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Original Paper

Parathyroid Hormone-related Protein (PTHrP) and Hypercalcaemia

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INTRODUCTION

HYPERCALCAEMIA COMPLICATES many cancers, especially squamous cell cancer of the lung and other sites, breast cancer, renal cortical carcinoma and a number of haematological malignancies. The term, humoral hypercalcaemia of malignancy (HHM), described a clinical syndrome caused by secretion by a tumour of a calcaemic factor acting on the skeleton to increase bone resorption and on the kidney to increase conservation of calcium. This syndrome has now been explained, with the isolation, cDNA cloning and expression of parathyroid hormone-related protein (PTHrP). The discovery of PTHrP, followed by the establishment of assays that enable its detection in the circulation, has provided new insights into the mechanisms for hypercalcaemia and the skeletal complications in cancer.

DISCOVERY OF PTHrP

Hypercalcaemia has been recognised as a metabolic complication of malignancy since 1924 [1]. In 1941 Albright proposed that hypercalcaemia in a patient with renal carcinoma, which resolved after irradiation of a single bone metastasis, might be due to production by the cancer of parathyroid hormone (PTH) [2]. In succeeding years this idea gained acceptance and the term, 'ectopic PTH syndrome', was widely used to apply to patients with cancer who had a high plasma calcium level, low phosphorus and minimal or no bony metastases [3]. Support for this came in 1966 when Berson and Yalow [4] published results with the first radioimmunoassay (RIA) for PTH, in which they found significant elevations of the PTH level in a number of unselected patients with lung cancer. Over the next several years until the early 1970s, several reports were published of measurable PTH (by radioimmunoassay) in extracts of cancer from such patients [5].

Throughout this time it was evident, however, that the RIA of PTH presented technical problems and in none of the above instances were circulating levels of PTH convincingly very high—certainly not at the levels frequently found with corresponding degrees of elevation of plasma calcium in patients with primary hyperparathyroidism. In the early 1970s some doubt arose regarding the nature of the hormone(s) producing the biochemical features of this cancer syndrome. In 1971, Riggs and associates [6] studied hypercalcaemic patients with non parathyroid cancer or primary hyperparathyroidism and noted that, although hypercalcaemia was greater in the tumour group, the latter had lower serum immunoreactive concentrations of an apparently different immunological entity to the PTH measured in primary hyperparathyroidism. A crucial study by Powell and associates [7] used multiple RIAs, which enabled detection of PTH, proPTH or PTH fragments, in the study of tumour extracts and sera from patients with hypercalcaemia and malignancy. PTH immunoreactivity was not detectable despite significant PTH-like bioactivity in tumour extracts and it was concluded that a humoral substance other than PTH must be responsible for the hypercalcaemia. Roof and colleagues [8] used several RIAs to demonstrate heterogeneity and a lack of immunological identity of PTH-like peptides in sera from both normo- and hypercalcaemic cancer patients and concluded that 'some tumours elaborate peptides with an amino acid sequence different from that of normal parathyroid hormone'.

The more comprehensive clinical and biochemical investigations that followed [9–11] indicated that the manifestations of HHM were mediated via PTH receptors in kidney and bone [5] and the development of sensitive bioassays for PTH led to exciting developments in the understanding of the syndrome. The bioassays revealed that these tumour extracts could stimulate adenylate cyclase in PTH-responsive renal cortical membranes [12]. A sensitive cytochemical assay for PTH in kidney cells could detect PTH-like bioactivity in the serum of patients in whom immunoreactive PTH was undetectable [13]. Studies in PTH-responsive osteogenic sarcoma cells showed that tumour extracts of rat and human origin could also stimulate adenylate cyclase in this system [14]. Peptide antagonists of PTH blocked biological activity, but pre-incubation with PTH antisera was ineffective in blocking biological activity [14], indicating that the active material acted on PTH receptors but was immunologically distinct from PTH. Messenger RNA for PTH could not be detected in any of a series of tumours associated with the HHM syndrome [15]. These observations led to the identification and

isolation of the factor responsible for the syndrome of HHM. Experimental animal models were developed and cell cultures established from animal and human tumours [16, 17]. Purification of the active factor to homogeneity on gels was reported [18, 19] and PTHrP was finally purified, sequenced and cloned from a cultured human lung cancer cell line (BEN) [18, 20] and from other sources [21, 22].

