH (OnLine Mendelian Inheritance in Man (OMIM) 100800), the most frequent form of dwarfism in humans, is characterized by a disproportionate shortness of long bones. TD, a lethal condition that closely resembles homozygous ACH, is characterized by very short limbs, platyspondyly, and narrow thorax. Based on radiological differences, TD has been divided in two subgroups: TD1 (OMIM 187600), defined by the presence of curved femurs, and TD2 (OMIM 187601) defined by the association of straight femurs and cloverleaf skull resulting from premature fusion of all cranial sutures. Virtually all cases of ACH have been ascribed to a G380R amino acid substitution in the transmembrane domain of FGFR3, whereas TD2 is accounted for by a K650E mutation in the FGFR3 tyrosine kinase 2 domain. TD1 is caused by missense mutations, that mostly create cysteine residues in the extracellular domain¹¹ but mutations leading to a loss of the stop codon have also been described.¹²

Histologically, growth plates in ACH and TD patients present a specific disruption of their architecture, with a disorganization and shortness of the chondrocyte columns, and a reduced size of the hypertrophic zone.^{13–17} Additional morphological abnormalities, including increased bone collar thickness, and presence of numerous large vascular canals have been reported. An inward extension of mesenchymal fibrous tissue from the perichondrium has been detected in TD, but not in ACH.

In vitro and *in vivo* studies in humans^{18–20} and in mice^{21–23} have documented FGFR3 expression in proliferative and prehypertrophic chondrocytes, and have shown that FGFR3-activating mutations affected chondrocyte proliferation and differentiation. Depending on the model, cell type, or age, FGFR3 activation either does not modulate,^{20–22} inhibits,^{21,23–26} or stimulates cell proliferation,²² while it systematically alters chondrocyte differentiation into hypertrophic cells. Conversely, targeted deletion of FGFR3 in mice leads to skeletal overgrowth.^{27,28} These results together with clinical observations have established FGFR3 as a negative regulator of endochondral bone growth.

Mouse models have revealed the key role played by the IHH/PTHrP/PTHR1-signaling pathway in controlling endochondral bone formation.^{29–32} Prehypertrophic chondrocytes secrete IHH, which signals to periarticular chondrocytes or perichondrial cells and up-regulates their PTHrP synthesis. Secreted PTHrP binds to the PTHR1 expressed by prehypertrophic chondrocytes to delay their differentiation into hypertrophic chondrocytes. IHH, thereby, indirectly slows the pace of chondrocyte hypertrophy. Furthermore, analyses of IHH-deficient mice have shown that IHH stimulates chondrocyte proliferation independently of PTHrP/PTHR1,³³ and determines the site of bone collar formation.³⁴

Two rare chondrodysplasias namely Blomstrand's lethal chondrodysplasia (OMIM 215045) and Jansen's metaphyseal chondrodysplasia (OMIM 156400) have been ascribed, respectively, to inhibitory and activating PTHR1 mutations.³⁵ Blomstrand's lethal chondrodysplasia and Jansen's metaphyseal chondrodysplasia are, respectively, characterized by advanced and delayed endochondral bone formation. In addition, analysis of endochondral growth plate from fetuses with Blomstrand's lethal chondrodysplasia has shown increased bone collar (cortical bone) thickness and/or thickening of subperiosteal ossification overgrowing the growth plate, but no inward growth of bone-perichondrial ring (personal data).³⁶ Conversely, although not histologically documented, defective bone-perichondrial ring formation in patients with Jansen's metaphyseal chondrodysplasia is strongly suggested by the striking widening of the metaphysis associated with an irregular metaphyseal border on X-ray analysis.³⁵ Thus, PTHR1 mutations in humans, as FGFR3 mutations, are associated with defective perichondrial bone formation in addition to abnormal endochondral ossification.

Relatively few studies have focused on both FGFR3- and IHH/PTHR1-signaling pathways, yet they are both acting on the same developmental bone process. Analysis of possible interactions between the two pathways in mouse models expressing different FGFR3-activating mutations has lead to conflicting results. Although reduced IHH and/or PTHR1 expression has been reported by some authors,^{37,38} IHH and PTHR1 mRNA levels were found to be normal in a TD2 mouse model.²²

The aim of the present study was to assess the possible interaction between IHH/PTHR1- and FGFR3-signaling pathways during physiological and pathophysiological development of the growth plate in human. For this purpose we correlated growth plate histological lesions with FGFR3 mutations and compared PTHR1 and IHH expression with respect to type 1 and 10 collagen expression in cartilage samples from fetuses carrying activating FGFR3 mutations and age-matched controls. Here we show that expression of PTHR1 and IHH is not affected by FGFR3 mutations even though the size of the proliferative, prehypertrophic, and hypertrophic zones is markedly reduced in the most severe conditions (TD1 and TD2). Furthermore, we show that cells within the bone-perichondrial ring in controls and patients express IHH, PTHR1, and type 10 and 1 collagen transcripts, suggesting that bone-perichondrial ring formation involves cells of both chondrocytic and osteoblastic phenotypes.

Materials and Methods

Tissue Sampling and Patients

All bone samples for the present studies were obtained after the informed consent of the parents, and according to the French Ethical Committee recommendations.

