Development 127, 543-548 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV3077

Indian Hedgehog coordinates endochondral bone growth and morphogenesis via *Parathyroid Hormone related-Protein*-dependent and -independent pathways

Seth J. Karp[1], Ernestina Schipani[2], Benoit St-Jacques[1,*], Joy Hunzelman[2], Henry Kronenberg[2] and Andrew P. McMahon[1,‡]

[1]Harvard University, Department of Molecular and Cellular Biology, The Biolabs, Cambridge, MA 02138, USA
[2]Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA
*Present address: Ontogeny, Inc., Cambridge, MA 02138, USA
‡Author for correspondence (e-mail: amcmahon@biosun.harvard.edu)

Accepted 15 November 1999; published on WWW 12 January 2000

SUMMARY

***Indian hedgehog (Ihh)* and *Parathyroid Hormone-related Protein (PTHrP)* play a critical role in the morphogenesis of the vertebrate skeleton. Targeted deletion of *Ihh* results in short-limbed dwarfism, with decreased chondrocyte proliferation and extensive hypertrophy, features shared by mutants in *PTHrP* and its receptor. Activation of *Ihh* signaling upregulates *PTHrP* at the articular surface and prevents chondrocyte hypertrophy in wild-type but not *PTHrP* null explants, suggesting that *Ihh* acts through *PTHrP*. To investigate the relationship between these factors during development of the appendicular skeleton, mice were produced with various combinations of an *Ihh* null mutation *($Ihh^{-/-}$)*, a *PTHrP* null mutation *($PTHrP^{-/-}$)*, and a constitutively active *PTHrP/Parathyroid hormone Receptor* expressed under the control of the Collagen II promoter *(PTHrPR*)*. *PTHrPR** rescues *$PTHrP^{-/-}$* embryos, demonstrating this construct can completely compensate for *PTHrP* signalling. At 18.5 dpc, limb skeletons of *Ihh, PTHrP* compound mutants were identical to *Ihh* single mutants suggesting *Ihh* is necessary for *PTHrP* function. Expression of *PTHrPR** in chondrocytes of *$Ihh^{-/-}$* mice prevented premature chondrocyte hypertrophy but did not rescue either the short-limbed dwarfism or decreased chondrocyte proliferation. These experiments demonstrate that the molecular mechanism that prevents chondrocyte hypertrophy is distinct from that which drives proliferation. *Ihh* positively regulates *PTHrP*, which is sufficient to prevent chondrocyte hypertrophy and maintain a normal domain of cells competent to undergo proliferation. In contrast, *Ihh* is necessary for normal chondrocyte proliferation in a pathway that can not be rescued by *PTHrP* signaling. This identifies *Ihh* as a coordinator of skeletal growth and morphogenesis, and refines the role of *PTHrP* in mediating a subset of *Ihh*'s actions.**

Key words: *Indian hedgehog* (*Ihh*), *Parathyroid Hormone-related Protein* (*PTHrP*), Endochondral bone, Chondrocyte, Cartilage

INTRODUCTION

Vertebrate long bones form through a process called endochondral ossification in which a cartilage template is replaced by a bony matrix (Erlebacher et al., 1995). Chondrocytes arise out of mesenchymal condensations and establish the skeletal element (Erlebacher et al., 1995). As the element grows, cells in the center become hypertrophic, transitioning from a mitotically active, *type II collagen* (*Col II*)-expressing state to a postmitotic, *type X collagen* (*Col X*)-expressing state (Kosher et al., 1986; Swalla et al., 1988; Elima et al., 1993). Hypertrophic cells then undergo apoptosis, which is accompanied by vascular invasion and bone deposition (Erlebacher et al., 1995). Continued elongation of the element requires the establishment of a growth plate, where the stage of maturation is correlated with the distance from the articular surface (Hunziker, 1988). Proliferating chondrocytes are closest, followed by prehypertrophic cells, hypertrophic cells and, finally, the central area where trabecular bone is laid down.

The growth of a skeletal element depends on precise regulation of chondrocyte proliferation and hypertrophy. An important determinant of the growth rate is the total number of proliferating chondrocytes (Hunziker, 1988), which depends on the size of the mitotically active pool of cells and the rate at which these cells proliferate. Correct morphogenesis requires integration of proliferation and hypertrophy over the entire element and, for the long bones, this includes defining the long axis of the element along which growth is directed. Understanding growth and morphogenesis of the long bones, therefore, requires elucidation of the mechanisms that (1) maintain a domain of mitotically active chondrocytes, in part through the regulation of chondrocyte maturation, (2) control

the rate of proliferation of these cells, (3) drive growth primarily along the long axis of the element, and (4) ossify the element.

