



## Direct evidence that PTHrP expression promotes prostate cancer progression in bone<sup>☆</sup>

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### Abstract

Parathyroid hormone-related protein (PTHrP) is an oncoprotein that is expressed in many malignancies as well as normal tissues. At essentially every site of expression, PTHrP regulates cell growth and proliferation. We and other investigators have previously reported that PTHrP is widely expressed by prostate cancer. For this tumor, there are substantial *in vitro* and correlative data that PTHrP expression regulates the progression of the tumor, especially in bone, but little direct data. We studied the effects of PTHrP expression on prostate cancer behavior directly in a mouse model of human prostate cancer cells that were transfected to express different forms of the polypeptide and then injected intraskeletally. Skeletal progression of the prostate cancer cells was evaluated radiologically and by measurement of serum tumor markers. PTHrP transfection converted a non-invasive 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, and this effect was also influenced by non-amino terminal peptides of PTHrP. Serum measurements of PTHrP, IL-6, IL-8, and calcium reflected tumor burden. Our experiments provide direct *in vivo* evidence that PTHrP expression results in the skeletal progression of prostate cancer cells.

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Originally discovered as a product of cancers that produce hypercalcemia, parathyroid hormone-related protein (PTHrP) has been demonstrated to be a product of many malignant tissues, including prostate cancer [1]. Studies of PTHrP in prostate cancer support the hypothesis that PTHrP expression regulates prostate

cancer progression and metastasis in bone [1–4]. We along with other investigators have demonstrated that PTHrP is robustly expressed by prostate cancers and that PTHrP levels are greater in malignant tissue than in hyperplastic and normal prostate [2–5]. Furthermore, PTHrP expression correlates with increasing tumor grade [2–4]. PTHrP expression in malignant prostate cell lines also correlates with tumor invasiveness and metastatic potential, well exemplified by the PC-3 prostate cancer cell line. PC-3 cells, derived from a prostatic bone metastasis, produce greater levels of PTHrP than DU 145 prostate cancer cells, derived from brain metastases [6,7]. In the prostate, as in other tissues,

<sup>☆</sup> *Abbreviations:* PTHrP, parathyroid hormone-related protein; SCID, severe combined immunodeficiency; IL, interleukin; PBS, phosphate-buffered saline; *i.v.*, intravenous; SEM, standard error of the mean.

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PTHrP can be processed into distinct peptides that have unique biological effects [7,8]. And prostatic expression of PTHrP is associated with regulatory matrices and molecular interactions that are important in the development and skeletal progression of the [1,9,10]. But while there are substantial *in vitro* and correlative data that PTHrP expression promotes the skeletal progression of many cancers, notably prostate, there are little direct *in vivo* data to support the hypothesis. We provide direct *in vivo* evidence of such an effect for PTHrP in prostate cancer with studies of tumor progression with a mouse model for human prostate cancer [11].

## Materials and methods

**Cells.** The DU 145 and PC-3 human prostate cancer cell lines [6,7] were obtained from American Type Culture Collection (Manassas, VA) and grown in monolayer in RPMI 1640 media (MediaTech, Herndon, VA) supplemented with 10% fetal bovine serum (Gemini Bio Products, Woodland, CA) at 37 °C in a humidified incubator with 95% air, 5% CO<sub>2</sub>. The DU 145 cell line was selected because it has a low constitutive PTHrP expression and does not grow or metastasize well in mouse tumor models, in contrast to PC-3 cells [6,7]. The PC-3 cell line, which was originally isolated from a prostate adenocarcinoma that had metastasized to the bone, has an osteolytic phenotype in the immunocompromised mouse models [1,6].

**Plasmid construction.** PTHrP expression plasmids used in this study were human prepro-PTHrP1-173 and prepro-PTHrP1-87; the prepro forms were used to facilitate PTHrP secretion. The constructs were directionally subcloned in the pCI-neo expression vector (Promega, Madison, WI) and the fidelities of all plasmids were confirmed by DNA sequencing and site-specific PTHrP immunoassays [1,7].

