PTHrP and Skeletal Development

HENRY M. KRONENBERG

Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts 02114, USA

ABSTRACT: Parathyroid hormone-related protein (PTHrP) participates in the regulation of endochondral bone development. After the cartilage mold is established in fetal life, perichondrial cells and chondrocytes at the ends of the mold synthesize PTHrP. This ligand then acts on PTH/PTHrP receptors on chondrocytes. As chondrocytes go through a program of proliferation and then further differentiation into postmitotic, hypertrophic chondrocytes, PTHrP action keeps chondrocytes proliferating and delays their further differentiation. Indian hedgehog (Ihh) is synthesized by chondrocytes that have just stopped proliferating and is required for synthesis of PTHrP. The feedback loop between PTHrP and Ihh serves to regulate the pace of chondrocyte differentiation and the sites at which perichondrial cells first differentiate into osteoblasts. Activation of the PTH/PTHrP receptor leads to stimulation of both G_s and G_q family heterotrimeric G proteins. Genetic analyses demonstrate that G_s activation mediates the action of PTHrP to keep chondrocytes proliferating, while G_q activation opposes this action. Downstream from G_s activation, synthesis of the cyclin-cdk inhibitor, p57, is suppressed, thereby increasing the pool of proliferating chondrocytes. PTHrP's actions to delay chondrocyte differentiation are mediated by the phosphorylation of the transcription factor, SOX9, and by suppression of synthesis of mRNA encoding the transcription factor, Runx2. These pathways and undoubtedly others cooperate to regulate the pace of differentiation of growth plate chondrocytes in response to PTHrP.

KEYWORDS: parathyroid hormone-related protein; growth plate; chondrocytes; Indian hedgehog

INTRODUCTION

Investigators studying the hypercalcemia of malignancy discovered parathyroid hormone-related protein (PTHrP) as the cause of that syndrome, when the protein is secreted into the circulation.[1] That discovery led to a series of studies exploring the physiologic roles of PTHrP. PTHrP was shown to be synthesized in many types of cells, to activate the same receptor activated by PTH, and, to

Address for correspondence: Henry M. Kronenberg, Endocrine Unit, Massachusetts General Hospital, Thier 501, 50 Blossom St., Boston, MA 02114. Voice: 617-726-3966; fax: 617-726-7543.
e-mail: hkronenberg@partners.org

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exhibit a variety of actions ranging from relaxation of many smooth muscle beds to regulation of cellular proliferation and differentiation.[2] The importance of many of these actions of PTHrP has been established by analyses of the phenotypes of mice missing the *PTHrP* gene,[3] the *PTH/PTHrP receptor* gene,[4] and the *PTH* gene.[5] The mice missing PTHrP die at birth because all bones formed by endochondral bone formation develop improperly.[6,7] As a result, the rib cage is small and inappropriately mineralized, leading rapidly to death. Further studies have shown that PTHrP regulates the proliferation and differentiation of growth plate chondrocytes. These actions typify local actions of PTHrP in many organs. The similarities of the growth plates of *PTHrP* (−/−) mice and those of *PTH/PTHrP receptor* (−/−) mice bring genetic support for the idea that the PTH/PTHrP receptor mediates the actions of PTHrP on the growth plate. Here I will review the actions of PTHrP in the growth plate, the way that PTHrP signaling is coordinated with signaling in other pathways, and initial genetic studies that explore the intracellular mechanisms of PTHrP action.

ENDOCHONDRAL BONE FORMATION

The development of all bones begins with the formation of mesenchymal condensations. Groups of mesenchymal cells draw close together and initiate a characteristic genetic program. In a few bones, such as the flat bones of the skull, these condensations then differentiate directly into osteoblasts. In most bones, however, mesenchymal cells differentiate into chondrocytes and an adjacent perichondrium. The chondrocytes then direct the differentiation of perichondrial cells into osteoblasts, through a process called endochondral bone formation.[8] FIGURE 1 illustrates the steps in endochondral bone formation. After the formation of condensations (FIG. 1B), the cells enlarge and form round chondrocytes (FIG. 1C). These chondrocytes proliferate and secrete a matrix rich in collagen type II and aggrecan. 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 the surrounding matrix, signal to adjacent perichondrial cells to direct their differentiation into osteoblasts, and also stimulate the invasion of blood vessels. With vascularization, preosteoblastic cells from the perichondrial region also invade the cartilage to begin formation of the first true bone inside the cartilage mold called the primary spongiosa (FIG. 1E,F). Osteoclasts, cells derived from the hematopoietic lineage, also enter the cartilage mold and, together with the cells of the osteoblastic lineage, digest the matrix that had been synthesized by the hypertrophic chondrocytes. These chondrocytes die through apoptosis at the border of the cartilage and primary spongiosa. At the ends of the cartilage mold, round chondrocytes continue to

