BoneKEy-Osteovision. 2005 November;2(11):7-15
http://www.bonekey-ibms.org/cgi/content/full/ibmske;2/11/7
DOI: 10.1138/20050181

PERSPECTIVES

How PTHrP Controls Growth Plate Chondrocytes

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Parathyroid hormone-related protein (PTHrP) was discovered because of its role in causing humoral hypercalcemia associated with malignancy (1), but that discovery only provided partial clues about the normal physiologic roles and mechanisms of PTHrP action (2). Shortly after the discovery of PTHrP, a large number of studies showed that it was synthesized in many types of cells, activated the same receptor that is activated by PTH, and, in various assays, modulated cell proliferation and differentiation, and smooth muscle relaxation. The generation of mice missing the *PTHrP* gene (3), the *PTH/PTHrP receptor* gene (4), or the *PTH* gene (5) then provided important models for testing the hypothesized physiologic roles of PTHrP. Mice lacking PTHrP have abnormalities in many organ systems, but skeletal abnormalities are the most striking morphological abnormalities in these mice and are the cause of their death. The mice die at birth because all bones resulting from endochondral bone formation develop improperly. As a result, the rib cage is small and inappropriately mineralized. Further studies have shown that abnormalities in the *PTHrP* (-/-) growth plates result from aberrant control of chondrocyte proliferation and differentiation (6;7). Such actions typify local effects of PTHrP in many organs. The growth plates of *PTHrP* (-/-) and *PTH/PTHrP receptor* (-/-) mice exhibit similar abnormalities. These similarities support the idea that PTHrP affects the growth plate by activating the PTH/PTHrP receptor. Here I will review the actions of PTHrP on the growth plate and emphasize how these actions are regulated in the context of multiple interacting signaling systems.

Endochondral Bone Formation

A review of normal endochondral bone formation will place the abnormalities of the *PTHrP (-/-)* mouse in a useful context**.** All bone development begins with the formation of mesenchymal condensations, groups of mesenchymal cells that draw close together and initiate a characteristic genetic program. In a few bones, notably the flat bones of the skull, these condensations then differentiate into osteoblasts (intramembranous bone formation). In most bones, however, a more convoluted pathway is followed, called endochondral bone formation (8). Mesenchymal cells differentiate into chondrocytes and adjacent perichondrium. Figure 1 illustrates the steps in endochondral bone formation. After condensing (Fig. 1B), the cells enlarge and form round chondrocytes (Fig. 1C) that secrete a matrix characterized by substantial production of collagen type II and aggrecan. These chondrocytes proliferate until, in response to a still unknown signal, chondrocytes in the middle of the developing bone stop proliferating, enlarge (hypertrophy), and change their genetic program to secrete a matrix rich in collagen type X (Fig. 1D). These hypertrophic chondrocytes then direct the mineralization of their surrounding matrix, signal to adjacent perichondrial cells to direct their transformation into osteoblasts, and also direct blood vessel invasion. Upon vascularization, perichondrial cells or other nearby cells that become osteoblasts also invade the cartilage to begin formation of the primary spongiosa (Fig. 1E;F). Osteoclasts, cells derived from the hematopoietic lineage, also invade and, together with the cells of the osteoblastic lineage, digest the matrix left behind by the hypertrophic chondrocytes, which undergo apoptotic death at the border of the cartilage and

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primary spongiosa. Away from that border, round chondrocytes continue to proliferate and then flatten out to form orderly columns of proliferating chondrocytes (Fig. 1G). These cells then stop proliferating (pre-hypertrophic cells) and undergo hypertrophy. Bone lengthening occurs because of increased chondrocyte number, synthesis of chondrocyte matrix, and substantial enlargement of chondrocytes during hypertrophy (9). Post-natally in the mouse, secondary sites of ossification form when certain chondrocytes in the region of round proliferative chondrocytes stop dividing, undergo hypertrophy, and attract a new, vascularized primary spongiosa, thereby recapitulating the endochondral sequence (Fig. 1H).

