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The multifaceted actions of PTHrP in skeletal metastasis

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Abstract

PTHrP, identified during the elucidation of mediators of malignancy-induced hypercalcemia, plays numerous roles in normal physiology as well as pathological conditions. Recent data support direct functions of PTHrP in metastasis, particularly from tumors with strong bone tropism. Bone provides a unique metastatic environment because of mineralization and the diverse cell populations in the bone marrow. PTHrP is a key regulator of tumor–bone interactions and regulates cells in the bone microenvironment through proliferative and prosurvival activities that prime the ‘seed’ and the ‘soil’ of the metastatic lesion. This review highlights recent findings regarding the role of PTHrP in skeletal metastasis, including direct actions in tumor cells, as well as alterations in the bone microenvironment and future perspectives involving the potential roles of PTHrP in the premetastatic niche, and tumor dormancy.

Keywords

bone; breast cancer; metastasis; parathyroid hormone-related protein; premetastatic niche; prostate cancer; PTHrP

PTHrP was first discovered as an etiological factor of humoral hypercalcemia of malignancy (HHM), commonly found in patients with certain types of advanced-stage cancers such as breast, lung, renal, ovarian and pancreatic carcinomas and myeloma [1,2]. An association between hypercalcemia and malignancy was first postulated in the 1920s with the development of the calcium assay [3], which allowed calcium measurement in patients with cancer. In 1941, Albright raised a hypothesis that ectopic parathyroid hormone (PTH) could be the tumor-derived factor responsible for HHM [4], owing to its important role in calcium homeostasis, which is a tightly regulated process involving coordination of bone, kidney, gut and parathyroid glands [5]. PTH is an 84-amino acid hormone secreted by the parathyroid glands and it plays an essential role in regulating phosphate, vitamin D, extracellular fluids and calcium balance. Albright’s hypothesis that PTH was the etiological factor in HHM was true from a functional standpoint but did not prove true clinically [6]. It was only in 1987 that three independent groups identified a protein with similar biological activities and structure to PTH, hence named PTHrP. An 18-kDa protein was isolated from a human lung cancer cell line with biological activities and high homology to the amino-terminal region of PTH in which eight residues in positions 1–13 were identical to the human PTH [7].

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Interestingly, two other independent groups also isolated and purified the same PTH-like factor from human renal carcinoma cells [8] and from human breast cancer cells [9,10].

Subsequently, PTHrP was found to be expressed in numerous types of cancer and its role in HHM was elucidated. Activation of the PTH/PTHrP receptor (PPR) in the skeleton evokes calcium release via bone resorption and activation of the PPR in the kidney to restrict calcium excretion [2]. Indeed, the main causes of hypercalcemia, primary hyperparathyroidism and HHM, show as-yet unexplained clinical differences, even though PTH and PTHrP have similar biological activities. For example, HHM patients present lower levels of the active form of vitamin D (calcitriol), metabolic alkalosis, and uncoupling responses of bone resorption and formation in contrast to what is observed with primary hyperthyroidism [5,11,12]. Other potential mediators of HHM are tumor-associated factors with systemic or local actions. Systemic factors, such as calcitriol, are increased in lymphomas and act on organs responsible for calcium homeostasis (kidney and intestine), resulting in elevated calcium levels [13]. Tumor-secreted factors with local actions that stimulate bone resorption such as IL-1, IL-6, TGF- α , TNF and granulocyte colony-stimulating factor (G-CSF) also promote increased calcium levels [5]. In addition to its role in hypercalcemia, further investigation demonstrated that PTHrP also plays important roles in tumor progression and metastasis, which is the main topic of this article.

PTHrP resembles PTH, sharing eight out of the 13 initial amino acids at the N-terminus, and binds to the PTH receptor type 1 known as the PPR. The PTHrP gene *PTHrP*, which is located on chromosome 12, spans more than 15 kb including nine exons and at least three promoters. Alternative splicing gives rise to three isoforms containing 139, 141 and 173 amino acids [14]. Furthermore, PTHrP has several functional domains; an N-terminal domain, a midregion domain and a C-terminal domain. The N-terminal domain (amino acids 1–36) has a binding site to activate the PPR, acting in autocrine, paracrine and endocrine manners, and leading to different biological effects and cell autonomous functions (Figure 1). The mid-region (amino acids 37–106) includes a nuclear localization sequence (NLS) that is important for the intracrine signaling of PTHrP in the nucleus and cytoplasm, regulating cell proliferation, survival and apoptosis. Lastly, the C-terminal domain (amino acids 107–139), also known as osteostatin, is associated with inhibition of osteoclastic bone resorption and anabolic effects in bone [14,15].

Along with tumorigenic functions, PTHrP also participates in normal physiology, acting as a hormone in calcium transportation in the fetus, late pregnancy and lactation [2]. PTHrP is also highly expressed in human tissues and plays an important role in the developmental stages of mammary glands, hair follicles and teeth [2]. The biological function of PTHrP is very important in development during endochondral bone formation. Deletion of PTHrP in mice results in chondrodysplasia and early death, and heterozygous *PTHrP*^{+/-} mice have an early osteoporotic phenotype with reductions in trabecular volume [16–18]. Altogether, these studies demonstrate the key role that PTHrP plays in normal physiology and developmental biology.