Indian hedgehog (Ihh), a member of the *hedgehog* family of secreted signaling molecules (Echelard et al., 1993), and *Parathyroid Hormone-related Protein* (*PTHrP)*, the factor responsible for humoral hypercalcemia of malignancy (Suva et al., 1987), are both required for normal skeletal development (Karaplis et al., 1994; Amizuka et al., 1996; St-Jacques et al., 1999). In mice, *Ihh* is initially expressed widely within the cartilage primordium of the long bones but, by birth, becomes localized to a zone of postmitotic, prehypertrophic chondrocytes immediately adjacent to the zone of proliferating chondrocytes (Bitgood and McMahon, 1995; Vortkamp et al., 1996; Lanske et al., 1996; St-Jacques et al., 1999). *PTHrP* is expressed in the periarticular perichondrium, and the mRNA for its receptor is expressed in a region that includes proliferating cells and extends into the prehypertrophic zone (Vortkamp et al., 1995; Lee et al., 1995, 1996). Null mutations in *PTHrP* (*PTHrP*$^{-/-}$) result in decreased numbers of mitotically active chondrocytes (Karaplis et al., 1996), whereas overexpression of *PTHrP* increases this pool (Weir et al., 1996), establishing a role for *PTHrP* in determining the size of the population of proliferating chondrocytes. Activation of *hedgehog* signaling can increase the pool of proliferating cells, but this effect requires intact *PTHrP* signaling (Vortkamp et al., 1996; Lanske et al., 1996). Combined with the ability of *Ihh* to upregulate *PTHrP*, this work suggested a model in which *Ihh* upregulates *PTHrP* to delay chondrocyte hypertrophy. In this model, *Ihh* produced by newly postmitotic chondrocytes as they initiate hypertrophic cartilage formation induces *PTHrP* expression in the periarticular perichondrium. This results in activation of the Parathyroid Hormone/ Parathyroid Hormone-related Protein Receptor (PTHrPR) on proliferating cells, and maintains them in a proliferative state. In this way, the rate of cells leaving the proliferating zone and therefore the rate of long bone growth is carefully controlled by an *Ihh/PTHrP* negative feedback mechanism (Vortkamp et al., 1996; Lanske et al., 1996). This model could explain the action of *Ihh* solely through a *PTHrP*-dependent mechanism in which delay of hypertrophy is sufficient to allow normal growth. Analysis of *Ihh* null mutant embryos (*Ihh*$^{-/-}$) demonstrated that expression of *PTHrP* at the periarticular surfaces of the long bones is indeed dependent on *Ihh* (St-Jacques et al., 1999). The unexpected finding that the short-limbed dwarfism observed in *Ihh*$^{-/-}$ mice is clearly more severe than that of *PTHrP* mutants suggested that the relationship between these two pathways might be more complicated and that either signal might have independent function. To address these issues, we performed a genetic analysis of the role of *Ihh* and *PTHrP* signaling in morphogenesis of the long bones.

MATERIALS AND METHODS

Mice generation and genotyping

Embryos were generated by crosses between mice carrying various combinations of three alleles, an *Ihh* null allele (St-Jacques et al., 1999), a *PTHrP* null allele (Karaplis et al., 1994) and a transgene in which an activated PTH/PTHrP receptor (PTHrPR*) is expressed under the regulation of the Collagen α1(II) promoter (TgA, Schipani et al., 1997). Informative litters were collected between 12.5 and 18.5 dpc and embryos analyzed as described. *Ihh* genotyping was performed by PCR using standard buffers on DNA extracted from pieces of liver or skin. For the mutant allele, a 307 bp fragment was generated using a 5′ primer (AGGAGGCAGGGACATGGATAGGGTG) and a 3′ primer (AGGAACAGACAGAACCGCAGTCGGG) over 35 cycles (94°C ×1 minute, 66°C ×1 minute, 72°C ×1 minute). For the wild-type allele, a 600 bp fragment was generated using the same 5′ primer and a 3′ primer (TACCGGTGGATGTGGAATGTGTGCG) over 41 cycles (94°C ×1 minute, 62°C ×1 minute, 72°C ×1 minute). 5% DMSO was added to this reaction and deazaGTP was substituted for dGTP. Genotyping for the transgene and the *PTHrP* wild-type and mutant alleles were performed as previously described (Schipani et al., 1997).

Skeletal staining

Alizarin red and Alcian blue staining was modified after McLeod (1980). Limbs were fixed for 48 hours in ethanol and then 48 hours in acetone. Staining was performed overnight at 37°C for 15.5 dpc limbs, and an additional 2 days at room temperature for 18.5 dpc limbs. Limbs were washed in 95% ethanol, cleared in 1% KOH and taken through graded steps into 80% glycerol.

Histology

Limbs were fixed overnight at 4°C in paraformaldehyde, embedded in paraffin using standard procedures, sectioned at 5 μm, and stained with Hematoxylin and Eosin.

In situ hybridization

In situ hybridization was performed according to Wilkinson (1992). Specimens were fixed in 4% paraformaldehyde at 4°C overnight. ^{35}S-labeled riboprobes for Colα1(II), Colα1(X) were used as previously described (Lee et al., 1996).

BrdU incorporation

Pregnant mice received intraperitoneal injections of 50 μg BrdU/gram of body weight and were killed one hour later. Limbs were dissected and fixed in 4% paraformaldehyde overnight at 4°C. Embedding and sectioning were performed using standard procedures. BrdU was detected as described (Nowakowski et al., 1989). Sections were counterstained with hematoxylin and eosin.

Linear regression analysis

Length-to-width ratios for wild-type tibias were plotted from 12.5 to 18.5 dpc and a line was fitted to the data using least squares method with Cricket Graph 1.3 software. This line was used to predict the length-to-width ratio of an element with a given width.