**PTHrP transfection.** The DU 145 prostate cells were seeded at a density of  $2 \times 10^4$  cells/cm<sup>2</sup> in 12-well cell culture dishes and transfected with 1 µg plasmid per well. Individual G418 resistant colonies (800 µg G418/ml) were isolated 21–30 days later. The conditioned media from the picked cell colonies were evaluated for PTHrP expression by site-specific immunoassays and the PTHrP expressing stable DU 145 cells were expanded [7].

**Animals.** Six-month-old male, severe combined immunodeficiency (SCID) mice were housed in a barrier filter room and fed Purina rodent chow ad lib. The animals were bled at the end of the study using a retro-orbital method. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH Publication Number 85-23) under assurance number A3873-01.

**Tumor implantation and experimental course.** Subconfluent DU 145 and PC-3 cells were freshly trypsinized, counted, and placed on ice immediately before injection. The SCID mice were injected with  $10^6$  cells in 15 µl sterile PBS into the bone marrow of the right femur using a 28-gauge needle and a Hamilton glass syringe [6,11]. This allows direct study of skeletal tumor progression, since skeletal injection bypasses the metastatic variables that are present in other animal models of human cancer. The left femur served as the negative control. We studied four types of stably transfected DU 145 cells: (1) wild-type cells, (2) vector (pCI-neo) transformed cells, (3) PTHrP1-87 transformed cells, and (4) PTHrP1-173 transformed cells. The PC-3 cell line was also used as PTHrP-expressing prostate cancer cell line that produces severe bone lesions in immunocompromised mice. The mice were evaluated 60 days after the injection of the cells for skeletal abnormalities by X-ray and biochemical changes in the sera [11].

**Immunoassays.** The conditioned media from the prostate cancer cells and the mouse sera were measured for PTHrP by radioimmu-

noassay [13]. In brief, human PTHrP 38–64 peptide was used as standard and PTHrP 1–86 peptide was radioiodinated by the chloramine T method. Rabbit antiserum to PTHrP 38–64 was used in 3-day non-equilibrium immunoassay format. All samples were assayed in multiple dilutions that paralleled the corresponding PTHrP standard curve. The intra- and inter-assay variations were approximately 7–12%, and the limits of assay sensitivity were 4 pmol/liter. The cytokine (IL-6 and IL-8) immunoassays used antibodies purchased from BioSource International (Camarillo, CA). These two-site immunoassays were detected with a streptavidin labeled β-D-galactosidase enzyme reaction using the fluorescent substrate, 4-methylumbelliferyl-β-D-galactopyranoside (Calbiochem, San Diego, CA), and had assay sensitivities of 3 and 4 pg/ml, respectively, and the intra- and inter-assay variations were approximately 7–12% [1,7,11]. The human IL-6 and IL-8 immunoassays do not cross-react with mouse IL-6 or IL-8 (communication from BioSource International).

**Calcium measurement.** Serum calcium was determined by the reaction of calcium with *o*-cresolphthalein to produce a red complex at pH 10 (Sigma Chemical, St. Louis, MO). The plates were scanned at 570 nm in a plate reader. A reference standard curve was generated to convert the sample optical density values into calcium concentrations [6,11].

**Radiographic analyses.** For skeletal studies, the mice were exposed to 40 keV for 20 s in an HP Faxitron 5000 series X-ray cabinet [12]. The Kodak X-Omat TL films were processed in a Kodak film processor. The radiographic images were quantitated visually using a 10× De-luxe loop objective by two observers. The semiquantitation scoring method was formulated as 0 = no lesions, 1 = minor changes, 2 = small lesions, 3 = significant lesions (minor peripheral margin breaks, 1–10% of bone surface disrupted), and 4 = significant lesions (major peripheral margin breaks, >10% of bone surface disrupted).

**Statistics.** Statistical analyses were performed using Microsoft Excel (Microsoft) software. Differences among treatment groups were assessed using ANOVAs and two-tailed Student's *t* tests. Correlation coefficient significance was determined using Documenta Geigy Scientific Tables, 6th Edition. The X-ray scoring differences were tested using Kruskal–Wallis ANOVA and the Dunn test for post hoc analyses. A value of  $P \leq 0.05$  was considered to be statistically significant. The data are reported as means ± standard error of the mean (SEM).