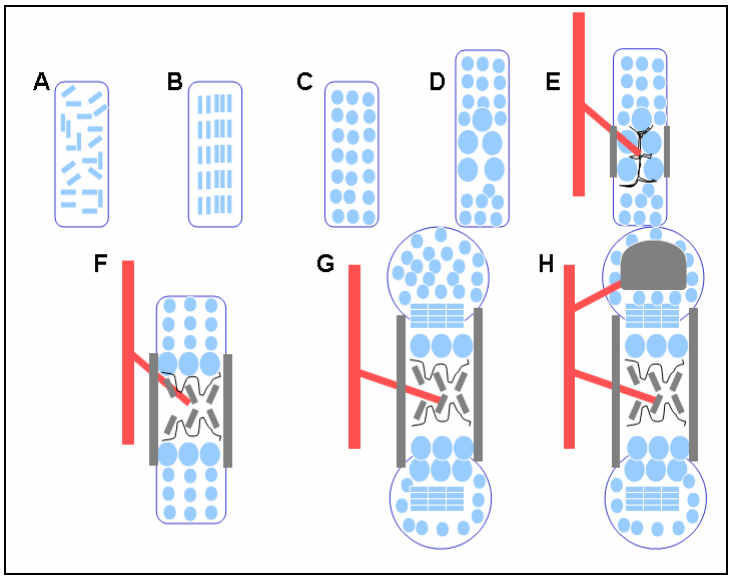

Figure 1: Endochondral Bone Formation. A. Mesenchymal cells before condensation. B. Mesenchymal cells form a condensation. C. Mesenchymal cells differentiate into chondrocytes. D. Chondrocytes in the middle of the cartilage mold stop proliferating and enlarge to become hypertrophic chondrocytes. E. Hypertrophic chondrocytes induce vascular invasion and formation of a bone collar in the adjacent perichondrium. F. Osteoblasts differentiate from cells brought into the cartilage mold with vascular invasion. G. Chondrocytes continue to proliferate, forming columns of flat chondrocytes. H. Secondary ossification centers form.

Chondrocytes between the primary and secondary ossification center form a true growth plate of cells that continues as the engine of bone lengthening in post-natal life. Though the fetal growth region is not a true "plate" of cells, here I will refer to the fetal growth region as the growth plate, since this usage has become common. This overall process, thus, requires careful coordination of cell migration, proliferation, differentiation, and death among groups of cells of varying lineages. Not surprisingly, these processes are tightly regulated by a series of signaling pathways, including those directed by fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), Indian hedgehog (Ihh), C-type natruretic protein (CNP), insulin-like growth factors (IGFs), wingless-int family members (Wnts), and PTHrP. Here I will focus on PTHrP, and mention this long but not exhaustive list to emphasize that the actions of PTHrP can only be understood in the context of the effects of multiple interacting pathways.

PTHrP Actions on the Growth Plate

In fetal bone, PTHrP is synthesized exclusively by perichondrial cells and chondrocytes at the ends of the growing bones (10) (shown schematically in Figure 3). Even when PTHrP production is stimulated, for example by genetic ablation of the PTH/PTHrP receptor or by

overexpressing Indian hedgehog throughout the growth region, increased PTHrP production is only detected in perichondrial cells and round chondrocytes at the ends of bones (11). Thus, a still unknown genetic program specifies which cells in the growing bone are competent to synthesize PTHrP. PTHrP then diffuses away from the site of production and binds to PTH/PTHrP receptors on nearby chondrocytes. How precisely this diffusion is constrained is not clear, but the identification of PTHrP on prehypertrophic cells expressing large numbers of PTH/PTHrP receptors suggests that such a diffusion process occurs (7). Proliferating chondrocytes express low numbers of PTH/PTHrP receptors, but increase receptor expression dramatically as chondrocytes stop proliferating. PTHrP acts on chondrocytes bearing PTH/PTHrP receptors to keep the chondrocytes proliferating and delay their conversion into prehypertrophic and then hypertrophic chondrocytes. To a lesser extent, PTHrP also increases the rate of chondrocyte proliferation, though this modest effect is only seen early during fetal bone development (12). Several genetic models support this formulation. Mice missing either the *PTHrP* or *PTH/PTHrP receptor* genes undergo the normal endochondral sequence, except that columns of proliferating chondrocytes are much shorter than normal or even non-existent. Conversely, mice overexpressing PTHrP in chondrocytes through a transgenic strategy are born with bones containing few hypertrophic chondrocytes and show a profoundly delayed endochondral sequence (13). Mice expressing a constitutively active PTH/PTHrP receptor in chondrocytes show similar effects (14). Both of these transgenic constructs prevent the neonatal death of mice lacking PTHrP in all cells (14;15). This observation justifies the conclusion that the *PTHrP (-/-)* mice die at birth because of skeletal abnormalities. Though no humans lacking PTHrP production have been identified, human fetuses with defective or absent PTH/PTHrP receptors (Blomstrand chondro-osteodystrophy) die *in utero* with skeletal abnormalities that closely resemble those of the *PTH/PTHrP receptor (-/-)* mouse (16;17). Further, humans with Jansen chondro-osteodystrophy have point mutations that render the PTH/PTHrP receptor constitutively active (18). Such people have growth abnormalities like those predicted from the actions of PTHrP on chondrocytic PTH/PTHrP receptors. Thus, it is likely that the lessons learned about the fetal actions of PTHrP in regulating the mouse growth plate are generally applicable to humans.