The PPR is a class II G-protein-coupled receptor comprised of seven transmembrane-spanning domains. The gene that encodes the PPR is highly conserved and homologous in rats, mice and humans, and the multiple exons that encode the gene are subjected to alternative splicing [19]. PTH and PTHrP amino-terminal regions bind to and activate the PPR, which is expressed in the main target cells of PTH and PTHrP: osteoblasts in bone and renal tubular cells in the kidney. Remarkably, PPR was also found to be expressed in many tumor types, such as prostate and breast, and in many other cancers [20,21], regulating tumor cell autonomous processes and contributing to tumor progression and growth. Consequently, PTHrP supports dual roles in skeletal metastasis: modulating the bone and

priming the metastatic microenvironment; and promoting tumor cell autonomous function, contributing to growth and progression. In bone, the PPR is primarily expressed in osteoblasts, osteocytes and bone marrow stromal cells such as osteoblast precursor cells. Osteoclasts do not express the PPR, as demonstrated by the lack of response to PTH [22]. The actions of PTH and PTHrP in osteoclasts are mediated by osteoblasts and osteocytes responsible for secretion of factors that activate osteoclasts. The PTH and PTHrP amino terminals interact with the J-domain functional portion of the PPR in osteoblasts, stimulating multiple signaling cascades, including the adenylate cyclase–protein kinase A pathway, the phospholipase C–protein kinase C pathway and the MAPK pathways, leading to anabolic and catabolic responses in bone [23].

Tumor-derived PTHrP can act in different ways to modulate tumor growth, progression and metastasis. For example, in HHM, PTHrP is secreted from primary tumors and acts in an endocrine manner, inducing bone resorption. When tumors metastasize to bone, PTHrP acts in a paracrine manner, secreting PTHrP in the bone microenvironment, activating osteoblasts and inducing bone remodeling. In addition, tumor cells also express the PPR, facilitating autocrine actions of PTHrP and contributing to cell proliferation and growth. Finally, PTHrP also acts in an intracrine manner, increasing cell survival and apoptosis resistance [24]. Although PTHrP plays multifunctional roles in skeletal metastasis, most investigations have focused on PTHrP's function as a tumor-promoting factor. However, emerging evidence supports the hypothesis that PTHrP also alters the tumor microenvironment, potentially contributing to metastasis development.

Roles of PTHrP in skeletal metastasis of cancer

According to Stephen Paget's 'seed and soil' hypothesis, disseminated tumor cells ('the seed') can produce metastases only when they are seeded in the correct 'soil' [25,26]. Therefore, metastasis is a multistep process that requires coordination of two different subsets: tumor cells and the metastatic organ. The tumor cells must acquire the ability to invade the surrounding tissue, gain access to the circulation by the lymphatic or blood circulation, survive and extravasate into a secondary site [27]. The second subset is the metastatic compartment that has to enable tumor invasion, colonization and growth. In other words, the metastatic organ is the fertile soil that favors tumor cell growth. PTHrP in skeletal metastases has the capacity to act on both parts of the process, nurturing the seed (tumor cells) and priming the soil (bone microenvironment).

PTHrP expression is commonly found in many types of cancer and increased expression is observed with tumor progression, with the highest expression being found in metastatic lesions [28–33]; however, the use of tumor-produced PTHrP as a prognostic factor is still controversial. Clinical studies in breast and lung cancers have implicated PTHrP expression in primary tumors as a good prognostic factor [34,35]. In a recent clinical study in non-small-cell lung carcinoma, PTHrP expression was associated with increased survival in females with either early or advanced stages of disease [34]. Henderson *et al.* demonstrated, in a prospective study of 526 patients with breast cancer followed for a 10-year period, that positive PTHrP expression in the primary tumors was correlated with improved survival and reduced development of bone metastases [35]. They concluded that PTHrP expression in the primary tumors conferred a less invasive phenotype that is distinct from its known osteolytic roles, which support skeletal metastasis.

By contrast, clinical studies in invasive human breast tumors indicated that PTHrP was detected in 60% of the tumors but not in the normal breast tissues and expression was greater in bone metastases, with PTHrP expression detected in approximately 90% [28,29]. A recent exciting study identified *PTHrP* as a breast cancer risk locus. In this large

investigation, which included two independent genome-wide association studies from 41 case-control studies and nine breast cancer genome-wide association studies, three new breast cancer loci were identified, including *PTHLH* (12p11). These data form highly convincing evidence implicating PTHrP in breast cancer pathogenesis. Still, the genes identified must be proven to be causal for cancer pathogenesis and the mechanisms need to be explored [36]. PTHrP is also expressed in more than 90% of colon cancer patients [30]. Extensive investigation of PTHrP function in prostate tumors demonstrated that expression contributes to tumor growth and progression [37]. Studies in human prostate cancer observed that PTHrP was differentially expressed depending on the cancer stage. PTHrP was expressed in 33% of benign prostate hyperplasia, 87% in well-differentiated prostate cancer and 100% in poorly differentiated and metastatic tumors [21,33].

The general consensus is that PTHrP is a supportive factor for cancer growth and progression. Differences in its prognostic applicability may reflect temporal aspects and/or downstream events that have been difficult to elucidate in the context of cancer. Moreover, PTHrP is a polyhormone with multiple biologically active domains, which may explain the variability seen in cancer prognosis and the necessity to further elucidate PTHrP actions in cancer. Alternative splicing and post-translational proteolysis generate different PTHrP isoforms and fragments that can elicit various cellular responses. The variety of PTHrP fragments and different actions (autocrine, paracrine, endocrine and intracrine) portrays the complexity of PTHrP-induced responses. The roles of the different PTHrP fragments and the cell biological responses that PTHrP generates are still not fully clarified.

PTHrP has been extensively investigated as an important bone factor in cancers that have significant bone tropism, especially in prostate and breast cancers. Indeed, bone is a common site for tumor metastases, and skeletal metastasis is the leading cause for mortality and morbidity among breast, prostate and lung cancer patients [38]. Postmortem examination demonstrated that approximately 70% of patients dying with breast cancer and approximately 90% of prostate cancer patients had evidence of bone metastases [38,39]. Other cancers that also metastasize to the skeleton include renal tumors, melanoma and multiple myeloma [38,40].

