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TITLE: Prostatic Acid Phosphatase Plays a Causal Role in Prostate Cancer Bone Metastases

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Bone metastases are the major cause of morbidity and mortality in prostate cancer (PCa). While there are treatments for the osteolytic phase of PCa bone metastases, there are no therapies that inhibit the later, osteoblastic phase. Prostatic acid phosphatase (PAP) is a protein secreted by PCa cells and is highly expressed in PCa osteoblastic bone metastases. We previously demonstrated that PAP secreted by PCa cells induces the proliferation and differentiation of osteoblasts (OB). We hypothesized that prostatic acid phosphatase (PAP) secreted by prostate cancer (PCa) cells in bone metastases plays a causal role in osteoblastic bone metastases. As the RANK/RANKL/OPG system coordinately controls the balance of osteoclasts vs. osteoblasts in bone, we determined the effects of PCa-derived PAP on RANKL and OPG expression in both PCa and OB cells. We demonstrate that PAP secretion by PCa cells modulates RANKL/OPG secretion in both PCa and bone cells, favoring more OPG and less RANKL which would promote an osteoblastic phenotype. We utilized three different human PCa cell lines: 1. VCaP (PAP+, induces osteoblastic lesions) 2. PC3 (PAP negative, induces osteolytic lesions) and PC3ML (more aggressive subline of PC3, also PAP negative and induces osteolytic lesions). We successfully generated cell lines that transiently knockdown PAP expression using RNAi technology (PAP-siRNA) in high PAP-expressing human PCa cells (VCaP-luc). Conversely, for the gain-of-function experiments, PAP-negative human PCa cells (PC3-luc) and a more aggressive subline (PC3-ML-luc) were stably transfected to overexpress secretory PAP by utilizing the retroviral expression vector pLenti-GIII-CMV-hACPP (ACPP is PAP) Lentiviral Vector (PC3-pPAP) and the pLenti-III-HA Blank Control (PC3-luc-control) vector as control. Stable cell clones were selected with puromycin and characterized by qRT-PCR and western blotting. Knockout of PAP in VCaP cells induced cell cycle arrest. Knock-in of PAP in PC3 and PC3ML cells increased OPG expression. We are currently performing in vivo studies (intratibial inoculation in nude mice) with our newly generated lines to study the effects of PAP on bone phenotype.					
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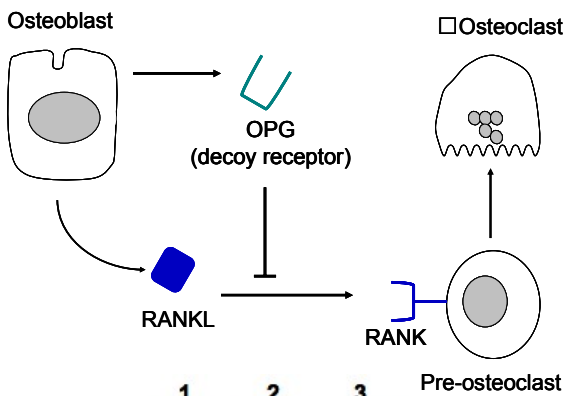
INTRODUCTION

The high morbidity and mortality associated with prostate cancer (PCa) derive from its tendency to metastasize to bone. There are currently no effective therapies that prevent or treat prostate cancer bone metastases. PCa cells preferentially metastasize to areas of bone where there is ongoing bone resorption. In addition, although PCa bone metastases are eventually osteoblastic, there is accumulating evidence that there is an initial and ongoing osteolytic phase that is essential for both PCa bone-targeting, initial osteolytic lesions and advanced osteoblastic lesions (1). PCa cells produce soluble factors that activate osteoclastic bone resorption, thereby aiding their entry into the bone microenvironment which is rich in growth factors from osteoclast-dissolved bone matrix. One of the soluble factors produced by PCa cells is PAP (2). Osteoclast-induced bone resorption is dependent upon the secretion of bone acid phosphatase by osteoclasts (3). PAP is similar to bone acid phosphatase in terms of its substrates, actions and pH optimum but the two enzymes can be distinguished by tartrate-inhibition (PAP) vs. tartrate-resistance (bone acid phosphatase). We have ample preliminary evidence that PAP plays a *causal* role in PCa bone metastases including (a) high expression of PAP in human PCa bone metastases and (b) *in vitro* evidence that PAP secreted by a human PCa cell line induces osteoblast and osteoclast growth/differentiation and enhances osteoclast bone-resorbing activity (4). We had preliminary *in vitro* evidence that prostatic acid phosphatase (PAP) secreted by PCa cells enhances their ability to target and grow in the bone microenvironment.

