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14. ABSTRACT Osteoblastic bone metastasis is a common complication of advanced prostate cancer, resulting in pain and pathologic fracture. Dickkopf homolog 1 (DKK1) is a secreted inhibitor of osteoblast Wnt signaling pathway and hypothesized to be a central regulator of prostate cancer osteoblastic bone metastasis. The purpose of this proposal is to examine mechanisms of DKK1 regulation by prostate cancer cells and determine whether DKK1 overexpression in bone blocks the formation of osteoblastic bone lesions in animal models of bone metastasis. We have now shown that human prostate cancer cell lines that produce osteolytic, but not osteoblastic, bone lesions in animal models of bone metastasis express significant amounts of DKK1 and this expression is correlated with the absence of DNA methylation at the DKK1 promoter CpG island. Our preliminary data points to a central role of DKK1 in prostate cancer bone metastasis and expect this work to translate into the development of novel therapeutic targets to treat this malignancy complication.					
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INTRODUCTION

Osteoblastic bone metastasis is a common complication of advanced prostate cancer, resulting in pain and pathologic fracture (1). In mouse models and human clinical studies of prostate cancer, tumor-produced endothelin-1 (ET-1) activates the osteoblast endothelin A receptor and increases new bone formation (2). In previously published work from our group, we demonstrated that dickkopf homolog 1 (DKK1), a negative canonical Wnt signaling regulator, is reduced by ET-1 resulting in enhanced canonical Wnt signaling activity and new bone formation (3). Others have shown that DKK1 secretion from prostate cancer cells themselves also contribute to bone microenvironment DKK1 (4). We hypothesized that DKK1 is a central regulator of prostate cancer bone metastasis. The purpose of this proposal is to examine mechanisms of DKK1 regulation by prostate cancer cells and determine whether DKK1 overexpression in bone blocks the formation of osteoblastic bone lesions in animal models of bone metastasis. Understanding the role of DKK1 in bone metastasis will facilitate the development of modulators of this factor and other Wnt signaling members. The development of such novel and targeted therapies to bone would represent a significant advancement in the treatment of prostate cancer metastasis to bone.

BODY

The PI moved from the University of Virginia to the University of Alabama at Birmingham on November 6, 2009. Although the tasks have not significantly changed, the physical move and transition to a new institution have created a temporary period of low research productivity. A no-cost extension will be requested at the end of the granting period.

Task 1: Determine if the osteoblastic response to ET-1 is blocked by *Dkk1* transgenic overexpression targeted to bone in mouse models of prostate cancer bone metastasis

A reliable and reproducible mouse model of bone metastasis utilizes the athymic nude mouse in the Balb/C genetic background. Immunodeficient nude mice are necessary to avoid rejection of human cancer cell lines. In Task 1, mice that overexpress osteoblast DKK1 (*Dkk1*^{Ob}) will be bred to athymic nude mice to test whether increased DKK1 in the bone microenvironment will block the formation of osteoblastic lesions. As described in the original proposal, *Dkk1*^{Ob} mice in the C57Bl/6 genetic background were to be bred to C57Bl/6 nude animals. But, it was unclear whether LuCaP23.1 cells form osteoblastic lesions similarly in C57Bl/6 vs. Balb/C animals. A pilot experiment was performed; the tumor take was 9/10 for Balb/C nude mice and 1/10 for C57Bl/6 nude mice (Fisher's exact test; p=0.0011). This significant difference suggests that *host* factors are regulating the formation of bone metastasis. This unexpected result may serve as the foundation for future hypotheses and grant proposals since most bone metastasis studies are aimed at investigating *tumor* factors that regulate bone metastasis. A similar pilot experiment examining PC3 (osteolytic) prostate cancer cells is planned.

Because of these results, the cells for the osteoblastic bone metastasis model will be changed to ARCaPm prostate cancer cells. This recently available prostate cancer cell line has the advantage over LuCaP23.1 xenograft cells in that they grow in culture, form osteoblastic bone lesions in both athymic nude and SCID mice, form lesions more rapidly within 8 weeks after intratibial injection, and reportedly grow in the C57Bl/6 genetic background (5).

Task 2: Determine how DKK1 production from bone cells and tumor is regulated *in vivo* in osteoblastic bone metastasis

The work proposed in this task is dependent on Task 1 and will be performed concurrently.