Radiographic manifestations of bone metastases show different characteristics; osteoblastic lesions demonstrate exacerbated activity of osteoblasts evidenced by abnormal bone formation, whereas osteolytic lesions show intensified osteoclast activity evidenced by abnormal bone resorption [41,42]. However, most of the tumors present mixed lesions, with the presence of both osteolytic and osteoblastic aspects [41]. Osteolytic lesions are associated with bone fractures and HHM, a common finding with advanced bone destruction. They are most common in breast, multiple myeloma, melanoma, lung, thyroid, renal and gastrointestinal malignancies. By contrast, prostate cancer metastatic lesions are predominantly osteoblastic. Tumor-derived factors, such as Wnt family ligands, BMPs, PDGF and endothelin-1, activate osteoblastic bone formation, contributing to skeletal metastasis [41].

The interplay of tumor cells with the bone microenvironment results in tumor growth and bone remodeling in skeletal metastasis [41]. Tumor cells secrete factors, such as PTHrP, TNF- α , IL-1, IL-6, IL-8 and IL-11, that stimulate bone cells. In turn, activated osteoblasts and osteoclasts secrete other factors that promote tumor growth, feeding a destructive cascade of metastatic growth [43]. Therefore, skeletal metastasis depends on both priming the seed (tumor cells) as well as nurturing the soil (bone). PTHrP is a pivotal tumor-derived factor, playing a role in both steps. For instance, it can act in priming the seed, participating in cell autonomous processes such as tumor cell proliferation, apoptosis, survival and anoikis, which enhance the capacity for tumor growth, dissemination and metastasis.

Importantly, PTHrP can act as an endocrine or paracrine factor, modulating bone responses and cellular aspects of the bone microenvironment, thus contributing to the formation of a conducive environment for cancer establishment in bone.

Tumor cell autonomous functions of PTHrP

Tumor-derived PTHrP can act in different ways to modulate tumor growth and progression in a cell-autonomous manner. PTHrP can act in paracrine, autocrine and intracrine modes to modulate diverse cell processes (Figure 1). The paracrine and autocrine actions of PTHrP derive from PPR activation through binding of amino-terminal PTHrP but are not the only mechanism. Post-translation protease cleavage generates biologically active mid-region and C-terminal PTHrP fragments that can act in a paracrine and autocrine fashion through activation of presumably novel cell surface receptors. The C-terminal fragment, also known as osteostatin, has a role in osteoclast inhibition and bone anabolic actions, suggesting an important role in skeletal metastasis, although its function in cell autonomous processes in cancer cells is not well defined [15]. The intracrine action of PTHrP depends on its NLS within the 87–107 amino acid region and regulates cell apoptosis, proliferation and the cell cycle. The PTHrP NLS interacts with importin- β 1 – independently of importin- α – transporting the protein through the nuclear pore complex and is dependent on microtubule integrity [44,45]. Alternatively, evidence suggests another pathway exists through internalization of the PPR–PTHrP complex in an endocytosis-dependent manner to the cytosol and rapid transport into the nucleus [46,47]. PPR–PTHrP complexes have been found in the nucleus of osteoblasts in bone and cells in other organs, such as kidney, liver, gut and ovary, although the functional mechanisms of PPR–PTHrP complexes are still not fully understood [46,47]. In addition, proteins smaller than 40 kDa may be translocated through the nuclear pore complex through mechanisms that are, as yet, unknown owing to the difficulty of visualizing and quantifying the transport [48]. The possibility of PTHrP peptides (<40 kDa) without the NLS interaction with importin proteins translocating directly through the nuclear pore complex cannot be ruled out, owing to the small size of the molecule, though this would likely be at much slower rates [48]. Nuclear PTHrP localization can then exert differential cellular responses than those seen with paracrine and autocrine PTHrP, highlighting the great diversity of PTHrP actions. Additional information on intracrine mechanisms of PTHrP can be found in detailed reviews [24,49]. Altogether, PTHrP differential actions can promote proliferation, evasion of apoptosis and anoikis, and invasion and migration, contributing to tumor growth and progression.

Proliferation

PTHrP stimulates tumor cell proliferation in different types of cancer. Recently, a study on breast cancer demonstrated that PTHrP is involved with tumor initiation, growth and metastasis [50]. In a spontaneous breast cancer model, *PTHrP* deletion significantly delayed tumor initiation and tumor growth. Reduced PTHrP expression resulted in reduced proliferation, as demonstrated by lower Ki67 and cyclin D1 staining as well as cell cycle arrest, suggesting an important PTHrP role for breast tumor proliferation [50]. In prostate cancer, PTHrP also promotes proliferation: prostate cancer cells that overexpressed PTHrP had enhanced tumor growth and tumor size in bone [37]. Another study demonstrated that transfected cells that overexpressed PTHrP (1–87) stimulated cell proliferation and the intracrine production of IL-8, a known growth-promoting and angiogenic factor [51]. The contribution of PTHrP to proliferation is also evident in renal carcinoma. Burton *et al.* demonstrated that autocrine PTHrP induced renal carcinoma cell proliferation and tumor growth, whereas antiserum and antagonists to PTHrP inhibited tumor growth *in vitro* [52]. Therefore, PTHrP contributes to tumor cell proliferation, promoting tumor growth, which is an important step for subsequent tumor progression and metastasis.