Femoral bone fragments originated from medically aborted fetuses with ACH or TD after ultrasonographic and X-ray *in utero* detection of severe dwarfism. Confirmation of diagnosis was subsequently achieved by post-

mortem X-ray, histological study of bone sections, and molecular detection of FGFR3 mutations. Screening for FGFR3 mutations was performed on genomic DNA extracted from white blood cells or cultured skin fibroblasts using standard procedures.^{12,39} Our series of patients comprised: 1) 9 fetuses with ACH (G380R mutation), 1 was 12 weeks, 1 was 15 weeks, and 7 had an age ranging from 32 to 38 developmental weeks; 2) 21 TD1 fetuses (16 to 27 weeks), their mutant genotypes included the R248C or S249C mutations (13 of 21), the Y373C mutation (5 of 21), and the X807R or X807G mutations (3 of 21); 3) 8 TD2 fetuses carrying the K650E mutation (22 to 23 weeks).

Control tissues from 14 normal fetuses, showing no evidence of skeletal abnormalities, were obtained from spontaneously or voluntarily terminated pregnancies. The age of fetuses ranged, in developmental weeks, from 9 to 16 (five cases), 17 to 25 (eight cases), and 32 to 38 (seven cases).

Histology and in Situ Hybridization

Tissues were fixed with 4% paraformaldehyde then embedded in paraffin and sectioned. Unless otherwise specified, all histological and *in situ* hybridization analyses were performed on serial sections of bone samples originating from the upper femoral ends, to allow reliable comparisons. Sections were stained with hematoxylin/eosin/safran (HES) using standard procedures.^{18,19}

In situ hybridization was performed as previously reported.^{18,19,40} Serial sections were hybridized with previously described ³⁵S-labeled riboprobes for human type 10 collagen (386-bp COL10A1 cDNA fragment), human type 1 collagen (2-kb COL1A2 cDNA) and human PTHR1 receptor transcripts (312-bp PTHR1 cDNA fragment). Primers located in exons 1 and 2 of human IHH gene were used to amplify a 704-bp fragment that was cloned into pGEM-T Easy vector (Promega France, Charbonnières, France). Sequencing of the cloned polymerase chain reaction product confirmed the originally reported human sequence.

Histological analysis was performed on samples from all fetuses with FGFR3 mutations and all control fetuses. Hybridization analysis with each probe was performed at least three times on samples obtained from the following fetuses: ACH, <15 developmental weeks (2 of 2 cases); ACH, 32 to 38 developmental weeks (77 cases); TD1, S249C/R248C (6 of 13 cases); TD1, Y373C (1 of 1 case); TD2 (3 of 8 cases); control, <16 developmental weeks (3 to 6 cases); and control, 17 to 25 developmental weeks (3 to 6 cases); and control, 35 developmental weeks (1 case). All hybridizations were performed using anti-sense and sense riboprobes. In all experiments, no signal was obtained after hybridization with the sense probes, confirming the specificity of reactions performed using anti-sense probes (data not shown). One example of the signal obtained by hybridization with each anti-sense probe is shown for each genotype at the different developmental ages studied. These results are representative of those obtained for the other samples in each group.

Results

Growth Plate Abnormalities in Fetuses Carrying FGFR3 Mutations

Histological examination of the growth plate from affected fetuses and comparison with age-matched controls disclosed the histomorphological abnormalities that characterize FGFR3-related skeletal dysplasias (Figure 1).^{13–17} Qualitatively, most of these abnormalities were similar irrespective of the mutant genotype, including reduced height of proliferating and hypertrophic zones, irregular columnar arrangement, and decreased number and size of hypertrophic chondrocytes. In contrast, the resting chondrocyte zone looked normal. The defective hypertrophic chondrocyte differentiation was associated with rare, short, thick, and distorted primary bone trabeculae enriched in cartilaginous mineralized matrix core. Bone-perichondrial ring thickness was increased, and in most cases associated with an inward overgrowth of fibrous tissue extending from the epiphyseal tip of the perichondrial groove of Ranvier that disrupted the growth plate at the bone-cartilage junction (Figure 1, G and K). Its development was asymmetrical, and, in the upper femoral epiphysis, seen only on the external side.

Phenotype-Genotype Correlation of Growth Plate Abnormalities

Careful examination of the phenotype as a function of age and FGFR3 mutation, revealed specific phenotype-genotype correlation as regards the severity of cartilage defects.

G380R Mutation (ACH)

The growth plate of prenatally diagnosed ACH fetuses early in development (Figure 1, C and D) was almost undistinguishable from age-matched controls (Figure 1, A and B), and the diagnosis was suspected on the bone collar thickening (Figure 1, A and C). Later in development the growth plate from ACH fetuses was clearly abnormal compared to that of age-matched controls (Figure 1, compare G and H to E and F), with a shortened proliferating zone and an abundant matrix between chondrocyte columns. Insufficient hypertrophy seemed to be associated with thick and highly reticulated trabeculae of residual mineralized cartilage and primary bone. Inward overgrowth of fibrous tissue extending from the perichondrial ring was also obvious (Figure 1G). The ossification that occurred on the inferior metaphyseal side of this tissue was less intense than in TD fetuses (Figure 1, compare G to K). This aspect illustrated the characteristic radiological image of the femoral head. Unlike TD, large vascular canals interrupting the ossification line were not observed.