Mechanisms of PTHrP Action on Chondrocytes

Because the defects in *PTHrP* (-/-) mice and *PTH/PTHrP receptor* (-/-) mice so closely resemble each other, one is tempted to conclude that most and perhaps all of the morphological consequences of PTHrP action on growth plate chondrocytes result from activating the PTH/PTHrP receptor. Having said that, PTHrP also clearly activates receptors distinct from the PTH/PTHrP receptor (19) and also has activities directly within cell nuclei (20). Further studies will be needed to determine whether these other pathways contribute importantly to the actions of PTHrP on the growth plate. Activation of the PTH/PTHrP receptor stimulates multiple heterotrimeric G proteins (21), including G_s, the G_q family, and $G_{12,\ 13}$ (22). The relative activation of each of these pathways varies among cell types, as do the physiologic consequences of activation. We have used genetic tools to identify the effects of activating the G_s and G_q pathways by PTHrP *in vivo* in the fetal growth plate. To determine the role of G_s signaling, we took advantage of technology that allows generation of chimeric mice with cellular contributions from genetically engineered embryonic stem (ES) cells and from normal hosts. Bastepe *et al.* (23) produced chimeras containing normal chondrocytes, as well as chondrocytes missing the second exon of the gene encoding $G_s\alpha$. Chondrocytes missing $G_s\alpha$ stopped proliferating prematurely and became hypertrophic inappropriately early. In that way they closely resembled chondrocytes missing the PTH/PTHrP receptor (24). Even heterozygous deletion of $G_s\alpha$ led to a modest, but detectably early, differentiation of hypertrophic chondrocytes. These and other experiments lead to the conclusion that the PTH/PTHrP receptor

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uses G_s activation to maintain chondrocyte proliferation. Subsequent studies using mice having mutant PTH/PTHrP receptors that cannot activate the G_q family of G proteins but can activate G_s normally (25) showed that activation of G_q by the PTH/PTHrP receptor mildly accelerates chondrocyte differentiation. Thus, activation of G_s and G_q by activating the PTH/PTHrP receptor in chondrocytes leads to actions that oppose each other. The usefulness of this seemingly wasteful opposition of two pathways is uncertain. As in many other settings in which a ligand activates opposing pathways, this complicated pattern may allow regulatory interactions with other pathways that are useful. Further, since activation of the G_q pathway often requires higher concentrations of ligand than does activation of the G_s pathway (26), one might predict that chondrocytes nearer to the source of PTHrP might respond relatively more dramatically with G_q activation than chondrocytes further from the source of PTHrP. Thus, the "antagonistic" actions of G_q might be strong near the top of the growth plate but fall off more quickly than the actions stimulated by G_s, which do not require a high ligand concentration. These contrasting responses of chondrocytes depending on their location in the growth plate may help determine precisely where chondrocytes stop proliferating and initiate further differentiation.

