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Expression of Indian Hedgehog, Parathyroid Hormone–Related Protein, and Their Receptors in the Postnatal Growth Plate of the Rat: Evidence for a Locally Acting Growth Restraining Feedback Loop After Birth

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ABSTRACT

A locally acting growth restraining feedback loop has been identified in the murine embryonic growth plate in which the level of parathyroid hormone–related peptide (PTHrP) expression regulates the pace of chondrocyte differentiation. To date, it is largely unknown whether this feedback loop also regulates the pace of chondrocyte differentiation in the growth plate after birth. We therefore characterized the spatio-temporal expression of Indian hedgehog (IHH), PTHrP, and their receptors in the postnatal growth plate from female and male rats of 1, 4, 7, and 12 weeks of age. These stages are representative for early life and puberty in rats. Using semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) on growth plate tissue, IHH and components of its receptor complex, patched (PTC) and smoothened (SMO), PTHrP and the type I PTH/PTHrP receptor messenger RNA (mRNA) were shown at all ages studied irrespective of gender. Using in situ hybridization, IHH, PTHrP, and PTH/PTHrP receptor mRNA were detected in prehypertrophic and hypertrophic chondrocytes in both sexes during development. In addition, especially in the younger age groups, faint expression of PTH/PTHrP receptor mRNA also was shown in stem cells and proliferative chondrocytes. Immunohistochemistry confirmed the observations made with in situ hybridization, by showing the presence of IHH, PTC, PTHrP, and PTH/PTHrP receptor protein in prehypertrophic and hypertrophic chondrocytes. In addition, staining for hedgehog, PTC, and PTHrP also was observed in growth plate stem cells. No differences in staining patterns were observed between the sexes. Furthermore, no mRNA or protein expression of the mentioned factors was detected in the perichondrium. Our data suggest that in contrast to the proposed feedback loop in the early embryonic growth plate, which requires the presence of the perichondrium, a feedback loop in the postnatal growth plate can be confined to the growth plate itself. In fact, two loops might exist: (1) a loop confined to the transition zone and early hypertrophic chondrocytes, which might in part be autocrine and (2) a loop involving the growth plate stem cells. (J Bone Miner Res 2000;15:1045–1055)

Key words: growth plate, chondrocyte, parathyroid hormone–related protein, Indian hedgehog, in situ hybridization, immunohistochemistry, reverse-transcription polymerase chain reaction

INTRODUCTION

LONGITUDINAL GROWTH results from proliferation and differentiation of growth plate chondrocytes. During growth, stem cells initiate their program of differentiation by entering the proliferating zone where they frequently divide and arrange in typical columns. Subsequently, cells stop dividing and start to hypertrophy. These chondrocytes are referred to as prehypertrophic chondrocytes. They mature further, become hypertrophic, and produce matrix components extensively. The matrix around the hypertrophic chondrocytes calcifies and finally the chondrocytes undergo apoptosis and are replaced by bone from the underlying metaphysis.[(1)]

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During the past years various growth factors and receptors have been identified that play a role in the regulation of chondrocyte proliferation and differentiation within the embryonic growth plate, for example, basic fibroblast growth factor (bFGF), transforming growth factor β (TGF-β), bone morphogenetic proteins (BMPs), and their receptors.[2–6]

Recently, parathyroid hormone–related peptide (PTHrP), the type I PTH/PTHrP receptor, and the morphogen Indian hedgehog (IHH) have been associated with the regulation of chondrocyte proliferation and differentiation.[7–9] PTHrP originally is identified as the causative agent of humoral hypercalcemia of malignancy.[10] Numerous studies have shown that this peptide plays an important role in multiple differentiation processes during embryonic development. Mice with a homozygous null mutation in the gene for PTHrP or, depending on its genetic background, the type I PTH/PTHrP receptor die around birth.[7,8,11] These mice have severe malformations in the skeleton, which displays an advanced state of maturation caused by accelerated chondrocyte differentiation and premature ossification. The role of the PTH/PTHrP receptor in skeletal development also is shown by mutations found in two human skeletal dysplasias: Jansen-type metaphyseal chondrodysplasia, caused by a gain of function mutation of the PTH/PTHrP receptor, and Blomstrand lethal osteochondrodysplasia, caused by a loss of function mutation in the PTH/PTHrP receptor gene.[12,13]

IHH, originally discovered in *Drosophila*, belongs to a family of morphogens involved in patterning, limb bud development and endochondral bone formation.[14] Overexpression of IHH in fetal chicken long bones blocks chondrocyte differentiation.[9] This is caused by an up-regulation of PTHrP expression in the periarticular perichondrium in fetal long bones. IHH is expressed in the transition zone, the receptor complex patched (PTC) and smoothened (SMO) in the perichondrium, at least in the first steps of endochondral bone formation, and PTHrP is expressed in the periarticular perichondrium.[9,15] These and other observations have led to the hypothesis of a locally acting growth restraining feedback loop, which regulates the rate of chondrocyte differentiation in the early embryonic growth plate.[9] Chondrocytes making the transition from the proliferative into the hypertrophic zone express IHH. Via yet unknown mechanisms involving the perichondrium, IHH increases the expression of PTHrP in the periarticular perichondrium. PTHrP in turn binds to its PTH/PTHrP receptor on late proliferating chondrocytes and inhibits their further differentiation. This results in less IHH producing cells, which closes the feedback loop. Thus, the level of PTHrP determines the rate of chondrocyte differentiation. This is underscored by ablation or overexpression of PTHrP in the growth plate in which chondrocyte differentiation is accelerated or delayed, respectively.[7,16] Very recently, additional evidence has been provided concerning the interaction between PTHrP and IHH. It was shown in chick sternal chondrocytes that PTHrP down-regulates bone morphogenetic protein 6 (BMP-6) messenger RNA (mRNA) expression in prehypertrophic chondrocytes.[17,18] Furthermore, exogenously added BMP-6 stimulates IHH expression. Therefore, it was concluded that BMP-6 is an intermediary in the PTHrP/IHH signaling pathway.

Whether the growth restraining feedback loop also is active in the postnatal growth plate and involved in growth plate closure at the end of puberty is presently unknown. In mouse long bones, expression patterns of IHH, PTC, and the PTH/PTHrP receptor have been studied, postnatally.[19–20] Here, we show that components of the IHH-PTHrP growth restraining feedback loop also are present in the postnatal rat growth plate from 1 week after birth until the end of puberty at the mRNA and protein level in a spatial patterning, which is distinct from the situation in early embryonic endochondral bone formation. Our results might imply a role for a similar feedback loop in regulating longitudinal growth postnatally.