S249C or R248C Mutations (TD1)

Lesions associated with these genotypes were the most severe (shown for S249C; Figure 1, K and L). The

Figure 1. HES-stained histological sections of femoral ends (upper epiphyses, except in **G**, which were lower epiphysis) from age-matched control fetuses (**A**, **B**, **E**, **F**, **I**, **J**) and fetuses carrying FGFR3 mutations (**C**, **D**, **G**, **H**, **K**, **L**, **M**, **N**, **O**, **P**) at two magnifications, illustrating the growth plate abnormalities associated with FGFR3 mutations. For each fetus, age in developmental weeks (dw), phenotype, and mutation are indicated. The severity of the abnormalities varies as a function of age and mutation. In the 13-week-old ACH fetus (**C**, **D**) the growth plate architecture closely resembles that of an age-matched control (**A**, **B**), whereas moderate abnormalities are observed in older ACH fetuses (compare **G** and **H** to **E** and **F**). The disorganization is more severe in TD1 (**K**, **L**, **M**, **N**) and TD2 (**O**, **P**). Note that the inward growth of fibrous tissue characteristic for the TD1 (**K**, arrow) is also obvious in an ACH case (**G**, arrow). Scale bars: 2.5 mm (**E**, **G**, **I**, **K**); 500 μ m (**A**, **C**, **M**, **O**); 130 μ m (**B**, **D**, **F**, **H**, **J**, **L**, **N**); 45 μ m (**P**).

chondro-osseous junction was irregular and serrated; the proliferative zone was short, disorganized, and poorly delineated from the resting and hypertrophic zones. At this level, cells were smaller than typical hypertrophic chondrocytes, retained a round aspect, and were surrounded by an abundant matrix. The mineralization zone was short; degeneration of hypertrophic chondrocytes occurred at different levels leaving thick and reticulated residual cartilage trabeculae. Primary bone trabeculae were rare, thick, and organized as a network rather than parallel to the diaphysis. Disruption of the growth plate by vascular canals was present in some sections (not shown). Typical tongue of fibrous tissue arising from the perichondrial area and narrowing externally to the growth plate was present.

TD1 fetuses carrying a Y373C mutation exhibited similar abnormalities except that hypertrophic chondrocytes looked wider with a slightly more regular columnar arrangement (not shown).

X807R and X807G Mutations (TD1) (Shown for X807G; Figure 1, M and N)

The columnar organization of the growth plate resembled that of fetuses with ACH. The proliferative zone was clearly distinguishable from the resting and hypertrophic zones, the latter being well developed. The chondro-osseous interface was regular and the number of vascular canals was in the same range as that in age-matched controls. The residual mineralized cartilage trabeculae were numerous, enlarged, and reticulated. The primary trabeculae were regularly oriented, thick, and slightly thinner than in ACH.

K650E Mutation (TD2) (Figure 1, O and P)

The columnar arrangement was better preserved than in TD1 patients carrying mutations in the extracellular domain of the receptor. The chondro-osseous junction

was frequently disrupted by enlarged ossifying vascular canals. The hypertrophic zone was clearly visible. The size of hypertrophic cells was similar to that observed in ACH fetuses and some cells had a polyhedral shape. However, ossification more closely resembled that observed in TD1 than in ACH. Bone-perichondrial ring, although thicker than that in control fetuses, was not as developed as that in TD1 (Figure 1, compare O to K and M).

Comparative Expression of PTHR1, IHH, and COL10A1 Genes in Growth Plates from Controls and FGFR3-Mutated Fetuses

In situ hybridization studies of growth plates from control fetuses showed that, as expected, COL10A1 transcripts were specifically detected in hypertrophic chondrocytes (Figure 2, A4 and C4). Little overlap between PTHR1 and COL10A1 was observed (Figure 2; A2 and C2 and A4 and C4), whereas IHH and COL10A1 strongly overlapped (Figure 2; A3 and C3 and A4 and C4). These results demonstrated PTHR1 mRNA expression in prehypertrophic chondrocytes, in the upper hypertrophic chondrocytes and in osteoblasts of the bone trabeculae (Figure 2, A2). The area of IHH expression was more extended than that of PTHR1 and involved prehypertrophic and hypertrophic chondrocytes (Figure 2, A3). PTHR1 and IHH partly co-localized in the prehypertrophic zone (Figure 2, A2 and A3). In the region of overlap between COL10A1, IHH, and PTHR1 mRNA expression, cell labeling by the PTHR1 probe was uneven. A similar pattern of PTHR1 and IHH gene expression was observed at 35 weeks of development (data not shown).

PTHR1 and IHH gene expression pattern in fetuses carrying FGFR3 mutations did not differ significantly from controls (Figure 2; B1 to B4 and D1 to E4), irrespective of the mutant genotype and age. Smaller areas of expression in FGFR3-mutated fetuses were directly related to the narrowing of prehypertrophic and hypertrophic zones seen on HES-stained sections. In certain cases, the layer of PTHR1-expressing cells (Figure 2, D2) or IHH-expressing cells (Figure 2, D3) appeared closer to the chondro-osseous junction than in controls. However, this was because of the reduced height of the hypertrophic zone and not to an ectopic expression of PTHR1 in hypertrophic cells.

In addition, PTHR1 and IHH mRNA signal intensity in fetuses carrying FGFR3 mutations appeared similar to, or more intense than, that in controls irrespective of the mutant genotype and age (Figure 2; compare A2 and A3 to B2 and B3, and C2 and C3 to D2 and D3 and E2 and E3). This indicated that no decrease in PTHR1 or IHH expression had occurred. As observed in controls, PTHR1 expression was uneven in the region of overlap with type 10 collagen.