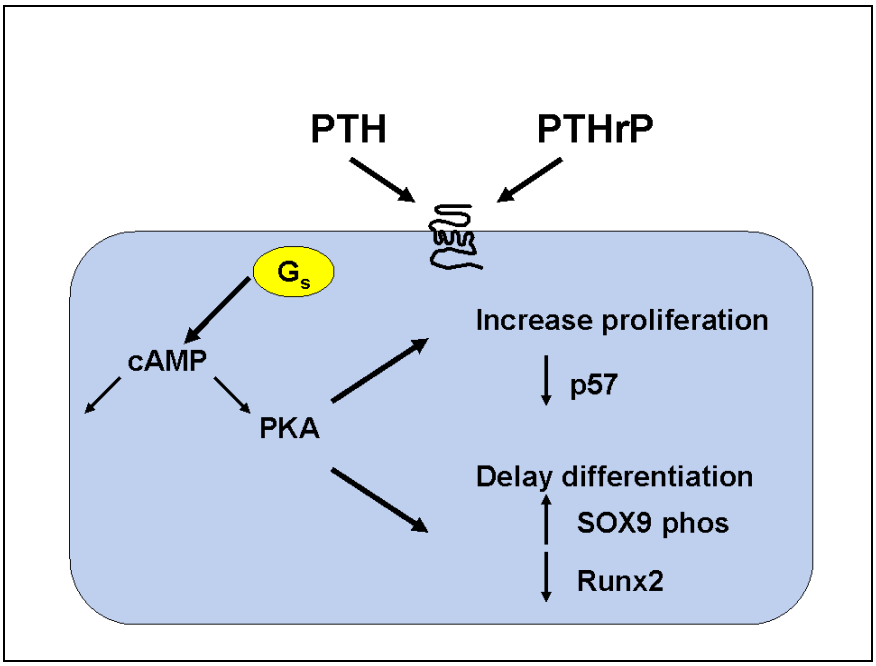

Figure 2. Intracellular signaling by the PTH/PTHrP receptor. Activation of the PTH/PTHrP receptor leads to activation of multiple G proteins. The activation of G_s leads to subsequent activation of adenylate cyclase and generation of cyclic AMP. Cyclic AMP has several actions, including the activation of protein kinase A (PKA). PKA then, by unknown mechanisms, leads to decreases in both p57 levels and in Runx2 mRNA and protein. PKA also phosphorylates SOX9, increasing its activity.

G_s activation leads to cyclic AMP production and protein kinase A (PKA) activation. The specific targets of cyclic AMP and PKA that lead to chondrocyte proliferation and the delay of differentiation are incompletely understood. One possible target is SOX9. Phosphorylation of the chondrocyte transcription factor, SOX9 (27), is regulated by PTHrP and PKA. Phosphorylated SOX9 activates target genes more efficiently than does unphosphorylated SOX9. Since SOX9 acts to slow the differentiation of chondrocytes, SOX9 phosphorylation probably contributes to the action of PTHrP. PTHrP also decreases expression of the *p57* gene in chondrocytes (28). p57 is a

member of the CIP/KIP family of inhibitors of cyclin-dependent kinases, which also includes p21 and p27. Ablation of p21 or p27 has little effect on the fetal growth plate, but ablation of p57 leads to increased proliferation and delayed differentiation of chondrocytes in a way that partly resembles the actions of PTHrP (29). Strikingly, in mice missing both the *PTHrP* and the *p57* gene, many growth plates, including those of the ribs, sternum, and ulna, exhibit proliferation and differentiation patterns much closer to normal than mice lacking only *PTHrP* (28). This result, combined with the action of PTHrP to lower levels of p57 mRNA and protein in chondrocytes, is consistent with the hypothesis that suppression of p57 synthesis is a major mechanism used by PTHrP to maintain chondrocyte proliferation and delay chondrocyte differentiation. Recent experiments demonstrate that PTHrP also decreases production of the transcription factor, Runx2, in fetal chondrocytes (but not in fetal osteoblasts) (30)**.** Since Runx2 is required for the hypertrophic differentiation of chondrocytes in most bones, the suppression of Runx2 production by PTHrP probably contributes to the delayed differentiation of chondrocytes. Figure 2 summarizes the actions of PTHrP just discussed. Undoubtedly, this figure is incomplete, since, as in many other systems, activation of cell membrane G protein-coupled receptors activates a complicated array of genetic programs.

Regulation of PTHrP Production

Indian hedgehog (Ihh) is a major regulator of PTHrP production in the growth plate. In the absence of Ihh, no synthesis of PTHrP mRNA can be detected in fetal bones, and chondrocytes in these bones exhibit the accelerated differentiation expected in the absence of PTHrP (31). Further, the genetic introduction of constitutively active PTH/PTHrP receptors into *Ihh* (-/-) mice reverses the accelerated differentiation of chondrocytes (12). Exactly how Ihh stimulates PTHrP production is uncertain. Ihh is synthesized by prehypertrophic and hypertrophic chondrocytes just after chondrocytes have stopped dividing (32). Ihh binds to Patched (Ptc), a receptor on the cell surface. Ptc, in the absence of Ihh, acts to suppress the activity of Smoothened (Smo), a second membrane protein. When Ihh binds Ptc, Smo then initiates further intracellular signaling (33). Ihh clearly activates proliferating chondrocytes that are adjacent to prehypertrophic cells that synthesize Ihh. These flat, proliferating chondrocytes do not synthesize PTHrP, however. Whether Ihh reaches chondrocytes at the ends of bones that do synthesize PTHrP, or instead initiates a more complicated cascade that indirectly leads to PTHrP synthesis at the ends of fetal bones is unknown. Whatever the pathway, the interactions of Ihh and PTHrP provide a powerful mechanism for precisely regulating chondrocyte proliferation and differentiation (Figure 3). PTHrP is produced by cells at the ends of bones and acts on nearby chondrocytes bearing PTH/PTHrP receptors to maintain their proliferation and delay their differentiation (10). Chondrocytes sufficiently far away from the ends of bones, however, stop proliferating and then and only then synthesize Ihh. Directly or indirectly, this Ihh signals back to the end of the growth plate to stimulate PTHrP synthesis. Ihh also has a number of actions that are independent of PTHrP (Figure 3). Ihh stimulates chondrocyte proliferation and directs adjacent perichondrial cells to become osteoblasts (34;35). In the absence of such signaling, perichondrial cells instead can become chondrocytes. Further, Ihh accelerates the conversion of round proliferating chondrocytes to flat columnar chondrocytes (11). Thus, together, PTHrP and Ihh regulate multiple steps in the development of the growth plate. Ihh stimulates PTHrP production and accelerates the conversion of round chondrocytes to flat columnar chondrocytes. PTHrP acts to maintain chondrocyte proliferation and delay Ihh production and chondrocyte differentiation. PTHrP and Ihh together, therefore, determine both the entry of chondrocytes into, and the exit of chondrocytes out of, the pool of flat proliferating chondrocytes. Further, by determining the site of Ihh production, PTHrP also determines the site of osteoblast production by the perichondrium.