MATERIALS AND METHODS

Animals

Female and male Wistar rats were obtained from Harlan (Horst, The Netherlands) and were killed at 1, 4, 7, and 12 weeks of age. One tibia was removed for immunohistochemistry, either for cryostat sections or for paraffin sections, while the other tibia was removed in order to dissect the proximal growth plate. This was done by cutting away all sides of the cartilage-bone borders to avoid contamination with fibrous tissue or bone tissue. The obtained growth plate material was rapidly frozen and stored until use at −80°C.

RNA isolation and complementary DNA synthesis

Total RNA was extracted from growth plate material according to Chomzcinsky and Sacchi.[21] Subsequently, RNA samples were treated with RNase-free DNase for 15 minutes at 37°C to remove residual DNA contamination (Promega, Leiden, The Netherlands). Samples were purified further by using RNeasy (Qiagen, Milden, Germany). The concentration of RNA was determined spectrophotometrically.

One microgram of RNA was denatured (10 minutes at 70°C followed by 5 minutes on ice) and reverse transcribed in a 20-μl reaction containing first strand buffer (75 mM KCl, 3 mM $MgCl_2$, and 50 mM Tris-HCl, pH 8.3), 5 mM dithiothreitol (DTT), 0.375 mM deoxynucleoside triphosphates (dNTPs), 200 ng of random hexanucleotides (all from Gibco BRL, Breda, The Netherlands), 1 unit of RNasin (Promega, The Netherlands), and 2.5 unit of M-MLV reverse transcriptase (Gibco BRL, The Netherlands) at 37°C for 60 minutes and denatured again at 70°C for 10 minutes. A second addition of 1 Unit of RNasin and 2.5 U of M-MLV reverse transcriptase was performed in each tube and the reaction was allowed to proceed at 37°C for 30 minutes followed by inactivation of the enzymes by incubation at 70°C for 10 minutes. Samples were diluted to a final concentration of 5 ng/μl (assuming a 100% efficiency of the reverse transcription) and stored until use at −20°C.

RT-PCR

To correct for differences in amounts of complementary DNA (cDNA), samples were equalized on their β-actin

TABLE 1. PRIMERS USED IN THIS PAPER WITH THEIR ORIENTATION, SEQUENCE, MELTING TEMPERATURE (TM), LENGTH OF AMPLICON (BP) AND NUMBER OF CYCLES USED IN THE RT-PCR PROTOCOL

Name	*S/AS*	*Sequence*	*Tm (°C)*	*bp*	*Cycles*
Indian hedgehog	S	TGGATATCACCACCTCAGAC	56	396	30
Indian hedgehog	AS	GATTGTCCGCAATGAAGAGC	56	396	30
Patched	S	GCATCGGAGTGGAGTTCACC	56	450	35
Patched	AS	CTCACTGATGCCAGACACCG	56	450	35
Smoothened	S	AACTATCGGTACCGTGCTGG	56	607	35
Smoothened	AS	CATCATGGGAGACAGTGTGC	56	607	35
PTHrP	S	AAAGCCAAGAGAAACGGTGGGCAT	56	400	35
PTHrP	AS	GCCAATCATGTGCACCAGTTCCTT	56	400	35
PTH/PTHrP receptor	S	TGCTTGCCACTAAGCTTCG	56	264	30
PTH/PTHrP receptor	AS	TCCTAATCTCTGCCTGCACC	56	264	30
β-actin	S	TGGAATCCTGTGGCATCCATGAAAC	56	350	30
β-actin	AS	TAAAACGCAGCTCAGTAACAGTCCG	56	350	30

bp, base pair.

content by competitive polymerase chain reaction (PCR; Table 1). This method has been described in detail elsewhere.[22] In short, 5 ng of cDNA was coamplified in the presence of 4-fold serial dilutions of internal standard plasmid pMCQ.[23] The standardized cDNA samples were used for semiquantitative PCR using specific primer sets for IHH, PTC, SMO, PTHrP, and the PTH/PTHrP receptor (Table 1). In all experiments, water and RNAs were used as negative controls in the PCR reactions. As a positive control cDNA of a mouse metacarpal was used. The PCR reaction volume (25 μl) contained reaction buffer (75 mM Tris-HCl, pH 9.0, 200 mM $(NH_4)_2SO_4$, and 0.01% Tween 20; Gibco BRL), 2.0 mM $MgCl_2$, 300 μM dNTPs (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), 0.4 μM of sense and antisense primer, and 0.25 U Goldstar DNA polymerase (Gibco BRL). The PCR reaction was performed on a Hybaid Omnigene (Biozym, Landgraaf, The Netherlands) for 30–35 cycles depending on the abundance of the amplicon. Each cycle consisted of 30 s at 94°C, 30 s at 56°C, and 1 minute at 72°C. The amplified products were loaded on a 1.5% agarose gel and analyzed under UV.

Generation of complementary RNA probes and Digoxigenin labeling and dot-spotting

A rat collagen X probe was kindly provided by Prof. E. Hunziker (Berne, Switzerland). A mouse IHM probe was made via ligation of a PCR product into a pCR™3 vector (Invitrogen, Groningen, The Netherlands). PTHrP and the PTH/PTHrP receptor probes were kindly provided by Dr. K. Lee and Dr. A.-B. Abou-Samra, respectively (Massachusetts General Hospital, Boston, MA, U.S.A.). Vectors (10 μg) were linearized with the required restriction enzymes. The probes were labeled with Digoxigenin (DIG) at 37°C for 1 h using the DIG RNA labeling kit (Boehringer Mannheim, Almere, The Netherlands), followed by a DNase treatment and addition of yeast transfer RNA (tRNA; 10 mg/ml). After having extracted twice with phenol, RNA was precipitated with 1/10 vol of 3 M NaAc, pH 5.4, and 2.5 vol of 100% ethanol for 1 h at -80°C, centrifugated, and this was repeated once. Finally, the pellet was dissolved in 10 mM Tris/HCl, pH 7.5, and 0.1 mM EDTA. The PTH/PTHrP receptor probe was hydrolyzed to reduce the probe size. For this, the volume was adjusted to 160 μl with TE buffer supplemented with 20 μl, 0.4 M $NaHCO_3$,, and 20 μl, 0.6 M Na_2CO_3, and was incubated at 60°C for 18 minutes. This was neutralized with 292.5 μl, 3 M NaAc, pH 5.4, and 21.5 μl acetic acid and 500 μl 100% ethanol was added. After precipitation for 1 h at −80°C centrifugation probes were resuspended with 50 μl TE buffer and stored at −20°C. Labeling of the probes was controlled by dot-spotting dilutions ranging from 1:2 to 1:32 on a nylon membrane, followed by UV treatment for 5 minutes. The membrane was blocked in 5% blocking buffer (blocking powder (Boehringer Mannheim) in 100 mM maleic acid and 150 mM NaCl, pH 7.5) for 1 h and alkaline phosphatase (ALP)–conjugated anti-DIG (1:1000) was applied in maleate buffer with 0.3% Tween for 30 minutes at room temperature. Finally, the membrane was equilibrated in staining solution (100 mM Tris/HCl, pH 9.5, and 1 mM $MgCl_2$) for 2 minutes and stained for 10 minutes in the dark at room temperature in staining solution containing 0.338 mg/ml nitroblue tetrazolium and 0.175 mg/ml 5-bromo-3-indolylphosphate.