ACTIONS

The amino acid sequence of PTHrP bears 60% homology with that of PTH over the first 13 amino acids. The gene codes for a protein of 141, 139 or 173 amino acids determined by alternate 3′ splicing of mRNA. A pre-pro sequence of 36 amino acids contains potential cleavage points at residues -8 or -6 leaving a short pro-sequence. The PTH-like biological activity of PTHrP is contained within the first 34 amino acids. Beyond this region, the two molecules have unique sequences.

The receptor binding and activation domains of PTH and PTHrP are contained within the first 34 amino acids [23]. Both have essentially identical actions through a common receptor for PTH/PTHrP [24], elevating plasma calcium by promoting bone resorption and decreasing calcium excretion. Synthetic and recombinant PTHrP peptides have been shown to have similar potency to PTH in stimulating adenylate cyclase in both bone and renal cells in addition to promoting resorption of cultured fetal rat long bones and neonatal mouse calvaria, whilst *in vivo* studies have shown them to be potent hypercalcaemic agents [25]. Histomorphometric analysis revealed increased osteoclastic activity in mice repeatedly injected with PTHrP(1-34) [26], whilst infusion of PTHrP(1-34) into nephrectomised, thyroparathyroidectomised rats confirmed that PTHrP acts to promote bone resorption *in vivo* [27].

Transplantation of PTHrP-producing tumours or cell lines into rats or athymic mice has provided models of humoral hypercalcaemia of malignancy [28, 29]. Passive immunisation of these rodents with either polyclonal or monoclonal antibodies against PTHrP has been shown to decrease serum calcium concentrations markedly, prolong the longevity of the animals and cause histomorphometric changes analogous to those produced by tumour resection (Figure 1) [28–30]. These studies provided conclusive evidence for the central role of PTHrP in the hypercalcaemia of malignancy, with amino-terminal directed antibodies blocking binding of PTHrP to the PTH/PTHrP receptor, thus blocking both the renal and bone actions of PTHrP.

THE ROLE OF PTHrP IN HYPERCALCAEMIA OF CANCER

Further confirmation of the aetiological link between PTHrP and hypercalcaemia associated with malignancy has been achieved by measurement of circulating levels by RIA. A large number of immunoassays for PTHrP have been developed using antibodies directed against amino-terminal, mid-molecule and carboxy-terminal sequences, as well as two-site assays spanning amino-terminal and mid-molecule sequences. Assays for PTHrP have provided further insight into the prevalence of PTHrP as a cause of hypercalcaemia of cancer and have the potential to become useful diagnostically. RIAs [31–34] and two-site immunoradiometric assays (IRMAs) [35–38] have documented circulating levels of PTHrP in high proportions of subjects with malignancy-associated hypercalcaemia. The possibility that PTHrP might circulate and contribute to hypercalcaemia in primary hyperparathyroidism is raised by the demonstration of PTHrP mRNA in parathyroid adenomas [39] and the demonstration of PTHrP by immunohistochemistry and Western blotting in parathyroid adenomas [40] and also in hyperplastic parathyroid tissue associated with chronic renal failure [40]. However, circulating levels of PTHrP are not generally elevated in primary hyperparathyroidism. Results so far suggest that PTHrP circulates at extremely low levels in healthy subjects, if at all, and may only rarely be detected by N-terminal RIAs. In contrast to assays directed against the amino-terminal portion of PTHrP, assays directed against the carboxy-terminal portion of the PTHrP molecule [35, 41] measure markedly elevated levels in patients with renal failure. This points out the similarity of PTHrP to native PTH with respect to metabolism of carboxy-terminal fragments that depend upon renal mechanisms for their clearance. The accumulation of C-terminal fragments in renal failure suggests that PTHrP also originates in non-malignant tissues. The source of circulating PTHrP in the absence of malignancy remains unclear.

