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Skeletal metastasis: Established and emerging roles of parathyroid hormone related protein (PTHrP)

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Abstract Parathyroid hormone related protein (PTHrP) is a well characterized tumor derived product that also has integral functions in normal development and homeostasis. PTHrP is produced by virtually all tumor types that metastasize to bone and numerous studies have demonstrated a correlation between PTHrP expression and skeletal localization of tumors. PTHrP has prominent effects in bone via its interaction with the PTH-1 receptor on osteoblastic cells. Through indirect means, PTHrP supports osteoclastogenesis by upregulating the receptor activator of NFκB ligand (RANKL) in osteoblasts. PTHrP also regulates osteoblast proliferation and differentiation in manners that are temporal and dose dependent. Bone turnover has been implicated in the localization of tumors to bone and PTHrP increases bone turnover. Bone turnover results in the release of growth factors such as TGFβ and minerals such as calcium, both of which impact tumor cell growth and contribute to continued PTHrP production. PTHrP also has anabolic properties and could be in part responsible for osteoblastic type reactions in prostate cancer. Finally, emerging roles of PTH and PTHrP in the support of hematopoietic stem cell development in the bone marrow microenvironment suggest that an interaction between hematopoietic cells and tumor cells warrants further investigation.

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The skeleton is the favored target site of cancer metastasis. With skeletal metastasis comes, at the cellular level, a new microenvironment for tumors to grow, and at the clinical level, devastating complications. Parathyroid hormone related protein (PTHrP) was first identified as a tumor-derived humoral factor with similar biological actions as parathyroid hormone (PTH). PTH and PTHrP are two major peptide mediators of bone development and remodeling. When tumors metastasize to bone and produce PTHrP the impact is dramatic with well characterized actions on bone remodeling and emerging roles in altering the bone marrow microenvironment. Bone is hard calcified tissue with collagen as a major component. Bone not only provides physical support, but also involves hematopoietic cell homeostasis. Certain tumors from breast, prostate, thyroid, lung and kidney particularly prefer to spread to bone [1]. On the one hand, bone provides a favorable environment with nourished blood flow and numerous growth factors. On the other hand, tumor cells release multiple factors including PTHrP that affect bones and cells in the bone marrow. Through these factors, tumors interact with the bone microenvironment to gain growth advantages and cause cancer associated bone lesions and complications. The bidirectional nature of tumor lesions in bone with tumor derived factors impacting the bone and hematopoietic marrow have been widely considered but the entire picture of these interactions is not yet clear.

1 PTHrP and gene functions

PTHrP was first discovered in patients with cancer associated hypercalcemia [2, 3]. PTHrP shares structural

similarity with PTH and both are potent stimulators of osteoclastgenesis. PTHrP overexpression is frequently detected in tumors with skeletal metastasis. Expression of PTHrP has correlated to bone metastasis and prognosis in tumor patients. Although originally identified as a tumor derived factor, in the past 20 years, information of its structure, regulation and function have dramatically expanded our knowledge of the roles of PTHrP in normal physiology and in pathologic conditions such as cancer and skeletal metastasis.

PTHrP is a peptide that forms as a transcriptional product of the PTHrP gene located on chromosome 12. Three splice variants result in products of 139, 141, and 173 amino acids in length [4]. Species conservation of PTHrP is evident with high homology across mammalian species [5]. PTHrP mRNA is typically unstable with a short half life but certain cytokines including transforming growth factor β (TGFβ) increase PTHrP mRNA stability [6]. PTHrP was originally named for its biological activity that resembles parathyroid hormone (PTH) and evolutionarily, the PTH and PTHrP genes arose by duplication although they are immunologically distinct proteins. PTH and PTHrP both bind to the same receptor in bone and kidney, the PTH-1 receptor (PTH-1R) (Fig. 1). PTHrP shares homology with PTH in the first 13 amino acids (70%) but the protein sequence is different thereafter. The region of amino acids 15–34 is critical for peptide binding to the receptor whereas the N-terminal 3 amino acids are critical for signaling through the cAMP, protein kinase A (PKA) pathway. PTHrP also contains a nuclear localization sequence (NLS) at the junction of the proximal two thirds and distal one third of the molecule. PTH and PTHrP have been most prominently studied for their signaling through the PKA pathway but have also been found to signal through the PKC pathway and have interactions with the MAPK pathway as well.

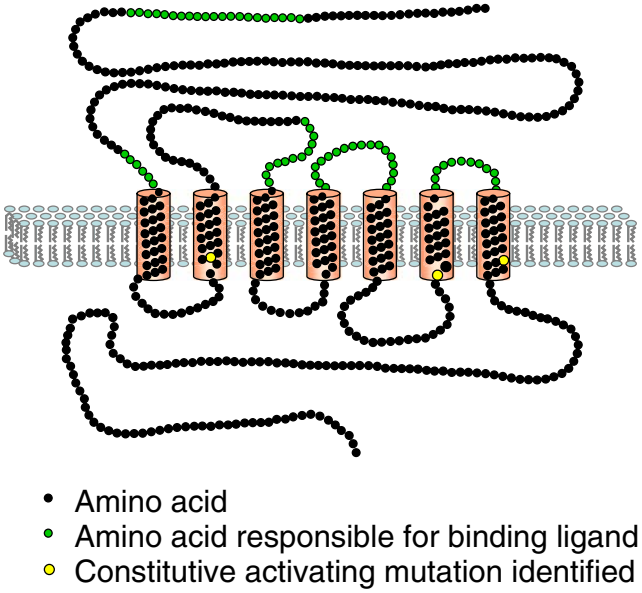

Fig. 1 Diagram of PTH-1R. The PTH-1R is a seven-transmembrane domain G-protein linked receptor. PTH and PTHrP bind to the N-terminal extracellular region of the receptor and signal through G-proteins to effect secondary messengers and downstream genes