In situ hybridization

We used the method described by Sommer and coworkers with some modifications.[24] In short, rats were fixed in vivo with a fixative consisting of 2% paraformaldehyde (PFA) and 0.2% glutaraldehyde in 0.1 M phosphate buffer supplemented with 75 mM lysine monohydrochloride and 10 mM Na-periodate. After decalcification (5 weeks in 10% EDTA supplemented with 0.5% PFA), tibias were embedded in paraffin and sections (6 μm) were cut with a Reichert Jung 2055 (Leica, Rÿswÿk, The Netherlands), mounted on Superfrost+ slides, (Fischer Scientific, Zoetermeer, The Netherlands) and deparaffinized in xylene (3–5 minutes) and graded ethanol in diethylpyrocarbonate (DEPC)-treated water followed by one wash in DEPC-treated phosphate-buffered saline (PBS) and twice in 2× SSC (0.3 M NaCl

and 0.03 M Na-citrate, pH 7.5) for 5 minutes each. Slides were incubated with 5 μg/ml proteinase K (Gibco BRL) in 100 mM TRIS/HCl and 50 mM EDTA for 15 minutes at 37°C. Sections were postfixed in 4% PFA for 5 minutes, followed by two washes with 2× SSC (1 minute each) and 0.1 M triethanolamine (TEA) for 5 minutes. Slides were acetylated with acetic anhydride and after a single wash in DEPC-treated water for 5 minutes, prehybridization was performed for 1 h at 50°C. The prehybridization mix consisted of 50% deionized formamide, 4× SSC, 50× Denhardt solution (5 g Ficoll, type 400; Amersham Pharmacia Biotech), 5 g polyvinylpyrrolidone, 5 g bovine serum albumin (BSA, fraction V; Sigma, St. Louis, MO, U.S.A.), and 250 μg/ml yeast tRNA. Hybridization was performed overnight at 50°C. The hybridization solution consisted of all components in the prehybridization mix supplemented with another 250 μg/ml yeast tRNA and 1% dextran sulphate (Amersham Pharmacia Biotech) in which the probes were diluted and covered with gelbond film (FMC Bioproducts, Rockland, ME, U.S.A.). After hybridization, slides were washed in 2× SSC for 30 minutes. Nonspecific hybridization was removed by 1 U/ml RNase T1 (Boehringer Mannheim) in 2× SSC and 10 mM EDTA for 30 minutes at 37°C. After having washed twice in 2× SSC and twice in 0.2× SSC at 55°C, nonspecific binding was blocked in 1% blocking buffer (Blocking Reagent; Boehringer Mannheim; in 100 mM maleic acid and 150 mM NaCl) for 30 minutes at room temperature. Slides were placed in a humid chamber and were incubated with peroxidase-conjugated anti-DIG (Boehringer Mannheim; 1:1000) in blocking buffer containing 0.2% Tween overnight at 4°C. The next day, biotin-labeled tyramides, prepared according to the method of Kerstens and coworkers, were applied (1:400 in PBS with 0.025% H_2O_2) at room temperature for 10 minutes followed by addition of ALP-conjugated streptavidin (1:300) in blocking buffer at room temperature for 45 minutes.[25] Slides were washed in maleate buffer containing 0.2% Tween, followed by equilibration in ALP buffer (100 mM Tris/HCl pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) for 5 minutes. Finally, sections were stained in a ALP buffer containing 10% polyvinylalcohol (MW 30–70 kDa), 0.338 mg/ml nitroblue tetrazolium, 0.175 mg/ml 5-bromo-3-indolylphosphate, and 1 mM levamisole (all from Sigma) at 30°C in the dark varying from 10 minutes to overnight depending on the mRNA abundancy.

