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<b>14. ABSTRACT</b> 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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## Table of Contents

	<u>Page</u>
<b>Introduction.....</b>	<b>1</b>
<b>Body.....</b>	<b>2-8</b>
<b>Key Research Accomplishments.....</b>	<b>8</b>
<b>Reportable Outcomes.....</b>	<b>8</b>
<b>Conclusion.....</b>	<b>8</b>
<b>References.....</b>	<b>8-10</b>
<b>Appendices.....</b>	<b>N/A</b>
<b>Supporting Data.....</b>	<b>N/A</b>

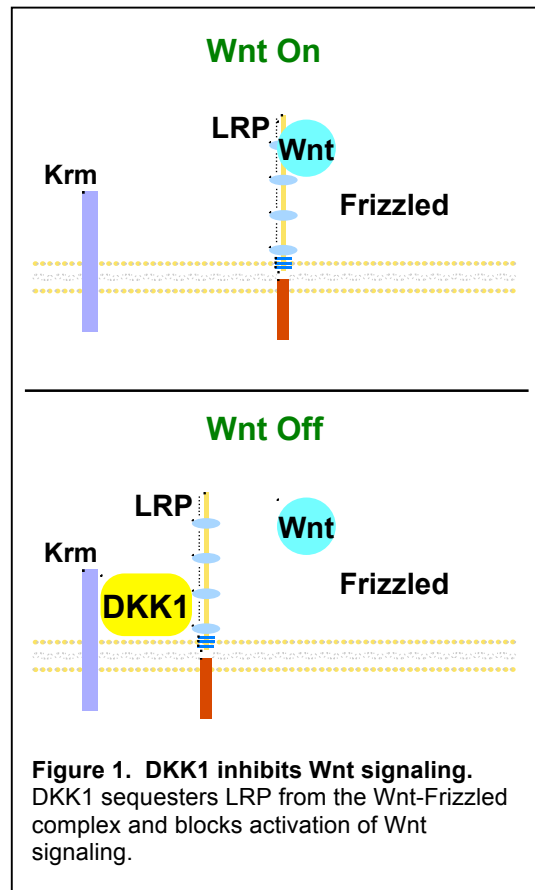
## INTRODUCTION

Bone metastasis is a common and unfortunate complication of advanced prostate and breast cancer. The arrival of cancer cell to bone defines a point in the disease when cure is unlikely. The invasion of tumor cells into bone irrevocably alters the bone microenvironment and initiates a skeletal response that is dependent on the type of tumor (1). Breast cancer bone metastasis typically results in massive osteolysis from the secretion of osteoclast-activating factors, such as parathyroid hormone-related protein and others (2). Prostate cancer classically forms osteoblastic lesions under the direction of osteoblast-activating factors that include endothelin-1 (ET-1), Wnt signaling proteins and bone morphogenetic proteins (3,4). Both osteolytic and osteoblastic bone metastases represent heightened states of bone turnover but differ in the extent to which osteoblast bone formation or osteoclast bone resorption predominates.

Dickkopf homolog 1 (DKK1) is a secreted inhibitor of canonical Wnt signaling that may predict cancer cell behavior in bone. Emerging and mounting evidence suggests that DKK1 dictates whether bone metastases have osteoblastic or osteolytic features (5). During normal bone homeostasis, DKK1 is secreted from mature osteoblasts that then feeds-back to inhibit Wnt signaling of osteoblast precursors (6). DKK1 operates by sequestering the LDL-related proteins 5 and 6 co-receptors from the G protein-coupled protein receptor Frizzled and thus blocks Wnt signaling activation (7) (**Figure 1**). Negative feedback of DKK1 supports tight control of bone formation and thus prevents excessive osteoblast activity. This role of DKK1 is illustrated by the osteopenic phenotype with DKK1 transgenic overexpression in mice (8,9).

DKK1 regulates the osteoblastic response to invading cancer cells in bone and therefore influences the balance between bone formation and resorption (5,6,10). This idea was first proposed when DKK1 was identified as a causal factor in osteoblast suppression characteristic of multiple myeloma bone disease (11). Since this first report, DKK1 has been implicated in other forms of cancer and bone metastasis. In animal models of prostate cancer bone metastasis, DKK1 overexpression in the prostate cancer cell line C4-2B, which normally forms mixed osteoblastic-osteolytic bone lesions, resulted in the formation of primarily osteolytic lesions (5). Conversely, knockdown of DKK1 expression in the PC3 prostate cancer cell line resulted in increased osteoblastic potential (5).