RESULTS

Long bone development in *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ double mutants is identical to *Ihh*$^{-/-}$ mice but distinct from *PTHrP*$^{-/-}$ mice

To understand the genetic interaction between *Ihh* and *PTHrP*, *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ double mutant mice were compared with each of the single mutants at 18.5 dpc. Development of the skeleton was assessed by staining with Alcian blue to indicate immature cartilage, and Alizarin red, which identifies the mineralized matrix secreted by mature hypertrophic chondrocytes and osteoblasts during bone formation. This staining identifies the cartilagenous epiphysis of the long bones and the central shaft, which is mineralized bone (Fig. 1A). *PTHrP*$^{-/-}$ limbs are shorter than wild type, with only a minimal zone of immature cartilage at the articular surface and an expanded region of mineralized bone (Fig. 1B). In *Ihh*$^{-/-}$ limbs, mineralization extends close to the articular surface, but the

elements are even smaller than those of *PTHrP*$^{-/-}$ embryos and do not display differentially increased longitudinal length (Fig. 1C). Limbs from *PTHrP*$^{-/-}$; *Ihh*$^{-/-}$ embryos were identical in size, morphology and mineralization pattern to those of *Ihh* mutant embryos, except for slightly less Alcian blue staining at the articular surface in one element, the femur (Fig. 1D). Sections from the proximal tibia were used to determine cellular morphology and to examine marker gene expression. In the wild type at 18.5 dpc, non-hypertrophic chondrocytes occupy the space at the articular surface and the first hypertrophic cells occur many cell diameters away from the ends of the long bones (Fig. 1F). *PTHrP* mutant elements demonstrate a decreased zone of non-hypertrophic cells (Fig. 1G). *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ and *Ihh*$^{-/-}$ elements displayed similar histology. Both exhibited hypertrophic cells abnormally close to the articular surface. In addition, the growth plate, trabecular bone and bone collar were absent (Fig. 1H,I). Expression of collagens was characteristic. In the wild type, non-hypertrophic chondrocytes express *Col II* mRNA, hypertrophic cells *Col X* mRNA, and there is a small transition region in which both are expressed (Fig. 2A,D). *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ and *Ihh*$^{-/-}$ elements exhibit expression patterns that are identical to each other but distinct from the wild type. There is a marked decrease in *Col II* expression, which was confined to the periphery of the element, whereas *Col X* expression occupied the entire center of the element (Fig. 2B,C,E,F).

Activation of *PTHrP* signalling has no effect on chondrocyte proliferation or limb size but rescues chondrocyte hypertrophy in *Ihh*$^{-/-}$ mice

To examine which functions of *Ihh* were mediated by *PTHrP*, we activated *PTHrP* signaling in immature chondrocytes of *Ihh*$^{-/-}$ mice and determined which aspects of the *Ihh*$^{-/-}$ phenotype were rescued. To accomplish this, we made use of the *α1* (*II*) *collagen* promoter to drive a ligand-independent activated *PTHrP* receptor (*PTHrPR**). When expressed in *PTHrP*$^{-/-}$ mice, this transgene completely rescues the extensive chondrocyte maturation and associated dwarfism of the *PTHrP* mutant embryos (Schipani et al., 1997). This demonstrates that the construct faithfully reproduces *PTHrP* signalling; we expressed it in *Ihh*$^{-/-}$ mice for this purpose.

Comparison of skeletal preparations from *Ihh*$^{-/-}$ and *Ihh*$^{-/-}$; *PTHrPR** mice at 18.5 dpc demonstrated that the activated receptor suppressed hindlimb mineralization, as judged by increased Alcian blue staining in the femur, tibia and fibula (Fig. 1C,E). In contrast, there was no significant difference in the lengths of these limb elements, though the *Ihh*$^{-/-}$; *PTHrPR** elements were slightly thicker. Elements from mice with two copies of the activated receptor were precisely the same length, but slightly thicker and with more extensive Alcian blue staining than mice with a single copy (data not shown). Histological analysis (Fig. 1H,J) demonstrated a general delay in chondrocyte hypertrophy in the elements of embryos expressing the activated receptor. The region of non-hypertrophic cells at the articular surface was larger in these elements, and non-hypertrophic cells were interspersed with hypertrophic cells in the center of the element. These results suggests that whereas *PTHrP* signaling is sufficient to delay chondrocyte hypertrophy and matrix mineralization, it is not able to substitute for *Ihh* in driving growth of the endochondral skeleton.

PTHrP signaling is sufficient to maintain a population of mitotically active chondrocytes in *Ihh*$^{-/-}$

To explain the effect of *PTHrPR** in the *Ihh*$^{-/-}$ background, we examined growth plates at 15.5 dpc. Wild-type limbs demonstrated discrete zones of non-hypertrophic and hypertrophic cells. In *PTHrP* null limbs, the area of non-hypertrophic cells was markedly decreased. The *PTHrPR** transgene completely inhibited chondrocyte hypertrophy in the *Ihh*$^{-/-}$ background at this time (Fig. 3A-D). Bromodeoxyuridine (BrdU) incorporation was used to assess DNA synthesis as an indicator of mitotic activity. The *Ihh*$^{-/-}$ elements demonstrated an absence of cells undergoing DNA synthesis over a large central region, whereas elements from *Ihh*$^{-/-}$; *PTHrPR** mice contained mitotically active cells throughout (Fig. 3G,H). To examine these results with respect to marker expression, in situ hybridization was carried out on serial sections. Compared with *Ihh* mutants, *Ihh* mutants expressing the *PTHrPR** transgene demonstrated extensive *Col II* expression and almost entirely absent *Col X* expression (Fig. 4A-D). These results extend the findings at 18.5 dpc and suggest that *PTHrP* signaling is sufficient to prevent chondrocyte hypertrophy and maintain a population of mitotically active cells in the absence of *Ihh*. Thus, by preventing hypertrophy, *PTHrP* signaling expands the number of cells undergoing basal levels of proliferation. This offsets the absence of hypertrophy resulting in skeletal elements which are similar in size between *Ihh*$^{-/-}$ and *Ihh*$^{-/-}$; *PTHrP*R* embryos, but dramatically smaller than wild type.