Task 3: Determine if *Dkk1* is inactivated by promoter CpG island hypermethylation in prostate cancer

Continued progress has been made in 1) correlating DKK1 expression of prostate cancer cells with behavior in bone (osteoblastic vs. osteolytic) and 2) uncovering mechanisms of DKK1 expression by promoter methylation. DKK1 is a secreted inhibitor of the Wnt signaling pathway, a critical signaling pathway for normal osteoblast differentiation. We hypothesized that prostate cancer cells with high DKK1 secretion will have suppressed osteoblast new bone formation, tipping the balance towards osteoclastic bone resorption and bone osteolysis. DKK1 expression was examined in prostate cancer cells. The prostate cancer cell line PC3, which produces large osteolytic lesions in an animal model of bone metastasis, exhibited marked DKK1 production. Conversely, C4-2B, C4-2 and LnCaP cell lines, which exhibit osteoblastic/mixed lesions, displayed essentially no DKK1 expression (**Fig. 1**). Methylation-specific PCR was performed on these cell lines examining the DKK1 CpG island located in the promoter and first exon. DNA methylation is an epigenetic mechanism to downregulate gene expression. PC3 cells showed no methylation of the promoter while the other prostate cancer cell lines had some degree of methylation (**Fig. 2**). Promoter methylation is therefore correlated with both DKK1 expression and bone metastasis phenotype.

To prove methylation was the principal factor in downregulation of DKK1 expression in the osteoblastic prostate cancer cells lines, C4-2B (low DKK1, methylated) and PC3 cells (high DKK1, unmethylated) prostate cancer cells were treated with the demethylating agent 5-aza-2'-deoxycytidine. This demethylating agent increase DKK1 mRNA in the C4-2B cells by ~500 fold but only slightly in PC3 cells (**Fig. 3**).

Since DKK1 is itself a transcriptional target of Wnt signaling, the Wnt signaling activity of the high DKK1 expressing PC3 prostate cancer cells were examined. The assumption was made that since DKK1 is highly expressed, Wnt signaling would be downregulated. The degree of Wnt signaling activity was estimated by how much this could be suppressed by a dominant negative (dnTCF) construct to T-cell factor (the end-target of Wnt signaling). In PC3 cells, dnTCF reduced Wnt signaling activity by at least 85% (**Fig. 4**). This suggested that Wnt signaling was active in these cells. How could Wnt signaling be elevated when these cells also expressed so much of the Wnt inhibitor DKK1?

The DKK1 receptor Kremen (Krm) may be responsible for this finding. In the absence of DKK1, Krm was recently shown to stabilize LRPs (Wnt co-receptors) at the cell membrane enhancing Wnt activation in addition to being the receptor for the Wnt inhibitor DKK1 (6). Therefore, in the absence of Krm, high DKK1 is unable to completely block Wnt signaling; and with low DKK1, Wnt signaling is attenuated.

Two Kremens have been identified and appear to have redundant actions. Kremen 1 and 2 expression was determined in the prostate and breast cancer cell lines that produced blastic, mixed and lytic bone lesions in animal models of bone metastasis. Total Kremen expression was then estimated in these cells. The lytic cell lines such as the prostate cancer PC3 expressed the least amount of Kremens (**Fig. 5**). And so, Kremen expression is inversely related to DKK1 expression. A model was formulated such that Kremens act as a switch in the osteolytic cancer cell lines regulate DKK1 expression and cooperate with DKK1 and Wnt signaling to control the bone phenotype of metastatic prostate cancer. Future studies include overexpressing Kremen in PC3 cells and examining whether 1) high DKK1 restores Wnt suppression, 2) reduced Wnt signaling activity decreases DKK1 expression, and 3) the tumor phenotype in bone changes in an animal model of bone metastasis.

KEY RESEARCH ACCOMPLISHMENTS

- Osteolytic, but not osteoblastic, prostate cancer cells express DKK1
- Osteolytic, but not osteoblastic, prostate cancer cells have unmethylated DKK1 promoter
- Demethylation corrects DKK1 suppression
- Low Kremen (DKK1 receptor) blocks Wnt suppression from high DKK1 in PC3 prostate cancer cells

REPORTABLE OUTCOMES

Invited Oral Presentation, 39th International Sun Valley Skeletal Biology Workshop, Sun Valley, Idaho, 2009.