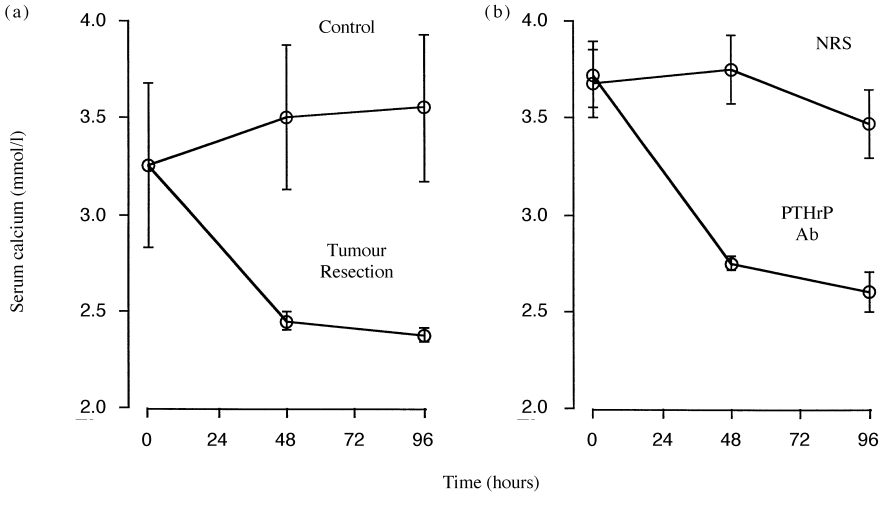


Figure 1. Effect of tumour resection (a) or injection of neutralising antiserum to PTHrP (b) on serum calcium in tumour-bearing athymic mice (NRS, Normal Rabbit Serum). Modified from *Endocrinology* 1990, 127, 305–310 with permission.

In some normocalcaemic patients with malignancy, levels close to the detection limit of various assays have been reported [31, 35, 38, 42]. We showed by immunohistochemistry that PTHrP was present in 100% of a series of squamous cell cancers of various origins [43]. This suggests that further sensitivity is needed for PTHrP assays to be applied to the early identification of those patients with cancer in whom circulating PTHrP levels are rising and who are, therefore, at risk of the development of hypercalcaemia.

HUMORAL HYPERCALCAEMIA OF MALIGNANCY (HHM)

The term 'humoral hypercalcaemia of malignancy' (HHM) was introduced to describe patients with certain cancers in whom blood calcium is elevated in the absence of skeletal metastases. Tumour factors are secreted that act on the skeleton generally to increase bone resorption and on the kidney to reduce calcium excretion and increase phosphorus excretion [5]. Nephrogenous cyclic AMP excretion is also increased [9–11] and there is often a mild hypokalaemic, hypochloraemic alkalosis. Using an N-terminal RIA [34], we detected circulating PTHrP in 100% of hypercalcaemic patients with solid tumours and no bone metastases (Figure 2). A group of hypercalcaemic patients have been observed with undetectable PTHrP by IRMA [37] and suppressed PTH, but elevated nephrogenous cyclic AMP excretion. Some of these patients had small cell carcinomas of the lung, a tumour type which has been shown to produce PTHrP [44, 45] but which rarely causes hypercalcaemia [44]. Small cell carcinoma cell lines produce amino-terminal species of PTHrP, which would not be detected by IRMA [44]. This suggests that *in vivo* tumour specific processing of PTHrP may limit the diagnostic utility of some PTHrP IRMAs.

There is little doubt that PTHrP is the major, if not the sole mediator of hypercalcaemia in patients with the HHM syndrome. It is still possible that, in some cases, other bone resorbing factors could contribute to the development of hypercalcaemia on a humoral basis. Colony stimulating factors, epidermal growth factor, interleukins 1α, 1β and 6, transforming growth factors α and β and tumour necrosis factor have all been shown to promote bone resorption *in vitro* [46–53] and/or be associated with hypercalcaemia [54–59]. The significance of these factors and their possible interplay with PTHrP should become clear as our knowledge of the role of cytokines in bone metabolism is increased.

HYPERCALCAEMIA AND SKELETAL METASTASES

Although for many years it was considered that the main mechanism of hypercalcaemia in patients with breast cancer was the release of calcium from bone by osteolytic deposits [5], there is now evidence for a humoral contribution in these patients also. The extent of metastatic bone disease correlates poorly with both the occurrence and the degree of hypercalcaemia in malignancy [60]. In 80–90% of cases of unselected solid tumour patients with hypercalcaemia, irrespective of whether bone metastases are present, there is evidence of an underlying humoral mechanism [60]. Measurement of plasma PTHrP concentrations by direct RIA confirmed this finding, with 65% of a series of hypercalcaemic breast cancer patients having detectable levels [32, 34, 42] (Figure 2). PTHrP levels above those of normal subjects were also found in 64% of patients with hypercalcaemia and metastatic malignancy to bone, from primary sources other than breast (Figure 2). This latter group included several patients in whom the mechanism of hypercalcaemia was likely to be humoral. Consistent with this is the finding that in the metastatic group, all squamous cell cancer patients had elevated PTHrP levels, as did one patient with a pancreatic neuroendocrine tumour in this group. The presence or absence of bone metastases, a feature that was used to distinguish between humoral and osteolytic mechanisms of hypercalcaemia, can no longer be used to define the syndrome of HHM. It is now well established that a high proportion of patients with hypercalcaemia and skeletal metastases have high circulating levels of PTHrP.