## Results

We studied the effects of PTHrP expression on the skeletal progression of prostate cancer cells with stable PTHrP transformants of the non-invasive DU 145 prostate cancer cell line that does not express substantial amounts of PTHrP, especially when compared to the invasive PC-3 cells, which robustly express PTHrP [6,7]. The PTHrP expression plasmids used in this study were human prepro-PTHrP1-173 and prepro-PTHrP1-87, absent in the carboxy terminal moieties of full-length PTHrP [7]. Transfection with both PTHrP plasmids changed the DU 145 cells to robust PTHrP producers (Table 1). And intraskeletal injection of both clones of PTHrP-expressing prostate cancer cells and control cells (wild-type and vector transfected DU 145 cells) into SCID mice demonstrated that the expression of PTHrP resulted in tumor progression in bone (Figs. 1 and 2). Furthermore, PTHrP secreted into the blood of tumor-bearing animals served as a tumor biomarker by correlating with prostate cancer burden in bone (Fig. 3).

Table 1  
In vitro and in vivo PTHrP expression levels in a mouse model of prostate cancer

Group	PTHrP species	PTHrP production in vitro (pg/ml/10 <sup>6</sup> cells/96 h)	PTHrP production in vivo (serum, pg/ml)
DU 145 (wild-type)	Endogenous (low)	101	0
DU 145—7A10 (vector)	Endogenous (low)	122	0
DU 145—3D9	1–87	4337	38
DU 145—5A1	1–173	1010	22
PC-3	Endogenous (high)	3233	883

At least eight mice (SCID) were used for each group. The left column shows the human prostate cell line used and the specific clone designation, the second column shows the PTHrP species used to transform the cell line, the third column shows the PTHrP production secreted by the cells in culture, and the right column shows the average PTHrP production in the mice sera 60 days after implantation of  $1 \times 10^6$  tumor cells into the bone marrow of the right femur of each mouse. Serum PTHrP generally correlated with in vitro PTHrP levels.

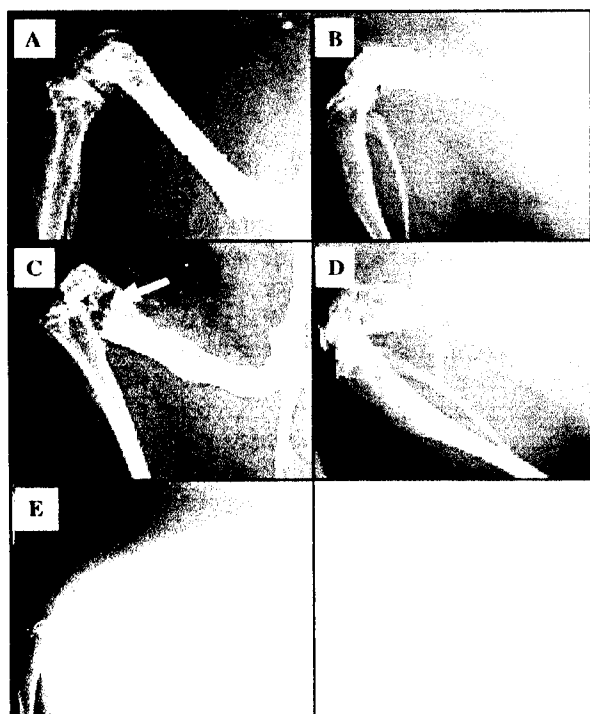


Fig. 1. Mouse model of PTHrP producing prostate cancer demonstrates increased formation of bone lesions. Four groups of DU 145 prostate cancer cells and PC-3 wild-type prostate cells were injected into the right femoral bones of nude mice and allowed to grow for 60 days. Representative femur X-rays for each group are shown in the panels. (A) DU 145 wild-type group. (B) DU 145-vector (7A10) group. (C) DU 145-PTHrP1–87 (3D9) group. (D) DU 145-PTHrP1–173 (5A1) group. (E) PC-3 wild-type group. The arrows in (C)–(E) demonstrate sites of osteolytic (arrows) and osteoblastic (arrowheads) bone lesions. The extent of the lesions generally correlated with their PTHrP expression. See Figs. 2 and 3.