The importance of PTHrP in normal development has been substantiated through gene targeted models of PTHrP and the PTH-1R ablation. PTHrP has noted roles during development through its prominent action in endochondral bone growth, mammary gland development and tooth eruption [7, 8]. Ablation of PTHrP results in neonatal lethality secondary to defective endochondral bone growth that renders the rib cage incapable of expansion and respiratory demise ensues. Ablation of the PTH-1R results in a similar phenotype and supports the PTH-1R as a key receptor for PTHrP actions during development. PTHrP regulates the differentiation and proliferation of growth plate chondrocytes and lack of PTHrP results in premature mineralization suggesting an important role of PTHrP is to inhibit mineralization. Mice overexpressing PTHrP have a delay in endochondral bone growth and mineralization with bones composed of excessive proliferating chondrocytes [9]. PTHrP is made in the epithelial cells of the mammary gland and is critical for mammary gland development [10]. Loss of PTHrP signaling results in a failure of mammary duct and nipple formation [10]. During lactation PTHrP plays an important role in the mobilization of calcium for mineralization of the skeleton of the offspring [11]. The mammary epithelium senses drops in serum calcium and facilitates PTHrP secretion via a feedback loop with the calcium sensing receptor. Interestingly, PTHrP is found at 100-fold higher levels in breast milk than in the serum of patients with hypercalcemia of malignancy [12]. The placenta is another source of PTHrP and the calcium sensing receptor may be triggered in the placenta to increase PTHrP secretion and hence regulate placental calcium transport. PTHrP also plays a unique role during tooth eruption [8]. As the developing tooth prepares to move through the bone and soft tissue into the oral cavity, the cells of the epithelial tissue known as the stellate reticulum on the coronal aspect of the tooth secrete PTHrP. The PTHrP causes recruitment and differentiation of osteoclasts that carve out the eruption pathway in the alveolar bone and facilitate eruption of the tooth. Mice with ablation of the PTHrP gene and rescue via targeted overexpression of PTHrP in the chondrocytes lack tooth eruption. PTHrP gene expression driven by the keratin K14 promoter is successful in rescuing the tooth eruption phenotype [8]. The roles of PTHrP in these developmental processes high-lights the impact of PTHrP in epithelial–mesenchymal interactions.

At the cellular level, PTHrP has been found to have a wide variety of effects on cells via intracrine, autocrine and paracrine manners that could impact its role in cancer progression and metastasis. PTHrP inhibits apoptosis via its intracrine activity dependent on its nuclear localization sequence [13]. Initial studies were performed in chondrocytes where the region of amino acids 87–107 had the characteristics of a nuclear localization site and cells expressing PTHrP with a deletion of this region were more likely to undergo apoptosis [13]. Subsequently this nuclear localization sequence was found to be protective for prostate carcinoma apoptosis [14]. PTHrP localizes to the nucleus and nucleolus during the G1 phase of the cell cycle a phenomenon thought to be dependent on the activity of the cyclin-dependent kinases cdc2 and cdk2 [15]. Cdc2 and cdk2 phosphorylate PTHrP increasingly as cells progress from G1 to G2+M phases of the cell cycle and limit the nuclear transport. The NLS of PTHrP is recognized with high affinity by importin β and comprises the region of amino acids 66–94 [16] The nuclear localization has been reported to occur via both intracrine and paracrine means. PTH-1R expressing cells incubated with fluorescently labeled PTHrP were shown to localize the PTHrP in the nucleus upon fresh incubation but not after receptor desensitization validating a paracrine and PTH-1R mediated process [16].

PTHrP stimulates smooth muscle relaxation and exerts vasodilatory responses. In response to mechanical stretch, PTHrP is produced and functions via the PTH-1R to relax vasoconstrictive agents or events and causes vasodilation. PTHrP causes dilation of coronary vessels via endothelial cell hyperpolarization [17]. There is strong evidence that PTHrP inhibits angiogenesis [18, 19] however opposing effects have also been reported [20, 21].

One of the actions of PTH and PTHrP that has been less well characterized is its ability to inhibit osteoblast differentiation. PTH and PTHrP are anabolic in bone and in current use clinically and experimentally for the treatment of osteoporosis, hence an inhibition of mineralization is somewhat counterintuitive. In search of a gene/genes that is key for anabolic actions of PTH/PTHrP in bone, many labs have assumed that because osteoblasts have PTH-1R receptors that its action on these bone forming cells would be a positive regulator of osteoblast matrix synthesis. Instead, PTH acts on osteoblasts to inhibit collagen synthesis and limit osteoblast differentiation [22]. One of the matrix associated proteins that is known for its ability to inhibit mineralization, matrix gla protein (MGP) is elevated in response to PTH and PTHrP, evidence found in both *in vitro* and *in vivo* systems [23]. Work performed in the early 1990s demonstrated the inhibition of osteoblast differentiation that occurs in response to PTH and several studies since that time have supported this finding [24]. However, there are a few reports of a temporal dependence of PTH administration that supports *in vitro* bone formation in osteoblasts [25].