“Wnt Signaling and Bone Metastasis”

Abstract:

In the United States, it is estimated that there will be 192,000 new cases of prostate cancer and 27,000 prostate cancer-related deaths this year. Similarly high numbers of breast cancer cases will occur with 194,000 new diagnoses and 40,000 breast cancer-related deaths. The vast majority of these patients who die will have skeletal metastases. Bone metastases are described as osteoblastic or osteolytic based on radiographic appearance. Prostate cancer bone metastases classically form osteoblastic lesions. Osteolytic bone disease is common in breast cancer metastasis and multiple myeloma bone disease. Bone metastases cause significant morbidity, most notably pain and fracture due to poor bone quality. The most devastating consequence is that once tumor has metastasized to bone, the disease is incurable. Bone targeted antiresorptive therapies such as bisphosphonates and denosumab reduce the progression of bone metastases but do not lead to cure. Dissection of the molecular mechanisms underlying bone metastases may translate into targeted therapies that not only treat the bone response to metastatic cancer cells but also eradicate cancer cells residing in bone.

The canonical Wnt signaling pathway plays a critical role in bone homeostasis, regulating osteoblast differentiation and indirectly regulating osteoclastogenesis via OPG/RANKL. Upon binding of a Wnt ligand to the frizzled receptor/LRP co-receptor complex, the signaling cascade is initiated leading to nuclear translocation of β -catenin and transcriptional activation of gene targets. Downregulation of this signaling pathway in the osteoblast results in low bone mineral density in mice and humans. Secreted factors that block Wnt signaling have been identified, and play an even larger role than either of the 19 identified Wnt ligands in regulating osteoblast activity and bone formation. Two such factors, dickkopfs and sclerostin, bind to and sequester LRPs away from the Wnt-frizzled activation complex and downregulate canonical Wnt signaling.

Dickkopf homolog 1 (DKK1) is a central factor in determining osteoblast behavior when tumor cells metastasize to bone. DKK1 is increased in bone marrow plasma in patients with myeloma osteolytic bone disease and in serum of patients with breast cancer bone metastasis. Similarly, cancer cell lines that produce DKK1, such as PC-3 and MDA-MB-231, suppress osteoblast activity and produce osteolytic lesions in animal models of bone metastasis. Cancer cell lines with low DKK1 expression (ZR-75-1 and T-47D) are associated with an osteoblastic response in animal models. However, low DKK1 production by these osteoinductive cancer cells alone is unlikely to produce the characteristic strong osteoblast stimulus. During normal bone remodeling, the osteoblast itself is the principal source of DKK1 and downregulation of this secreted factor is essential in the formation osteoblastic bone lesions. ZR-75-1 and T-47D

abundantly produce endothelin-1 (ET-1), a secreted factor that activates the osteoblast endothelin A receptor and reduces DKK1 secretion. This strategy ensures low DKK1 metastasis microenvironment concentration, which favors pathologic osteoblast activation. A model is proposed where the osteoblastic bone response to cancer metastasis is dually dependent on cancer cell and osteoblast regulation of DKK1. DKK1 therefore represents a unique therapeutic target for bone metastasis and understanding the regulation of this secreted factor is critical.

A CpG island is positioned within the promoter and first exon of *DKK1*. CpG islands are short stretches of DNA, with a GC content greater than 60%, and with a larger than expected frequency of the CpG dinucleotide. Methylation of CpG islands is often dysregulated in cancer and contributes to misexpression of genes and to tumorigenesis. This phenomenon of epigenetic gene regulation has also been described during prostate cancer progression and other types of cancer cell tumorigenesis. *DKK1* promoter hypermethylation has been correlated with colon cancer aggressiveness. Progressive *DKK1* promoter hypermethylation and transcriptional downregulation may in fact occur during prostate cancer metastasis and during the transformation from androgen-dependent to androgen-independent growth. *DKK1* expression and CpG island methylation was examined in prostate and breast cancer cell lines. C4-2B, C4-2 and LnCaP prostate, and T47D breast lines exhibited low *DKK1* expression while ZR-75.1, MCF-7 and MDA-MB-231 breast, and PC3 prostate lines had significant *DKK1* expression. Methylation-specific sequencing of the *DKK1* CpG island was performed on the human cancer cells. Hypermethylation at this promoter correlated with the lowest *DKK1* expression in cancer cell lines. *DKK1* expression was restored with the DNA demethylating agent 5-aza-2'-deoxycytidine, confirming that epigenetic mechanisms regulate expression.