Evasion of apoptosis &/or promotion of survival

PTHrP intracrine actions have been under investigation for their roles in intracellular biology, especially cell survival, growth and apoptosis. In prostate cancer, PTHrP and its NLS were found to prevent tumor cell apoptosis [37]. Prostate cancer cells that overexpressed PTHrP had enhanced tumor growth. In addition, cells with deletion of the NLS were more susceptible to undergo apoptosis than full-length PTHrP-transfected cells or controls. These findings indicated a role of PTHrP in prostate cancer cell survival via an intracrine manner. Similar results were also observed in a breast cancer cell line, demonstrating a critical role for nuclear targeting in the antiapoptotic and cell cycle regulatory effects of PTHrP [53]. MCF-7 breast cancer cells that overexpressed PTHrP with an intact NLS sequence were protected from apoptosis induced by serum starvation and presented cells in G2-M stage of the cell cycle compared with cells overexpressing a mutated NLS sequence, indicating an intracrine role for PTHrP in apoptosis and cell cycle regulation. The role of PTHrP autocrine/paracrine actions in cell growth and cell death *in vivo* was demonstrated in renal carcinoma cells, in which anti-PTHrP antibody treatment reduced tumor growth by inducing cell death [54]. A neutralizing antibody for PTHrP was also used against different renal carcinoma cell lines, and strategies blocking both PPR and PTHrP signaling decreased tumor growth by inducing apoptosis [55]. These studies highlight PTHrP as an important growth factor and a survival signal that contributes to tumor growth. Moreover, acquiring apoptosis resistance is an important quality for the survival of cells that eventually enter the circulation and colonize different organs, therefore establishing metastatic foci.

Invasion & migration

Intracrine PTHrP signaling is also thought to influence tumor invasion and metastasis. In a prostate cancer study, PC-3 cells that overexpressed intact PTHrP upregulated the expression of the $\alpha 1$, $\alpha 5$, $\alpha 6$ and $\beta 4$ integrin subunits [56]. The presence of NLS signaling was necessary for the increase in integrin expression, which is known to facilitate cancer cell adhesion, migration and invasion – requirements necessary for cancer cell colonization in skeletal metastasis [56]. Interestingly, integrin $\alpha 6$ and $\beta 4$ levels are also increased in colon cancer, suggesting a role for PTHrP in integrin expression in different types of cancers [31]. PTHrP also positively regulates LoVo cells' (human colon cancer cells) proliferation, migration and invasion *in vitro* [57]. Overexpression of PTHrP augmented xenograft growth and expression of integrins $\alpha 6$ and $\beta 4$, as well as PI3K pathway components. PTHrP mediates upregulation of integrin $\alpha 6\beta 4$ expression, activating the PI3K–Akt pathway [57]. A recent study investigated the link between PTHrP expression and Rac1, a GTPase. The authors demonstrated that the PTHrP positive effect on Rac1 activity was via the guanine nucleotide exchange factor Tiam1. Interestingly, the effects of PTHrP expression were mediated by integrin $\alpha 6\beta 4$ activation of the PI3K pathway, which regulates both Rac1 and Tiam1 activity [58]. Therefore, PTHrP expression in prostate and colon cancer is associated with tumor growth, migration and invasion. In addition, PTHrP also influenced the expression of the chemokine receptor CXCR4, an adhesion factor expressed in breast cancer that binds to SDF-1/CXCL12 and is present in bone [50]. In this study, PTHrP was coexpressed with CXCR4 and was crucial for the metastatic spread. The role of PTHrP in facilitating cell invasion and migration consequently contributes to metastatic spread, by increasing cell motility, enabling cell invasion to the surrounding tissue and facilitating the access of tumor cells to the blood. Tumor cells can then intravasate into the bloodstream and disseminate into different organs where adhesion molecules would facilitate tumor cell adhesion and colonization into the metastatic organ.

Evasion of anoikis

Anoikis is a phenomenon of cell apoptosis resulting from detachment with loss of cell–matrix interactions. Evasion of anoikis is an essential step in the metastatic process so that the cells can survive and colonize a distant organ [59]. The PTHrP intracrine pathway plays an important role in tumor apoptosis evasion; however, little is known regarding its role in anoikis. Recent studies suggest that PTHrP could be important for anoikis. Bhatia *et al.* demonstrated, in an *in vitro* study, that the PTHrP intracrine pathway protected prostate cancer cell lines PC-3 and C4-2 from doxorubicin-induced apoptosis, and promoted anchorage-independent cell growth [60]. The intracrine effects of PTHrP were mediated via integrin $\alpha 6\beta 4$ -mediated activation of the PI3K–Akt pathway, since knockdown of integrin $\alpha 6\beta 4$ decreased the PTHrP-mediated activation of the PI3K–Akt pathway. PTHrP also increased NF- κ B activity via a PI3K-dependent pathway. This study suggested a role for PTHrP in anoikis and activation of survival pathways.

Most recently, Park and McCauley investigated the participation of PTHrP and its NLS in the anoikis of prostate cancer [61]. Here, downregulation of PTHrP in PC-3 cells conferred increased apoptosis of cells cultured in suspension. On the other hand, overexpression of the gene resulted in protection from anoikis. LNCaP cells that expressed full-length PTHrP or NLS-defective cells were generated and cultured under an anoikis challenge. Interestingly, only full-length PTHrP expression was able to rescue cells from anoikis. Investigation of an apoptosis-related gene array demonstrated that expression of TNF- α , a proapoptotic protein, was increased when PTHrP was downregulated and decreased with PTHrP overexpression, but not in NLS-defective PTHrP-overexpressing cells. This suggests that the PTHrP-mediated reduction in proapoptotic TNF- α is dependent on full-length PTHrP to confer anoikis resistance. Moreover, *in vivo* low-PTHrP-expressing cells resulted in fewer metastatic lesions compared with cells overexpressing PTHrP, suggesting an anoikis role due to loss of intracrine PTHrP activity. These findings suggest that PTHrP nuclear localization confers resistance to anoikis and delineate a new mechanism associated with prostate cancer metastasis [61]. Tumor cells can survive after detachment from the primary tumor, and overcome the physical obstacles of not having a protective matrix and neighboring cell interactions, as well as surviving in the bloodstream; these are essential steps for metastasis onset.