PTHrP both stimulates and inhibits cell proliferation dependent on the cell differentiation state. Many of the initial studies of PTHrP activity during cell proliferation were identified via its role in stimulating chondrocyte proliferation. In the developing growth plate, PTHrP is synthesized by chondrocytes and perichondral cells and in an autocrine manner keeps chondrocytes in a proliferative state while inhibiting their differentiation to hypertrophic chondrocytes [26]. In osteoblastic cells a differentiation stage dependent effect of PTHrP has been well characterized where in confluent differentiated osteoblasts, PTHrP results in a cell cycle arrest and resulting inhibition of proliferation [27]. In contrast in proliferating osteoblasts under low serum conditions PTHrP increases cell numbers via its transcriptional upregulation of the cyclin D1 promoter [28]. It is not surprising then that the net effect of PTHrP in the bone microenvironment of the metastatic lesion is complexed by the plethora of cells at various differentiation stages. One could speculate that in certain conditions PTHrP could act in concert with another tumor-derived factor to augment a proliferative effect and result in a net osteoblastic condition while in other situations the balance could outweigh toward an inhibition of osteoblast activity and a predominance of the catabolic nature of PTHrP.

2 PTHrP signaling

The PTH/PTHrP receptor (PTH-1R) is a seven-transmembrane Class II G-protein linked receptor found predominantly in the target tissues of bone and kidney. The PTH-1R couples to Gs and Gq leading to activation of the PKA and PKC pathways [29]. Like other G-protein linked receptors, the PTH-1R undergoes cyclical receptor activation desensitization and internalization [30]. After ligand binding and endocytosis the PTH-1R is either recycled to the cell membrane or targeted for degradation. Arrestins contribute to the desensitization of both Gs and Gq mediated PTH-1R signaling. Interestingly, PTH-1R activation and internalization can be selectively dissociated since analogs of PTH that do not activate cAMP are capable of receptor internalization [31]. Signaling of the PTH-1R is also modified by scaffolding proteins such as the Na+/H+ exchanger regulatory factor (NHERF) 1 and 2 through PDZ1 and PDZ2 domains [32, 33].

Most of the work of PTH-1R signaling has centered on the cAMP pathway leading to PKA activation and phosphorylation of the cyclic AMP response element binding protein (CREB). Downstream from this, CREB

binds to the cyclic AMP response element (CRE) in the promoter region of many genes including the c-*fos* proto-oncogene and transcriptionally activates its expression. The Fos protein is a member of the AP-1 transcription factor that binds to the promoter region of various target genes. Work including both *in vitro* and *in vivo* studies has suggested that c-*fos* is a key mediator of PTH and PTHrP actions in development [34, 35]. Relative to other AP-1 family members and PTH and PTHrP action, all the FOS family members are upregulated in response to PTH and PTHrP but only the JunB member of the JUN family is upregulated [36]. The AP-1 protein is a heterogeneous transcription factor composed of a dimer of Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra-1 and Fra-2) family members. The Jun and Fos family members interact through leucine zipper motifs and bind DNA through a basic region. There are a wide array of genes important in bone and hematopoiesis that have AP-1 sites in their promoters and hence could be targets of PTHrP action in the bone microenvironment [37].

3 PTHrP and cancer

3.1 Breast cancer

The expression of PTHrP has been identified in a large number of primary breast cancers [38] and expression may be even higher in skeletal metastatic lesions. PTHrP was found positive in 60% of primary breast cancers in a series of 102 consecutive invasive breast tumors removed surgically from normocalcemic women [38]. Further study demonstrated that PTHrP was localized by immunohistochemistry in 92% of breast cancer metastases in bone but only 17% of metastases in non-bone sites [39]. Similar results were reported by other groups. Kohno et al. reported that 57% of breast cancer specimens showed positive PTHrP immunoreactivity [40]. Of these, the PTHrP positivity was 83% in the patients who developed skeletal metastases, 38% in those who developed lung metastases, and 38% in those without recurrence, respectively. Interestingly, the level of positive PTHrP staining was strongly related to expression of the estrogen and progesterone receptors [40]. A recent study showed PTHrP protein was expressed in 68% of early breast cancer compared with 100% of bone metastases [41]. Bouizar et al. investigated the differential expression of PTHrP isoforms in different stages of breast cancer. The amount of the 1–139 isoform mRNA was much higher in the tumors of patients who later developed metastases than in those of patients who developed no metastases. This isoform mRNA was also more abundant in breast tumors from patients who developed bone metastases than in those of patients who developed metastases in soft tissues. By contrast, the amounts of the 1–141 isoform mRNA in these three groups of tumors were similar [42]. In addition, elevated levels of circulating PTHrP, as measured by RIA were also detected in 65% of breast cancer patients [43]. These findings raise the question as to whether PTHrP may contribute to the ability of breast cancers to develop into bone metastases.