The *overall purpose* of these studies was to investigate our *hypothesis* that prostatic acid phosphatase (PAP) secreted by prostate cancer (PCa) cells in bone metastases plays a *causal* role, particularly in the osteoblastic phase of the disease. Specifically, we investigated the role of PAP in the paracrine growth and differentiation of osteoclasts and osteoblasts. In addition we determined the effects of PAP knockdown and overexpression on the growth and bone phenotype of human PCa cells.

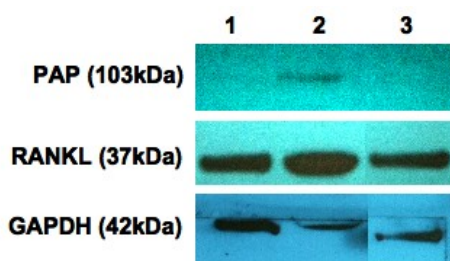
BODY

1. Effects of secretory PAP on osteoblasts, osteoclasts and the RANK/RANKL/OPG system



Osteoblasts mediate osteoclast differentiation and function by secreting two proteins (Figure 1): (1) RANK ligand (RANKL), which promotes osteoclastic activity when bound to the RANK receptor on osteoclasts, and (2) Osteoprotegerin (OPG), a decoy receptor that binds to RANKL and inhibits the activation of osteoclasts.

Figure 1. Osteoblasts mediate osteoclast activity by RANKL and OPG.



To evaluate the baseline expression of PAP, RANK, RANKL, and OPG, protein from VCaP (PAP+ PCa cells), PC3 (PAP- PCa cells), and MC3T3 (osteoblasts) cultures were analyzed by Western blot. RT-PCR was used to study the role of PAP on the mRNA expression of RANKL and OPG in VCaP and PC3 with and without L-tartrate, an inhibitor of PAP enzymatic activity.

Figure 2. Baseline PAP and RANKL expression: 1: PC3 (PAP- PCa cells), 2: VCaP (PAP+ PCa cells), 3: MC3T3 (osteoblasts). Both PCa cell lines secreted RANKL, which could influence the balance between osteoclast and osteoblast activity.

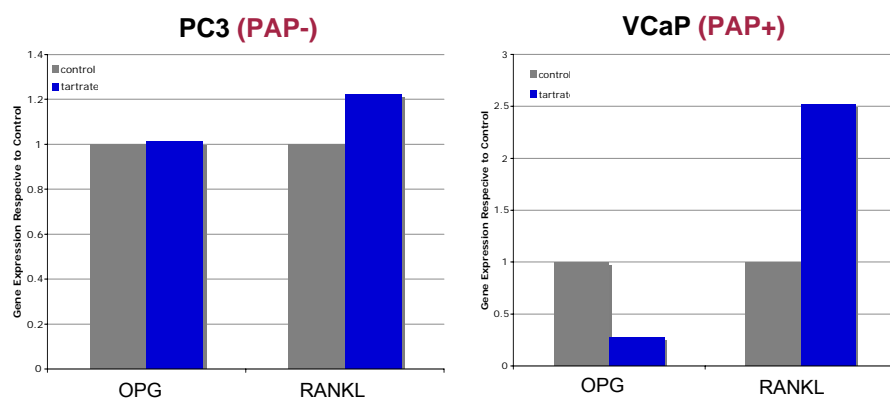


Figure 3. Autocrine effect of PAP: grey: without L-tartrate, blue: with L-tartrate inhibition. PAP increased OPG and decreased RANKL mRNA expression. Inhibition of PAP enzymatic activity in VCaP decreased OPG and increased RANKL mRNA expression.

We next determined the paracrine effect of PAP on RANKL expression in osteoblasts. MC3T3 mouse preosteoblast cells were cultured for 2 days in conditioned media from 3-day cultures of PC3 and VCaP cells, followed by immunohistochemical staining.