Gene Expression in Bone-Perichondrial Ring from Control and FGFR3-Mutated Fetuses

The bone-perichondrial ring surrounds the metaphysis and extends from the proliferating chondrocyte layer to the zone of primary trabeculae at the metaphyso-diaph-

Figure 2. PTHR1, IHH, and COL10A1 mRNA expression in proximal femoral growth plates from control fetuses (A1 to A4 and C1 to C4) and fetuses carrying different FGFR3 mutations (B1 to B4 and D1 to E4). For each fetus, age in developmental weeks (dw), phenotype, and mutation are indicated. Serial sections were stained with HES (A1 to E1) or used for *in situ* hybridization using anti-sense ³⁵S-labeled PTHR1 (A2 to E2), IHH (A3 to E3), and COL10A1 (A4 to E4) riboprobes. Dark-field photomicrographs of *in situ* hybridization are shown. The same pattern and signal intensity of gene expression is observed in control fetuses and fetuses affected with FGFR3 mutations. Scale bars: 100 μ m (A1 to A4, C1 to E4); 200 μ m (B1 to B4).

yseal junction (Figure 3, A1). Early in development, the bone-perichondrial ring is thin. As development proceeds, its thickness increases, and cells become clearly visible inside the osseous matrix (Figure 3, C1).

Analysis of gene expression in the control perichondrial ring region showed IHH, PTHR1, and type 10 collagen transcripts in growth plate hypertrophic chondrocytes lining the internal side of the ring (Figure 3; A2 to A4 and C2 to C4). PTHR1 and COL1A2 (but not IHH) transcripts co-localized in osteoblasts present in the loose

connective tissue lining the periosteal side of the bone-perichondrial ring (Figure 3, A2 and A5). Fibroblasts expressing only type 1 collagen formed the thick layer of fibrous periosteum. At later stages of development (from 23 to 24 weeks of development), most sections showed an enlarged perichondrial ring (Figure 3, C1 to C5). The type 1 collagen expression domain was predominant and seemed to wrap the germinative zone of the ring (Figure 3, C5). At the marginal germinative zone, osteoblasts secreting bone matrix were positive for PTHR1 and COL1A2 (Figure 3; arrows in C2 and C5). The tip of the ring was bordered on the external and internal sides by COL1A2-expressing cells, which formed a thin layer descending toward the cartilage-bone junction on the internal side of the bone-perichondrial ring (Figure 3; arrows in C5 and D5). In addition, PTHR1, IHH, COL10A1, and COL1A2 genes were expressed in cells entrapped within the mineralized matrix (Figure 3, C1 to C5). Four types of cells were recognizable: 1) cells expressing PTHR1, IHH, and COL10A1 (hypertrophic chondrocyte phenotype); 2) cells expressing either PTHR1 and COL10A1 or 3) PTHR1 and COL1A2 (osteoblastic phenotype); and 4) cells expressing only COL1A2 at a lower level.

The bone-perichondrial ring in FGFR3-mutated fetuses was thicker than that in age-matched controls; the increase in thickness was more pronounced as a function of age and phenotype severity (Figure 3). Importantly, the same pattern of gene expression was observed in bone-perichondrial ring from FGFR3-mutated fetuses irrespective of the mutation and age, a pattern that was similar to that of age-matched controls (Figure 3). No ectopic expression was noticed. In some FGFR3 mutant perichondrial rings, expression of COL10A1 appeared increased and associated with a decreased COL1A2 mRNA. The presence of PTHR1, IHH, COL10A1, and COL1A2 mRNA in cells within the mineralized matrix of the perichondrial ring was particularly visible in the thickened bone-perichondrial ring of FGFR3-mutated fetuses in the second or third trimester of gestation (Figure 3; D2 to D5 and E2 to E5).

Expression of the Four Genes in Vascular Canals

Disruption of the growth plate by numerous enlarged vascular canals are typically observed in TD fetuses, as

shown in Figure 4. The epiphyseal extremities of the canals are formed by mesenchymal tissue while mineralized matrix covered by an osteoblast layer is present at the metaphyseal ends. Despite the canal's striking enlargement, gene expression was in keeping with cell phenotypes. The vascular canals were surrounded by PTHR1- and IHH-positive prehypertrophic and upper hypertrophic chondrocytes (Figure 4, B and C) and COL10A1-positive hypertrophic chondrocytes (Figure 4D). Osteoblasts lining the mineralized matrix inside the vascular canal expressed PTHR1 and COL1A2 (Figure 4B). Fibroblasts present in the connective tissue at the epiphyseal end of the canal expressed COL1A2 (Figure 4E). No overlap between type 1 and 10 collagens, or IHH and type 1 collagen, was observed.

Discussion

The goal of the present study was to analyze the respective role of FGFR3- and IHH/PTHR1-signaling pathways and their possible interaction in the control of physiological and pathophysiological human endochondral bone formation. For this purpose, we performed growth plate histological analysis and investigated the pattern of expression of PTHR1 and IHH in bone samples from a series of fetuses affected with FGFR3-activating mutation as compared to age-matched controls. Type 10 collagen was used as a specific marker of hypertrophic chondrocytes and type 1 collagen for osteoblast and fibroblast labeling. Results were analyzed as a function of phenotype severity.