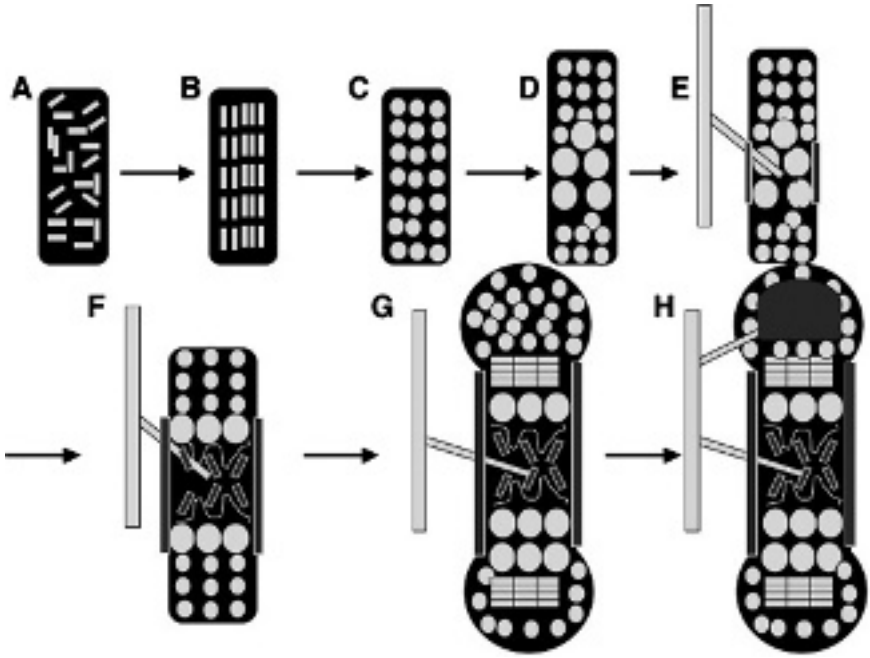

FIGURE 1. Endochondral bone formation. The sequential steps in endochondral bone formation are illustrated. (**A**) Mesenchymal cells. (**B**) Mesenchymal cells move close together to form a condensation. (**C**) Mesenchymal cells differentiate into chondrocytes. (**D**) Chondrocytes in the middle of the cartilage mold stop proliferating and become hypertrophic chondrocytes. (**E**) Hypertrophic chondrocytes induce vascularization and formation of a bone collar in the adjacent perichondrium. (**F**) Osteoblasts differentiate from cells brought into the cartilage mold with vascular invasion, forming the primary spongiosa. (**G**) Chondrocytes continue to proliferate, forming columns of flat chondrocytes. (**H**) Secondary ossification centers form.

proliferate. Those closest to the hypertrophic chondrocytes flatten out and form orderly columns of still proliferating chondrocytes (FIG. 1G). The cells closest to the hypertrophic chondrocytes stop proliferating (prehypertrophic cells) and subsequently hypertrophy. Bone lengthening is caused by the increase in chondrocyte number, the synthesis of chondrocyte matrix, and the substantial enlargement of chondrocytes that occurs during hypertrophy. Subsequently, secondary sites of ossification form. Chondrocytes in the center of the region of round proliferative chondrocytes stop dividing, hypertrophy, and attract a new, vascularized primary spongiosa. In this way, the sequence of endochondral bone formation is recapitulated (FIG. 1H). Chondrocytes between the primary and secondary ossification center form a true growth plate that persists 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, multiple signaling pathways are required to regulate these complicated processes. These pathways include those directed by fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), Indian hedgehog (Ihh), C-type natriuretic protein (CNP), insulin-like growth factors (IGFs), wingless-int family members (wnts), ligands for nuclear receptors (retinoic acid, glucocorticoids, thyroid hormone, estrogens, androgens, and PTHrP). Here I will focus on PTHrP, but mention other pathways to emphasize that the actions of PTHrP can only be understood in the context of a complicated network of interacting signaling pathways.