Cancer cells not only secrete DKK1 but also are able to manipulate the secretion of DKK1 from the osteoblast. This is mediated by tumor-secreted ET-1, which activates the osteoblast endothelin A receptor (ETAR) and downregulates osteoblast DKK1 (12). ET-1 therefore promotes pathologic bone formation by ensuring DKK1 is quelled, permitting excessive osteoblast activity and bone formation. ETAR antagonists slow progression of osteoblastic lesions in animal models of osteoblastic bone metastasis and demonstrated in human clinical trials, which suggests an important role of DKK1 in bone metastasis (3,13,14). Collectively,



DKK1 secreted by both cancer cells and mature osteoblasts contribute to bone microenvironment DKK1, and influences osteoblast development and pathologic bone formation in bone metastasis.

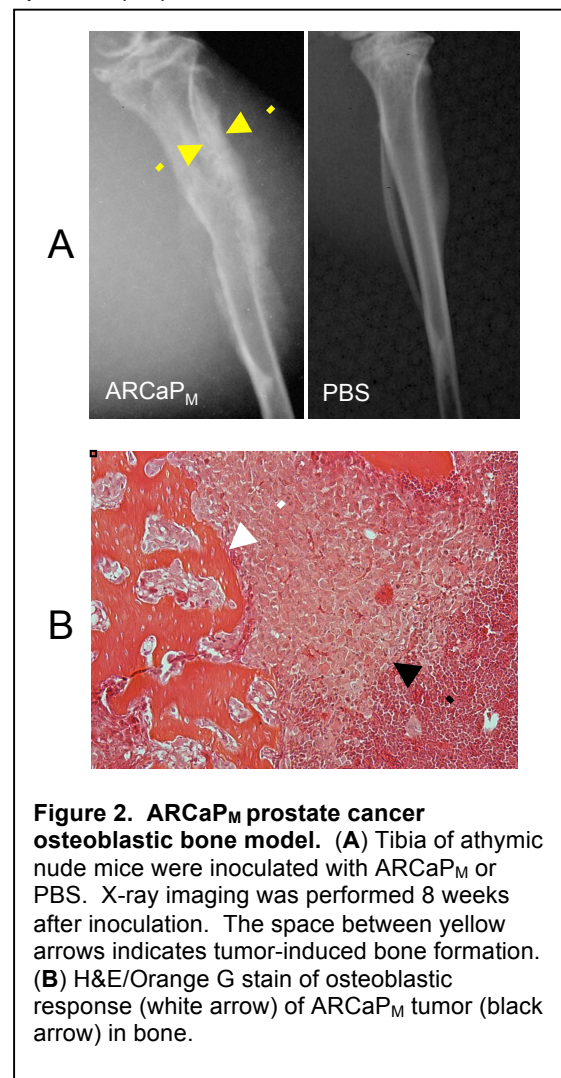
We hypothesized that DKK1 is a central regulator of prostate cancer bone metastasis. The purpose of this proposal was to examine mechanisms of DKK1 regulation by prostate cancer cells and determine whether DKK1 overexpression in bone blocks the formation of osteoblastic bone lesions in animal models 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

### 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

The *Dkk1*<sup>Ob</sup> transgenic mouse was generated with *Dkk1* under the transcriptional control of the 2.3 kb Collagen 1a1 promoter that specifies *Dkk1* expression to the osteoblast. The osteopenic bone phenotype of this mouse has been reported (15). *Dkk1*<sup>Ob</sup> mice were in the C57BL/6 genetic background and were to be bred to C57BL/6 nude animals to accept human prostate cancer cells. However, it remained unclear whether human cancer cells commonly used in mouse metastasis models (BALB/C nude) form bone lesions similarly in the C57BL/6 background. In addition since mice carrying the nude mutation are poor breeders, the SCID mutation (*Prkdc*<sup>scid</sup>) was a preferred immunodeficient model. A pilot experiment was performed which demonstrated that the tumor take of the LuCaP23.1 xenograft was 9/10 for BALB/C nude mice and 1/10 for C57BL/6 nude mice (Fisher's exact test;  $p=0.0011$ ). A similar pilot experiment was performed using the ARCaP<sub>M</sub> prostate cancer cells. Tumor take was 6/6 for BALB/C nude vs. 1/6 for C57BL/6 SCID. Based on the low tumor appearance in the C57BL/6 backgrounds in both prostate cancer bone metastasis models, mice in the BALB/C background were used.