ET-1 secreted by osteoinductive cancer cells is a causal factor in osteoblastic bone metastasis. Osteoblast activation occurs through downregulation of DKK1 secretion and subsequent canonical Wnt signaling activation. Mechanisms of DKK1 down-regulation by ET-1 in the osteoblast were explored using *DKK1*/luciferase reporter constructs. ET-1 did not change *DKK1* promoter activity in murine primary osteoblasts, but destabilized *DKK1* mRNA via the 3'UTR. Putative AUF1 binding elements and species conserved miRNA consensus sequences may mediate these actions of ET-1.

The observations by our group and others are consistent with a bone metastasis model in which osteoblast activity is dually regulated by cancer cell and osteoblast DKK1 secretion. The mechanisms involve both static epigenetic promoter silencing and dynamic control of *DKK1* mRNA stability via its 3'UTR. Understanding the regulation of *DKK1* gene expression in tumor and bone may lead to novel therapies for osteoblastic bone metastases.

Invited Oral Presentation, The IX International Meeting on Cancer Induced Bone Disease, Arlington, Virginia, 2009

“Molecular Mechanisms of Bone Metastasis in Prostate Cancer”

Abstract:

Normal bone homeostasis and remodeling are disrupted with the arrival of metastatic prostate cancer cells to bone. Through cooperative and cross-talking signals that foster the formation of bone metastasis, bone provides a hospitable microenvironment for the invading cells and in turn prostate cancer cells manipulate existing pathways that stimulate the osteoblast and bone formation. The osteoblast canonical Wnt signaling serves as one critical pathway that supports the osteosclerotic response to prostate cancer by increasing osteoblast differentiation and indirectly reducing osteoclastogenesis via increased OPG expression. Secreted factors that antagonize Wnt signaling have been identified, and play an even larger role than any of the 19 identified Wnt ligands in regulating osteoblast activity and bone formation. Dickkopf homolog 1 (*DKK1*) is one such secreted Wnt signaling antagonist that determines osteoblast behavior

when prostate cancer cells metastasize to bone. It is dually downregulated in both osteoblasts and cancer cells. Cancer cell DKK1 expression is therefore inversely correlated with osteoblast activity.

Prostate cancers abundantly secrete endothelin-1 (ET-1), a causal factor in osteoblastic bone metastasis. ET-1 activates the osteoblast endothelin A receptor which downregulates paracrine DKK1 leading to activation of Wnt signaling. Mechanisms of DKK1 down-regulation by tumor-secreted ET-1 in the osteoblast were investigated. ET-1 did not change *DKK1* promoter activity in murine primary osteoblasts or RNA PolII promoter occupancy, but destabilized *DKK1* mRNA via the 3'UTR. Putative AUF1 binding elements and species conserved miRNA consensus sequences that may mediate the actions of ET-1 are being examined.

DKK1 regulation in cancer cells was also investigated. A CpG island is positioned within the promoter and first exon of *DKK1*. Methylation of CpG islands is often dysregulated in cancer and contributes to misexpression of genes and to tumorigenesis. Progressive *DKK1* promoter hypermethylation and transcriptional downregulation may in fact occur during prostate cancer bone metastasis. *DKK1* expression and CpG island methylation was examined in prostate cancer cell lines. C4-2B, C4-2 and LnCaP prostate cancer lines exhibited low *DKK1* expression while PC3 and DU145 prostate lines had significant *DKK1* expression. Methylation-specific sequencing of the *DKK1* CpG island was performed on the human cancer cells. Hypermethylation at the *DKK1* promoter correlated with the lowest *DKK1* expression in cancer cell lines. *DKK1* expression was restored with the DNA demethylating agent 5-aza-2'-deoxycytidine, confirming that epigenetic mechanisms regulate its expression.

The observations by our group and others are consistent with a bone metastasis model in which osteoblast activity is dually regulated by cancer cell and osteoblast *DKK1* secretion. The mechanisms involve both static epigenetic promoter silencing and dynamic control of *DKK1* mRNA stability via its 3'UTR. Understanding the regulation of *DKK1* gene expression in tumor and bone may lead to novel therapies for osteoblastic bone metastases.