PTHrP signaling does not affect the chondrocyte proliferation rate in *Ihh*$^{-/-}$ mice

The failure of *PTHrPR** to rescue the growth defect in *Ihh* mutants despite its ability to increase the pool of mitotically active cells suggests that the *PTHrP* pathway is unable to restore proliferation rates in the *Ihh*$^{-/-}$ embryos to wild-type levels. To address this, we determined the percentage of chondrocytes incorporating BrdU by analyzing non-hypertrophic, mitotically active cells which do not express *Col X*. In wild type, *PTHrP*$^{-/-}$ and *Ihh*$^{-/-}$ embryos, these cells are restricted to an area near the articular surface (Fig. 3E-G), whereas in *Ihh*$^{-/-}$; *PTHrPR** elements, all cells are non-hypertrophic and do not express *Col X* (Fig. 3H). The presence of the activated receptor had no significant effect on the percentage of immature chondrocytes in S phase (Table 1, wild type versus *Ihh*$^{-/-}$ P=0.003, *Ihh*$^{-/-}$ versus *Ihh*; *PTHrPR** P=0.71). This suggests an absolute requirement for *Ihh* in establishing normal levels of chondrocyte proliferation.

PTHrP$^{-/-}$ mice display decreased proliferation rates

Interestingly, *PTHrP* mutants that demonstrated a reduced domain of proliferating cells also displayed a small but significant decrease in the rate of chondrocyte proliferation compared to wild type at 13.5 and 15.5 dpc (data not shown, and Fig. 3A,B,E,F, Table 1, P=0.04), which was restored to wild-type levels by expression of the transgene (Table 1, *PTHrP*$^{-/-}$ versus *PTHrP*$^{-/-}$; *PTHrPR** P=0.03, wild type versus *PTHrP*$^{-/-}$;*PTHrPR** P=0.40) providing additional evidence of the ability of the transgene to completely reproduce *PTHrP* signalling, including its proliferative effect. Importantly, proliferation rates in the *Ihh*$^{-/-}$ were significantly less than in the *PTHrP*$^{-/-}$ elements (Table 1, P=0.03).

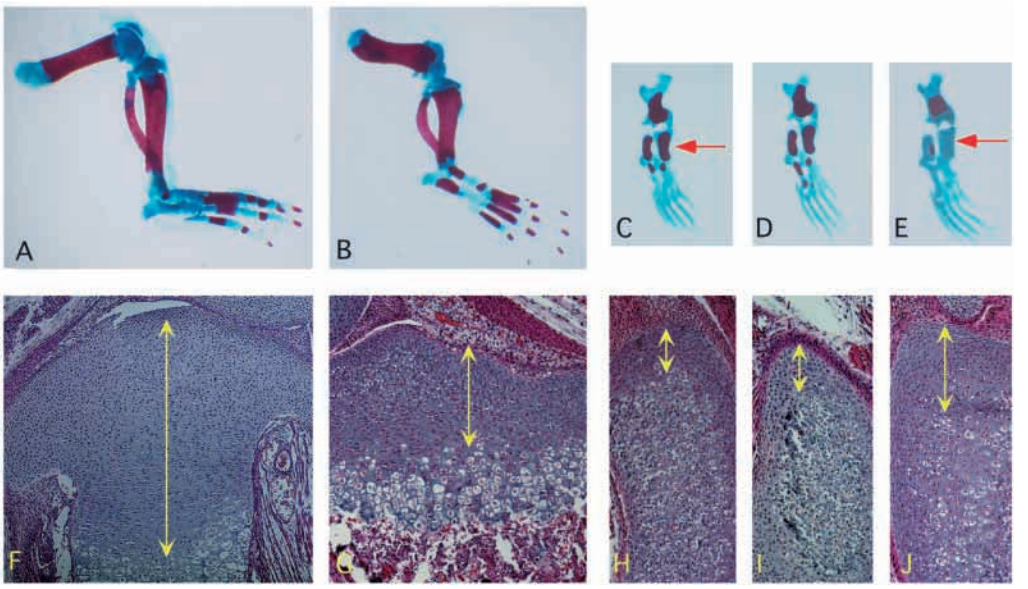

Fig. 1. Effects of manipulating *PTHrP* signaling in *Ihh* mutants. (A,F) Wild type, (B,G) *PTHrP*$^{-/-}$, (C,H) *Ihh*$^{-/-}$, (D,I) *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ and (E,J) *Ihh*$^{-/-}$; *PTHrPR**. (A-E) 18.5 dpc skeletal preparations taken at the same magnification, and (F-J) is corresponding histology taken at the same magnification. Loss of *PTHrP* in *Ihh*$^{-/-}$ elements results in limbs that are identical by skeletal staining (C,D) and histology (H,I). Activation of *PTHrP* signaling in *Ihh*$^{-/-}$ using *PTHrPR** significantly decreases red staining, which is entirely absent in the tibia (red arrows in C,E). This is confirmed by histology (H,J), which demonstrates reduced chondrocyte hypertrophy. Yellow arrows mark the regions of non-hypertrophic chondrocytes.