Another modification, as stated in the 2010 Annual Report, utilized the ARCaP<sub>M</sub> prostate cancer cell line instead of the LuCaP23.1 prostate cancer xenograft. The ARCaP<sub>M</sub> 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, and form lesions more rapidly within 8 weeks after intratibial injection (**Figure 2**). Therefore, the ARCaP<sub>M</sub> prostate cancer cell line was utilized in Aim 1.

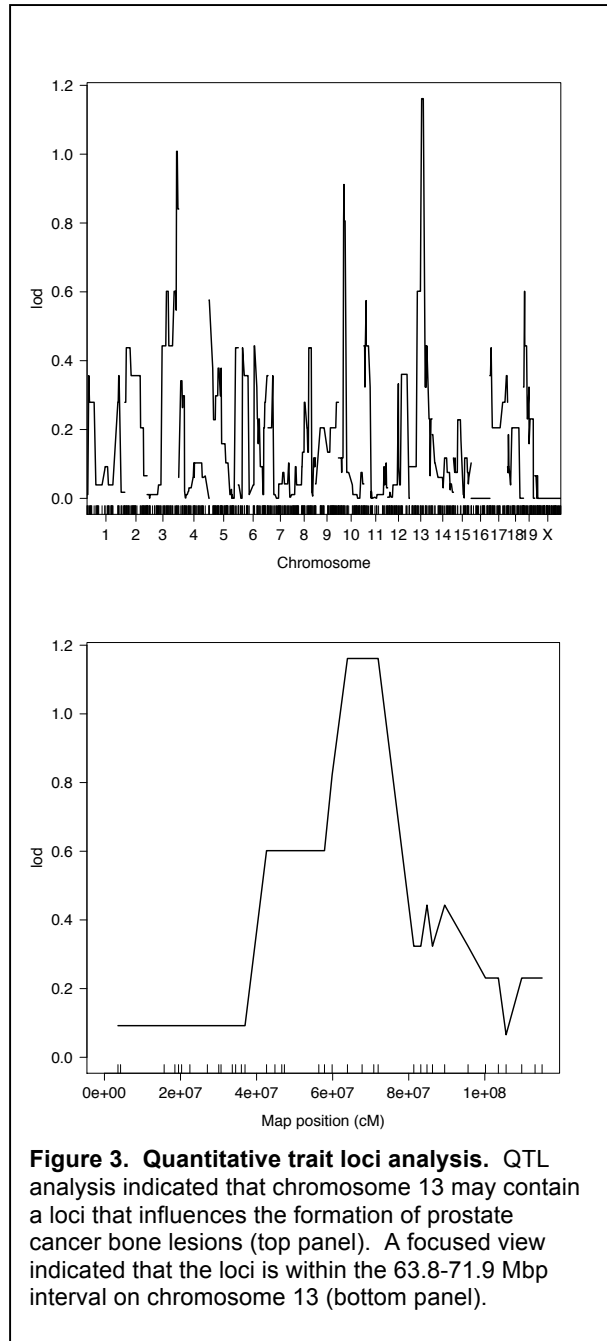


**Figure 2. ARCaP<sub>M</sub> prostate cancer osteoblastic bone model.** (A) Tibia of athymic nude mice were inoculated with ARCaP<sub>M</sub> or PBS. X-ray imaging was performed 8 weeks after inoculation. The space between yellow arrows indicates tumor-induced bone formation. (B) H&E/Orange G stain of osteoblastic response (white arrow) of ARCaP<sub>M</sub> tumor (black arrow) in bone.

A modified breeding strategy was proposed so that the study mice would possess at least 87.5% of the BALB/C background to maximize the number of mice that accept tumor. ARCaP<sub>M</sub> prostate cancer cells were inoculated into the left cardiac ventricle of WT and *Dkk1*<sup>Ob</sup> male SCID mice. At 12 weeks of age when radiographic evidence of bone lesions appeared evident, the mice were euthanized and bones were harvested as detailed in the original proposal. Regrettably, histomorphometric analyses demonstrated that only four animals possessed detectable bone lesions. These mice were equally distributed between WT and *Dkk1*<sup>Ob</sup> animals. Another separate bone metastasis experiment was performed in parallel with the proposed experiment. The parallel experiment demonstrated nearly 100% tumor take to bone, which essentially rules out other technical issues with intracardiac inoculation. Because of the lower tumor take, we were unable to perform further tumor analysis.