PTHrP-dependent expression of growth factors

When osteolytic tumors metastasize to bone, they promote a destructive cascade of events also known as ‘vicious cycle’. PTHrP secreted by tumor cells increases bone resorption, and induces bone matrix release of calcium and numerous growth factors, such as TGF- β , promoting tumor growth in bone. TGF- β signaling is a very important aspect of PTHrP osteolytic actions in bone. Mutation of TGF- β type II receptor in MDA-MB-231 cells resulted in less bone destruction, decreased osteoclasts and prolonged survival in mice [62]. Conversely, constitutively active TGF- β type II receptor breast cancer cells increased PTHrP production in tumors and enhanced osteolytic bone metastasis [62]. In this context, a destructive cascade of tumor and bone interactions is established where PTHrP binds to and stimulates the PPR present in osteoblasts and osteocytes to express RANKL, leading to osteoclast differentiation and bone loss. Osteoclast-mediated bone resorption then releases factors such as calcium, TGF- β , IGF-1 and FGFs that favor tumor proliferation and augment PTHrP production. In addition, PTHrP can also induce expression of CCL2/MCP-1, thus contributing to tumor growth. Li *et al.* demonstrated *in vitro* and *in vivo* that prostate cancer-derived PTHrP induced osteoblastic secretion of CCL2 in bone and that PTHrP antagonist treatment inhibited the secretion of CCL2 [63]. Thus, CCL2 supports tumor growth, progression and metastasis by different means. It can directly stimulate tumor cell migration, proliferation and survival, or indirectly establish an appropriate niche for growth, eliciting

angiogenesis and macrophage recruitment and polarization to the M2 type (protumorigenic macrophages) [64,65]. CCL2 also participates in skeletal metastasis, promoting increased osteoclastic numbers and activity, which are important for tumor growth in bone [66]. These studies provided evidence that PTHrP in bone metastasis is an important modulator for the release and secretion of growth factors such as TGF- β and CCL2, which will further support tumor growth and skeletal metastasis progression.

Overall, PTHrP is a tumor-promoting factor involved in each step of metastasis. First, PTHrP contributes to tumor growth in the primary tumor site, promoting cell proliferation, survival and evasion of apoptosis. Subsequently, PTHrP participates in the cell invasion and migration required to penetrate the surrounding tissue and gain access to the circulation. Next, PTHrP participates in anoikis evasion, so that tumor cells can survive and extravasate into a secondary site, where they can establish metastatic growth. Finally, when tumors metastasize to bones, PTHrP still acts on the bone microenvironment to induce a destructive cascade with release of numerous growth factors that contribute to tumor growth and enhanced PTHrP expression. Hence, PTHrP participates in all steps of the metastatic processes; from tumor growth, progression, invasion, migration and survival to bone modulation, in order to support tumor growth, as summarized in Figure 1.

Role of PTHrP in the metastatic microenvironment

PTHrP actions in skeletal metastasis are not only restricted to the tumor cell autonomous functions but also act in the modulation of the bone marrow microenvironment. Extensive evidence demonstrates that PTHrP is a tumor-promoting factor. However, emerging evidence supports the hypothesis that PTHrP can also modulate the bone microenvironment, providing a congenial 'soil' for tumor metastasis. Evidence suggests that PTHrP nurtures the 'soil' to house and subsequently 'feed' the disseminated cells, leading to metastatic onset and growth. Evolving PTHrP participation in the modulation of the bone metastatic environment includes modulation of cellular contents and promotion of angiogenesis, all of which are known to contribute to metastasis (Figure 2). In this section, recent findings reporting PTHrP actions in the bone microenvironment will be discussed.

PTHrP actions in bone: direct & indirect effects

PTHrP binds to PPRs primarily expressed in osteoblasts, osteocytes and bone marrow stromal cells such as osteoblast precursor cells. However, the net effects of PTH/PTHrP on bone (i.e., anabolic or catabolic) are dependent on the duration and exposure. For example, intermittent administration of PTH *in vivo* results in bone formation, while continual infusion of PTH causes significant bone loss [23]. In a recent study, Horwitz *et al.* investigated the effect of continuous infusion of human PTH (1–34) or human PTHrP (1–36) at low doses (2 and 4 pmol/kg/h, respectively) in healthy adult volunteers for 7 days [67]. Continuous infusion induced hypercalcemia and hypercalciuria and rapidly increased bone resorption. Interestingly, bone formation was suppressed by 30–40%, causing sustained arrest in the osteoblast maturation program. Indeed, PTHrP has a direct effect on the osteoblast cell cycle that is dependent on the developmental stage [68,69]. PTHrP upregulated JunB in osteoblasts with reduction in cyclin D1 and G1 cell cycle arrest [68]. Such findings suggest that PTHrP may influence the life span and activity of osteoblasts in bone.

Osteoblasts are not the only cell responsible for RANKL production and bone remodeling. Osteocytes are cells located within bone matrix, are embedded and surrounded by mineral tissue, and are the major cells present in bone, comprising 90–95% of all bone cells in adults. They are also the longest-lived bone cells, being able to survive for up to decades [70]. During the past 10 years, much attention has been placed on osteocyte functions,

shifting their status from bystander cells into key players of the bone microenvironment. Recently, two independent groups investigated the role of osteocytes *in vivo* and found an important role for them in bone remodeling – they are the main source of RANKL for osteoclastogenesis [71,72]. These studies challenged the dogma in bone biology that osteoblasts are the key cells that modulate bone remodeling and bone coupling. For example, PTH and PTHrP actions were believed to be mediated in great part, if not exclusively, by osteoblast activation. However, osteocytes also express PPRs, therefore suggesting a significant role in the actions of both peptides. In a recent study, specific osteocyte deletion of PPR resulted in mild osteopenia, increased sclerostin expression and impaired homeostatic calcemic response, demonstrating a significant role of PTH/PTHrP signaling in bone remodeling and homeostasis [73]. Since bone metastasis requires interactions between tumor cells and bone cells, the osteocytes, as potential PTHrP-responsive cells, could be playing a role in the modulation of the microenvironment, with the secretion of different growth factors mediating not only the tumor growth, but also the bone microenvironment. These events would favor metastatic growth and progression; yet, such a role for PTHrP in osteocytes has not been delineated.