CONCLUSION

Bone metastasis is a significant complication of advanced prostate cancer that causes pain and pathologic fracture. This work is aimed at uncovering the role of *DKK1* in prostate cancer bone metastasis. We have discovered a correlation between behavior of prostate cancer in bone, *DKK1* expression and DNA methylation of the *DKK1* promoter. We will extend this work and examine *DKK1* promoter methylation patterns in human prostate cancer bone metastasis and whether this pattern correlates with risk for and progression of bone metastasis. Studies that are in progress will examine whether overexpression of *DKK1* in the bone microenvironment blocks bone metastasis in an animal model. Medical and social costs of bone metastasis are high. This work is expected to translate into improved treatments for prostate cancer bone metastasis and facilitate the development of therapeutic targets to *DKK1*.

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APPENDICES

None

SUPPORTING DATA

Normalized DKK1 in conditioned media

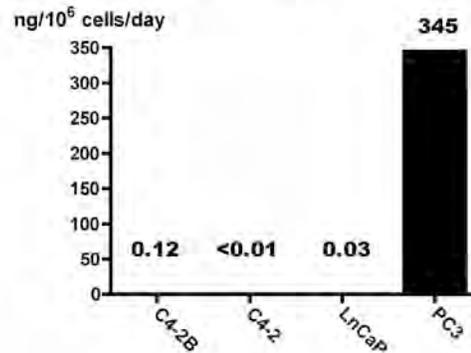


Fig. 1. DKK1 secretion correlates with prostate cancer bone metastasis phenotype. C4-2B, C4-2, LnCaP and PC3 human prostate cancer cell lines were grown in culture and conditioned media was collected for DKK1 ELISA analysis. DKK1 concentration was normalized to cell number. The osteolytic cancer cell line PC3 exhibited marked DKK1 secretion.

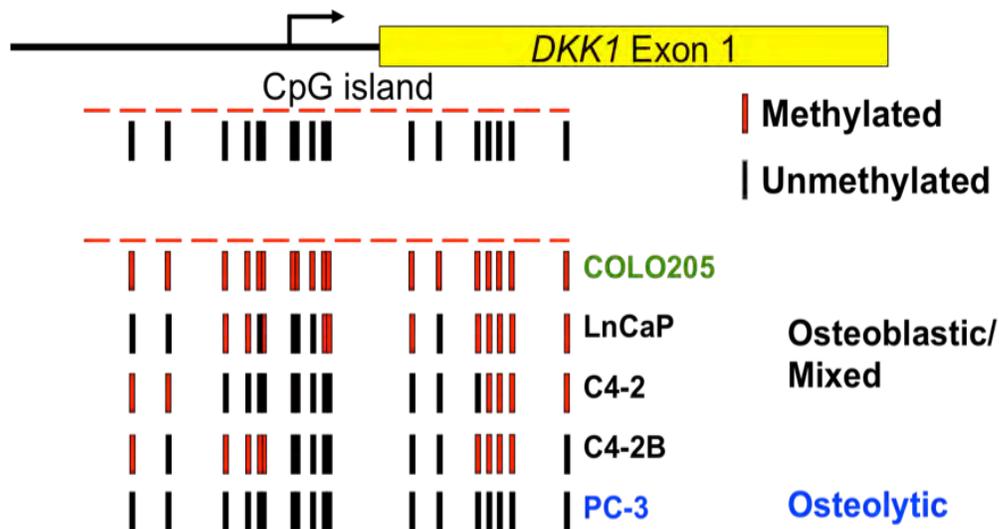


Fig. 2. DKK1 promoter methylation correlates with prostate cancer bone metastasis phenotype. The DKK1 promoter contains a 233 bp CpG island with 18 potential methylation sites (bars). Methylation-specific sequencing of this CpG island was performed in human cell lines. The COLO205 colon cancer cell line served as a control for methylation. The prostate cancer cell lines that produce osteoblastic or mixed lesions (LnCaP, C4-2, C4-2B) displayed different degrees and patterns of methylation. The osteolytic cell line PC3 displayed no methylation.