In vitro studies using cell lines transfected with either wild-type or mutant FGFR3 cDNAs have shown that TD1 and TD2 mutations (R248C and K650, respectively) lead to stronger constitutive activation of the receptor than the ACH mutation (G380R).⁴¹ These findings provided a biochemical rationale to explain the higher clinical and radiological severity of TD and were further supported by molecular, radiological, and histopathological correlations in TD.⁴² Our results confirm and extend this study through additional FGFR3 mutations. Fetuses carrying mutations in the extracellular domain (TD1) exhibited more severe growth plate histological abnormalities than TD2 cases. In addition, we show that mutations deleting the stop codon (X807R and X807G) resulted in a less

Figure 3. PTHR1, IHH, COL10A1, and COL1A2 mRNA expression in bone-perichondrial ring regions of upper femoral ends from two control fetuses (A1 to A5 and C1 to C5), two fetuses with ACH (B1 to B5 and D1 to D5), and one TD2 fetus (E1 to E5). For each fetus, age in developmental weeks (dw) and phenotype are indicated. Serial bone sections were stained with (A1 to E1) or hybridized with riboprobes for PTHR1 (A2 to E2), IHH (A3 to D3), COL10A1 (A4 to E4), and COL1A2 (A5 to E5) then stained with H&E. Bright-field photomicrographs are shown, except in A2, A3, A5, and D2 which are dark-fields. Note the increase in bone-perichondrial ring thickness as a function of age (compare A1 and C1), and in FGFR3-mutated fetuses as compared to controls (compare B1 to A1, and D1 and E1 to C1). Note that the scale of C1 to C5 differs from that of other panels. The same pattern of gene expression is observed in controls and FGFR3-mutated fetuses. PTHR1, IHH, and COL10A1 mRNA are expressed by cells on the internal side (i) of the bone-perichondrial ring; note the flat elongated appearance of some cells directly in contact with the internal surface of the bone ring (C1). Cells forming the loose connective layer (lo) covering the periosteal side of the bone ring express PTHR1 and COL1A2 mRNAs, and cells present in the thick fibrous layer (th) continuous with the perichondrium and periosteum express COL1A2 mRNA only. PTHR1, IHH, COL10A1, and COL1A2 mRNA are observed in cells within the mineralized matrix of the perichondrial ring in control (C1 to C5) and FGFR3-mutated fetuses (D1 to E5). Note that cells forming the marginal germinative zone (M) at the tip of the bone-perichondrial ring are positive for PTHR1 and COL1A2 (arrows in C2 and C5), and are bordered on the periosteal and endosteal sides by COL1A2-expressing cells; these COL1A2-expressing cells formed a thin layer descending toward the cartilage-bone junction on the endosteal side of the bone-perichondrial ring (arrowhead in C5 and D5). C, cartilage; B, bone; M, marginal germinative zone; i, internal layer of the bone ring; r, bone-perichondrial ring; lo, loose connective layer covering the periosteum side of the bone ring; th, thick fibrous layer in continuity with the perichondrium and periosteum; N.A., not available. Scale bars: 200 μ m (A1 to B5 and D1 to E5); 100 μ m (C1 to C5).

Figure 4. PTHR1, IHH, COL10A1, and COL1A2 mRNA expression in vascular canals of the growth plate from a TD2 fetus (22 weeks). Sections encompassing vascular canals were HES stained (**A**) or hybridized with PTHR1 (**B**), IHH (**C**), COL10A1 (**D**), and COL1A2 (**E**) riboprobes then stained with H&E. Bright-field photomicrographs are shown in **B**, **D**, and **E** and dark-field in **C**. Arrows in **B** indicate PTHR1 mRNA expression in osteoblasts lining the mineralized matrix. **A** and **B** show two serial sections of the same vascular canal and **C**, **D**, and **E** are from different vascular canals. Scale bars, 100 μ m.

severe phenotype than mutations creating cysteine residues (S249C and R248C). As expected, the G380R ACH mutation led to milder histological abnormalities, consistent with the clinical phenotype and molecular classification. So far, correlation between clinical severity and the extent of receptor activation *in vitro* has been restricted to three mutations namely R248C, K650E, and G380R.⁴¹ Whether a similar correlation could be established between growth plate histopathological abnormalities and the constitutive activation induced by the whole set of FGFR3 mutations causing skeletal dysplasias, remains to be experimentally confirmed.

The present study also provides information regarding bone-perichondrial ring formation in controls and patients and substantiates the genotype/phenotype correlation between bone-perichondrial ring histology and FGFR3 mutations. The bone-perichondrial ring is thought to play an important role in the regulation of long bone growth at the growth plate level.¹⁻³ However, little is known about factors regulating bone-perichondrial ring formation at the site of hypertrophic chondrocyte differentiation. Analysis of IHH-deficient mice revealed that, contrary to a current view, bone-perichondrial ring formation differs from membranous bone formation, and is under the control of at least one key gene, Indian hedgehog, that

appeared to determine the site of bone collar formation.^{33,34} Our demonstration of type 1 and 10 collagen expression in cells within the mineralized matrix of bone-perichondrial ring further suggested that the process involved in its formation is distinct from endochondral or membranous bone ossification. It is noteworthy that the presence of cells surrounded by a matrix containing both type 1 and type 10 collagens has been described in osteochondromas (exostoses) and mesenchymal chondrosarcomas^{43,44} supporting the hypothesis that posthypertrophic differentiation of terminally differentiated chondrocytes into osteoblast-like cells synthesizing type 1 collagen might be a physiological process.⁴⁵ The association of an overgrowth of the bone-perichondrial ring with a high number of cells expressing type 10 collagen, PTHR1, and IHH in the internal part of the ring as observed both in TD and ACH cases also raised the possibility that bone-perichondrial ring formation involves cells of both chondrocytic and osteoblastic phenotypes.

