COMMENTARIES

A Cup Half-Full or Half-Empty? When PTHrP Levels Matter

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Parathyroid hormone (PTH)-related protein (PTHrP), the protein encoded by *PTHLH*, is a developmental regulatory molecule first isolated from tumors associated with the paraneoplastic syndrome of humoral hypercalcemia of malignancy (1). PTHrP shares homology with PTH 1–34 and, as a consequence, this peptide binds and activates PTHR1 (2). PTHR1 is responsible for mediating the pleiotropic paracrine effects of PTHrP as well as the endocrine actions of PTH on calcium and skeletal homeostasis (1;3;4). As a result, PTHR1 mutations, but not *PTHLH* mutations, can be associated with abnormal regulation of calcium and phosphorus metabolism. The role of the PTHrP/PTHR1 signaling pathway as an essential regulator of endochondral bone development became apparent through the analysis of genetically manipulated mice (5;6). Analysis of PTHrP gene-manipulated mice has demonstrated that, in addition to endochondral bone formation, PTHrP acts by regulating epithelial-mesenchymal interactions during organogenesis of certain epithelial organs, including the skin, mammary glands, and teeth (1;3;4). PTHR1 mutations have been identified in several skeletal disorders. Dominant activating mutations cause Jansen's metaphyseal chondrodysplasia (JMC) (7), whereas recessive inactivating mutations are found in both Blomstrand's lethal chondrodysplasia (BLC) (7), and Eiken syndrome (8). Heterozygous PTHR1 mutations (either germline or somatic mutations in tumor tissue) have also been found in some, but not all, cases of Ollier disease (9;10). In contrast, *PTHLH* had not yet been directly linked to human disease. Two groups have now identified *PTHLH* mutations that render PTHrP haploinsufficient, resulting either from *cis*-directed *PTHLH* downregulation (11) or loss-of-function mutations within the *PTHLH* gene (12), causing autosomal-dominant Brachydactyly Type E (BDE) with short stature. As predicted from the rare human syndromes associated with mutations in the *PTHR1* gene, BDE caused by *PTHLH* haploinsufficiency leads to a similar phenotype, albeit less severe with variable expressivity. These results extend and refine our understanding of the phenotypic consequences of defects in the PTHrP/PTHR1 signaling pathway, and underscore the need for biallelic PTHrP expression for normal pre- and post-natal bone development and growth.

BDE is characterized by a general shortening of metacarpals and metatarsals and/or phalanges (13;14). The phenotype is variable within families, ranging from a moderate shortening of individual metacarpals to a shortening of all bones in the hands and/or feet. BDE can be isolated or occur within a syndrome. Isolated BDE has been associated in sporadic cases with microdeletions of 2q37, as well as with mutations in HOXD13, while syndromic familial BDE is frequently associated with *GNAS* haploinsufficiency (15). However, the genetic cause of the majority of BDE cases remains unexplained.

In a family affected with autosomal-dominant BDE, Maass *et al*. (11) identified a balanced t(8;12)(q13;p11.2) translocation with breakpoints (BPs) upstream of *PTHLH* on chromosome 12p11.2. Through a series of elegant experiments, the group clearly demonstrated that the t(8;12)(q13;p11.2) translocation leads to a *cis*-regulated down-regulation of *PTHLH* expression that causes BDE in the family. First, the authors determined by nucleotide sequence analysis that the derivative chromosome 8 BP [der(8) BP] is located approximately 86,000 kb upstream of *PTHLH* exon 1 in a highly conserved region harboring a putative Activator protein 1 (AP-1) binding site together with a bidirectional EBS/C-ets-1 binding motif translocated from 8q13. Indeed, they showed by EMSA that AP-1 and C-ets-1 bind at the der(8) BP, raising the possibility that the introduction of the novel C-ets-1 site may influence differential regulation of *PTHLH* during chondrogenesis. They subsequently confirmed this possibility using several complementary strategies. They showed: (i) an enrichment in regulatory histone modifications H3K4me1 and H3K4me3 in fibroblasts of a BDE patient comparing the der(8) BP affected and wt(12) unaffected alleles, indicating the specific occurrence of epigenetic modifications at the translocation BP; (ii) a significant enrichment in the transcription factors AP-1 (c-Jun) and C-ets-1 *in vivo* binding at der(8) BP compared to wt(12) alleles in chondrogenically differentiated fibroblasts of a BDE patient; (iii) a down-regulation of *PTHLH*, ADAMS-7 and ADAMS-12

expression in fibroblasts undergoing chondrogenic induction from a BDE patient compared to that of fibroblasts from unaffected controls; (iv) a decrease of *PTHLH* promoter activity by der(8) BP or C-ets-1 using human and murine chondrocytes.