PTHrP actions are restricted not only to direct effects on bone cells such as osteoblasts and osteocytes; through the activation of these cells, PTHrP induces the release of a variety of growth factors and cytokines derived from activated cells as well as the bone matrix with the effect of modulating other cellular components, such as stromal cells and immune cells, which could be playing important roles in the metastatic ‘soil’. Emerging evidence suggests that PTHrP may also play a role in inflammatory responses associated with HHM. Studies found that concomitant PTHrP expression of inflammatory cytokines, such as TNF, IL-1 α and IL-6, augment bone resorption activity [74–76]. More evidence is necessary to delineate the regulation of PTHrP and cytokine expression in a cancer context. However, substantial advances have linked PTHrP actions with inflammatory responses and diseases [77], highlighting a possible role in cancer – often considered the wound that never heals – with an inflammatory aspect strongly implied in its progression. Further studies are needed to explore PTHrP function in the cellular milieu of the bone microenvironment, the growth factors and cytokines expressed, and how these may contribute to tumor growth and metastasis.

Angiogenesis

Angiogenesis is a well-studied process supporting tumor growth and progression. Growing evidence proposes that PTHrP can affect skeletal metastasis progression via stimulation of angiogenesis. Akino *et al.* first described a direct effect of tumor-derived PTHrP in angiogenesis, after observing that a metastatic pituitary tumor cell line (GH3) that expressed high levels of PTHrP had increased vascularity in xenografts. Using *in vitro* studies, they demonstrated that PTHrP did not affect endothelial cell proliferation and migration but dose-dependently stimulated capillary tube formation [78]. Although a contradictory study argued that PTHrP was an angiogenesis inhibitor functioning by activation of protein kinase A, little evidence exists to support this hypothesis [79]. In fact, a recent study, in a spontaneous breast cancer mouse model with specific *PTH1LH* gene deletion, demonstrated that PTHrP expression not only affected tumor initiation, progression and metastasis but also influenced tumor angiogenesis. PTHrP ablation resulted in reduced angiogenesis [50]. In addition, Gujral *et al.* investigated the role of PTHrP in IL-8 production in prostate cancer cells, which is a known contributing factor to tumor angiogenesis and growth. Transfected cells that overexpressed PTHrP (1–87) and (1–173) stimulated cell proliferation and the production of IL-8, but not VEGF, suggesting a specific IL-8 response. Surprisingly, the PTHrP (65–87) region was required for PTHrP (1–87) to robustly stimulate IL-8 in prostate cancer cells. Since exogenous PTHrP (1–36 and 1–87) did not affect IL-8 expression, they concluded that

PTHrP (1–87) was required for intracrine enhanced IL-8 production by PTHrP [51]. A PTHrP paracrine effect in angiogenesis in bone metastasis has also been investigated. Liao *et al.* showed, *in vitro*, that the PTHrP pro-angiogenic effect was dependent on the presence of bone marrow stromal cells [80]. A potential mechanism could be through PTHrP-mediated osteoblastic secretion of CCL2, a known angiogenic factor [63,81,82]. Indeed, recent data demonstrate that the PTHrP angiogenic effect is dependent on osteoclast activity and MMP9 production [83]. Further studies are necessary to elucidate PTHrP's role in tumor angiogenesis, especially in bone metastasis.

In summary, PTHrP activates cells in the bone microenvironment, promoting angiogenesis and thus priming the bone microenvironment to be conducive to metastatic onset and growth in bone. There is convincing evidence that PTHrP participates in angiogenesis in bone, yet the precise role of angiogenesis in skeletal metastasis needs further elucidation.

PTHrP as a therapeutic target

Given the multiple roles PTHrP has in HHM, in cell autonomous tumor cell activity and in the metastatic tumor environment of bone, PTHrP is a potential therapeutic target. Strategies utilizing neutralizing antibodies or small molecular inhibitors, or targeting the signaling pathways that PTHrP elicits, are promising. Neutralizing antibodies demonstrated positive responses in animal models, reducing skeletal metastasis, bone lesions and also hypercalcemia [84,85]. However, human clinical data are lacking.

In addition to direct inhibition of PTHrP actions, chemotherapeutic drugs also result in suppression of PTHrP production. Furugaki *et al.* demonstrated that erlotinib, an EGF receptor tyrosine kinase inhibitor, reduced osteolytic bone resorption induced by lung cancer cells through its effect on the RANKL production by osteoblasts/stromal cells [86]. Interestingly, erlotinib also suppressed the production of osteolytic factors, including PTHrP production. Lorch *et al.* also demonstrated the role of the EGF receptor in two different squamous lung carcinoma xenografts that had reduced PTHrP expression after treatment with the EGF receptor tyrosine kinase inhibitors PD153035 and gefitinib [87]. Moreover, targeting cells that release growth factors known to stimulate PTHrP production such as TGF- β may also reduce PTHrP [62].

In concert with the need to develop new strategies to inhibit PTHrP actions is the need to fully define PTHrP's roles in the different stages of cancer. Controversy about PTHrP and cancer prognosis raises concerns regarding the optimum time that PTHrP therapy should be applied. Moreover, the variety of PTHrP isoforms and fragments generated by post-translational proteolysis highlights the different potential roles PTHrP can have in multiple cell types. Many answers are needed to safely test potential therapies against PTHrP.