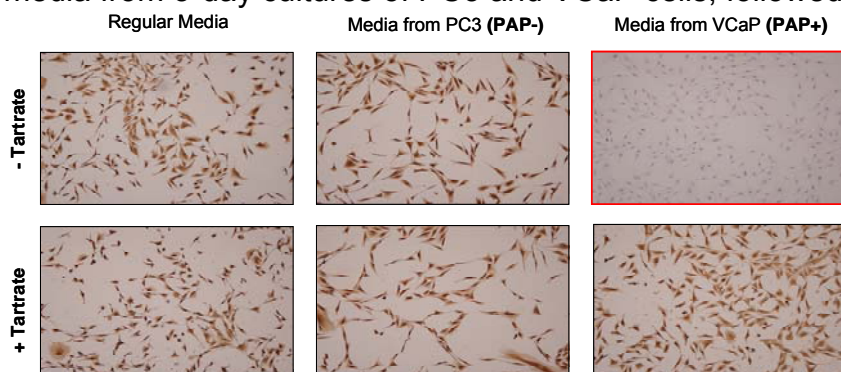
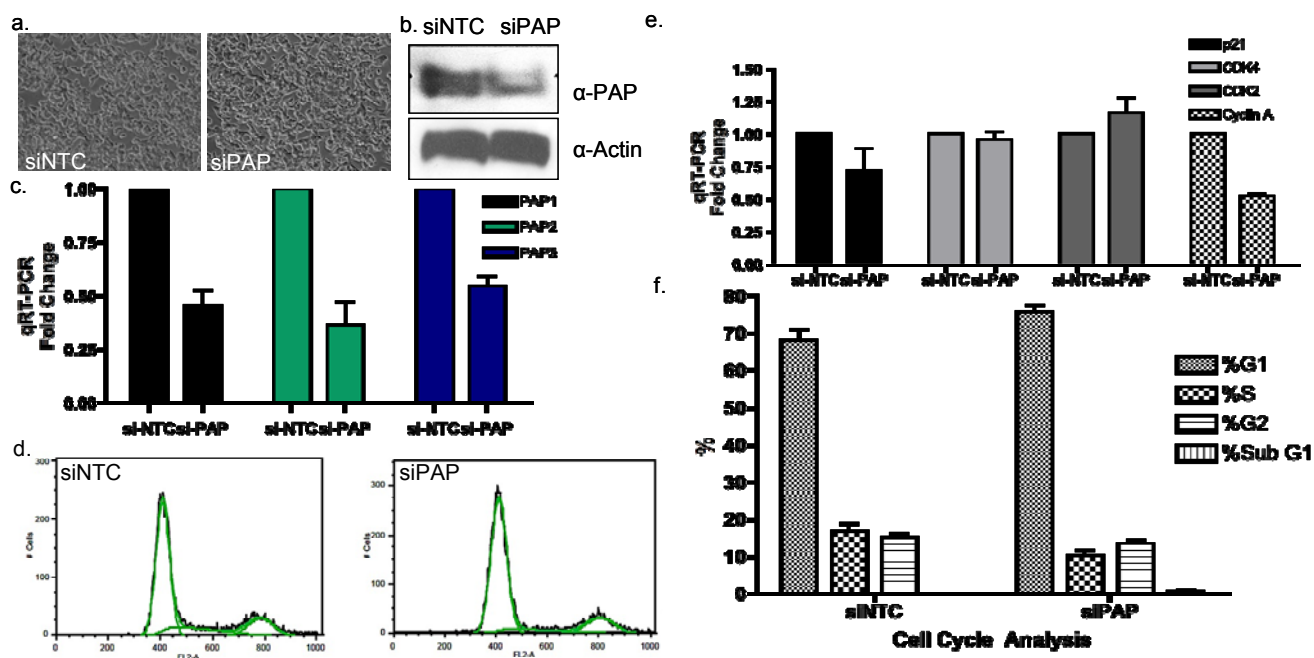


Figure 4. RANKL expression in osteoblasts +/- PAP from PCa conditioned medium. RANKL stains brown. PAP decreased RANKL expression. Inhibition of PAP activity in VCaP increased osteoblast RANKL expression

2. Generation of human PCa cell lines with variable PAP expression. We utilized two different PCa cell lines, which differ in their expression and secretion of PAP. The VCaP cell line, originally derived from a vertebral metastases in a patient with hormone-resistant PCa, expresses and secretes PAP. VCaP cells induce an osteoblastic phenotype in bone metastases. In contrast, the PC3 human PCa cell line, although also derived from a patient with bone metastases, does not express appreciable amounts of PAP and induces an osteolytic phenotype in bone. We successfully generated cell lines that transiently knockdown PAP expression using RNAi technology (PAP-siRNA) in high PAP-expressing human PCa cells (VCaP-luc). Conversely, for the gain-of-function experiments, PAP-negative human PCa cells (PC3-luc) and a more aggressive subtype (PC3-ML-luc) were stably transfected to overexpress secretory PAP by utilizing the retroviral expression vector pLenti-GIII-CMV-hACPP (ACPP is PAP) Lentiviral Vector (PC3-pPAP) and the pLenti-III-HA Blank Control (PC3-luc-control) vector as control. Stable cell clones were selected with puromycin and characterized by qRT-PCR and western blotting.