The relationship of PTHrP with prognosis in breast cancer is still uncertain. Using immunohistochemistry and *in situ* hybridization, PTHrP was observed in 69% of breast adenocarcinomas [44]. Expression of immunoreactive PTHrP was inversely correlated with tumor stage and extent of nodal involvement at the time of diagnosis. A similar trend was documented by another group who reported that among 177 surgically resected breast carcinoma specimens, 64% of the breast tumors were positive for PTHrP immunohistochemical staining [45]. When the PTHrP expression was divided into three categories, a significant positive relationship was found between PTHrP expression and histological grade of tumor. The patients with positive PTHrP tended to have a poor outcome proportional to the degree of positive staining. Another study reported positive PTHrP staining in 56% of the cancers and although PTHrP immunostain was related to bone metastases and hypercalcemia, it was unrelated to standard prognostic factors, recurrence or survival [46]. In contrast, another group recently reported PTHrP as an indicator of improved prognosis of breast cancer [47]. A prospective study was done in a large group of 526 consecutive patients with operable breast cancer. The significance of positive PTHrP staining by immunohistochemistry was evaluated for a median of 10-year follow-up. Improved survival was observed for the 79% of tumors which stained positively for PTHrP. Patients with PTHrP-positive primary tumors were less likely to develop bone metastases. The PTHrP status was positively associated with the estrogen receptor and progesterone receptor status, but not with tumor grade. In general, the appearance of metastases to the bone was higher in patients with PTHrP negative primary cancers compared to PTHrP positive primary cancers (22% vs. 10%). They also found that patients with PTHrP negative primary cancers could have PTHrP positive metastatic lesions which highlights that PTHrP status may be modified during the process of metastasis or in the bone marrow microenvironment. The differences in this study versus other reports may be due to the usage of different PTHrP antibodies in immunohistochemistry. Whole PTHrP protein is processed by cleavage and converted to multiple peptides. The amino-terminal PTHrP (1–34) is the most active component that can bind to PTH/PTHrP receptor and enhance osteoclastogenesis whereas the carboxyl terminal may inhibit bone resorption by osteoclasts [48]. Hence the selection of antibodies that

target specific epitopes of the peptide could provide differing results amongst studies.

3.2 Prostate cancer

Normal prostate expresses PTHrP at a low level [49]. By immunohistochemistry and in situ hybridization, PTHrP expression was detected in a few epithelial cells in normal prostate. Double staining with chromogranin A (a generic neuroendocrine marker) revealed that PTHrP was present in a subpopulation of neuroendocrine cells [49]. Radioimmunoassay demonstrated that PTHrP is present in seminal fluid [50]. The fact that no PTHrP expression is detectable in the seminal vesicles suggests that PTHrP is secreted by the prostate. PTHrP expression is found in malignant prostate tissues and our group initially reported its expression in human metastatic lesions [14]. In the University of Michigan SPORE warm autopsy program tissue procurement [51], PTHrP has been detected strongly in bone metastatic lesions and co-localizes with PSA positive tumor tissue (Fig. 2). In primary tumors, one study examined PTHrP in 33 radical prostatectomy specimens in patients with clinically localized carcinoma using immunohistostaining [52]. PTHrP was localized in the cytoplasm of the tumor cells and the intensity of the staining correlated with increasing tumor grade. A more comprehensive study showed that expression of PTHrP was associated with the progression of prostate carcinoma [53]. In normal prostate tissue, PTHrP was barely detected. In benign prostatic hyperplasia, 13 of 15 tissues were positive for PTHrP and an average of 33% cells stained positive. In contrast, all prostate cancer specimens were positive for PTHrP. In well-differentiated and prostate caner, an average of 87% cells stained positive, whereas, 100% cells were positive in poorly differentiated and metastatic tumors. The intensity of staining for PTHrP was scored at 3+ in well differentiated tumor and 4+ in poorly differentiated tumors [53]. By northern blot analysis, a robust amount of PTHrP mRNA was found in a highly metastatic prostate cancer cell line PC-3 derived from bone metastases [14]. In contrast, PTHrP was hardly detected in LNCaP, a low metastatic prostate cancer cell line derived from lymph node metastases.

The receptor for PTHrP, the PTH-1R has also been reported to be present in prostate cancer cells. The PTH-1R was reported to be highly expressed in bone metastases from prostate cancer [54]. Using *in situ* hybridization, it was reported that the receptor appears to co-express with PTHrP in prostate cancer. PTHrP was found positive in 13 out of 14 primary tumors and all 14 metastases. The receptor was positive in all primary tumors and 12 out of 14 metastases. The co-expression of PTHrP and its receptor in prostate cancer suggests an autocrine/paracrine mechanism that may contribute to prostate cancer progression.

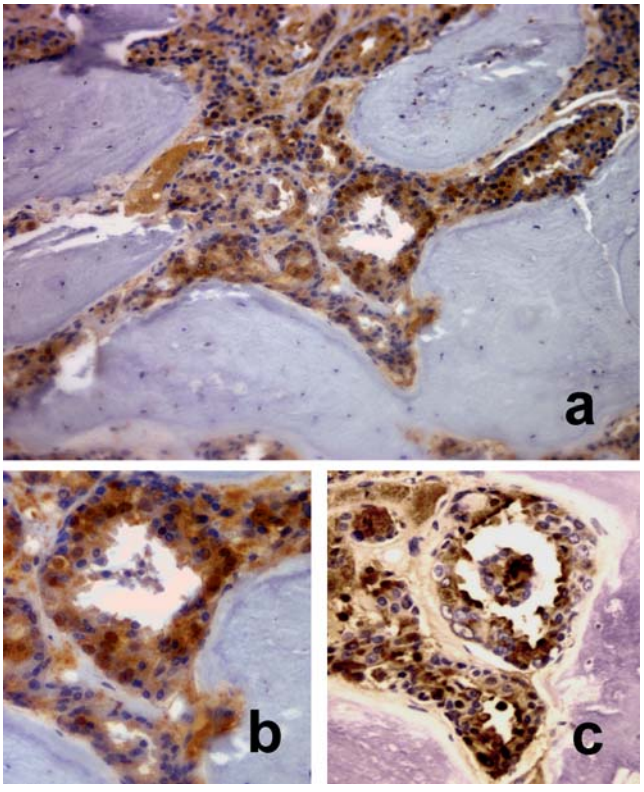

Fig. 2 PTHrP expression in prostate cancer bone metastases. (a), (b) Immunostaining for PTHrP in prostate cancer metastatic lesion at the vertebrae. Low power (20×) in (a) and magnified region in (b) demonstrate cytoplasmic and nuclear staining. (c) Co-localization of PSA in the same region as PTHrP immunostaining verifies prostate tissue metastasis

3.3 Lung cancer

PTHrP expression is common in all major lung cancer cell types [55] More than 50% of human lung tumors express PTHrP [55–57]. PTHrP expression may be more common in squamous cell carcinoma and less common in adenocarcinoma than in other lung cancer types [57, 58]. Increased expression of PTHrP in lung cancer patients could be also detected in serum and urine [58]. One report showed direct evidence that lung cancer cells might obtain the ability to produce more PTHrP with progression [59]. Although increased methylation in the 5' region of PTHrP is associated with neoplasia, over expression of PTHrP in lung cancer appears unrelated to DNA methalation [60].