PTHrP, the premetastatic niche & tumor dormancy

PTHrP is a potent and efficient tumor-promoting factor, acting in two different compartments; the tumor and the bone microenvironment. Although extensive studies have investigated the actions of PTHrP in cellular autonomous tumor function, PTHrP involvement in modulating the metastatic microenvironment warrants extensive investigation. For instance, PTHrP, functioning as an endocrine factor secreted by primary tumors with its regulation in the bone microenvironment at a distance, could support the formation of a premetastatic niche. Such a premetastatic niche would provide a permissive microenvironment for the recruitment of tumor cells, leading to micrometastasis initiation and establishment [88]. Studies in lung metastasis demonstrated the participation of bone marrow-derived cells in the formation of the premetastatic niche before the arrival of tumor cells in the lungs [89–91]. Hematopoietic progenitor cells expressing VEGFR1 and

fibronectin clustered in tumor-specific metastatic sites, suggesting the formation of a premetastatic niche that contributes to disseminating tumor cell engraftment in the lungs [89]. In addition, conditioned media from distinct tumor types with different patterns of metastatic dissemination redirected the formation of these clusters and transformed the metastatic profile, therefore dictating organ-specific tumor metastasis. In addition to hematopoietic progenitor cells, macrophage recruitment has also been implicated in premetastatic niche formation [90,91]. In 2006 Hiratsuka *et al.* demonstrated, in a lung premetastatic and metastatic phase, that factors released by subcutaneous tumors induced expression of inflammatory proteins S100A8 and S100A9 in lungs, which triggered macrophage recruitment to the site [91]. Antibodies targeting S100A8 and S100A9 resulted in an 80–90% reduction of colonized tumor cells to the lungs. Later, they demonstrated that serum amyloid A3, acting through Toll-like receptor 4 on macrophages and tumor cells, mediated S100A8 and S100A9 expression specifically in the lung [90].

In bone, the formation of a premetastatic niche is not well defined. The lack of spontaneous skeletal metastasis models challenges advances in this area. However, most evidence is focused mainly in the context of endocrine-like actions that modulate the bone microenvironment. Factors other than PTHrP that are secreted by tumors and that can modulate the bone microenvironment from a distance provide evidence of potential premetastatic niche formation in skeletal metastasis. For example, heparanase is an enzyme produced by breast cancer cells that cleaves heparan sulfate to produce syndecan-1. Tumor-derived syndecan-1 that is shed in the primary tumor acts in bone, increasing osteoclastogenesis and contributing to osteolysis [92,93]. Other factors, such as osteopontin and matrix metalloproteinase, may also play a role in promoting tumor growth and skeletal metastasis [94,95].

Indeed, PTHrP is also an attractive potential factor for premetastatic niche formation in bone. For instance, PTHrP can modulate the production of CCL2 in bone by osteoblasts, inducing macrophage recruitment and activation into M2 tumor-promoting cells as well as stimulating osteoclastogenesis, which will altogether enhance tumor growth and progression [63–66]. This suggests a potential mechanism for a premetastatic niche formation in the bones, where tumor-derived PTHrP induces CCL2 expression in osteoblasts, contributing to modulation of the bone microenvironment into a conducive niche. In conclusion, although its role in bone metastasis is not yet defined, PTHrP is a potential candidate for endocrine actions in bone modulation and premetastatic niche formation (Figure 2).

Bone consists of an assorted cellular profile and PTHrP actions in this context are inadequately explored. For example, myeloid cells, such as macrophages, have been associated with tumor progression and metastasis of different types of cancer, as well as contributing to premetastatic niche formation [90,91,96]. Since macrophages share the same precursors as osteoclasts, PTHrP may indirectly regulate the myeloid population in bone and skeletal metastasis. A possible mechanism would be by PTHrP-mediated osteoblastic secretion of CCL2 [63]. Another cell type that is likely to be involved in tumor progression and metastasis is myeloid-derived suppressor cells (MDSCs), which are immature myeloid cells involved in immune suppression and tumor escape from host control, as well as angiogenesis and tumor growth [97]. MDSCs are identified by the expression of myeloid cell (CD11b) and granulocyte (Gr-1) markers and are increased in bone marrow, spleen and peripheral blood in tumor-bearing hosts [97]. Unfortunately, their role in skeletal metastasis is not yet defined, but possible roles have been suggested as a potential source for angiogenesis and osteoclastogenesis as well as contributing to the development of osteolytic lesions and the progression of metastasis [98]. Tumor-derived PTHrP is involved in the expansion and potentiation of MDSCs in the bone marrow that are recruited to the tumor tissue, contributing to tumor angiogenesis and growth [99]. Myeloid-derived cells, such as

macrophages and MDSCs, have been implicated in tumor growth, angiogenesis and immunosuppression, as well as mechanistic aspects of a premetastatic niche. PTHrP modulation of these cells in bone, and how they can contribute to skeletal metastasis, is an inadequately studied area with future potential.

Another evolving area of study is tumor dormancy. Tumor cell dormancy is one of the biggest problems in skeletal metastasis and is believed to be associated with tumor relapse [100]. Metastasis is a very inefficient process in which less than 0.01% of the tumor cells that engage the circulation form metastatic foci [25,101]. The skeleton is a very complex environment with constant bone remodeling, hematopoiesis and a very rich milieu, with growth factors and mineral components being continually released. Therefore, the fact that bone is one of the common sites of metastasis is not surprising. It is thought that tumor dissemination is a process that occurs earlier, but in this process most of the cells fail to overcome the challenges imposed by entering the circulation [25]. However, it has also been proposed that, when cells circumvent this challenge and find a distant organ to colonize, they can stay dormant for decades [102]. This may be the reason that patients with solid tumors that were completely excised present with bone metastasis decades later, even if the primary tumors are not present any longer. Another problem with cell dormancy is that its detection presents difficulties of feasibility. Most skeletal metastases diagnoses are made only when tumors are visible and in advanced stages. Cell quiescence is also a big challenge in cell dormancy since most anticancer therapies target the highly proliferative cells. Detection of circulating tumor cells in the bone marrow has raised awareness that bone could also be a potential tumor cell-housing environment [100]. In this case, bone could offer a shelter where tumor cells attach and stay quiescent until they can be recruited to other sites or even go back to their place of origin. PTHrP makes an interesting candidate for abetting cell dormancy because it not only acts via intracrine and autocrine modes to modulate gene expression and cellular responses in tumors, but also engages paracrine modulation of the bone microenvironment. The action of PTHrP, in the regulation of cell cycle and expression of integrins, could be useful for the tumors to attach to the bone and acquire quiescence until they are activated to proliferate and form detectable metastases. As little is known about PTHrP in cell dormancy, this is an area of potential promise for elucidation and therapeutic targeting.