Both ACH and TD fetuses exhibited a reproducible inward growth of the bone-perichondrial ring, usually localized on the external side of the femoral head. To our knowledge, inward growth of the bone-perichondrial ring had not been reported in ACH. Our observation might be because of the opportunity that we had to study intact

bones from ACH fetuses, and not only bone biopsies. The molecular mechanisms leading to overgrowth of the bone-perichondrial ring are not known, but most likely involve increased proliferation of germinative cells, and increased differentiation of osteoblast-like cells. In agreement with this hypothesis, we observed COL1A2 mRNA expression in cells localized at the internal end of the fibrous band (data not shown). Surprisingly, bone-perichondrial ring overgrowth appeared to be independent of the FGFR3 mutation. No inward growth of the bone-perichondrial ring has been observed in Blomstrand fetuses carrying loss-of-function mutations in the PTHR1 gene although thickening of the bone collar and cortical diaphyseal bone has been described.³⁵ Histological analysis of the bone-perichondrial ring in Blomstrand fetuses revealed that in contrast to control or FGFR3-mutated fetuses, only cortical/periosteal bone was present and associated with defective bone-perichondrial ring development (personal data).³⁶ This observation is supported by data obtained in PTHrP and PTHR1 knockout mice^{46–48} and indicates that formation of the bone-perichondrial ring is not similarly affected by FGFR3 and PTHR1 mutations. Further analysis of genetically manipulated mice overexpressing both FGFR3 and PTHR1 genes should help define the specific defects.

In control fetuses PTHR1 and IHH were co-expressed in prehypertrophic and upper hypertrophic chondrocytes whereas lower hypertrophic chondrocytes expressed only IHH. Comparison of PTHR1 and IHH expression patterns between control and pathological samples failed to demonstrate defective or ectopic expression of both genes in the growth plate, bone-perichondrial ring, and vascular canals of FGFR3-mutated fetuses. Our inability to detect a significant reduction in PTHR1 and IHH expression at the growth plate level suggests that in humans, expression of these two genes is not down-regulated by the FGFR3-signaling pathway. Yet, we cannot exclude the possibility that FGFR3 could modulate expression of the ligand PTHrP in human cartilage as, indeed, we were unable to detect these transcripts in control or pathological fetuses after 10 weeks of development (not shown). Alternatively, FGFR3 mutations could promote overexpression of Patched (Ptc1), the cell surface receptor of IHH, on proliferating chondrocytes, and cells from the perichondrium and primary spongiosa, similar to what has been reported in a mouse TD2 model.²²

Indeed, IHH and PTHR1 expression studies in mouse models harboring various FGFR3-activating mutations have given rise to conflicting data. Hence, decreased expression of IHH and Ptc1 occurred in mice overexpressing the ACH mutation.²¹ TD mutations also appeared to down-regulate the IHH/PTHR1 pathway in mice carrying the K644E or S365C amino acid substitutions.^{37,38} By contrast mice carrying the TD2 FGFR3 mutation normally expressed IHH and PTHR1.²² It is worth noting that this latter model more closely mimicked the human phenotype than the previous and was associated with an intense and persistent expression of Ptc. Indeed, even though interaction between FGFR3- and IHH/PTHR1-signaling pathways would be compatible with the reported suppression of FGFR3 gene transcription by

cAMP,⁴⁹ our results do not support such an interaction in humans. Discordant effects on signaling molecules reported in several mouse models may be attributed to the procedure used to generate mutant animals. In transgenic mice, the phenotype is dependent on the promoter used for FGFR3 overexpression that may lead to variable and ectopic expression of the transgene.²¹ The inability of these mouse models to faithfully reproduce the human phenotype in most cases suggests that conclusions drawn with these models should be taken with caution.

Formation of the bone-perichondrial ring in FGFR3 mouse models has received little attention up to now. In one ACH model (mutation G369C), an advanced bone-perichondrial ring formation has been described,²³ whereas no bone-perichondrial ring defect was reported in mice carrying the most common ACH mutation.²¹ These results are at variance with our findings of an increased bone-perichondrial ring thickness irrespective of the FGFR3 mutation in patients and might be accounted for by the nonexpression of the transgene in prehypertrophic chondrocytes in the latter mutant mouse model.²¹ Indeed, accelerated formation of the bone-perichondrial ring and absence of premature ossification in FGFR3 fetuses argues against a down-regulation of the IHH/PTHR1 pathway by FGFR3. Support to this assertion is provided by radiographical data originating from Jansen patients carrying activating mutations in the PTHR1 gene. Defective bone-perichondrial ring formation in these patients is strongly suggested by the striking widening of the metaphysis associated with an irregular metaphyseal border³⁵ and seems to be consistent with the PTHR1 activation that would induce lower IHH expression.⁵⁰ It further indicates that signaling through FGFR3 is unable to compensate for defective perichondrial ring formation.

In summary, results from the present work indicate the absence of a direct interaction between FGFR3- and IHH/PTHR1-signaling pathways in humans. Furthermore, the observation that cells within the bone-perichondrial ring in controls and patients express markers for both chondrocytic and osteoblastic phenotypes supports the possibility that bone-perichondrial ring formation involves cells of both phenotypes.

Acknowledgment

We thank the Réseau de la Société Française de Foetopathologie for tissue specimens.