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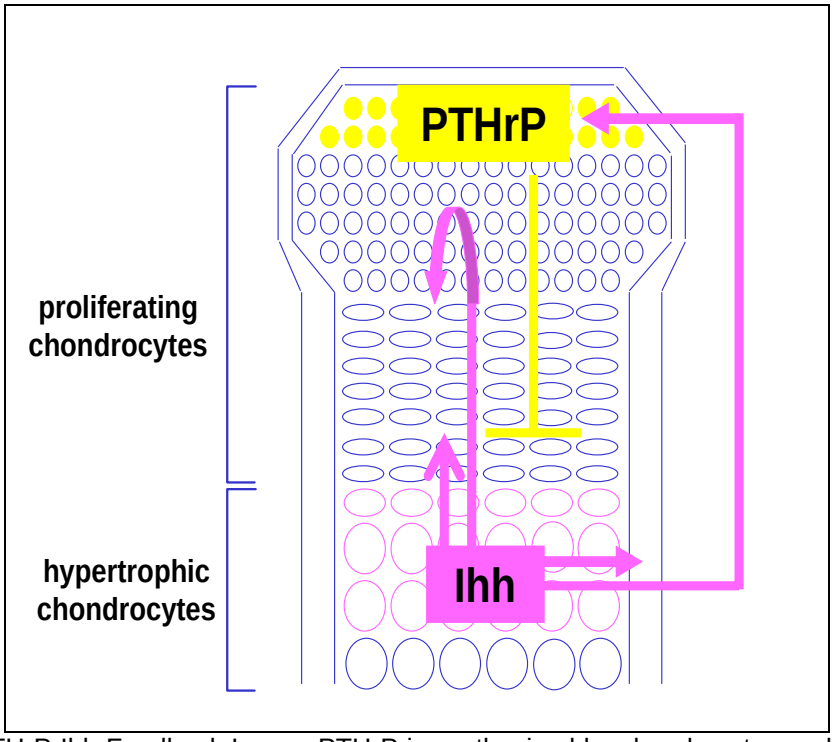

Figure 3: PTHrP-Ihh Feedback Loop**.** PTHrP is synthesized by chondrocytes and perichondrial cells at the ends of the developing bones. PTHrP maintains chondrocyte proliferation and delays chondrocyte differentiation into pre-hypertrophic and hypertrophic chondrocytes. After chondrocytes stop proliferating, they synthesize Indian hedgehog (Ihh). Ihh acts to increase the synthesis of PTHrP, to accelerate the differentiation of round proliferative chondrocytes into flat proliferating chondrocytes, to increase the rate of proliferation of adjacent chondrocytes, and to direct perichondrial cells to differentiate into osteoblasts.

Perspective

As noted earlier, multiple signaling pathways act together to regulate bone development. Pathways such as the fibroblast growth factor (FGF) pathway have profound effects on chondrocyte proliferation and differentiation at multiple stages. In addition to directly decreasing chondrocyte proliferation and accelerating chondrocyte differentiation, FGF signaling suppresses Ihh expression (36). In that fashion, the two signaling pathways act in concert. These complicated interactions emphasize the importance of studying the actions of pathways in the context of intact bone. Such studies have been made possible by genetic manipulations, such as those described here. The resulting picture of PTHrP actions is likely to typify some actions of PTHrP in other tissues as well. Determining the role of PTHrP in the human post-natal growth plate and in articular cartilage, both in health and in disease, are important topics for future research.

Conflict of Interest: The author has declared that no conflict of interest exists.

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