Immunohistochemistry

Tibias were fixed overnight in 4% PFA or a zinc-macrodex solution [4.8% dextran, 0.1% $CaCl_2$, and 0.5% of both $ZnCl_2$ and $Zn(Ac)_2$], substituted with 10% formalin, decalcified for 5 weeks in 10% EDTA, and embedded in paraffin. Six-micrometer paraffin sections were cut, mounted on Superfrost+ slides (Fischer Scientific), and deparaffinized through Paraclear (twice for 5 minutes; Earthsafe Technologies, Bellemead, NY U.S.A.) and graded ethanols. Sections were placed in a 60:40 PBS/methanol solution with 1% H_2O_2 for 30 minutes followed by three rinses with PBS. For digestion, either 0.04% pepsin, pH 2.0 (Sigma), for 8 minutes at 37°C (IHH, PTC, and PTH/PTHrP receptor) or 0.025 U/ml neuraminidase (Sigma) was used for 60 minutes at room temperature (PTHrP), followed by a triple wash in PBS. Then, sections were preincubated with 0.5% blocking buffer (Boehringer Mannheim, The Netherlands; in (TNT) 0.1 M TRIS, 0.15 M NaCl, and 0.05% Tween-20 containing 0.4% Triton X-100) for 60 minutes at 37°C. Sections were incubated overnight at 4°C with primary antibody in 0.5% blocking buffer. The following polyclonal antibodies were used: (1) goat anti-IHH raised against an amino acid sequence at the carboxy terminus of human IHH, which cross-reacts with mouse and rat IHH (1:25; Santa Cruz, U.S.A.); (2) Goat anti-IHH (1:250; Santa Cruz, Santa Cruz, CA U.S.A.) raised against an amino acid sequence at the amino terminus of human IHH, sonic hedgehog (SHH) and desert hedgehog (DHH), which also recognizes these hedgehogs in mouse and rat; (3) goat anti-PTC (1:100; Santa Cruz) raised against amino acids 18–36 of mouse PTC, which cross-reacts with rat PTC; (4) rabbit anti-PTHrP (1:10; Oncogene Science, Cambridge, MA U.S.A.) raised against amino acids 34–53 of human PTHrP, which also recognizes mouse and rat PTHrP; and (5) two rabbit anti-PTH/PTHrP receptor antibodies (kindly provided by L.H.K. Defize, Hubrecht laboratory, Utrecht, The Netherlands), one raised against an amino acid sequence at the C terminus of the mouse PTH/PTHrP receptor (PVU, 1:1000) and one against the N terminus (AVA).[26] Depending on the primary antibody, the second antibody was a rabbit anti-goat (Amersham Pharmacia Biotech) or donkey anti-rabbit (DAKO, Carpinteria, CA, U.S.A.), conjugated with biotin, at a dilution of 1:100 and was applied for 60 minutes at 37°C. This was followed by incubation with streptavidin conjugated with horse radish peroxidase (1:75; Amersham Pharmacia Biotech) at 37°C for 45 minutes. Biotin-labeled tyramides were applied (1:400 in PBS with 0.025% H_2O_2) at room temperature for 10 minutes followed by a second addition of peroxidase-labeled streptavidin (1:75) at 37°C for 45 minutes.[25] The peroxidase-labeled antibodies were then visualized with either 3-amino-g-ethylcarbazole (AEC) (0.2 mg/ml in acetate buffer pH 5.2 with 0.04% H_2O_2; Sigma) for 3 minutes or 0.05% Diaminobenzidine (Sigma) solution with 0.085% H_2O_2 for 5 minutes. After counterstaining in a 1% hematoxylin solution for 30 s, the sections were embedded in aquamount (BDH, Poole, U.K.). Control experiments included omission of the first antibody and/or competition of the first antibody with corresponding peptides, available for both hedgehog antibodies and PTC (Santa Cruz) and for PTHrP (Oncogene Science).

RESULTS

Expression of the mRNAs coding for IHH, PTC, SMO, PTHrP, and the PTH/PTHrP receptor in the postnatal growth plate

To analyze the expression of IHH, PTC, SMO, PTHrP, and the PTH/PTHrP receptor in the postnatal growth plate, we performed semiquantitative reverse-transcription (RT)–PCR. Three consecutive competitive RT-PCR experiments were performed for each gene. The mRNAs coding for IHH,

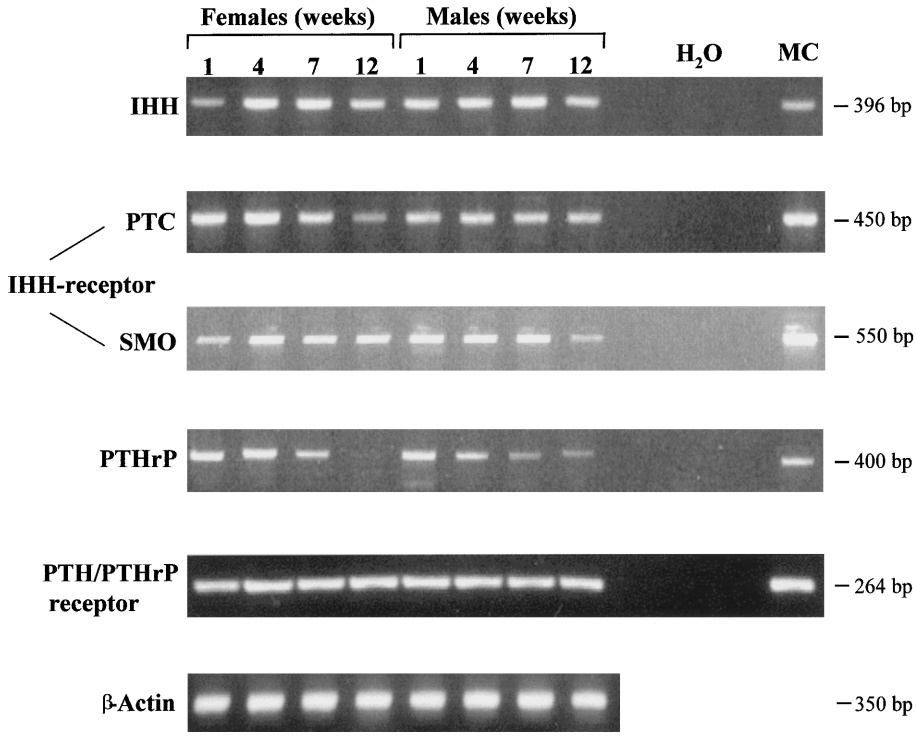

FIG. 1. Semiquantitative RT-PCR on mRNA isolated from tibial growth plate tissue of 1-, 4-, 7-, and 12-week-old female and male rats using primer sets specific for IHH, PTC, SMO, PTHrP, and the PTH/PTHrP receptor. β-Actin was used as an internal control for the amount of growth plate cDNA. An embryonic (E19) mouse metacarpal (MC) was used as a positive control and water was used as a negative control.

PTC, SMO, PTHrP, and the type I PTH/PTHrP receptor were detected in growth plate tissue isolated from female and male rats that were 1, 4, 7, and 12 weeks of age (Fig. 1). IHH mRNA was expressed throughout development in both sexes, as were the mRNAs coding to its receptor complex PTC and SMO. Although PTHrP mRNA was abundant in young rats, the expression seemed to decline with age and at 12 weeks expression of the mRNA was almost absent. In contrast, PTH/PTHrP receptor mRNA was expressed intensively throughout development.