ACTIONS OF PTHrP ON THE FETAL GROWTH PLATE

In fetal mouse bone PTHrP is synthesized by perichondrial cells and chondrocytes at the ends of the growing bones[9] (shown schematically in FIG. 2). Early in bone development, these cells appear to be the only cells in bone competent to synthesize PTHrP. Even when PTHrP production is stimulated,

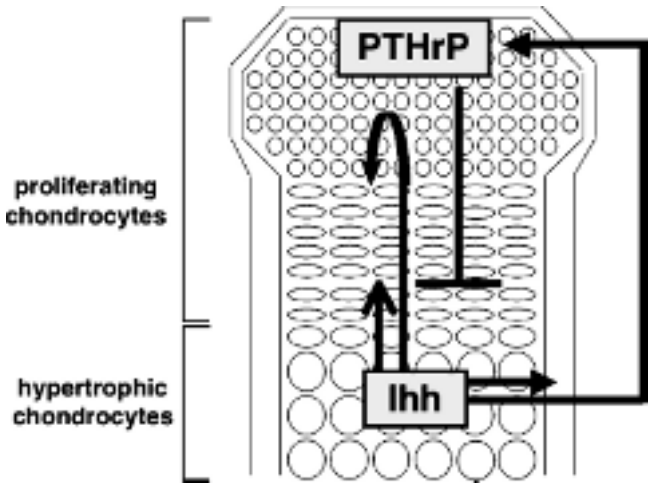

FIGURE 2. PTHrP-Ihh feedback loop. PTHrP is synthesized by chondrocytes and perichondrial cells at the ends of the developing bones. PTHrP acts on chondrocytes to keep the chondrocytes proliferating and to delay the differentiation of the chondrocytes into prehypertrophic and hypertrophic chondrocytes. After chondrocytes stop proliferating, they then synthesize (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.

for example, by various genetic tricks that increase the synthesis of Ihh in fetal bone, only perichondrial cells and round chondrocytes at the ends of the bones increase their production of PTHrP.[10] PTHrP then diffuses away from its site of production and binds to PTH/PTHrP receptors on nearby chondrocytes.[7] Proliferating chondrocytes express low numbers of PTH/PTHrP receptors, but increase receptor expression dramatically as the cells stop proliferating. PTHrP acts on chondrocytes bearing PTH/PTHrP receptors to keep the chondrocytes proliferating and to delay their conversion into prehypertrophic and then hypertrophic chondrocytes. PTHrP also modestly increases the rate of chondrocyte proliferation, though this action is only detectable early during fetal development.[11]

The phenotypes of mice with a variety of mutant genes support this model of PTHrP action. Mice missing either the PTHrP or the PTH/PTHrP receptor genes undergo the normal endochondral sequence of proliferation and hypertrophy, except that columns of proliferating chondrocytes are much shorter than normal or even nonexistent (FIG. 3). Further, in chimeric mice with some chondrocytes missing the PTH/PTHrP receptor, the mutant chondrocytes become hypertrophic much sooner than their normal counterparts.[12] Conversely, mice overexpressing PTHrP in chondrocytes through a transgenic strategy are born with bones composed almost exclusively of proliferating chondrocytes, with a profound delay in the endochondral sequence.[13] Similar findings occur in mice expressing a constitutively active PTH/PTHrP receptor in

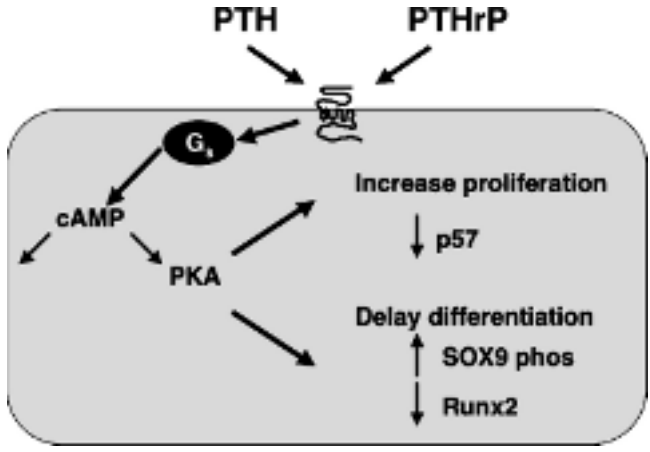

FIGURE 3. Intracellular signaling by the PTH/PTHrP receptor. Activation of the PTH/PTHrP receptor leads to activation of multiple G proteins. The activation of G_s leads to subsequent activation of adenylate cyclase and generation of cyclic AMP. Cyclic AMP has several actions, including the activation of protein kinase A (PKA). PKA then, by unknown mechanisms, leads to a fall in p57 and Runx2 mRNA and protein. PKA also phosphorylates SOX9, increasing the activity of SOX9.