3.4 Renal carcinoma

The kidney is one of the major targets of PTHrP action. In the adult kidney, PTHrP is expressed in the pre- and post-glomerular arterial tree in vascular smooth muscle cells and endothelial cells, in the glomerular podocytes and parietal

epithelial cells, in proximal and distal tubules including the macula densa and in collecting ducts [61]. PTHrP expression has frequently been observed in renal cell carcinoma. PTHrP is localized to the cytosol of tumor cells at various staining intensities [62]. Gotoh et al. reported that PTHrP was present in 95% of human renal cell carcinomas [63]. There was no correlation between the level of immunostaining and the patient's serum calcium level. Tumors of the granular cell type expressed PTHrP to a significantly greater extent than tumors of the clear cell type. Iwamura investigated PTHrP in 40 cases of renal cell carcinoma [62]. Seventy-five percent showed strong staining and 25% were weak or nonexistent. The intensity of staining showed no significant correlation with gender, tumor greatest dimension, stage, or grade. In contrast, tumors of the clear cell type were found to express PTHrP to a significantly greater extent than tumors of the granular cell type. Papworth et al. detected PTHrP in the sera of 15% of 243 renal cell carcinoma patients [64]. A trend of hypercalcemia was found in the patients with PTHrP, but although serum PTHrP positively correlated with serum calcium, it did not correlate with tumor stage. In another study high levels of PTHrP mRNA in renal cell carcinoma and secreted protein in sera correlated with increased levels of K-Ras mRNA suggesting a casual relationship [65]. Since Ras proteins have been implicated at many levels during tumorigenesis via their ability to transduce extracellular signals to a variety of intracellular events, this suggests that Ras may be upstream of PTHrP and contribute to hypercalcemia of malignancy.

3.5 Colon cancer

There have been several reports of colon cancer patients with hypercalcemia and documented high levels of PTHrP [66]. Malakouti et al. reported that up to 91.5% of colon cancers stained positive for PTHrP by immunostaining. In polyps of the colon 22.6% of the cells were PTHrP positive, while in normal colon, 5.7% of the tissue samples were PTHrP immuno-positive and the intensity of PTHrP staining was higher in tumors than polyps [67]. Amplification of the PTHrP gene was observed in leukocytes from a colon cancer patient with humoral hypercalcemia [68].

3.6 PTHrP mediates preferential localization of tumor cells to bone

Enhanced PTHrP expression is frequently identified in tumors with bone metastasis. The causal effect of PTHrP in tumor skeletal progression has been intensively explored.

With an intracardiac injection model, Iguchi et al. found human lung squamous cell carcinoma-derived cells (HARA) are more aggressive to develop bone metastasis than another line of human lung squamous cell carcinoma-derived cells (QG-56). The malignancy of these cell lines correlated to their PTHrP expression. Furthermore, treatment with anti-PTHrP neutralizing antibody was sufficient to reduce the incidence of bone metastases, number of tumor colonies and the tumor volume of HARA cells [69]. Similar work was performed in breast cancer. Four of eight established human breast cancer cell lines expressed PTHrP [70]. Among these cell lines, the MDA-MB-231 is the one that expresses the highest PTHrP. It developed typical osteolytic lesions after inoculated into left ventricles of nude mice. Anti-PTHrP (1–34) significantly decreased osteolytic lesions at bone.

Other than studies using neutralizing antibodies, the roles of PTHrP in bone metastasis have also been evaluated by stable transfection of the PTHrP gene into tumor cell lines. Ectopic expression of PTHrP in a breast cancer cell line resulted in greater osteolytic bone lesions in mice bearing tumors [71]. Overexpression PTHrP converted a non-invasive prostate cell line into one that progressed in the skeleton. Injection of the PTHrP transfected cells resulted in greater tumor progression in bone when compared to non-transfected cells [72].

3.7 PTHrP in bone metastasis

PTHrP can facilitate tumor bone metastasis either by enhancing metastatic potentials of malignant cells directly via autocrine, paracrine and intracrine mechanisms, or by converting bone to a much more fertile environment for tumor growth via tumor and bone interactions. PTHrP and its receptor are frequently coexpressed in tumors [54, 73]. Thus, the direct effect of PTHrP on tumor progression via autocrine and/or paracrine mechanisms cannot be ignored. PTHrP might stimulate cell proliferation, adhesion and survival by directly acting on tumor cells. In other cell systems, PTHrP stimulates cell proliferation by regulating cell cycle, via regulation of cyclin D1 expression [28, 74]. PTHrP resulted in an increase of DNA synthesis in MCF-7 breast cancer cell line with ectopic expression of its receptor [75]. PTHrP also stimulated proliferation in highly tumorigenic human SV40-immortalized breast epithelial cells [76] and clear cell renal carcinoma [77]. Overexpression of PTHrP by stable transfection with a PTHrP cDNA promoted colon cancer cell proliferation [78]. PTHrP also resulted in increased adhesion to collagen type I, fibronectin, and laminin [79]. The anti-apoptotic effect of PTHrP in cancer progression may also be responsible for increased cell numbers. Pretreatment with PTHrP-(1–34) and PTHrP-(140–173) suppress apoptosis induced by UV irradiation or treatment of PMA [14, 80]. An intracrine mechanism may also contribute to this anti-apoptotic effect of PTHrP [13, 81].