To identify the cells in the growth plate that expressed the mRNA coding for IHH, PTHrP, and the type I PTH/PTHrP receptor, nonradioactive in situ hybridization was performed. A collagen X probe was used as a positive control. As shown in Figs. 2A and 2B, collagen X mRNA was abundant in the hypertrophic chondrocytes in rats of 4 weeks and 12 weeks of age. Specific signals for IHH and PTHrP mRNA were detected in the prehypertrophic and hypertrophic chondrocytes in both genders at all age groups studied (Figs. 2D, 2E, 2G, and 2H). In the 4-week-old rats hybridization with the IHH and PTHrP probe resulted in some nonspecific staining, because it was observed in the growth plate matrix and not in the cell cytoplasm. Finally, PTH/PTHrP receptor mRNA was abundant in the prehypertrophic and hypertrophic zone and was expressed faintly in the stem cells and proliferating chondrocytes, especially in the younger age groups (Figs. 2J and 2K). Control sections in which the sense probe was used did not show significant signals above background (Figs. 2C, 2F, 2I, and 2L). None of the mRNAs were detected in the perichondrium at the lateral sides of the growth plate. Besides localization in the growth plate, PTH/PTHrP receptor mRNA also was abundantly present in osteoblasts lining the surfaces of the trabeculae and cortical bone. In contrast, we did not detect IHH and PTHrP mRNAs in other cells but we did detect them in chondrocytes.

Immunohistochemical evaluation of IHH, PTC, PTHrP, and the PTH/PTHrP receptor in the postnatal growth plate

To study the expression patterns of the corresponding proteins, we performed immunohistochemistry on tibial growth plates of female and male rats at 1, 4, 7, and 12 weeks of age. Sections were stained with specific antibodies against IHH, PTC, PTHrP, and the PTH/PTHrP receptor. Only slight differences were observed in staining patterns for all antibodies between age groups and sexes. Therefore, only growth plates of 4-week-old and 12-week-old rats are shown. Control experiments included omission of the first antibody and/or preincubation of the first antibody with corresponding peptides. These experiments did not show staining in growth plate tissues for the used antibodies (Figs. 3P and 3Q). Two different antibodies were used for staining of IHH. One antibody was directed against the C terminus and was specific for IHH (HH-C). The second was directed against the N terminus and cross-reacted with other hedgehog family members. Strong staining with the antibody against HH-C was observed in the prehypertrophic and hypertrophic chondrocytes in 1-week-old and 4-week-old rats (1 week: data not shown; 4 weeks: Fig. 3A). Thereafter, staining decreased and was undetectable in rats that were12 weeks of age (Fig. 3B). The staining was confined to cyto-

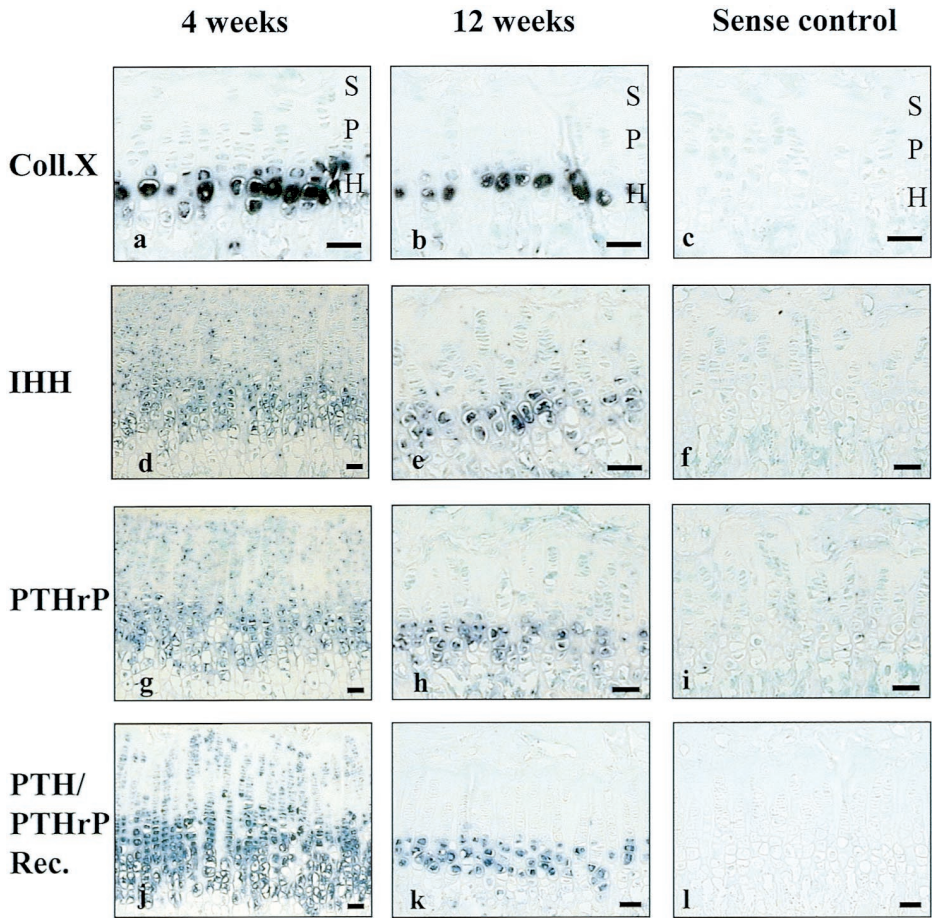

FIG. 2. In situ hybridization on tibial growth plates of 4-week-old and 12-week-old male rats. (A) Collagen X mRNA at 4 weeks and (B) at 12 weeks. (D) IHH mRNA at 4 weeks and (E) at 12 weeks. (G) PTHrP mRNA at 4 weeks and (H) at 12 weeks. (J) PTH/PTHrP receptor mRNA at 4 weeks and (K) at 12 weeks. Control sections (sense probe) are depicted for (C) Collagen X, (F) IHH, (I) PTHrP, and (L) the PTH/PTHrP receptor, respectively. Bar represents 200 μm. S, stem cells; P, proliferating chondrocytes; H, hypertrophic chondrocytes.

plasmic granules (Fig. 3C). Staining with the antibody against HH-N showed strong staining in the prehypertrophic and hypertrophic chondrocytes but also was abundant in the stem cells of the growth plate. In contrast to the antibody directed against HH-C, staining with the antibody directed against HH-N did not decrease with age *(*Figs. 3D and 3E). Similar to HH-C, HH-N staining was confined to cytoplasmic granules (Fig. 3F).