chondrocytes.[14] Both of these transgenic constructs can prevent the neonatal death of *PTHrP* (−/−)[14,15]; this observation justifies the conclusion that the PTHrP (−/−) mice die at birth because of their skeletal abnormalities. Presumably, the rib cage, with its smaller circumference than normal, does not allow adequate respiration. Though no humans have been identified with absent PTHrP production, human fetuses with defective or absent PTH/PTHrP receptors (Blomstrand chondro-osteodystrophy) die *in utero* with skeletal abnormalities closely resembling those of the PTH/PTHrP receptor (−/−) mouse.[16,17] Furthermore, humans with Jansen chondro-osteodystrophy have point mutations in the gene encoding the PTH/PTHrP receptor that render the receptor active even in the absence of ligand.[18] Such people have growth abnormalities like those predicted from the actions of PTHrP in fetal mouse bone. These changes lead to delay in hypertrophic chondrocyte differentiation and resultant short stature. Thus, it is likely that the fetal mouse growth plate resembles that of the human in the way that PTHrP regulates chondrocyte proliferation and differentiation.

INTERACTIONS BETWEEN IHH AND PTHrP

Ihh is a major regulator of PTHrP synthesis in the growth plate. In the absence of Ihh, PTHrP mRNA levels are undetectable in fetal bone.[19] The chondrocytes in these bones exhibit accelerated differentiation, as would be in bones that lack PTHrP. In addition, the genetic introduction of constitutively active PTH/PTHrP receptors, using a transgenic construct that uses the collagen IIα (1) promoter to drive expression of these receptors, into Ihh (−/−) mice leads to reversal of the accelerated differentiation of chondrocytes.[11] The pathway used by Ihh to stimulate the production of PTHrP is unknown. Ihh is synthesized by prehypertrophic and hypertrophic chondrocytes just after chondrocytes stop proliferating.[20] Ihh binds to patched (ptc), a membrane protein that keeps another membrane protein, smoothened (smo) from reaching the plasma membrane and mediating further actions of Ihh.[21] Smoothened action then leads to an increase of active forms of gli transcription factors in the nucleus and a decrease in proteolytically cleaved, inhibitory forms of gli in the nucleus. Activation of the hedgehog pathway usually leads to increased synthesis of ptc mRNA. Such an increase in ptc mRNA is easy to detect in proliferating chondrocytes adjacent to those synthesizing Ihh, but not clearly evident in cells synthesizing PTHrP at the ends of the fetal bones. This observation and the considerable distance between Ihh-producing and PTHrP-producing cells raise the possibility that Ihh might not act directly on chondrocytes synthesizing PTHrP. Some data, for example, suggest that blockade of transforming growth factor (TGF)-β signaling can decrease PTHrP synthesis in response to Ihh.[22] Thus, the TGF-β signaling pathway may partly mediate stimulation of PTHrP synthesis by Ihh. Further, the movement of Ihh must be regulated in a

complicated manner, given that the active form of Ihh is heavily lipid-modified, with a palmitate group at its amino terminus and a cholesterol moiety on its carboxy-terminus.[23] Whatever the mechanism whereby Ihh regulates PTHrP synthesis, the interactions of Ihh and PTHrP provide a powerful pathway for precisely regulating the proliferation and differentiation of chondrocytes in the growth plate (FIG. 2). PTHrP is produced by cells at the ends of bones[9] and acts on nearby chondrocytes bearing PTH/PTHrP receptors to keep them proliferating and delay their differentiation. Chondrocytes sufficiently far away from the source of PTHrP, however, stop proliferating and then and only then synthesize Ihh. This Ihh then, directly or indirectly, signals back to the end of the growth plate to stimulate the synthesis of more PTHrP. This feedback system, thus, determines the site at which chondrocytes stop proliferating and start making Ihh.

Such a feedback loop might be expected not only to determine the length of the zone of chondrocyte proliferation but might also contribute to the striking uniformity of chondrocyte morphology across the growth plate: the columns of proliferating chondrocytes quite uniformly end in a smooth line that separates the prehypertrophic and hypertrophic chondrocytes from the columns. In both the PTHrP (−/−) and the PTH/PTHrP receptor (−/−) mice, the early hypertrophic chondrocytes unevenly overlap with columnar chondrocytes.[6,24] This unevenness is an expected result of the absence of an effective feedback mechanism for determining the precise site at which proliferation stops. Presumably, in the normal setting, if, stochastically, a columnar chondrocyte stops proliferating sooner than otherwise expected, it will synthesize Ihh closer to the top of the growth plate than otherwise. This Ihh would be expected, then, to stimulate the production of an increased amount of PTHrP (because the Ihh-producing cell is closer to the top of the growth plate than average), and that PTHrP would act to keep nearby chondrocytes proliferating. This process would be expected to increase the uniformity of the sites at which the columns of proliferating chondrocytes across the growth plate choose to initiate the process of hypertrophy.

