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

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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 periacicular 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<sup>−/−</sup>) 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 periacicular perichondrium. This results in activation of the Parathyroid Hormone/Parathyroid Hormone-related Protein Receptor (PTHrP-R) 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<sup>−/−</sup>) demonstrated that expression of PTHrP at the periacicular 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<sup>−/−</sup> 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 (PTHrP<sup>R+</sup>) is expressed under the regulation of the Collagen 2α1 (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 (AAGAGGGAGGACATGGATGGGGTG) and a 3′ primer (AGGAACAGACAGACCAGTCGGG) 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 (TACGGTGGAATGTTGTGCG) 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 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 acetic acid. 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. 35S-labeled riboprobes for Col1α1(II), Col1α1(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<sup>−/−</sup>; PTHrP<sup>−/−</sup> double mutants is identical to Ihh<sup>−/−</sup> mice but distinct from PTHrP<sup>−/−</sup> mice

To understand the genetic interaction between Ihh and PTHrP, Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> limbs, mineralization extends close to the articular surface, but the
elements are even smaller than those of PTHrP<sup>−/−</sup> embryos and do not display differentially increased longitudinal length (Fig. 1C). Limbs from PTHrP<sup>−/−</sup>; Ihh<sup>−/−</sup> 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<sup>−/−</sup>; PTHrP<sup>−/−</sup> and Ihh<sup>−/−</sup> 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<sup>−/−</sup>; PTHrP<sup>−/−</sup> and Ihh<sup>−/−</sup> 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<sup>−/−</sup> mice**

To examine which functions of Ihh were mediated by PTHrP, we activated PTHrP signaling in immature chondrocytes of Ihh<sup>−/−</sup> mice and determined which aspects of the Ihh<sup>−/−</sup>-phenotype were rescued. To accomplish this, we made use of the α1(I) collagen promoter to drive a ligand-independent activated PTHrP receptor (PTHRPR*). When expressed in PTHrP<sup>−/−</sup> 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<sup>−/−</sup> mice for this purpose.

Comparison of skeletal preparations from Ihh<sup>−/−</sup> and Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> 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<sup>−/−</sup>; PTHrP<sup>−/−</sup> 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<sup>−/−</sup>**

To explain the effect of PTHrP<sup>−/−</sup> in the Ihh<sup>−/−</sup>-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 PTHrP<sup>−/−</sup> transgene completely inhibited chondrocyte hypertrophy in the Ihh<sup>−/−</sup>-background at this time (Fig. 3A-D). Bromodeoxyuridine (BrdU) incorporation was used to assess DNA synthesis as an indicator of mitotic activity. The Ihh<sup>−/−</sup>-elements demonstrated an absence of cells undergoing DNA synthesis over a large central region, whereas elements from Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> 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 PTHrP<sup>−/−</sup> 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<sup>−/−</sup> and Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> embryos, but dramatically smaller than wild type.

**PTHRP signaling does not affect the chondrocyte proliferation rate in Ihh<sup>−/−</sup> mice**

The failure of PTHrP<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> and Ihh<sup>−/−</sup> embryos, these cells are restricted to an area near the articular surface (Fig. 3E-G), whereas in Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> 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<sup>−/−</sup>; P<sub>0.003</sub>, Ihh<sup>−/−</sup> versus Ihh; PTHrP<sup>−/−</sup> P<sub>0.71</sub>). This suggests an absolute requirement for Ihh in establishing normal levels of chondrocyte proliferation.

**PTHRP<sup>−/−</sup> mice display decreased proliferation rates**

Interestingly, PTHrP<sup>−/−</sup> 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<sup>−/−</sup> versus PTHrP<sup>−/−</sup>; PTHrP<sup>−/−</sup> P<0.03, wild type versus PTHrP<sup>−/−</sup>; PTHrP<sup>−/−</sup> P<0.40) providing additional evidence of the ability of the transgene to completely reproduce PTHrP signaling, including its proliferative effect. Importantly, proliferation rates in the Ihh<sup>−/−</sup> were significantly less than in the PTHrP<sup>−/−</sup> elements (Table 1, P<0.03).

545Ihh and PTHrP in endochondral bone development
**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<sup>−/−</sup> and Ihh<sup>+/−</sup>; PTHrP<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> elements show striking differences with Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> 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. 1.** Effects of manipulating PTHrP signaling in Ihh mutants. (A,F) Wild type, (B,G) PTHrP<sup>−/−</sup>, (C,H) Ihh<sup>−/−</sup>; (D,I) Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> and (E,J) Ihh<sup>−/−</sup>; PTHrPR<sup>−/−</sup>. (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<sup>−/−</sup> elements results in limbs that are identical by skeletal staining (C,D) and histology (H,I). Activation of PTHrP signaling in Ihh<sup>−/−</sup> using PTHrPR<sup>−/−</sup> 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<sup>−/−</sup> limbs display isotropic growth, in contrast to PTHrP<sup>−/−</sup> 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<sup>−/−</sup> and PTHrP<sup>−/−</sup> 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 limbs 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<sup>−/−</sup> 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<sup>−/−</sup> 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.

**Fig. 2.** Loss of PTHrP does not affect chondrocyte identity in Ihh<sup>−/−</sup> mice. (A,D) wild type, (B,E) Ihh<sup>−/−</sup>; (C,F) Ihh<sup>+/+</sup>; PTHrP<sup>−/−</sup> 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<sup>−/−</sup> and Ihh<sup>+/−</sup>; PTHrP<sup>−/−</sup> mice. In contrast to wild type, in Ihh<sup>−/−</sup> and Ihh<sup>−/−</sup>; PTHrP<sup>−/−</sup> elements, Col II occupies a small rim at the periphery, while Col X occupies a large area in the center.
**Ihh and PTHrP in endochondral bone development**

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 the transgene reversed the premature maturation of chondrocytes observed in ihh−/− mice (St-Jacques et al., 1999), or (3) a combination of both. Our analysis of proliferation in non-hypertrophic regions of 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 that hypertrophy is characteristic of PTHrP signaling (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**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>BrdU incorporation (%)</th>
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<tr>
<td>Wild type</td>
<td>21.0±0.8</td>
</tr>
<tr>
<td>Ihh−/−</td>
<td>13.6±2.3</td>
</tr>
<tr>
<td>Ihh+/−; PTHrPR*</td>
<td>12.9±2.5</td>
</tr>
<tr>
<td>PTHrP+/−; PTHrPR*</td>
<td>18.2±1.5</td>
</tr>
<tr>
<td>PTHrP+/−: PTHrPR*</td>
<td>21.8±1.3</td>
</tr>
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Each result is derived from at least three separate sections from at least three different animals. Standard deviations are given.
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 embryos, 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.

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