PTHrP is an active regulator of bone resorption. In bone, PTHrP stimulates the expression of RANKL by osteoblasts. RANKL acts on preosteoclasts to cause the transition to mature osteoclasts. Eventually, PTHrP enhances the process of bone resorption. Bone is a reservoir of calcium and growth factors. These growth factors include TGF-β, IGFs, FGFs, PDGF, and BMPs. With bone resorption, local calcium is elevated and the stored growth factors such as TGF-β from the bone matrix are released. Elevated calcium increases tumor cell proliferation via the calcium sensing receptor and also stimulates PTHrP synthesis form the tumors, resulting in a cyclic feedback support system for tumor establishment and growth [82, 83]. These alterations by PTHrP could be critical for establishment of bone metastasis. Further evidence comes from studies of inhibition of bone resorption by bisphosphonates where skeletal progression was greatly reduced [84–88]. In addition to elevated calcium, TGF-β stimulates production of PTHrP by tumors [89]. The feedback loop thus amplifies the favorable signals for tumor growth (Fig. 3). A vicious cycle was thus proposed to depict the role of PTHrP in tumor skeletal progression [90].

3.8 PTHrP skeletal lesions and hypercalcemia

PTHrP is important not only for localization and the late expansion of tumor at bone, but also for pathology of tumor associated bone lesions and complications. Tumor growth at bone changes bone structure, and causes two types of lesions: osteoclastic and osteoblastic. Osteoclastic lesions are characterized by bone resorption and destruction, while osteoblastic lesions are characterized by new bone formation. Actually, all bone lesions show both osteoclastic and osteoblastic activity. Both processes are typically accelerated in the affected bones. The two different types of lesions result from different aberrations in the process of bone remodeling. Either of the lesions places the bone structure at risk of weakening and destruction.

Osteoclastic lesions are characteristic for most tumors. This type of lesion is common in breast, lung, and renal carcinoma. PTHrP expression has been shown closely associated with these lesions in patients. As a potent stimulator of osteoclastgenesis, PTHrP is a primary mediator of bone destruction by tumors. The vicious cycle model well describes the critical role of PTHrP in this type of lesion.

Osteoblastic lesions are relatively rare and most frequently seen in prostate cancer. This lesion is histologically characterized by increased newly formed woven bone. Numerous osteoblasts exist at the region near to the tumor. Nevertheless, the mechanism is still not well identified yet, probably because effective animal models are lacking. PTHrP (1–34) binds to same receptor as PTH. Both of them are stimulators of osteoclastgenesis, while anabolic actions are also observed [34, 35, 91, 92]. Interestingly, PTHrP was reported to be associated with tumor-induced calcification in breast cancer [44]. Tumor-associated calcifications were identified in 43% of PTHrP-positive carcinomas, but in only 12% of PTHrP-negative carcinomas. Thus, PTHrP may be implicated in the osteoblastic lesion.

Hypercalcemia is probably the most common complication of malignancy, and closely related to tumor morbidity. Though some non-PTHrP factors could also be involved, PTHrP appears to play central role in cancer associated hypercalcemia. The majority of hypercalcemia patients have high levels of PTHrP. Synthetic PTHrP amino-terminal fragments alone have been shown to cause severe hypercalcemia in animal models. Neutralizing antibodies to PTHrP can reverse hypercalcemia caused by hypercalcemic human tumors grown in nude mice. PTHrP thus seems necessary and sufficient to explain the key features of hypercalcemia of malignancy. The mechanisms of PTHrP could be (1) Overexpression PTHrP at bone metastases cause increase of mature osteoclasts and bone resorption. (2) Circulating PTHrP may also work through regulation of calcium metabolism in kidney and intestine. Pertinent to prostate carcinoma, PTHrP has been shown to be cleaved by prostate specific antigen (PSA) resulting in cleavage products that do not activate the cAMP pathway and hence would not lead to hypercalcemia [93]. Unfortunately this intriguing finding has only been shown *in vitro* and not yet in animals or humans.

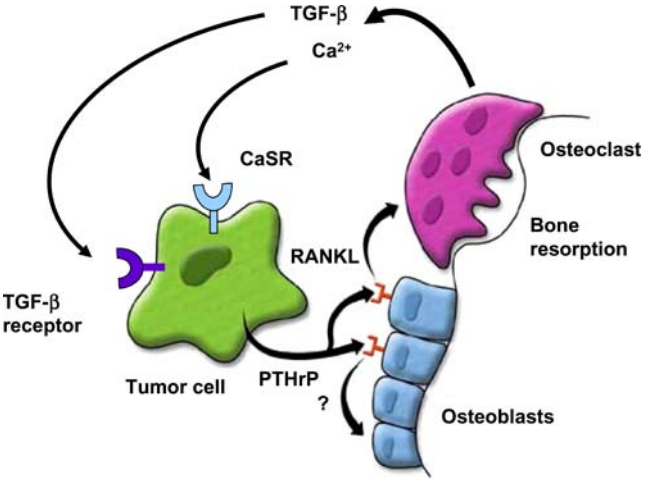

Fig. 3 PTHrP and a vicious cycle of bone metastasis. Tumor cells secrete PTHrP which stimulates bone resorption via receptor activator NFkB ligand (RANKL) expression in osteoblastic cells. Bone resorption results in release of growth factors such as TGFβ and calcium from the extracellular matrix. Calcium and TGFβ both feedback to tumor cells to increase PTHrP production. This unique interaction amplifies favorable signals for tumor localization in bone