Klopocki *et al*. (12) identified by CGH array a microdeletion on chromosome 12p encompassing six genes, among them *PTHLH*, in a family affected with autosomal dominant BDE and short stature. The phenotype in the family was variable, but complete penetrance was observed. Six of eight affected individuals presented with short stature; learning difficulties were present in all affected members. Because *PTHLH* was the only one of the deleted genes known to play a critical role in skeletal development, Klopocki *et al*. searched for mutations in the *PTHLH* gene in additional individuals with BDE and short stature, and were able to identify two missense (L44P and L60P), a nonstop (X178WextX*54), and a nonsense (K120X) mutation. The missense mutation L60P was tested by chicken limb bud micromass culture (16) and was shown to result in loss-of-function, as indicated by a reduced stimulation of alkaline phosphatase activity, used as an indicator of late-stage chondrocyte differentiation.

In both studies, the mutations segregated with the disorder and were not found in control alleles. Thus, mutations that lead to haploinsufficiency of *PTHLH* result in BDE with short stature.

PTHLH Haploinsufficiency and Skeletal Abnormalities

Studies in mice have established that during endochondral bone formation, PTHrP acting on its receptor PTHR1 facilitates the continuous proliferation of chondrocytes in the growth plate, and postpones their programmed differentiation into hypertrophic chondrocytes. Conversely, the lack of PTHrP accelerates the normal differentiation process of growth plate chondrocytes (5;6). Thus resting and proliferating chondrocytes undergo fewer cycles of cell division and

differentiate prematurely into hypertrophic cells, which then undergo apoptosis and are subsequently prematurely replaced by invading osteoblasts. Illustrating these functions, animals that are "null" for PTHrP or the PTHR1 receptor die *in utero* or shortly after birth, and show a profound acceleration of growth plate mineralization. In contrast, mice overexpressing PTHrP under the control of a growth plate-specific promoter are viable, but show a severe delay in chondrocyte maturation, which leads to impaired bone growth and elongation. In humans, activating and inactivating PTHR1 mutations cause JMC and BLC, characterized by delayed and advanced bone formation, respectively (7). Thus, too much or too little PTHrP/PTHR1 signaling in the growth plate leads to short-limbed dwarfism, but through entirely different mechanisms.

In the family studied by Maass *et al.* (11), the affected individuals had a final height in the lower range of normal, presented with a shortening of the extremities compared to the trunk, and had a reduction of the ratio of arm span to height. In addition, affected patients showed dysmorphic facies with macrocephaly, a prominent forehead and a depressed nasal root, reminiscent of the phenotype observed in acrodysostosis (13). These features indicate the synchondroses of the bones forming the base of the skull, and the margins of the foramen magnum (chondrocranium) were also affected by premature ossification and unification, as described in mice and humans lacking both alleles of either *PTHLH* or *PTHR1* (5-7). Of the 13 affected individuals described by Klopocki *et al.* (12), 10 had short stature. The growth velocity of these 10 affected individuals appeared to slow down and stop prematurely, resulting in small stature. It is interesting to note that the long bones that appear to be the most affected are the metacarpals, metatarsals, and phalanges, which are long bones with a single growth plate, either at the distal (metacarpal and metatarsal) or proximal end. Thus, the BDE phenotype is compatible with insufficient PTHrP levels in the growth plate, altering chondrocyte proliferation and differentiation, leading to abnormal ossification and length

reduction in affected bones and premature closure of the growth plate. PTHrP haploinsufficient mice (heterozygous mice, lacking one copy of the PTHrP gene) were previously shown to be normal at birth, but exhibit abnormal postnatal bone development starting by 3 months of age, and display histologic and morphologic abnormalities similar to those seen in homozygous mutants (17). These results in humans and mice underscore that one copy of PTHLP is not sufficient to insure normal bone development and growth. This is in contrast to results with PTHR1, as PTHR1 haploinsufficient mice and humans [(18) and personal observation] do not display evidence for osteochondrodysplasia. Interestingly, a *de novo* duplication comprising the *PTHLH* gene has been recently associated with symmetrical enchondromatosis in a patient (Collinson M, *et al.*, *American Journal of Medical Genetics*, in press).