Ihh$^{-/-}$ limbs display isotropic growth, in contrast to *PTHrP*$^{-/-}$ or wild-type limbs

Finally, we examined the contributions of *Ihh* and *PTHrP* to the establishment of differential growth along the long axis of the limb, a critical aspect of morphogenesis. To address this issue, we examined the ratio of the length to width of the tibia as a morphogenetic parameter. In wild type, *Ihh*$^{-/-}$ and *PTHrP*$^{-/-}$ limbs, the initial size and morphology of mesenchymal condensations are identical (St-Jacques et al., 1999; Karaplis et al., 1994); the ratio of length to width of the tibia begins at approximately 2:1 at 12.5 dpc. In wild-type limbs, this ratio increases to 8:1 by 18.5 dpc. In the *PTHrP*$^{-/-}$ embryos, the ratio increases to 6:1 at 18.5 dpc and, though growth is decreased, there is still a clear asymmetry, which favors growth along the long axis. In contrast, the ratio in the *Ihh*$^{-/-}$ mice remains approximately 2:1 at 18.5 dpc. This is the expectation if growth occurs isotropically, and represents a loss of directional growth. A wild-type tibia with the same width has a predicted ratio of 3.9:1. Thus, whereas growth in the presence of Ihh is directed along the long axis of the limb, in the absence of Ihh, residual growth is isotropic.

DISCUSSION

Ihh is necessary for *PTHrP* function

To determine which aspects of *PTHrP* signaling were independent of *Ihh*, we first compared endochondral elements from *Ihh* and *PTHrP* single mutants with those from *Ihh*; *PTHrP* double mutants. *Ihh*$^{-/-}$ and *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ elements were indistinguishable from one another, identical in size, morphology and ossification pattern. Histologically they display similar patterns of chondrocyte hypertrophy. This result suggests that *PTHrP* activity is lost in *Ihh* mutants. This could be due either to loss of expression of *PTHrP* in the *Ihh*$^{-/-}$ elements, or a requirement for *Ihh* in the response to *PTHrP*. Consistent with the former, other studies have been unable to demonstrate *PTHrP* expression in the *Ihh* mutants by section in situ hybridization (St-Jacques et al., 1999). Our results provide genetic evidence supporting the conclusion that *Ihh* positively regulates *PTHrP* during endochondral bone formation.

In contrast, *PTHrP*$^{-/-}$ elements show striking differences with *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ double mutants. The double mutants are smaller and dysmorphic. There are similarities in chondrocyte maturation with hypertrophic cells and mineralized matrix extending close to the articular surface. These results demonstrate that *Ihh* is necessary for growth and morphogenesis of the skeletal elements, but that *PTHrP* only mediates a subset of *Ihh* function.

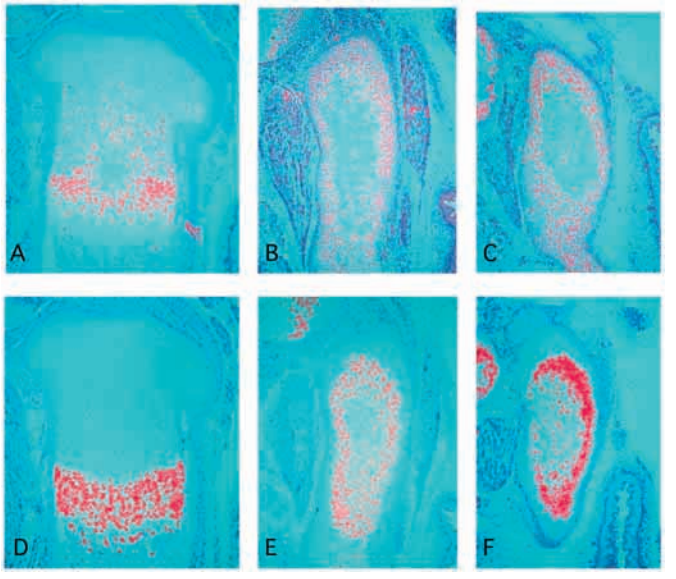

Fig. 2. Loss of *PTHrP* does not affect chondrocyte identity in *Ihh*$^{-/-}$ mice. (A,D) wild type, (B,E) *Ihh*$^{-/-}$, (C,F) *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ at 18.5 dpc. (A-C) *Col II* transcripts, (D-F) *Col X* transcripts. *Col II* is expressed primarily in non-hypertrophic cells (A), whereas *Col X* is expressed in hypertrophic cells (D). Patterns of *Col II* transcripts (B,C) and *Col X* transcripts (E,F) are identical in *Ihh*$^{-/-}$ and *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ mice. In contrast to wild type, in *Ihh*$^{-/-}$ and *Ihh*$^{-/-}$; *PTHrP*$^{-/-}$ elements, *Col II* occupies a small rim at the periphery, while *Col X* occupies a large area in the center.