3.9 Clinical implications of PTHrP in cancer

Multiple tumors produce PTHrP and high production of PTHrP is often detected in the serum or urine of cancer patients. Measuring serum or urinary PTHrP could be a simple and useful way for early tumor detection, imaging and prognostic evaluation. Several reports supported that increased PTHrP levels in serum and urine could be useful indicators of hypercalcemia, bone metastasis and prognosis in lung cancer patients [58, 94, 95]. Increased PTHrP in serum was also identified in sera of patients with prostate carcinoma [96], renal cell carcinoma [64], and melanoma [97]. The prognostic value isn't quite clear in breast cancer, since PTHrP is reported to be either positively or negatively associated with prognosis. PTHrP has also been tested as a marker to assess leukemia patients with hypercalcemia [98]. In experimental tumor biology, PTHrP has been used as a marker for tracking tumor and monitoring tumor growth [99, 100]. The clinical significance of PTHrP in diagnosis and prognosis needs more intensive and extensive evaluation.

The central roles of PTHrP in skeletal metastasis and associated lesions encourage the exploration of preventive and therapeutic measures by targeting PTHrP. Anti-PTHrP neutralizing antibodies have been shown to be effective to reduce skeletal metastasis, bone lesions and hypercalcemia [69, 71, 101]. Humanized anti-PTHrP antibody was engineered for therapeutic purposes [102, 103]. Small molecule inhibitors of PTHrP and its signaling have attractive prospects for clinic treatment and large-scaled industrial production. Two small nucleotide analogs were reported to inhibit production of PTHrP by tumor cells and reduce bone lesions with higher survival rates in animal models [104]. Vitamin D analogs are also inhibitors of PTHrP expression, and have inhibitory effects in metastasis of lung cancer [105]. PKA is the major signaling pathway that mediates PTHrP-induced production of RANKL by osteoblasts. Antagonists against PKA could be a promising target. Due to the high expression of PTHrP in tumors, PTHrP could also be a common target molecule in specific immunotherapy for patients with a wide variety of tumor types, particularly bone metastases. PTHrP-specific and cancer-reactive cytotoxic T lymphocytes were also generated from patients with different tumor types and may play a role in targeting tumor metastases [106].

PTHrP facilitates tumor bone metastasis and causes bone lesions predominantly via a vicious cycle. Tumor cells, osteoblasts, and osteoclasts interact with each other via multiple cytokines (like TGF-β, IL-6) and local elevated calcium. Any measure that may suppress the vicious cycle could be valuable for intercepting malignant progression in bone. It could be a promising strategy to combine anti-PTHrP drugs with other drugs targeted at different steps in the vicious cycle. The mixture could result in low toxicity and high effective therapy for tumor bone metastasis.

3.10 PTH/PTHrP, stem cell niche and bone metastasis

The bone marrow is the hematopoietic reservoir housed in the skeleton and is the site of generation of circulating blood cells (Fig. 4). Late during fetal development hematopoiesis occurs in the liver and spleen and by puberty most hematopoiesis occurs in the sternum, vertebrae, iliac bones and ribs that contain red marrow. The red marrow consists of a reticular framework located between trabecular bone. These spaces contain fat cells, stromal fibroblasts, and blood cell precursors. As the precursors mature they exit through vascular sinuses and enter the circulation. Support of blood cell proliferation and maturation comes from cytokines produced by stromal cells, macrophages in the bone marrow, and activated T lymphocytes. The most well characterized cytokines include those in the family of colony stimulating factors. The bone marrow is the site of generation of all circulating blood cells and the site where B lymphocytes mature. There has been a great deal of long term interest in the impact of bone cells, in particular how osteoclasts differentiate from hematopoietic cells and affect bone homeostasis. More recently interest is growing in the area of how osteoblasts impact hematopoiesis [107], but a relatively unexplored area is how blood cells in the marrow may impact bone formation. Cytokine production by hematopoietic cells impacts osteoblastic activity but wheth-

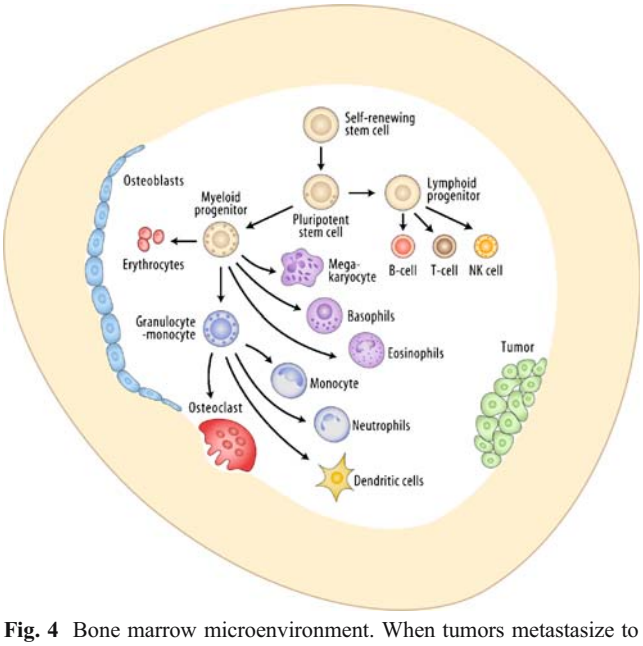

Fig. 4 Bone marrow microenvironment. When tumors metastasize to bone they have the potential for bidirectional interactions with cells in bone as well as the range of hematopoietic lineage cells in the bone marrow

er direct cell contact and juxtacrine factors play key roles is less clearly defined.