Tumor progression was also marked by increases in serum interleukin (IL)-6, IL-8, and calcium (Fig. 3); IL-6 and IL-8 are growth-regulating and angiogenic cytokines that also promote prostate cancer progression [10]. And there were correlations among tumor volume and the degree of tumor burden in bone and serum PTHrP as well as calcium and IL-8 (Fig. 3). Significant

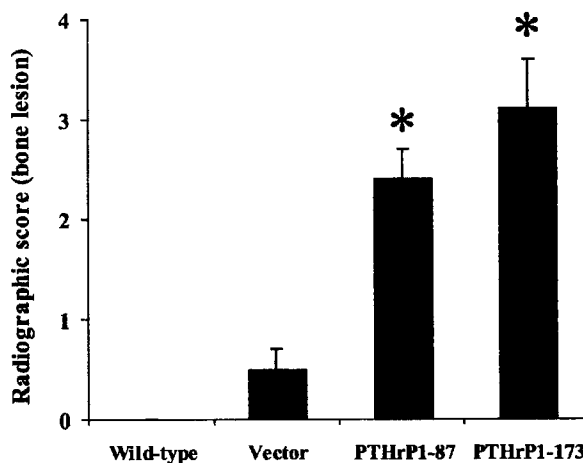


Fig. 2. PTHrP increases bone lesions in a mouse model of prostate cancer. Quantitation of the radiographic images of the mouse femurs injected with the four groups of DU 145 cells and the PC-3 group shown in Fig. 1. The results represent the average scores from two observers for eight mice per group and the error bars are SEM. The asterisks indicate a significant difference compared to the control groups (wild-type and vector) based on a two-tailed Student's *t* tests (\* $P < 0.001$ ).

correlations ( $P < 0.01$ ) of skeletal score (Figs. 1 and 2) vs the specific biomarkers were: with serum PTHrP,  $r = 0.835$ ; with serum calcium,  $r = 0.818$ ; with serum IL-6,  $r = 0.818$ , and with serum IL-8,  $r = 0.756$ . Significant correlations ( $P < 0.01$ ) between the other serum measurements were also demonstrated (IL-6 vs IL-8,  $r = 0.991$ ; PTHrP vs calcium,  $r = 0.493$ ; calcium vs IL-6,  $r = 0.935$ ; and calcium vs IL-8,  $r = 0.929$ ). Thus, PTHrP transfection converted the DU 145 cells from a non-invasive phenotype to one that progresses in bone (Fig. 1). These results provide direct evidence that PTHrP expression by prostate cancer cells promotes the development of skeletal lesions by this tumor, perhaps acting through IL-6 and -8 [1,10,11].

The PTHrP produced by the DU 145-PTHrP1–173 cells was of a lesser amount than the DU 145-PTHrP1–87 (Table 1 and Fig. 3), but the radiographs nevertheless showed more tumor damage in the

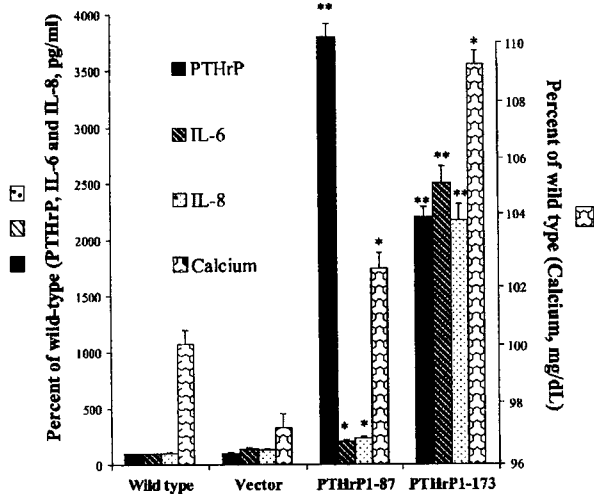


Fig. 3. Serum PTHrP, calcium, IL-6, and IL-8 levels were elevated in mice injected with PTHrP expressing prostate cancer cells. The SCID mice were bled 8 weeks after injection of DU 145 or PC-3 cancer cells and serum levels were measured. The asterisks indicate a significant difference compared to the control groups (wild-type and vector) based on a two-tailed Student's *t* test (\* $P < 0.05$ , \*\* $P < 0.001$ ).