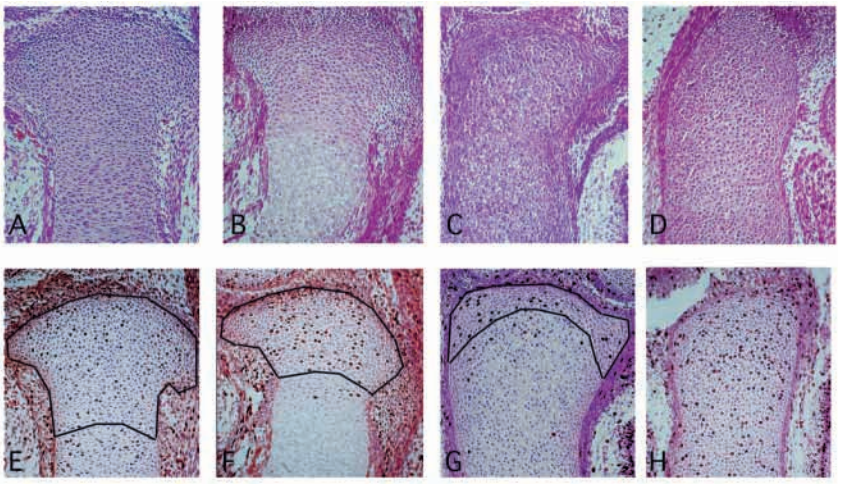

Fig. 3. Effects of *PTHrP* on chondrocyte proliferation and hypertrophy. (A,E) Wild type, (B, F) *PTHrP*$^{-/-}$, (C,G) *Ihh*$^{-/-}$, (D,H) *Ihh*$^{-/-}$; *PTHrPR** at 15.5 dpc. (A-D) Histology, (E-H) corresponding BrdU-labeled sections. *PTHrP*$^{-/-}$ mice have decreased numbers of non-hypertrophic cells (A,B), which proliferate at a decreased rate compared with wild type (E,F; Table 1). Activation of *PTHrP* signaling using *PTHrPR** in *Ihh*$^{-/-}$ mice increases the number of non-hypertrophic cells (C,D), but does not alter the rate of proliferation of these cells (G,H; Table 1). Black lines surround the non-hypertrophic population of cells that was analyzed.

To further clarify which aspects of *Ihh* signaling were mediated by *PTHrP*, we expressed *PTHrPR** using the *collagen II* promoter in *Ihh*$^{-/-}$ mice. *PTHrPR** is an H223R point mutation in the human *PTHrPR* gene which is responsible for Jansen's chondrodysplasia, a human genetic syndrome characterized by a disordered growth plate and short-limbed dwarfism (Schipani et al., 1995). The *α1* (*II*) *collagen* promoter is well characterized and directs expression of the transgene in cartilage (Metsaronta et al., 1995; Cheah et al., 1991; Yamada et al., 1990)). This construct rescues the skeletal phenotype of *PTHrP*$^{-/-}$ mice at the time of birth (Schipani et al., 1997), and is therefore able to completely reproduce *PTHrP* signalling. Consequently, examining the activity of this ligand-independent receptor in the *Ihh*$^{-/-}$ background allowed us to determine which aspects of the *Ihh* phenotype resulted from the loss of expression of the *Ihh* target, PTHrP, at the periarticular surfaces. Expression of the transgene reversed the premature maturation of chondrocytes observed in *Ihh* mutants at both 15.5 and 18.5 dpc. Since previous studies have demonstrated this maturation arrest is chracteristic of *PTHrP* signalling (Vortkamp et al., 1996; Weir et al., 1996), this marked phenotypic effect indicates that the activated receptor is indeed functional in Ihh$^{-/-}$ limbs as expected but can only rescue a subset of *Ihh*-dependent phenotypes. When combined with the genetic evidence that *PTHrP* signaling is absent in *Ihh* mutants (St-Jacques et al., 1999) these results lend strong support to a model in which *Ihh* maintains a zone of immature chondrocytes through the transcriptional regulation of *PTHrP* (Vortkamp et al., 1996).

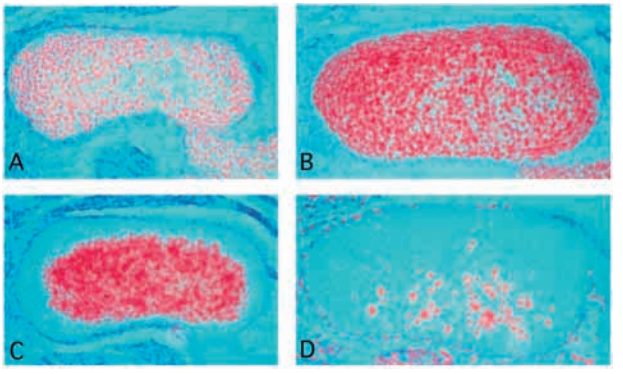

Fig. 4. Activation of *PTHrP* signaling delays chondrocyte hypertrophy in *Ihh*$^{-/-}$ mutants. (A,C) *Ihh*$^{-/-}$, (B,D) *Ihh*$^{-/-}$; *PTHrPR** at 15.5 dpc. (A,B) *Col II* transcripts, (C,D) *Col X* transcripts. While *Col II* expression occurs throughout both elements (A,B), *Col X* expression is markedly decreased by addition of the transgene (C,D).