It has long been known that tumors typically home to areas in the bone marrow that contain 'red marrow'. Areas of fatty marrow do not appear to be as attractive for tumors to home to. Recent interest has focused on the potential role of PTH and PTHrP as a stem cell factor that supports hematopoiesis in these sites. Work with a constitutively active PTH-1R revealed that PTH signaling resulted in increased bone formation in the trabecular compartment of bone at the same time that the cortical bone was reduced [108]. Further investigation revealed that PTH was capable of supporting hematopoiesis through its action on early hematopoietic stem cells and via the notch/jagged pathway. The current hypothesis is that PTH binds to receptors on osteoblasts and stromal cells and stimulates the expression of Jagged (Fig. 5). Jagged is a ligand for notch which supports many stages of hematopoietic stem cell differentiation including T and B cell lymphogenesis. Blocking notch with a gamma secretase inhibitor was successful at reducing the PTH mediated effect further validating this interaction. The link between the PTH stimulation of hematopoiesis and an anabolic action of PTH is suggested but not yet confirmed. Interestingly, this raises the possibility that PTHrP secreted from tumors in the bone marrow microenvironment may promote hematopoietic stem cell development and perhaps an increase in bone formation. In skeletal metastatic tumors, despite a prominent lytic component there is an increase in bone formation [84]. Whether this is secondary to a remodeling feedback loop or another mechanism such as an intermediate

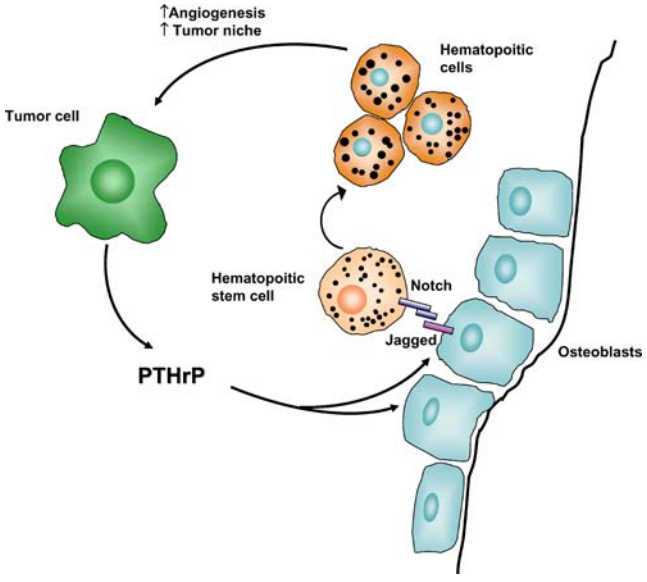

Fig. 5 Tumor cells have been reported to produce PTHrP that acts on osteoblastic cells to increase expression of Jagged 1. Jagged 1 supports hematopoietic stem cell development via interaction with Notch on hematopoietic cells. Increases in hematopoietic cells have the potential to turn on angiogenesis and support the tumor cell niche in bone

hematopoietic cell impacting osteoblastic cell activity is as yet unclear. PTH and/or PTHrP have also been found to regulate other genes associated with hematopoiesis including macrophage colony stimulating factor (M-CSF), monocyte chemotactic protein-1 (MCP-1), and vascular cell adhesion molecule 1(VCAM-1) [9, 109, 110]. Hence when tumors metastasize to the bone and produce PTHrP there is clearly a direct impact on not only the target osteoblastic cells but also on the hematopoietic cells and stem cells that impact the microenvironment through their potential to mediate cell communication, commitment and differentiation (Fig. 4).

3.11 Conclusions future directions

PTHrP is expressed by a wide variety of tumors that metastasize to the skeleton. Once in the skeleton, PTHrP is likely the most bone active cytokine produced by the tumor, and it acts via its receptor, the PTH-1R on osteoblastic cells. The actions of PTHrP on osteoblastic cells are to either stimulate or inhibit proliferation dependent on the cell stage, to inhibit mineralization and support osteoclastogenesis. The catabolic actions of PTHrP are well characterized and have been clearly implicated in lytic metastatic lesions. The potential for tumor derived PTHrP to impact bone formation with a resultant 'osteoblastic' type lesion is less clear but data is suggestive, and further study is warranted. Several aspects of the 'vicious cycle' exist between tumor cells and bone where PTHrP is a key intermediary factor. The manner and impact of PTHrP in the bone marrow microenvironment is emerging as an important activity of PTHrP in the stem cell niche. Tumor cell/hematopoietic cell communications require further investigation but evidence suggests that bidirectional exchanges of tumor cells and cells of the bone marrow exist and that tumor derived PTHrP may be central to these interactions in the microenvironment of the metastatic lesion. PTHrP has been implicated as a deleterious mediator of tumorigenesis in metastatic lesions, however its expression relative to patient outcomes has been controversial, and further study is necessary to precisely define correlations of PTHrP and tumor progression in humans. Numerous strategies have been explored to dysregulate PTHrP and hold promise with further verification as therapeutic strategies to lessen the burden of tumor metastasis.

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