DU 145-PTHrP1-173 mice (Figs. 1 and 2). The PTHrP1-87 plasmid was designed to express the hypercalcemic peptide (PTHrP1-34) but not the classical nuclear localization sequence peptide (PTHrP87-107), osteostatin (PTHrP107-141) or the non-classical nuclear localization and growth-regulatory sequences contained in the carboxy terminus of PTHrP1-173, notably PTHrP140-173 [1,12,13]. The PTHrP1-173 plasmid is able to produce all the potential functional PTHrP peptides [1,7]. The observation that the DU 145-PTHrP1-173 mice had more extensive bone lesions than the DU 145-PTHrP1-87 mice despite lower PTHrP expression (Fig. 1) indicates that there are important functional elements in the 88-173 region of PTHrP that promote tumor progression in bone.

## Discussion

While the most widely studied effects of PTHrP have been of its amino terminal peptide, which signals through the same receptor as PTH, there is accumulating evidence that non-amino terminal peptides of PTHrP can exert biological effects [1,12,13]. For example, PTHrP107-141 has in some studies been reported to inhibit osteoclast function [14]; and we have recently demonstrated that PTHrP140-173 has growth-regulatory actions [13]. These bioactive peptides can be derived through processing of native PTHrP at its many endoproteolytic processing sites [1,8]. In fact, the multiple processing sites in PTHrP predict over 90 peptides, and enzymes that seem to serve this function have been

recently identified from mammalian tissues, including prostatic PSA [8,14]. Since PTHrP contains peptides that may differentially regulate both osteoblasts (e.g., PTHrP1-34) and osteoclasts (e.g., peptides included in PTHrP107-141), the processing of prostatic PTHrP can effect the tumor's skeletal phenotype and progression of bone involvement [4,10,15]. Tissue-specific processing of PTHrP into such peptides may explain the osteoblastic nature of most prostate cancers and also account for the osteolytic component of the tumors. While osteoblastosis is the common phenotype of prostate cancer metastases, osteolysis is likely necessary precursor for prostate cells to colonize bone in that metastatic cells would be less able to invade and grow in mineralized tissue without bone resorption [1,4].

The effects of PTHrP, its isoforms, and processed peptides on prostate cancer are complex. They reflect the fact the native molecule can be processed into peptides that mediate pleiotropic actions through many molecular pathways, including cell growth and apoptosis [12,16]. The presence of moieties in native PTHrP that, respectively, stimulate and inhibit osteoclast-mediated bone resorption presents important molecular mechanisms for PTHrP's skeletal actions. And the effects of the carboxy terminus of PTHrP1-173, important in our experiments, have not been widely examined.

Although prostate cancer is usually characterized by osteoblastic metastases, the tumor also causes osteolysis [2-5]. In addition to PTHrP, a variety of factors produced by cancer cells can stimulate osteoclasts, including transforming growth factors, epidermal growth factors, interleukins, tumor necrosis factors, and prostaglandins. Furthermore, while most studies of PTHrP's regulatory interactions have focused on growth factors and cytokines, it has been recently appreciated that PTHrP also exerts its effects in concert with calcium-regulating and -responding agents [16-18].

While it is well established that PTHrP regulates prostate cell cancer growth, some controversy remains about the nature of this biological effect [1,19]. This is likely due to the differential processing and resulting heterogeneity of the expression of PTHrP in different experimental preparations of prostate cells. To overcome the variability of PTHrP expression that can occur even among established prostate cell lines in the hands of experienced investigators, we have genetically engineered cell lines with specific PTHrP characteristics that can address the hypothesis that we are testing [10].

Although several animal models for human tumor growth have been developed for studying metastatic development and progression in bone of prostate cancer, including orthotopic, systemic, and local inoculation of tumor cells, the direct injection model bypasses the metastatic variables that are present in these other animal models [6]. This allows direct study of skeletal tumor

progression. Studies in animal models such as those reported here can serve at the interface of *in vitro* studies and clinical research. These animal models can thus help to elucidate the role of PTHrP in human prostate cancer pathogenesis and progression, and to identify specific PTHrP species that can become diagnostics and therapeutic targets for this malignancy. For example, we have also developed stable high-expression green fluorescent protein (GFP) transductants of PTHrP expressing human cancer cell lines that can conveniently document cancer progression in the mouse model [20]. Such studies can have clinical import, since most patients with prostate cancer have skeletal metastases; and such metastases are responsible for much of the morbidity of the disease, especially in bone.

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