In addition to chondrocytes, PTHrP is also produced by osteoblasts and other bone cells (19;20) and is required for maintenance of adult skeletal mass in mice: haploinsufficiency (17) and conditional deletion of PTHrP in osteoblasts (21) in mice results in osteoporosis with apparently normal calcium and phosphorus homeostasis. In this context, PTHrP (N-terminal portion) has been proposed to exert specific and complementary effects to those of PTH in the control of bone remodeling (22). No evidence for osteoporosis was found in the families presented by Klopocki *et al.* (12) [measurement of bone density is not mentioned by Maass *et al.* (11)]. Assuming that PTHrP also regulates bone remodeling in humans, these findings suggest that, at least in these individuals, the reduced PTHrP levels are sufficient to maintain bone remodeling despite being insufficient for normal endochondral bone formation.

PTHLH Haploinsufficiency and Ectodermal Dysplastic Phenotypes

PTHrP gene-manipulated mice display ectodermal dysplastic phenotypes (3;4). Studies with transgenic mice, in which

PTHrP expression is targeted through a human keratinocyte-specific promoter (K14) to the developing epidermis and mammary gland, demonstrated that PTHrP also plays a critical role in hair follicle development and branching morphogenesis of the mammary gland. These conclusions were further supported by findings in PTHrP-null mice that had been rescued from neonatal death by targeting PTHrP expression to chondrocytes through the α1(II) collagen promoter. These rescued mice lack mammary epithelial ducts, due to a failure of the initial round of branching growth that is required for transforming the mammary bud into the primary duct system. Ablation of both copies of the *PTHR1* gene recapitulated the phenotype of PTHrP-ablated animals.

Using similar approaches, it was also demonstrated that PTHrP is required for normal tooth eruption. Teeth appeared to develop normally in rescued PTHrP knockout mice, but became trapped by the surrounding bone and underwent progressive impaction. In these tissues, PTHrP mRNA was identified by *in situ* hybridization in epithelial cells, while PTH/PTHrP receptor mRNA was found on mesenchymal or stromal cells. These observations led to the concept that the communication between epithelium and mesenchyme involves the PTH/PTHrP receptor, and that PTHrP signaling is essential for normal development of these tissues.

In humans, PTHR1 mutations that lead to haploinsufficiency of the receptor also result in defects in tooth eruption (18), while complete inactivation of PTHR1 causing BLC results in defects in mammary gland and tooth eruption (23). Klopocki *et al*. (12) report delayed tooth eruption and/or oligodontia and hypoplastic nails of the first fingers in some affected patients. In the study by Maass *et al*. (11), the index patient had no abnormalities in breast development or nursing, while this point is not discussed in the study by Klopocki *et al*. (12). However, we have identified a novel heterozygous mutation in the *PTHLH* gene in a mother and daughter affected with BDE; the

daughter presented with abnormal breast development (Agnès Linglart and Caroline Silve, unpublished results). Taken together, these studies establish that abnormalities in the described ectodermal functions of PTHrP on mammary gland development, tooth eruption and skin can also be present in patients with reduced expression of PTHrP, albeit with a variable expressivity.

Phenotype/Genotype Correlations

All patients affected with the *PTHLH* mutations in the study by Klopocki *et al*. (12) are expected to have "true" haploinsufficiency. The level of *in vivo* PTHrP expression in the patients explored by Maass *et al*. (11) cannot be assessed, and may be variable as a function of the tissues expressing PTHrP. Based on the results of their *in vitro* studies, however, it appears that at least chondrocytes display "true" haploinsufficiency. Thus, based on these reports, it is plausible that *PTHLH* haploinsufficiency is frequently associated with defects in endochondral bone formation and growth, albeit with a variable expressivity even within the same family. Ectodermal features linked to PTHrP/PTHR1 function are not obligatory and appear more variable. Contrary to what has been observed with the analysis of phenotypes associated with PTHR1 mutations in JMC and BLC, there does not seem to be a gradient of phenotypic signs correlated to levels of PTHrP expression.

Most PTHrP actions appear to be mediated following binding of the N-terminal portion to the PTHR1 receptor and stimulation of cAMP production (2;24). However, PTHrP most likely activates alternative receptors, and also exerts nuclear actions (25). Additional endocrine and paracrine functions have been ascribed to the mid-region and terminal portion of the molecule. Thus, it is possible that differences in the extent to which PTHrP mutations interfere with signaling through these pathways could contribute to the spectrum of phenotypic abnormalities seen in these patients. Alternatively, a host of other environmental, epigenetic or genetic factors may influence the phenotype. Further effort is required to

fully explain the variable phenotype seen in these patients.