Ihh has a number of actions that are independent of PTHrP (FIG. 2). Ihh stimulates proliferation of chondrocytes and directs adjacent perichondrial cells to become osteoblasts.[25,26] Interestingly, this action of Ihh is not needed during intramembranous bone formation in the skull. In the absence of such signaling during endochondral bone formation, however, perichondrial cells can become chondrocytes. Further, Ihh stimulates the conversion of round proliferating chondrocytes to flat columnar chondrocytes.[10] Since the flat proliferating chondrocytes form columns that extend in the longitudinal axis of long bones, this action of Ihh serves to reinforce the asymmetry of bone growth in the long bones. Thus, together, PTHrP and Ihh regulate multiple steps in the development of the growth plate. Ihh stimulates the production of PTHrP and accelerates the conversion of round chondrocytes to flat columnar chondrocytes. PTHrP acts to keep chondrocytes proliferating and delays the production

of Ihh and the further differentiation of chondrocytes. PTHrP and Ihh together, therefore, determine both the entry and the exit of chondrocytes from the pool of flat proliferating chondrocytes. Further, by determining the site of Ihh production, PTHrP thus also determines the site at which perichondrial cells first become true osteoblasts in the bone collar adjacent to the growth plate.

INTRACELLULAR PATHWAYS ACTIVATED BY PTHrP IN FETAL GROWTH PLATE 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 morphologically obvious actions of PTHrP to regulate the growth plate are via activation of the PTH/PTHrP receptor. Having said that, PTHrP also clearly activates receptors distinct from the PTH/PTHrP receptor in other settings (such as the stimulation of placental calcium transport[27]) and also has actions directly within cell nuclei that have been documented *in vitro*.[28] Additional studies will be needed to determine the contributions of these other pathways to the actions of PTHrP on the growth plate. Binding of PTHrP to the PTH/PTHrP receptor stimulates multiple heterotrimeric G proteins,[29] including G_s, the G_q family, and $G_{12,13}$.[30] Activation of each G protein triggers a series of downstream events that culminate in obvious biological consequences. The relative stimulation of each of the distinct signaling pathways varies among cell types, as do the physiologic consequences of this activation. Furthermore, the activities of each intracellular pathway are influenced by pathways triggered by signals from adjacent cells and from matrix proteins, each distributed asymmetrically in the growth plate. Therefore, to make sure that we study the actions of PTHrP in a setting relevant to the *in vivo* setting, we have used genetic tools to identify the roles of activation of the G_s and G_q pathways by PTHrP in the fetal growth plate. To determine to role of G_s signaling, we took advantage of technology that allows the generation of chimeric mice with cellular contributions from genetically engineered embryonic stem (ES) cells and from normal hosts. Ung-il Chung mated mice heterozygous for ablation of the second exon of the gene encoding $G_s\alpha$ and derived ES cells homozygous for the $G_s\alpha$ ablation from resultant blastocysts.[31] He then produced chimeras that contained normal chondrocytes, as well as chondrocytes missing the gene encoding $G_s\alpha$. The chondrocytes missing $G_s\alpha$ stopped proliferating prematurely and became hypertrophic chondrocytes close to the top of the growth plate. In that way they closely resembled chondrocytes missing the PTH/PTHrP receptor generated using analogous chimeric mice.[12] From this similarity and other experiments we conclude that the PTH/PTHrP receptor uses G_s activation to keep chondrocytes proliferating. Experiments were subsequently performed using mice that have mutant PTH/PTHrP receptors that cannot activate the G_q family of G proteins but can activate G_s normally.[32] These mice were generated