PTC, the receptor for IHH, was expressed intensively in the prehypertrophic and hypertrophic chondrocytes and in the stem cells. The expression was comparable for all age groups studied and was clearly visible in the cytoplasm and on the cell membrane (Figs. 3G–3I). PTHrP was detected in the stem cells of the growth plate but also in the prehypertrophic and hypertrophic chondrocytes throughout development (Figs. 3J and 3K). Just as for IHH, the staining pattern of PTHrP was restricted to cytoplasmic granules (Fig. 3L). The PTH/PTHrP receptor was abundantly expressed in the prehypertrophic and hypertrophic chondrocytes throughout development (Figs. 3M and 3N). The specific staining was confined to the cell membranes, whereas some nonspecific staining was present in the extracellular matrix (Fig. 3O). Identical staining patterns were observed using a PTH/PTHrP receptor antibody directed against the N-terminal epitope (AVA; data not shown) and the C-terminal epitope (PVU). The immunohistochemical data confirmed the observations made by in situ hybridization showing that none of the components were expressed at the lateral sides of the growth plate. We did not detect IHH, PTHrP, or PTC in osteoblasts and lining cells. In contrast, the PTH/PTHrP receptor was abundant in these cells. PTC was clearly visible in osteocytes throughout the bone, whereas none of the other factors were detected in these cells.

DISCUSSION

The identification of a feedback loop regulating the pace of chondrocyte differentiation in the embryonic growth plate prompted us to investigate whether such a loop also exists in the postnatal growth plate. Here, we show that all components of the proposed feedback loop occurring in the embryonic growth plate also are expressed in the postnatal growth plate of rats of 1, 4, 7, and 12 weeks of age in both genders. The expression patterns are at some points distinct from patterns observed in the embryonic mice and chicken growth plates and taking the species difference into account we therefore propose that in the growth plate of the rat a comparable but not identical feedback loop may regulate the balance of chondrocyte proliferation and differentiation.

We examined tibial growth plates of rats 1, 4, 7, and 12 weeks of age in both genders. These time points were

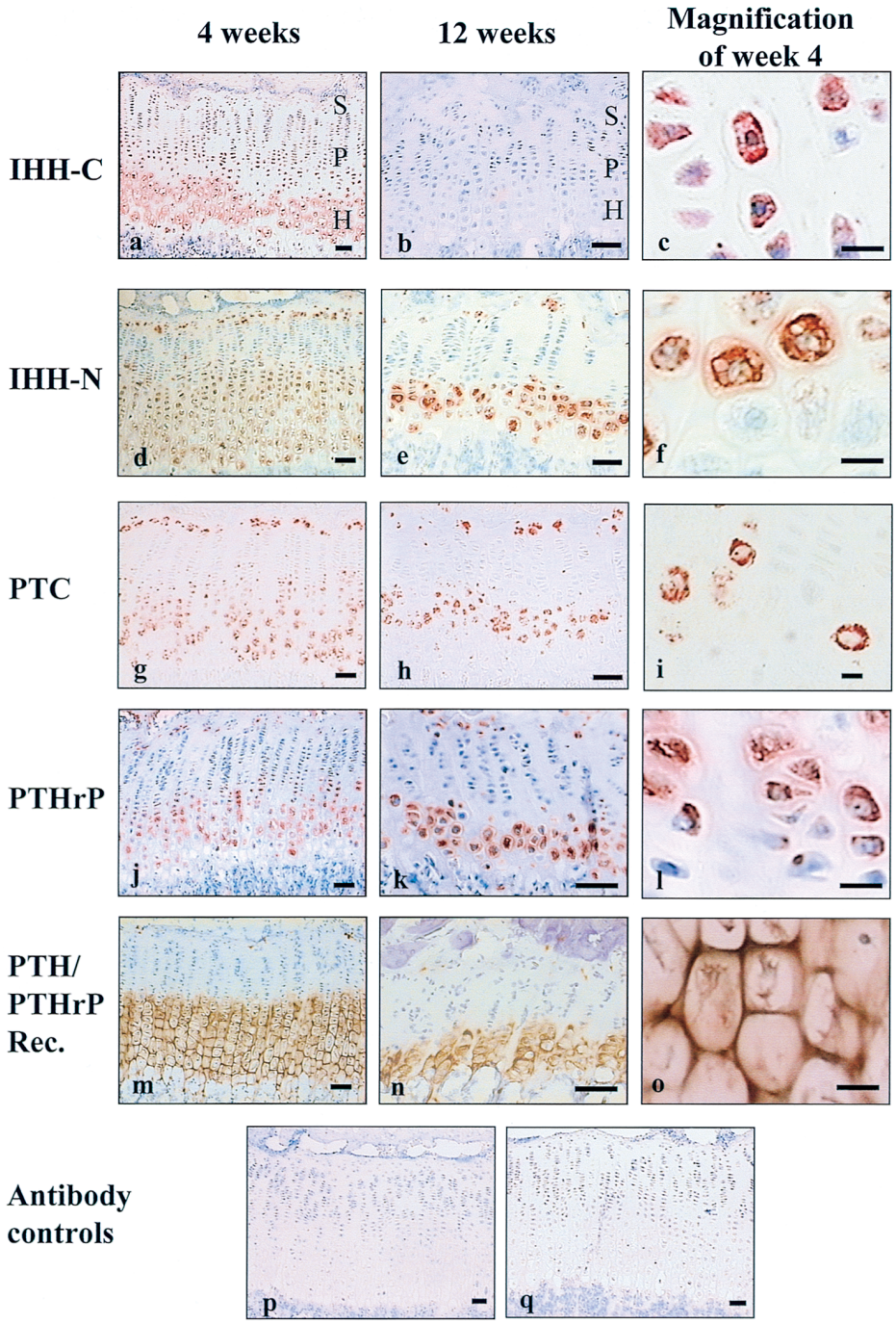

FIG. 3. Immunohistochemistry on tibial growth plates of 4-week-old and 12-week-old rats. Staining for IHH using an antibody against the C terminus (HH-C) (A) at 4 weeks and (B) at 12 weeks. Bar represents 200 μm. (C) magnification of (A) (bar represents 50μm). Staining for IHH using an antibody against the N terminus (HH-N) (D) at 4 weeks and (E) at 12 weeks. Bar represents 200 μm. (F) magnification of (D) (bar represents 50μm). PTC staining (G) at 4 weeks and (H) at 12 weeks. Bar represents 200 μm. (I) magnification of (G) (bar represents 50μm). PTHrP staining (J) at 4 weeks and (K) at 12 weeks. Bar represents 200 μm. (L) magnification of (J) (bar represents 50μm). PTH/PTHrP receptor staining (M) at 4 weeks and (N) at 12 weeks. Bar represents 200 μm. (O) magnification of (M) (bar represents 50 μm). (P and Q) Negative controls in which first antibody was omitted or first antibody was preincubated with a corresponding peptide, respectively. Bar represents 200 μm. S, stem cells; P, proliferating chondrocytes; H, hypertrophic chondrocytes.