Cell Cycle Analysis - FACS

Figure 5. Effect of knockdown of PAP expression in VCaP cells using RNAi.

PAP knockdown in VCaP cells was confirmed by Western blot and qRT-PCR using three different PAP primers (panels b and c). PAP knockdown had no effect on VCaP cell morphology (panel a) but did increase the proportion of cells in G1 and subG1 and decrease the proportion in S phase as compared to control (panel d, e). PAP knockdown also modulated the expression of cell cycle proteins involved in G1 and S phase (panel f).

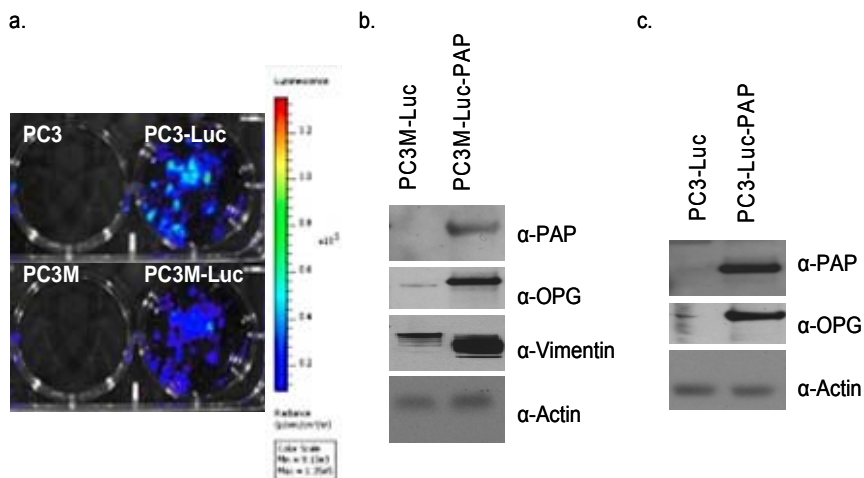


Figure 6. Effect of overexpression in PC3 and PC3ML cells. PC3-luc and PC3ML-luc cells were infected with virus particles containing PAP. Luciferase expression was confirmed using a xenogen platform (panel a) and for PAP protein expression with Western Blot (panels b and c). PAP overexpression led to increased protein expression of OPG by PC3 cells.

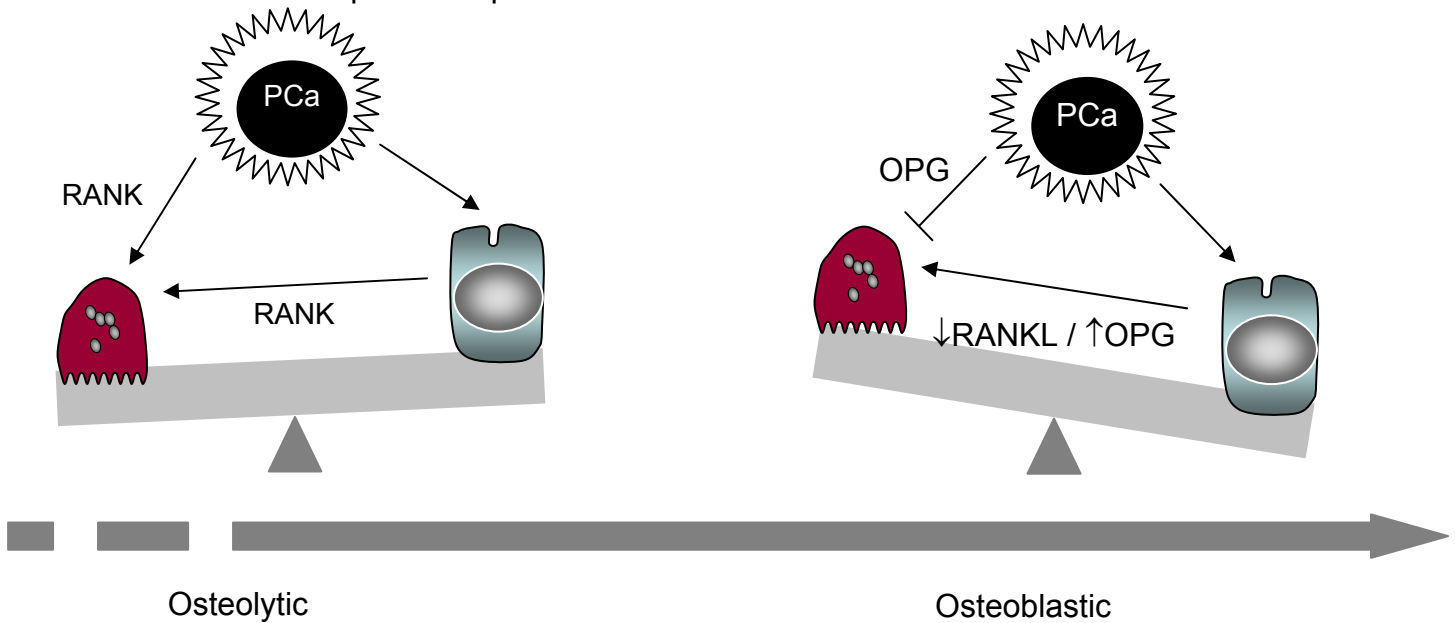
3. Effect of Forced Expression of PAP in PC3 cells on Bone Metastases *In Vivo*