Conditions with a Skeletal Phenotype Similar to That Observed in BDE and Other Defects in the PTHrP/PTHR1 Signaling Pathway

Defects upstream or downstream of PTHrP/PTHR1 interactions can be associated with a skeletal phenotype similar to that observed in BDE. It is interesting to compare the phenotype of these disorders with that seen in patients with abnormalities in expression or function of PTHrP.

Acrocapitofemoral dysplasia

PTHrP acting on PTHR1 is part of a tightly coupled signaling relay formed with Indian Hedgehog (IHH) acting on its receptor (PTCH1) that is critical for the regulation of chondrocyte differentiation and endochondral ossification (5). PTHrP regulates the switch from proliferating to hypertrophic chondrocytes, and thereby influences the number of cells expressing IHH. IHH is a member of a family of morphogen proteins that is highly expressed in the transition zone between proliferating and hypertrophic chondrocytes. PTHR1 and IHH signaling pathways are tightly coupled, although both exert functions independently of each other. A defect in IHH that affects its interaction with the PTHrP pathway may be expected to result in a phenotype similar to that in BDE. Mutations in the IHH gene have been identified in two chondrodysplasias, acrocapitofemoral dysplasia (ACFD), an autosomal recessive disorder with cone-shaped epiphyses in the hands and hips resembling some BDE features (26), and autosomal dominant brachydactyly type A1 (BDA1) (27). Interestingly, mutations causing ACFD and BDA1 have been identified in restricted non-overlapping regions of IHH, and are positioned on different domains of IHH in a tri-dimensional structural model (26;27).

Pseudohypoparathyroidism (PHP) and pseudoPHP (PPHP)

In target tissues, binding of PTH to PTHR1, its G protein-coupled receptor, activates Gsα, the alpha stimulatory subunit of the G protein, and induces the generation of cAMP, the major intracellular second messenger of the PTHrP/PTHR1 signaling pathway (2). Gsα is encoded by the *GNAS* locus, a complex imprinted locus (15). Epigenomic defects at the *GNAS* locus cause a series of diseases named pseudohypoparathyroidism (PHP) and pseudoPHP (PPHP). PHP type 1a (PHP1a), the most frequent form of PHP, and PPHP are caused, respectively, by maternally and paternally inherited Gsα loss-of-function mutations. These patients present with Albright Hereditary Osteodystrophy (AHO), a collection of physical features comprising brachymetacarpy, brachymetatarsy, short stature and ectopic ossification. Some variability can be observed in the clinical symptoms that define AHO. For instance, patients affected with PHP1a, but not with PPHP, also present with obesity and some degree of mental retardation (28;29). However, some degree of skeletal dysplasia (*i.e.*, the metacarpal and metatarsal abnormalities and short stature) is always present and is strongly reminiscent of that seen in BDE, including in its variable expressivity. Thus, Gsα and *PTHLH* haploinsufficiencies lead to similar skeletal phenotypes.

Acrodysostosis

In addition to BDE and short stature, affected patients described by Maass *et al*. (11) present with a chondrocranium phenotype that is not reported in the patients with PTHrP mutations, but that is also observed in acrodysostosis (13). The mechanism whereby the *cis*-regulatory site identified in the upstream portion of the *PTHLH* gene in these patients leads to this specific phenotype has not been elucidated, but may be related to a specific level of *PTHLH* downregulation occurring in these bones. The cause of acrodysostosis is not known; the similarities in the phenotypes for

patients affected by acrodysostosis or BDE indicate that the PTHrP/PTHR1 signaling pathway may be involved.

Conclusion

Dysregulation of the exquisite equilibrium in the PTHrP/PTHR1 signaling pathway, an important regulator of endochondral bone formation, appears to lead to a chondrodysplasia with short stature. Short stature can be the result of too much or too little signaling, and illustrates the absolute requirement for a controlled balance between chondrocyte proliferation and differentiation for harmonious growth. The skeletal phenotype illustrates the tight control exerted by the PTHrP/PTHR1 signaling pathway on chondrocyte fate. Although some genotype/phenotype correlations have been documented, more work is needed. While this emphasizes the importance of the temporospatial and quantitative patterns of expression of the ligand and its receptor, it also indicates that additional factors need to be considered. Clearly, the studies by Maass *et al*. (11) and Klopocki *et al*. (12) demonstrate that the quantitative amount of PTHrP, not merely its presence, is critical for both normal endochondral bone development and epithelial-mesenchymal interactions during organogenesis of certain organs. These studies also underscore that the amount of PTHrP necessary for normal development may differ as a function of the target tissue, and that the expression of decision-making molecules upstream and downstream of the PTHrP/PTHR1 signaling pathway also appear to modulate the requirement for PTHrP signaling.

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