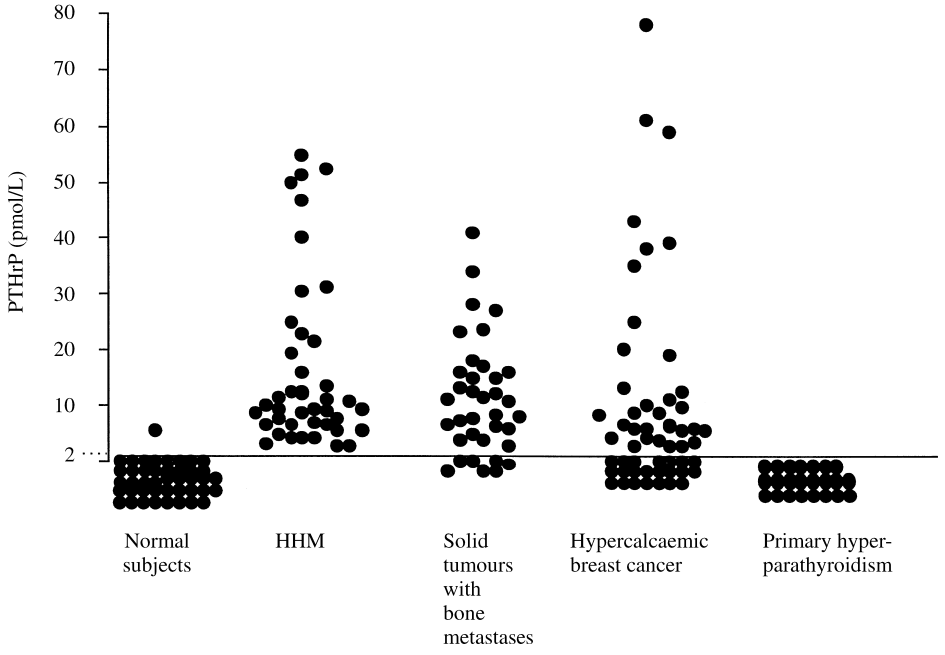


Figure 2. Circulating PTHrP levels by N-terminal RIA in different clinical groups.

In the case of breast cancer, another possible role arises for PTHrP. Approximately 70% of patients dying from breast cancer have bone metastases and, of these, 10–20% are hypercalcaemic [61]. The avidity of breast cancer metastases for bone has long been recognised. Currently, there is no single accurate predictor to identify which patients will develop bone metastases. PTHrP has been demonstrated by immunohistochemistry in approximately 60% of breast tumours [42, 62–64]. The presence of PTHrP in primary breast tumours correlates with positive progesterone receptor status [62, 63] and prognostic index [62] but not prognostic status [64]. PTHrP has been detected by immunohistochemical staining in 92% of breast cancer metastases to bone compared with only 17% of metastases to other sites [65]. PTHrP mRNA has been detected by *in situ* hybridisation in 73% of bone metastases from breast cancer and 20% of metastases to other sites [66]. One possibility which is of particular interest is that PTHrP production might contribute to the ability of breast cancers to erode bone and establish there as metastases. The clinical observations have been extended by using a mouse model of bone metastases in which inoculation of a human cancer cell line into the left ventricle of the mouse reliably produces osteolytic metastases. An antibody against PTHrP(1–34) blocked the formation of osteolytic bone lesions and the growth of metastatic deposits [67] in nude mice injected with a PTHrP producing breast cancer cell line. These data strongly suggest that PTHrP expression by breast cancer cells enhances their metastatic potential to bone.