References

1. Baldet P: Le cartilage de croissance, développement, structure et dynamique. *Notions Fondamentales en Orthopédie*. Edited by JG Pous, A Dimeglio, P Baldet, F Bonnel. Paris, Doin, 1980, pp 17–47
2. Caplan AL, Pechak DG: The cellular and molecular embryology of bone formation. *Bone and Mineral Research*, ed 5. Edited by WA Peck. New-York Elsevier Science Publishers BV, 1987, pp 117–183
3. Olsen BR, Reginato AM, Wang W: Bone development. *Annu Rev Cell Dev Biol* 2000, 16:191–220
4. Hall B, Miyake T: All for one and one for all: condensations and the initiation of skeletal development. *Bioessays* 2000, 22:138–147

5. Coutelier L: L'encoche d'ossification: aspect particulier de la croissance d'un os long. *Notions Fondamentales en Orthopédie*. Edited by JG Pous, A Dimeglio, P Baldet, F Bonnel. Paris, Doin Editeurs, 1980, pp 48–56
6. Uthoff H: The development of the limb buds. The growth of tubular bones. *The Embryology of the Human Locomotor System*. Berlin, Springer-Verlag, 1990, pp 7–24
7. Naski MC, Ornitz DM: FGF signaling in skeletal development. *Front Biosci* 1998, 3:D781–D794
8. Bonaventure J, Rousseau F, Legeai-Mallet L, Benoist C, Le Merrer M, Munnich A: Récepteurs des facteurs de croissance fibroblastiques (FGFR) et anomalies héréditaires de l'ossification endochondrale et membranaire. *Med Sci* 1996, 12:44–49
9. Ornitz DM: Regulation of chondrocyte growth and differentiation by fibroblast growth factor receptor 3. *Novartis Found Symp* 2001, 232: 63–80
10. Xu X, Weinstein M, Li C, Deng C: Fibroblast growth factor receptors (FGFRs) and their roles in limb development. *Cell Tissue Res* 1999, 296:33–43
11. Webster MK, Donoghue DJ: FGFR activation in skeletal disorders: too much of a good thing. *Trends Genet* 1997, 13:178–182
12. Rousseau F, Saugier P, Le Merrer M, Munnich A, Delezoide AL, Maroteaux P, Bonaventure J, Narcy F, Sanak M: Stop codon FGFR3 mutations in thanatophoric dwarfism type 1. *Nat Genet* 1995, 10:11–12
13. Horton WA, Hood OJ, Machado MA, Campbell D: Growth plate cartilage studies in achondroplasia. *Basic Life Sci* 1988, 48:81–89
14. Horton WA, Hood OJ, Machado MA, Ahmed S, Griffey ES: Abnormal ossification in thanatophoric dysplasia. *Bone* 1988, 9:53–61
15. Ornoy A, Adomian GE, Eteson DJ, Burgeson RE, Rimoin DL: The role of mesenchyme-like tissue in the pathogenesis of thanatophoric dysplasia. *Am J Med Genet* 1985, 21:613–630
16. Rimoin DL, Hughes GN, Kaufman RL, Rosenthal RE, McAlister WH, Silberberg R: Endochondral ossification in achondroplastic dwarfism. *N Engl J Med* 1970, 283:728–735
17. van der Harten HJ, Brons JT, Dijkstra PF, Barth PG, Niermeyer MF, Meijer CJ, van Geijn HP, Arts NF: Some variants of lethal neonatal short-limbed platyspondylic dysplasia: a radiological ultrasonographic, neuropathological and histopathological study of 22 cases. *Clin Dysmorphol* 1993, 2:1–19
18. Delezoide AL, Lasselini-Benoist C, Legeai-Mallet L, Brice P, Senée V, Yayon A, Munnich A, Vekemans M, Bonaventure J: Abnormal FGFR 3 expression in cartilage of thanatophoric dysplasia fetuses. *Hum Mol Genet* 1997, 6:1899–1906
19. Delezoide AL, Benoist-Lasselini C, Legeai-Mallet L, Le Merrer M, Munnich A, Vekemans M, Bonaventure J: Spatio-temporal expression of FGFR 1, 2 and 3 genes during human embryo-fetal ossification. *Mech Dev* 1998, 77:19–30
20. Legeai-Mallet L, Benoist-Lasselini C, Delezoide AL, Munnich A, Bonaventure J: Fibroblast growth factor receptor 3 mutations promote apoptosis but do not alter chondrocyte proliferation in thanatophoric dysplasia. *J Biol Chem* 1998, 273:13007–13014
21. Naski MC, Colvin JS, Coffin JD, Ornitz DM: Repression of hedgehog signaling and BMP4 expression in growth plate cartilage by fibroblast growth factor receptor 3. *Development* 1998, 125:4977–4988
22. Iwata T, Chen L, Li C, Ovchinnikov DA, Behringer RR, Francomano CA, Deng CX: A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos. *Hum Mol Genet* 2000, 9:1603–1613
23. Chen L, Adar R, Yang X, Monson EO, Li C, Hauschka PV, Yayon A, Deng CX: Gly369Cys mutation in mouse FGFR3 causes achondroplasia by affecting both chondrogenesis and osteogenesis. *J Clin Invest* 1999, 104:1517–1525
24. Henderson JE, Naski MC, Aarts MM, Wang D, Cheng L, Goltzman D, Ornitz DM: Expression of FGFR3 with the G380R achondroplasia mutation inhibits proliferation and maturation of CFK2 chondrocytic cells. *J Bone Miner Res* 2000, 15:155–165
25. Segev O, Chumakov I, Nevo Z, Givol D, Madar-Shapiro L, Sheinin Y, Weinreb M, Yayon A: Restrained chondrocyte proliferation and maturation with abnormal growth plate vascularization and ossification in human FGFR-3(G380R) transgenic mice. *Hum Mol Genet* 2000, 9:249–258