chosen because they define representative stages of longitudinal growth in humans, that is, fast growth during childhood (1 week and 4 weeks), puberty (7 weeks), and reduction of growth velocity at the end of puberty at which the rat tibial growth plate is reduced to only a few cell layers (12 weeks).[27] Expression patterns between male and female rats were compared because there is a clear distinction in growth velocity between males and females from the age of 4 weeks on. Using RT-PCR analysis of growth plate tissue, we showed that mRNA coding for IHH, PTC, SMO, PTHrP, and the PTH/PTHrP receptor were all expressed in growth plate tissue at all ages studied. In addition, strong signals were observed for the chondrocyte-specific markers collagen II and X (data not shown). Because markers, formerly believed to be specific for osteoblasts, like osteocalcin, osteopontin, bone sialoprotein, and core-binding factor α1 (Cbfa1) also are expressed in chondrocytes, we could not use RT-PCR to exclude the presence of osteoblasts in the growth plate samples.[28–31] Therefore, and furthermore to identify the cells that expressed

the various mRNAs and their proteins, we examined sections of the growth plate by nonradioactive in situ hybridization and immunohistochemistry. The results confirmed the data obtained by RT-PCR analysis and showed that all components were expressed in the postnatal growth plate at the mRNA and/or protein level.

IHH mRNA was present in prehypertrophic and hypertrophic chondrocytes of the growth plate in 4-week-old and 12-week-old female and male rats. Compared with the restricted expression of IHH mRNA to the transitional zone between the proliferative and hypertrophic chondrocytes in mouse and chicken fetal long bones, IHH mRNA expression in postnatal rats was extended to the hypertrophic chondrocytes.[9,19] In contradiction to observations made in mice in which IHH expression disappeared with the onset of puberty, we did not observe a decrease in IHH mRNA expression during puberty.[20] In fact, IHH expression in rats was relatively unaffected by puberty. The IHH mRNA expression corresponded to IHH protein localization, shown by immunohistochemistry using an antibody against the C terminus of IHH and an antibody against the N terminus, which also cross-reacts with other hedgehogs. Using the antibody against the C terminus, IHH protein was localized to cytoplasmic granules of prehypertrophic and hypertrophic chondrocytes in growth plates of 1-week-old and 4-week-old rats. At 7 weeks and 12 weeks of age, however, no staining was observed in contrast to the mRNA expression data. However, at all ages, staining with an antibody against the N terminus of hedgehog was observed in the prehypertrophic and hypertrophic chondrocytes. This discrepancy is most likely explained by the reduced affinity of the C-terminal antibody for the antigen compared with the N-terminal antibody. In contrast to the C-terminal antibody, the N-terminal antibody strongly stained growth plate stem cells in all age groups. These cells did not express IHH mRNA as shown by in situ hybridization, suggesting that growth plate stem cells express SHH or DHH. Alternatively, stem cells are target cells for transported/diffused hedgehog, which is derived from producing cells either in the growth plate or the epiphysis. SHH expression has been shown in growth plate tissue and chondrocyte cultures of 6-week-old chickens, especially in undifferentiated chondrocytes.[32] Using RT-PCR, we did not detect SHH or DHH mRNA in growth plate tissue (data not shown). DHH, on the other hand, has been studied in testes primarily where it has been implicated to play a role in spermatogenesis.[33] However, because growth plate stem cells were not excised by the isolation of growth plate tissue for RT-PCR analysis to avoid contamination of cells of the epiphysis, this did not exclude the presence of SHH or DHH. Other explanations for the discrepancy between the HH-N immunostaining and the expression of IHH mRNA could be diffusion of IHH from other production sites toward the growth plate stem cells or rapid transcription of IHH mRNA in the stem cells. Currently, we are performing in situ hybridization and immunohistochemical experiments with probes/antibodies for SHH to clarify this issue.

The expression of PTC protein overlapped the expression of IHH protein in the prehypertrophicand hypertrophic chondrocytes. Additionally, PTC protein was abundantly present in growth plate stem cells. The expression pattern did not change with age, was similar in both genders, and was comparable with the previously reported mRNA distribution in the postnatal murine growth plate, except that in mice expression it also was found in the early proliferating chondrocytes.[19] In sharp contrast with the mRNA expression in early stages of fetal mice and chicken long bone formation, PTC mRNA and protein were not detected in the perichondrium at the lateral sides of the growth plate postnatally. In both fetal mice and chicken, PTC mRNA is predominantly expressed in the perichondrium.[19] Furthermore, in mice, PTC expression is observed in proliferating and prehypertrophic chondrocytes whereas in chicken, PTC is expressed in resting chondrocytes.[9,19,34] These data could, besides the possibility of a species difference, suggest a spatio-temporal regulated shift in expression of PTC from perichondrial and prehypertrophic cells during embryonic endochondral bone formation to stem cells and prehypertrophic and hypertrophic chondrocytes during postnatal endochondral bone formation.

In the postnatal growth plate of the rat, PTHrP mRNA and protein were expressed in prehypertrophic and hypertrophic chondrocytes as well as in the stem cells, as shown by in situ hybridization and immunohistochemistry. We did not detect PTHrP protein in the perichondrium. The RT-PCR analysis suggested that PTHrP mRNA decreased during puberty. This observation was not corroborated by the results of the in situ hybridization and immunohistochemistry. However, because these methods are difficult to quantify, no attempts were done to make statements concerning mRNA and protein levels of any of the factors studied. The expression of PTHrP mRNA and protein in the postnatal growth plate differs from the patterns described during early embryonic endochondral bone formation in which PTHrP mRNA and protein was abundantly expressed in the periarticular perichondrium in mouse, rat, and chicken, while hardly any signal was present in prehypertrophic and hypertrophic chondrocytes.[9,35–37] In later embryonic stages, at least in mice, PTHrP mRNA and protein also are detected in hypertrophic chondrocytes.[38] These data suggest an age-dependent difference in PTHrP localization during endochondral bone formation.