Now that we have successfully generated PC3-luc and PC3ML-luc cell lines with overexpression of PAP, we have initiated studies in nude mice in which the cells are inoculated intratibially (utilizing the contralateral tibia for vector control cell inoculation). We have inoculated a total of 40 mice (10 per group, PC3-luc vector control, PC3-luc-PAP, PC3ML-luc vector control and PC3ML-luc-PAP) 3 weeks ago. We are utilizing the xenogen system to follow the bone disease progression during the course of the experiment. Mice will be euthanized and tissues collected

approximately 3 months after the inoculation and the tibiae compared using histomorphometry as to the extent of tumor volume as well as the bone reaction (osteoblastic vs osteolytic).

KEY RESEARCH ACCOMPLISHMENTS

1. PAP secretion by PCa cells modulates RANKL/OPG secretion in both PCa and bone cells. RANKL secretion by PCa and osteoblast cells shifts the balance of bone remodeling towards osteoclastic activity, whereas OPG secretion inhibits osteoclasts thereby shifting toward an osteoblastic phenotype. We demonstrated that inhibiting PAP (with tartrate) decreases RANKL secretion by osteoblast cells. We further demonstrated that forced PAP expression in PC3 and PC3 ML cells increases their protein expression of OPG.



2. Generated PAP knockdown and knock-in human PCa cell lines for in vitro and in vivo study.

3. Initiated studies with PAP-knock-in PC3-luc and PC3ML-luc cells in nude mice. The PAP-expressing cells have increased expression of OPG, which would inhibit osteolytic activity. As wtPC3 and PC3ML cells produce an osteolytic bone reaction, we expect that intratibial tumors with PC3-PAP and PC3ML-PAP cells will have an osteoblastic phenotype, similar to that induced with the PAP-expressing VCaP cell line (4). The osteoblastic PCa phenotype represents the end stage of PCa bone metastases for which there are currently no effective therapies. As PAP enzymatic activity is inhibited by L-tartrate, a common component of food and red wines, the results of these studies may lead to new treatments for osteoblastic bone metastases.

REPORTABLE OUTCOMES

Abstracts

1. Cohen, D, Yao, S, Nalamura Y, McNamara, K., Sasano H., Goldenberg, B, Kirschenbaum A., and **Levine, A.C.** The role of steroid receptor, steroidogenic enzymes and prostatic acid phosphatase immunohistochemical expression in the diagnosis and prognosis of prostate cancer. 94th Annual Meeting of the Endocrine Society, Houston, TX, June 2012.

2. Kirschenbaum A, Izadmehr S, Liao G, Leiter A, Chen W, Yao S, and **Levine AC**. Prostatic acid phosphatase in prostate cancer bone metastases: rank ligand as a mediator. 95th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2013.

CONCLUSIONS

Our results suggest that PAP secreted by PCa cells in bone metastases modulates RANKL/OPG expression in both PCa and osteoblast cells. The overall effect of PAP is to increase OPG and decrease RANKL thereby favoring the osteoblastic phenotype. If our ongoing in vivo studies confirm that PCa-derived PAP favors tumor growth in bone and induces an osteoblastic bone reaction, therapies that target PAP may be effective for the treatment of the osteoblastic phase of PCa bone metastases.

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