Ihh drives chondrocyte proliferation in a largely *PTHrP*-independent pathway

In principle, the failure of the activated receptor to rescue growth in the *Ihh*$^{-/-}$ mice might be due to an inability to (1) maintain a population of proliferating, non-hypertrophic cells, (2) increase the abnormally low level of proliferation in the *Ihh*$^{-/-}$ mice (St-Jacques et al., 1999), or (3) a combination of both. Our analysis of proliferation in non-hypertrophic regions (areas not expressing *collagen X*) in growth plates of *Ihh*$^{-/-}$; *PTHrPR** limbs at 15.5 dpc indicated that although *PTHrP* signaling is sufficient to maintain a pool of mitotically active chondrocytes, it is unable to increase proliferation above the 'basal' level found in *Ihh*$^{-/-}$ limbs. These results suggest that the rate of chondrocyte proliferation is controlled by an aspect of *Ihh* signaling which is independent of *PTHrP*.

Surprisingly, *PTHrP* itself can influence proliferation rates. In *PTHrP* mutants, the rate of chondrocyte proliferation is less that wild type, although significantly greater than in the *Ihh*$^{-/-}$ mice. Together, these results suggest that the main driving force for chondrocyte proliferation is *Ihh* signaling acting largely through a *PTHrP*-independent mechanism; *PTHrP* signaling may itself regulate proliferation to a lesser extent but the failure of the activated receptor to influence proliferation in the *Ihh*$^{-/-}$ mice indicates an absolute requirement for *Ihh*. Since *Ihh* expression does not seem to be altered in *PTHrP* mutants (Vortkamp et al., 1996; Lanske et al., 1996), but *Ptc-1*, the likely *Ihh* receptor, and *PTHrPR* are co-expressed in proliferating chondrocytes (St-Jacques et al., 1999), it is possible that *PTHrP* could modify the response to *Ihh* signaling. These results establish that the maintenance of a mitotically active population of chondrocytes can be uncoupled at a molecular level from the signals that determine their proliferation rates. Furthermore, these results

Table 1. Percentage of non-hypertrophic chondrocytes that incorporate BrdU at 15.5 dpc in the tibia

Genotype	BrdU incorporation (%)
Wild type	21.0±0.8
Ihh$^{-/-}$	13.6±2.3
Ihh$^{-/-}$; *PTHrPR**	12.9±2.5
PTHrP$^{-/-}$	18.2±1.5
PTHrP$^{-/-}$;*PTHrPR**	21.8±1.3

Each result is derived from at least three separate sections from at least three different animals. Standard deviations are given.

indicate that preventing chondrocyte hypertrophy is not of itself sufficient for normal growth of the skeleton.

Ihh is necessary to establish growth primarily along the long axis of the bone

Proper morphogenesis of the long bones requires anisotropic growth which is primarily directed along the long axis of the bone. How this is achieved is a fundamental problem in morphogenesis. Examination of the *Ihh*$^{-/-}$ limbs suggested that this information was lost in these elements, but was preserved in the *PTHrP*$^{-/-}$ limbs. *Ihh* mutants do not display the normal longitudinal stacking of maturing chondrocytes which is a prominent feature of the growth plate in wild-type animals. Whether this contributes to the isotropic growth, and whether stacking is directly controlled by *Ihh* signalling or indirectly by other parameters such as delayed hypertrophy in the skeletal element remains to be determined.

Ihh coordinates endochondral bone development

In summary, *Ihh* plays a pivotal role in coordinating events in the developing endochondral skeleton. *Ihh* regulates *PTHrP*, which delays chondrocyte hypertrophy. This ensures a supply of proliferating, non-hypertrophic chondrocytes. *Ihh* drives proliferation of these cells in a pathway that is largely independent of *PTHrP* signaling. Finally, *Ihh* may also be necessary to drive differential growth along the longitudinal axis of the long bone.

The mechanism by which *Ihh* drives its *PTHrP*-independent effects is an important and unanswered question. There is some evidence that Ihh may signal directly to proliferating chondrocytes. Two general target targets of this pathway, the Hedgehog receptor, *Patched*, and the downstream transcriptional effector *Gli-1* are both expressed in proliferating chondrocytes and their expression is lost in *Ihh* mutants (St-Jacques et al., 1999). However, both targets are also expressed in the adjacent perichondrial/periosteal region leaving open the possibility of a signaling relay. Further, it is currently unclear whether Ihh signals directly to maintain *PTHrP* expression at the periarticular surfaces, which are a considerable distance from the domain of *Ihh* expression within the growth plate. Clarification of these issues will require approaches to remove the ability to respond to *Ihh* signaling from relevant regions of the skeletal element. In conclusion, the results reported here lend further support to the model that *hedgehog* genes coordinate a diverse set of functions in part by recruiting molecules to perform specific tasks. Further defining the mechanism of *Ihh* and *PTHrP* action will help to unravel the intracellular mechanisms which produce the local responses of chondrocyte proliferation and hypertrophy, as well as the global coordination of these processes which is necessary for skeletal morphogenesis.

Work in A. P. M.'s laboratory was supported by National Institutes of Health grant NS33642 and DK56246. S. J. K. was supported by NIH grant HD08356 and a grant from the American College of Surgeons. H. K. is supported by NIH grant DK-47038. E. S. is supported by NIH grant DK-50708-01. We thank members of the McMahon Laboratory for helpful discussions, and D. Faria and J. Faxton for expert technical assistance.