Prostate cancers also have a predilection for metastasis to bone and may produce osteolytic and/or osteoblastic lesions [68, 69]. Unlike breast cancer, hypercalcaemia is a rare complication of prostate cancer (less than 2% of cases) and is usually associated with tumours of unusual histology [70]. Despite this, PTHrP has been detected immunohistochemically in 100% of prostate cancers [71]. PTHrP protein and mRNA have been detected in both the neuroendocrine and epithelial cells of the prostate [72, 73] and PTHrP is a prostate-derived component of seminal plasma [74]. PTHrP has been shown to be an autocrine growth regulator in prostate cancer cells [75] and a role for PTHrP in the progression of prostatic intraepithelial neoplasia has been suggested [76].

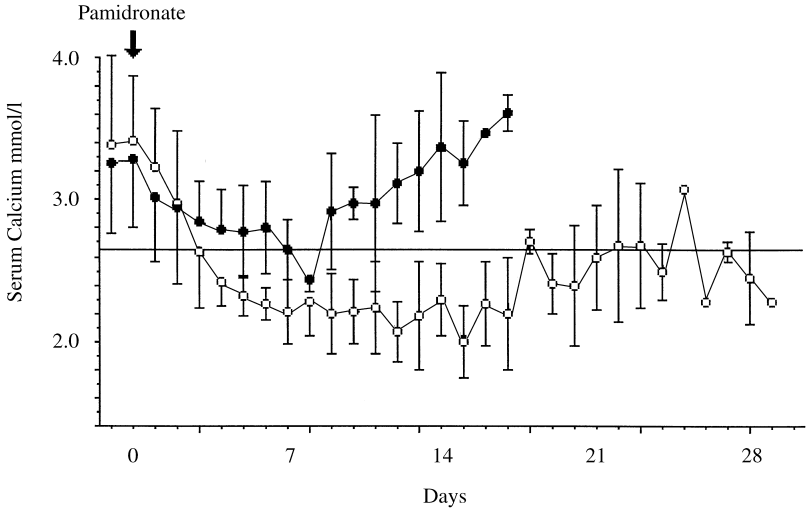
HYPERCALCAEMIA IN HAEMATOLOGICAL MALIGNANCY

Haematological malignancies may be associated with osteolytic bone destruction and with hypercalcaemia. Elevated PTHrP levels are detected by RIA in a proportion of patients with haematological malignancies and hypercalcaemia (Figure 2) [31, 32, 35]. Hypercalcaemia is relatively uncommon in both non-Hodgkin's and Hodgkin's lymphoma. In a series of 165 consecutive patients admitted to a haematology unit, we documented hypercalcaemia in 18. It was due to primary hyperparathyroidism in 3 cases. In the others, it was associated with multiple myeloma in 9 patients, high grade B-cell non-Hodgkin's lymphoma in 5 and with myeloid neoplasia in one [77]. A number of case reports have documented hypercalcaemia without lytic bone lesions in both Hodgkin's and non-Hodgkin's lymphoma, associated with elevated 1,25 $(OH)_2D$ levels and low PTH levels in plasma [78, 79]. We detected circulating levels of PTHrP of the order of those associated with HHM in cases of non-Hodgkin's lymphoma of B-cell lineage [77]. Immunohistochemical staining demonstrated intracellular PTHrP in some of the neoplastic cells from a lymph node section in one of the cases.

The regulation of *PTHrP* gene expression has been extensively studied in adult T-cell leukaemia/lymphoma, a malignancy associated with human T-cell leukaemia virus type I (HTLV-1) infection. This malignancy is frequently associated with the HHM syndrome [38, 80]. Expression of PTHrP mRNA within HTLV-1-infected T cells in culture has been demonstrated and immunohistochemical staining detected PTHrP in neoplastic lymph nodes. The HTLV-1 genome encodes a protein, tax, required for transcriptional activation of the HTLV-1 long terminal repeats. Tax also transactivates the PTHrP promoter [81]. This transactivation is restricted to T-cells [82] and another transcription factor, Ets1 acts in synergy with tax to increase *PTHrP* gene transcription [83].