Conclusion

PTHrP is an important protein not only for normal physiological processes such as in development and physiology, but also as an important player in different cancers and in metastasis to the skeleton. PTHrP is a key regulator of tumor–bone interactions and regulates cells in the bone microenvironment, through proliferative and prosurvival activities that prime the ‘seed’ and the ‘soil’ of the metastatic lesion. In summary, PTHrP has multifaceted actions as an endocrine, paracrine, autocrine and intracrine peptide that displays a variety of biological functions in tumorigenesis and the devastating cascade of tumor metastasis.

Future perspective

Advances in the area of bone biology, such as the identification of osteocytes as potential key players in bone regulation, bring novel concepts and expand our knowledge of the influence that PTHrP may have in bone. In fact, understanding of PTHrP actions in bone is a crucial step to dissect the mechanisms for tumor cell growth and bone metastasis. Moreover, novel concepts in cancer research need to be applied and tested for PTHrP functions. For example, the fact that PTHrP exerts an endocrine function in bone in the case of hypercalcemia of malignancy suggests that PTHrP could also modulate different organs via

an endocrine mode. Therefore, in bone, PTHrP has potential as a premetastatic niche factor and further investigations in this area are needed to dissect such early steps of cancer metastasis. Another under-investigated area is tumor cell dormancy and how this affects the onset of metastasis. Improved animal models and specific molecular markers are needed to investigate these novel theories and concepts. Understanding the earlier steps of tumor progression and metastasis will facilitate the development of improved therapeutic targets to overcome cancer.

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Executive summary

Background

- PTHrP was first identified as an etiological factor of hypercalcemia but plays important roles in normal physiology, bone development, and tumor growth, progression and metastasis.
- PTHrP can function in different manners: with endocrine, paracrine, autocrine and intracrine actions. Therefore, PTHrP plays different roles in various cellular processes.

Roles of PTHrP in skeletal metastasis

- PTHrP is highly expressed by cancers with high bone tropism and is associated with skeletal metastasis development and progression.
- The dual function of PTHrP applied in the 'seed and soil' hypothesis is demonstrated by its actions in the tumor cell autonomous processes (seed) and the modulation of the bone microenvironment (soil), contributing to tumor growth, progression and bone metastasis.

Tumor cell autonomous function of PTHrP

- PTHrP can promote tumor cell proliferation, evasion of apoptosis and anoikis, survival, invasion and migration, contributing to tumor growth and progression.

Role of PTHrP in the metastatic 'soil' microenvironment

- PTHrP participation in the modulation of the bone metastatic environment includes regulation of the cellular components such as macrophages and promotion of angiogenesis, both of which are known to contribute to metastasis.
- Further investigation of the role of PTHrP in the modulation of the bone microenvironment is necessary to elucidate the earlier steps in the mechanism and progression of skeletal metastasis.

Figure 1. Multiple actions of PTHrP in tumor growth in bone

Tumor-derived PTHrP acts by different modes in order to enhance tumor growth, progression and metastasis. PTHrP acts in an intracrine manner, increasing cell survival, apoptosis resistance and anoikis evasion. There are two potential pathways for nuclear localization of PTHrP: (A) translocation of PTHrP through the NPC in which the PTHrP nuclear localization sequence interacts with $\beta 1$ and is then transported to the nucleus; and (B) internalization of the parathyroid hormone/PTHrP receptor-PTHrP complex by an endocytosis-dependent pathway to the cytosol and rapid transport into the nucleus. Moreover, tumor cells also express the parathyroid hormone/PTHrP receptor facilitating the autocrine actions of PTHrP and contributing to cell proliferation and apoptosis resistance and growth. When tumors metastasize to bone, PTHrP acts in a paracrine manner, secreting PTHrP in the bone microenvironment, activating osteoblasts and inducing a destructive cascade with release of numerous growth factors that contribute to tumor growth and enhanced PTHrP expression. Therefore, PTHrP participates in all steps of the metastatic processes, from tumor growth, progression, invasion, migration and survival to bone modulation in order to support metastases.

$\beta 1$: Importin $\beta 1$; ER: Endoplasmic reticulum; NPC: Nuclear pore complex.

Figure 2. PTHrP actions in the modulation of bone microenvironment and a potential role for a premetastatic niche formation

Tumor-derived PTHrP endocrine actions in bone are an inadequately studied area. An evolving model suggests PTHrP modulates the bone microenvironment by inducing osteoblast and potentially osteocyte secretion of CCL2 (MCP-1) and/or IL-6, which in turn mediates expansion of myeloid cells, such as macrophages and myeloid-derived suppressor cells, which are recruited to the tumor site, contributing to tumor growth, angiogenesis and progression. Moreover, growing evidence demonstrates that PTHrP is a potential candidate for premetastatic niche formation in bone with the expansion of these myeloid cells, forming a convivial niche for metastatic growth in bone.

Solid lines: Known pathways; Dashed lines: Potential pathways.