REFERENCES

Amizuka, N., Henderson J. E., Hoshi, K., Warshawsky, H., Ozawa, H., Goltzman, D. and Karaplis, A. C. (1996). Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for Parathyroid Hormone-related Peptide gene deletion. *Endocrinology* **137**, 5055-5067.

Bitgood, M. J. and McMahon, A. P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol*. **172**, 126-138.

Cheah, K. S., Lau, E. T., Au, P. K. and Tam, P. P. (1991). Expression of the mouse alpha 1(II) collagen gene is not restricted to cartilage during development. *Development* **111**, 945-53.

Echelard Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). *Sonic hedgehog*, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-30.

Elima, K., Eerola, I., Rosati, R., Metsaranta, M., Garofalo, S., Perala, M., De Crombrugghe, B. and Vuorio, E. (1993). The mouse collagen X gene: complete nucleotide sequence, exon structure and expression pattern. *Biochem. J*. **289**, 247-253.

Erlebacher, A., Filvaroff, E. H., Gitelman, S. E. and Derynck, R. (1995). Toward a molecular understanding of skeletal development. *Cell* **80**, 371-378.

Hunziker, E. B. (1988). Growth plate structure and function. *Pathol. Immunopathol. Res.* **7**, 9-13.

Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L., Kronenberg, H. M. and Mulligan, R. C. (1994). Lethal skeletal dysplasia from targeted disruption of the Parathyroid Hormone-related Peptide gene. *Genes Dev*. **8**, 277-289.

Kosher, R. A., Kulyk, W. M. and Gay, S. W. (1986). Collagen gene expression during cartilage differentiation. *J. Cell Biol*. **102**, 1151-1156.

Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H. K., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Juppner, H., Segre, G. V. and Kronenberg, H. M. (1996). PTH/*PTHrP* receptor in early development and Indian hedgehog-regulated bone growth. *Science* **273**, 663-666.

Lee, K., Deeds, J. D. and Segre, G. V. (1995). Expression of Parathyroid Hormone-related Peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* **136**, 453-463.

Lee, K., Lanske, B., Karaplis, A. C., Deeds, J. D., Kohno, H., Nissenson, R. A., Kronenberg, H. M. and Segre, G. V. (1996). Parathyroid hormone-related Peptide delays terminal differentiation of chondrocytes during endochondral bone development. *Endocrinology* **137**, 5109-5118.

Metsaranta M., Garofalo, S., Smith, C., Niederreither, K., de Crombrugghe, B. and Vuorio, E. (1995). Developmental expression of a type II collagen/beta-galactosidase fusion gene in transgenic mice. *Dev. Dyn*. **204**, 202-10.

McLeod, M. J. (1980) Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. *Teratology* **22**, 299-301.

Nowakowski, R. S., Lewin, S. B. and Miller, M. W. (1989). Bromodeoxyuridine immunohistochemical determination of the lengths of the cell cycle and the DNA-synthetic phase for an anatomically defined population. *J. Neurocytol*. **18**, 311-318.

Schipani, E., Kruse, K. and Juppner, H. (1995). A constitutively active mutant PTH-*PTHrP* receptor in Jansen-type metaphyseal chondrodysplasia. *Science* **268**, 98-100

Schipani, E., Lanske, B., Hunzelman, J., Luz, A., Kovacs, C. S., Lee, K., Pirro, A., Kronenberg, H. M. and Juppner, H. (1997). Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide. *Proc. Natl. Acad. Sci. USA* **94**, 13689-13694.

St-Jacques, B., Hammerschmidt, M., McMahon, A. P. (1999). Indian hedgehog signaling regulates proliferation and differentiation of the chondrocytes and is essential for osteoblast differentiation during skeletal development. *Genes Dev.* **13**, 2072-2086.

Suva, L. J.,Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C., Kemp, P. B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J. and Wood, W. I. (1987). A Parathyroid Hormone-related Protein implicated in malignant hypercalcemia: cloning and expression. *Science* **237**, 893-896.

Swalla, B. J., Upholt, W. B. and Solursh. M. (1988). Analysis of type II collagen RNA localization in chick wing buds by *in situ* hybridization. *Dev. Biol.* **125**, 51-58.

Vortkamp, A., Lee, K., Lanske, B., Segre, G. V., Kronenberg, H. M. and Tabin, C. J. (1996). Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. *Science* **273**, 613-622.

Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. and Broadus, A. E. (1996). Targeted overexpression of Parathyroid Hormone-related Peptide in chondrocytes causes chondrodysplasia and delayed endochondral bone formation. *Proc. Natl. Acad. Sci. USA* **93**, 10240-10245.

Wilkinson, D. G. (1992). Whole mount in situ hybridization to vertebrate embryos. In *In Situ Hybridization: A Practical Approach* (ed. D. G. Wilkinson), pp. 75-83. IRL Press, Oxford, UK.

Yamada, Y., Miyashita, T., Savagner, P., Horton, W., Brown, K. S., Amramczuk, J., Xie, H. X., Kohno, K., Bolander, M. and Bruggeman, L. (1990). Regulation of the collagen II gene in vitro and in transgenic mice. *Ann. N. Y. Acad. Sci.* **580**, 81-87.