by taking advantage of information about a PTH/PTHrP receptor that exhibits such signaling after introduction of mutations in the sequence encoding the second intracellular loop of the receptor.[33] The mutant sequence was substituted for the normal sequence using homologous recombination to generate a "knock-in" mouse. By examining the bones of mice with normal G_s signaling but absent G_q signaling in response to activation of the PTH/PTHrP receptor, we could deduce actions of the G_q pathway in chondrocytes. These studies showed that activation of G_q by the PTH/PTHrP receptor mildly accelerates chondrocyte differentiation. Thus, activation of G_s and G_q by activation of the PTH/PTHrP receptor in chondrocytes leads to actions that oppose each other. The usefulness of this seemingly wasteful opposition of the two pathways is not certain. As in many other settings in which a stimulus activates opposing pathways, this complicated pattern may allow regulatory interactions with other pathways that are useful. Furthermore, the particular properties of the PTH/PTHrP receptor suggest another consequence of the activation of both G_s and G_q by the PTH/PTHrP receptor in chondrocytes. Since activation of the G_q pathway generally requires higher concentrations of PTHrP than does activation of the G_s pathway,[34] one might predict that, chondrocytes nearer to the source of PTHrP might respond more dramatically with G_q activation than chondrocytes further from the source of PTHrP. Thus, chondrocytes nearer to the top of the growth plate, where PTHrP is synthesized, would be expected to have G_q signaling oppose the actions of G_s. As the source of PTHrP moves further away from the proliferating chondrocytes, however, one would predict a more rapid loss of G_q activation than of G_s activation. The net effect would be to allow PTHrP to prolong proliferation more dramatically than otherwise expected, as the concentration of PTHrP falls. These contrasting responses of chondrocytes depending on their locations in the growth plate may help smooth the functional gradient of PTHrP action across the growth plate.

G_s activation leads to production of cyclic AMP and activation of protein kinase A (PKA). The actions of cyclic AMP and PKA that lead chondrocytes to continue proliferating and delay differentiation are incompletely understood. PTHrP and PKA are the major sources of phosphorylation of the chondrocyte transcription factor, SOX9.[35] 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 to delay chondrocyte differentiation. PTHrP also decreases expression of the p57 gene in chondrocytes both in bone explants and *in vivo*, as deduced from measuring levels of p57 mRNA and protein in mice missing the *PTHrP* gene.[36] p57 is a member of the CIP/KIP family of inhibitors of cyclin-dependent kinases; this family also includes p21 and p27. Ablation of the p21 or p27 genes has little effect on the fetal growth plate, but ablation of the gene encoding p57 leads to an increase in proliferation and delay of differentiation of chondrocytes that partly resembles the phenotype of *PTHrP* (−/−) mice.[37] Strikingly, in double knockout mice missing

both the *PTHrP* and the *p57* gene, many growth plates, including those of the ribs, sternum, and ulna, exhibit patterns of proliferation and differentiation much closer to normal than in the *PTHrP* (−/−) growth plates.[36] This result is consistent with the hypothesis that suppression of p57 synthesis is a major mechanism used by PTHrP to keep chondrocytes proliferating and delay their differentiation. Recent experiments demonstrate that PTHrP also decreases the production of the transcription factor, Runx2, in chondrocytes (but not in osteoblasts) in explants of fetal long bones.[38] In such explants, PTH administration rapidly leads to a fall in Runx2 mRNA and protein in chondrocytes. Since Runx2 is required for the hypertrophic differentiation of chondrocytes in most bones, the action of PTHrP to suppress Runx2 production probably contributes to the delay in the differentiation of chondrocytes caused by PTHrP. FIGURE 3 summarizes the actions of PTHrP just discussed. Undoubtedly, this figure is incomplete, since the multiple signaling cascades downstream from the PTH/PTHrP receptor must modify the actions of large numbers of pathways.

CONCLUSIONS

The focus here on the actions of PTHrP in the growth plate does not imply either that these actions are the most important actions of PTHrP or that PTHrP is the most important regulator in the growth plate. Certainly, multiple signaling systems are together required for proper coordination of bone development. The actions of PTHrP can only be understood in the context of these other signaling systems. Further, the presence of PTHrP and PTH/PTHrP receptors in virtually every organ at various times during development and adult life suggests that PTHrP signaling has actions in multiple physiologic settings. Whether hedgehog signaling regulates PTHrP signaling in other settings is currently unknown. It seems likely that actions of PTHrP to, for example, relax smooth muscle will use intracellular pathways that differ in important respects from those outlined here. Thus, while the growth plate may well serve as a useful model for the actions of PTHrP to regulate proliferation and differentiation in some settings, it seems likely that the varied sites of PTHrP action will reveal a broad array of cell-specific responses. The study of genetically altered mice will continue to provide powerful tools for establishing the physiologic relevance of proposed actions of PTHrP.

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