Hypercalcaemia occurs in approximately one third of all patients with multiple myeloma [5]. Histological examination of the lytic lesions in multiple myeloma has shown increased bone resorption by normal osteoclasts in the areas near myeloma cells [84]. This observation suggests that myeloma cells secrete a factor or factors which stimulate the osteoclast either directly or via the osteoblast to resorb bone. Since the initial demonstration of the secretion of an 'osteoclast activating factor' by myeloma cells [85], several cytokines have been postulated to be responsible for increased bone resorption, among them tumour necrosis factor β, interleukin-1 and interleukin-6. Early RIAs measured elevated PTHrP levels in some patients with hypercalcaemia and myeloma [31] and a subsequent number of case studies also reported elevated PTHrP in plasma of patients with myeloma and hypercalcaemia [86–88]. In our series of consecutive hypercalcaemic patients, admitted to hospital with haematological malignancies, elevated plasma levels of PTHrP were measured in one third of patients with hypercalcaemic multiple myeloma [77]. PTHrP mRNA has been shown in cultured human myeloma cell lines and in an iliac crest biopsy of a patient after PCR amplification [87, 89]. We also demonstrated *PTHrP* gene transcripts and protein in bone marrow plasma cells from a hypercalcaemic patient with elevated plasma PTHrP levels, indicating PTHrP production by myeloma cells. It seems likely that PTHrP is another cytokine contributing to hypercalcaemia and the skeletal complications of this disease. It is possible that PTHrP has a role as a local mediator of increased bone resorption in multiple myeloma and that it is at times produced in sufficient quantities to reach the circulation and produce an endocrine effect. Such a process could contribute to the osteoporosis in multiple myeloma as well as to hypercalcaemia. These observations indicate that PTHrP-mediated hypercalcaemia not only occurs in association with solid tumours with or without skeletal metastases, but can also occur with haematological malignancies.

Despite PTHrP having a dual action on bone and kidney, the agents available for the treatment of hypercalcaemia in cancer are primarily aimed at reducing bone resorption and no agent has a substantial effect on calcium excretion. A poor response to pamidronate occurs in cases of hypercalcaemia of malignancy that have evidence of renal tubular stimulation as indicated by a low tubular threshold for phosphate or high threshold for calcium [90]. It is therefore not surprising that, in studies of patients with tumour-induced hypercalcaemia,



PTHrP (pmol/L):

	Mean	Range
Good responders (open circles)	2.9	<2.0 - 11.6
Poor responders (closed circles)	29.0	4.4 - 162

Figure 3. PTHrP and calcium concentrations in poor and good responders to treatment of tumour-induced hypercalcaemia with pamidronate. Modified from the *Lancet* 1993, 341, 1611–1613 with permission.

the PTHrP level was the best determinant for the calcaemic response to pamidronate, with high levels correlating with poor response and vice versa [91] (Figure 3). Other parameters which indirectly indicated the presence of a humoral mechanism for hypercalcaemia also correlated with response. The presence of bone metastases also predicted a good response to treatment [91].

PTHrP AS A PARACRINE EFFECTOR

Whereas PTHrP clearly functions as a hormone in those cancers in which it is produced in excess, in normal circumstances it is produced locally in many tissues, where it is a paracrine effector. PTHrP is widely distributed in normal tissues, and there is a high degree of amino acid sequence conservation across species, implying that PTHrP has important physiological functions. The extensive distribution of the PTH/PTHrP receptor in non classical PTH target tissues supports the idea that PTHrP may mediate many of the extrarenal and extraskeletal actions previously ascribed to PTH. Since PTHrP is not readily detectable in the peripheral circulation of normal subjects, with the notable exception of during lactation [92], these functions are probably autocrine or paracrine in nature. PTHrP has been shown to have hypotensive actions and to relax vascular smooth muscle and also to have a stretch related relaxant effect upon the smooth muscle of hollow viscera such as urinary bladder and uterus [25]. In addition to the PTH-like actions of the amino-terminal portion of the molecule, additional actions have been shown to be located in specific regions of PTHrP beyond the N-terminal portion. Mid-molecule regions of PTHrP have been shown to stimulate placental calcium and magnesium transfer [93] and inhibit renal bicarbonate excretion [94]. *In vitro* studies have shown that carboxy-terminal peptides may inhibit bone resorption in an isolated rat osteoclast bone resorption assay [95, 96], but the true nature of the activity of PTHrP carboxy-terminal peptides remains controversial [97, 98]. The detection of PTHrP and the PTH/PTHrP receptor in murine embryonic carcinoma cell lines, embryonic stem cells and pre-implantation embryos [99, 100], throughout fetal tissues [101] and in pregnancy-related tissues [102–104] and the demonstration of autocrine/paracrine growth factor properties [25], strongly support a role for PTHrP in developmental cell biology and as an autocrine or paracrine mediator of cell growth and differentiation.

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