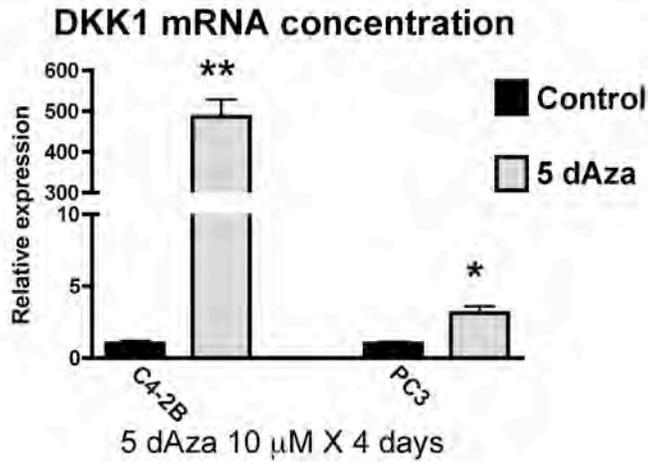


Fig 3. DNA demethylation increases DKK1 mRNA in C4-2B prostate cancer cells. C4-2B and PC3 prostate cancer cells were treated with the DNA demethylating agent 5-aza-2'deoxyctidine (5 dAza) for 4 days. 5 dAza markedly increased Dkk1 mRNA in the highly methylated DKK1 gene of C4-2B prostate cancer cells.

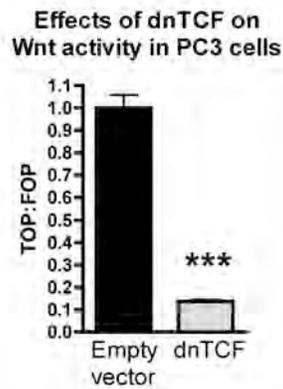


Fig 4. Dominant negative TCF downregulates Wnt signaling in PC3 prostate cancer cells. PC3 cells, producing large amounts of DKK1, were transfected with dnTCF to downregulate Wnt signaling resulting in significant reduction of Wnt signaling.

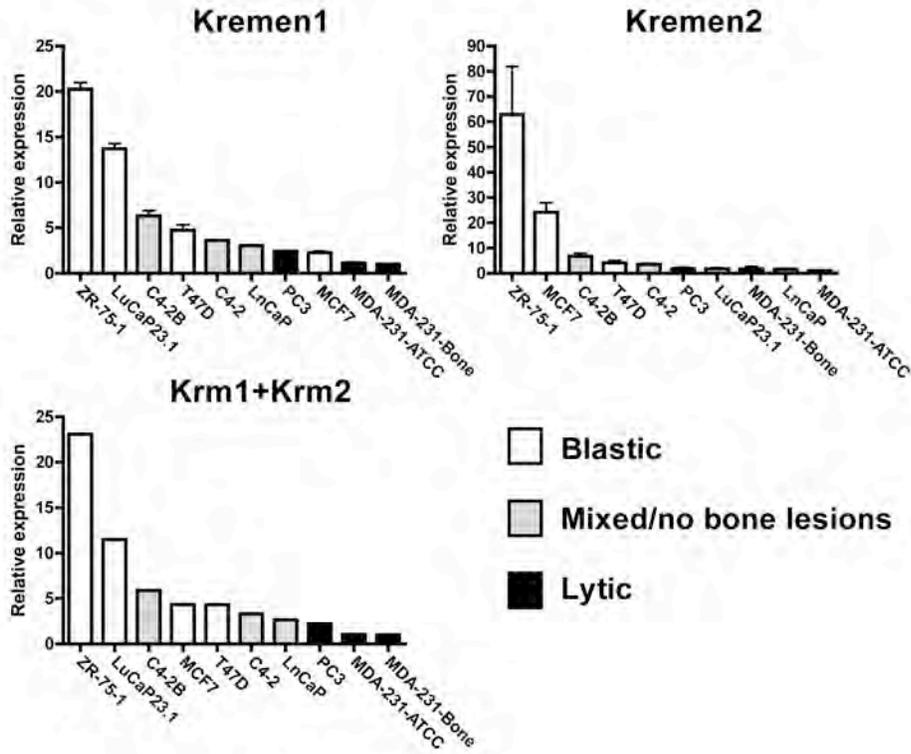


Fig 5. Kremen expression correlates with bone phenotype. Prostate and breast cancer cells used in models of bone metastasis were surveyed for Kremen mRNA expression. The cell lines that produce osteoblastic lesions expressed the most Kremen.