Using in situ hybridization, PTH/PTHrP receptor mRNA was expressed intensely in the prehypertrophic and hypertrophic chondrocytes and faintly in stem cells and proliferating chondrocytes during postnatal development. The intense expression in prehypertrophic and hypertrophic chondrocytes was confirmed by immunohistochemistry using two different polyclonal antisera. Protein staining was not detected in the proliferative chondrocytes, in line with a strongly reduced PTH/PTHrP receptor mRNA expression in these cells. Similar findings were published recently in the postnatal murine growth plate in which PTH/PTHrP receptor mRNA is expressed in late proliferating and prehypertrophic chondrocytes during development.[19] The postnatal expression patterns in mice and rats differed from the expression patterns described in fetal mice, chicken, and rat long bones. From the beginning of endochondral bone formation, PTH/PTHrP receptor mRNA is intensely expressed in all cartilage anlagen, which at this time of development

consist of undifferentiated chondrocytes.[37] At somewhat later stages, at least in mice and rats, PTH/PTHrP receptor mRNA expression is confined to late proliferative and early hypertrophic chondrocytes.[9,39]

We did not observe major differences in the expression patterns for IHH, PTC, PTHrP, and the type I PTH/PTHrP receptor between sexes. Furthermore, the expression patterns in growth plates of young fast-growing rats did not significantly differ from the patterns observed in growth plates of pubertal rats and of rats at the end of puberty when growth has almost ceased. This suggested that differences in regulation of growth between genders and the decrease in growth velocity at the end of puberty are perhaps not a direct consequence of changes in the expression of any of the aforementioned components. However, we cannot exclude that more subtle differences in expression patterns undetectable with the methods we have used cause the differences in growth between sexes.

Based on our results we conclude that IHH, PTC, SMO, PTHrP, and the PTH/PTHrP receptor are all expressed in the growth plate at the mRNA and/or protein level in an expression pattern, which at critical points differs from the situation in early embryonic endochondral bone formation. We, therefore, propose that refinement of the growth restraining feedback loop as postulated during embryonic endochondral bone formation is required for the postnatal situation. In contrast to the proposed loop occurring in the early embryonic growth plate in which hedgehog target cells are located in the perichondrium and do not express PTHrP, in the postnatal growth plate the IHH-PTHrP feedback loop can be confined to the prehypertrophic and hypertrophic chondrocytes, which express IHH, PTHrP, PTC, and the PTH/PTHrP receptor mRNA and protein (Fig. 4). In this situation the feedback loop may be in part autocrine suggesting that an as yet unknown intermediary factor by which hedgehog regulates PTHrP expression, as proposed in the early embryonic situation, may not be necessary. In fact, hedgehogs may directly regulate PTHrP expression, whereas PTHrP may directly control IHH expression. In analogy with the embryonic situation the loop may be involved in controlling the pace of chondrocyte differentiation in the transition zone between the late proliferative and hypertrophic chondrocytes, although we do not have functional data proving this. In addition, the loop also may function in controlling the synchrony of the hypertrophying chondrocytes. Such a role recently has been proposed for PTHrP based on experiments with chimeric mice consisting of wild type, PTH/PTHrP receptor expressing cells, and PTH/PTHrP receptor ablated cells.[34] Such a role would fit the abundant expression of IHH, PTC, PTHrP, and PTH/PTHrP receptor throughout the zone of hypertrophic chondrocytes in the postnatal growth plate. Because the PTH/PTHrP receptor is expressed essentially throughout the growth plate, albeit at different intensities, it seems likely that the modulating activity of the PTH/PTHrP receptor can take place in all zones, regulating both the pace as well as the synchrony of chondrocyte differentiation.

Based on the presence IHH, PTHrP, and PTC in growth plate stem cells we postulate the existence of a second IHH-PTHrP feedback loop ,which may be confined to the stem cell zone of the postnatal growth plate (Fig. 4). This loop is speculative and awaits functional testing.

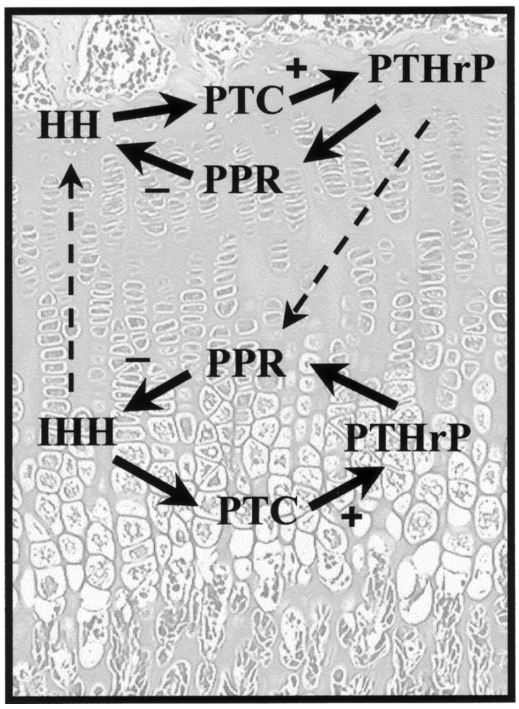

FIG. 4. Model for regulation of chondrocyte differentiation in the postnatal growth plate. Two growth restraining feedback loops may be distinguished. In the first loop, IHH binds PTC in the hypertrophic zone after which PTHrP production is stimulated. PTHrP then binds to its receptor in the hypertrophic zone which down-regulates IHH, closing the loop. This loop might in part be autocrine. In a second, more speculative loop, hedgehogs can bind PTC in the stem cell zone. This may result in PTHrP production stimulation, which then diffuses to its receptor and finally leads to IHH down-regulation. Cross-talk may occur between the two loops.

In addition, we cannot exclude that communication between hedgehog expressing prehypertrophic and hypertrophic chondrocytes and PTC expressing stem cells and vice versa might occur, although this seems unlikely because these regions are separated by a matrix-rich zone encompassing the proliferative chondrocytes, which may prevent free diffusion of hedgehogs. Functional studies will be needed to provide answers on the precise role of the IHH/PTHrP growth restraining feedback loop during postnatal growth and puberty and whether this loop is under control of systemic hormones involved in the regulation of longitudinal growth. In this respect, it is worthwhile to mention that receptors for growth hormone, estrogens, and thyroid hormone are expressed on growth plate chondrocytes, suggesting that these hormones may control chondrocyte proliferation and differentiation directly by interfering with the IHH/PTHrP growth restraining feedback loop in the postnatal growth plate.[40–42]

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