26. Wang Y, Spatz MK, Kannan K, Hayk H, Avivi A, Gorivodsky M, Pines M, Yayon A, Loni P, Givol D: A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Proc Natl Acad Sci USA* 1999, 96:4455–4460
27. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM: Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat Genet* 1996, 12:390–397
28. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P: Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* 1996, 84:911–921
29. Strewler GJ: The physiology of parathyroid hormone-related protein. *N Engl J Med* 2000, 342:177–185
30. Philbrick WM, Wysolmerski JJ, Galbraith S, Holt E, Orloff JJ, Yang KH, Vasavada RC, Weir EC, Broadus AE, Stewart AF: Defining the roles of parathyroid hormone-related protein in normal physiology. *Physiol Rev* 1996, 76:127–173
31. Kronenberg HM, Chung U: The parathyroid hormone-related protein and Indian hedgehog feedback loop in the growth plate. *Novartis Found Symp* 2001, 232:144–152
32. Karaplis AC, Deckelbaum RA: Role of PTHrP and PTH-1 receptor in endochondral bone development. *Front Biosci* 1998, 3:D795–D803
33. St-Jacques B, Hammerschmidt M, McMahon AP: Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. *Genes Dev* 1999, 13:2072–2086
34. Chung UI, Schipani E, McMahon AP, Kronenberg HM: Indian hedgehog couples chondrogenesis to osteogenesis in endochondral bone development. *J Clin Invest* 2001, 107:295–304
35. Silve C, Jüppner J: Genetic disorders caused by mutations in the PTH/PTHrP receptor: Jansen's metaphyseal chondrodysplasia and Blomstrand's lethal chondrodysplasias. *The Parathyroids*. Edited by JP Bilezikian, R Marcus, M Levine. San Diego, Academic Press, 2001, pp 707–728
36. Oostra RJ, van der Harten JJ, Rijnders WP, Scott RJ, Young MP, Trump D: Blomstrand osteochondrodysplasia: three novel cases and histological evidence for heterogeneity. *Virchows Arch* 2000, 436:28–35
37. Li C, Chen L, Iwata T, Kitagawa M, Fu XY, Deng CX: A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and ink4 cell cycle inhibitors. *Hum Mol Genet* 1999, 8:35–44
38. Chen L, Li C, Qiao W, Xu X, Deng C: A Ser(365)→Cys mutation of fibroblast growth factor receptor 3 in mouse downregulates Ihh/PTHrP signals and causes severe achondroplasia. *Hum Mol Genet* 2001, 10:457–465
39. Rousseau F, Bonaventure J, Legeai-Mallet L, Pelet A, Rozet JM, Maroteaux P, Le Merrer M, Munnich A: Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. *Nature* 1994, 371:252–254
40. Wysolmerski JJ, Cormier S, Philbrick WM, Dann P, Zhang JP, Roume J, Delezoide AL, Silve C: Absence of functional type 1 parathyroid hormone (PTH)/PTH-related protein receptors in humans is associated with abnormal breast development and tooth impaction. *J Clin Endocrinol Metab* 2001, 86:1788–1794
41. Naski MC, Wang Q, Xu J, Ornitz DM: Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. *Nat Genet* 1996, 13:233–237
42. Wilcox WR, Tavormina PL, Krakow D, Kitoh H, Lachman RS, Wasmuth JJ, Thompson LM, Rimoin DL: Molecular, radiologic, and histopathologic correlations in thanatophoric dysplasia. *Am J Med Genet* 1998, 78:274–281
43. Legeai-Mallet L, Rossi A, Benoist-Lasselini C, Piazza R, Mallet JF, Delezoide AL, Munnich A, Bonaventure J, Zylberberg L: EXT 1 gene mutation induces chondrocyte cytoskeletal abnormalities and defective collagen expression in the exostoses. *J Bone Miner Res* 2000, 15:1489–1500
44. Aigner T, Loos S, Muller S, Sandell LJ, Unni KK, Kirchner T: Cell differentiation and matrix gene expression in mesenchymal chondrosarcomas. *Am J Pathol* 2000, 156:1327–1335
45. Descalzi Cancedda F, Gentili C, Manduca P, Cancedda R: Hypertrophic chondrocytes undergo further differentiation in culture. *J Cell Biol* 1992, 117:427–435
46. Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM, Mulligan RC: Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes Dev* 1994, 8:277–289
47. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize LH, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV,

- Kronenberg HM: PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 1996, 273:663–666
48. Lanske B, Amling M, Neff L, Guiducci J, Baron R, Kronenberg HM: Ablation of the PTHrP gene or the PTH/PTHrP receptor gene leads to distinct abnormalities in bone development. *J Clin Invest* 1999, 104: 399–407
  49. McEwen DG, Green RP, Naski MC, Towler DA, Ornitz DM: Fibroblast growth factor receptor 3 gene transcription is suppressed by cyclic adenosine 3', 5'-monophosphate. Identification of a chondrocytic regulatory element. *J Biol Chem* 1999, 274:30934–30942
  50. Yoshida E, Noshiro M, Kawamoto T, Tsutsumi S, Kuruta Y, Kato Y: Direct inhibition of Indian hedgehog expression by parathyroid hormone (PTH)/PTH-related peptide and up-regulation by retinoic acid in growth plate chondrocyte cultures. *Exp Cell Res* 2001, 265:64–72