These results triggered a novel hypothesis with the potential to salvage this lengthy experiment. As stated above, my laboratory's success with intracardiac tumor inoculation is high in the BALB/C background. We speculated that the mixed BALB/C / C57BL/6 background influenced how the bone microenvironment interacted with potential circulating cancer cells. Few studies have examined how the host, or the metastatic microenvironment, influences bone metastasis. We therefore took this opportunity to study this novel aspect further. In collaboration with Dr. Charles Farber at the University of Virginia, we performed quantitative trait loci (QTL) analysis to determine whether the inheritance of particular chromosomal regions of BALB/C vs. C57BL/6 predicted the formation of bone lesions. Using the Illumina Mouse MD Linkage Chip containing 1449 SNPs, genomic DNA was analyzed in all mice that underwent intracardiac inoculation of the ARCaP<sub>M</sub> prostate cancer cell line.

QTL analysis revealed an interval on chromosome 13 spanning between 63.8 Mbp to 71.9 Mbp that correlated well to BALB/C / C57BL/6 heterozygosity (**Figure 3**). Investigation of that interval revealed a total of 30 genes. These include a cluster of zinc finger proteins and, most interesting, the steroid 5 alpha-reductase 1 gene (*Srd5a1*). This gene encodes for the major enzyme that converts weakly active testosterone to potent dihydro-testosterone. Finasteride used to treat early prostate cancer also targets this enzyme. More importantly, 5 alpha-reductase has been reported to be differentially expressed and correlates with severity of prostate cancer (16). Whether expression also correlates with the risk of bone prostate cancer bone metastasis is



**Figure 3. Quantitative trait loci analysis.** QTL analysis indicated that chromosome 13 may contain a loci that influences the formation of prostate cancer bone lesions (top panel). A focused view indicated that the loci is within the 63.8-71.9 Mbp interval on chromosome 13 (bottom panel).

unknown. However, this area is an exciting avenue of future investigation.

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

Because of the low tumor take in bone after intracardiac inoculation and the lack of correlation between the appearance of bone lesions and DKK1 expression, we were unable to complete this task. This outcome is regrettable since the successful results of this task would have likely identified targets and secondary effects of host-derived DKK1 in prostate cancer bone metastasis.

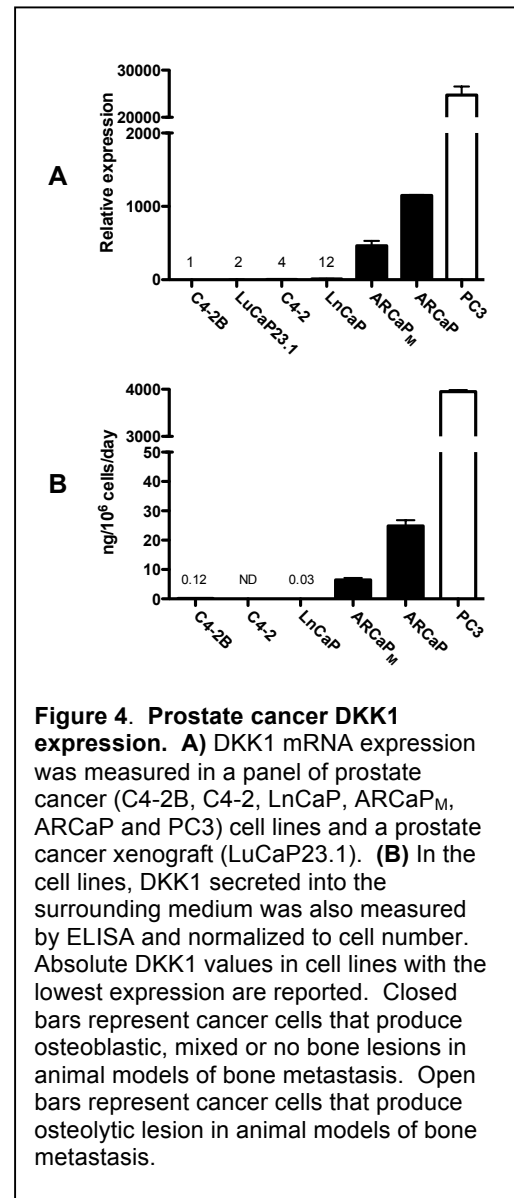
### Task 3: Determine if *Dkk1* is inactivated by promoter CpG island hypermethylation in prostate cancer

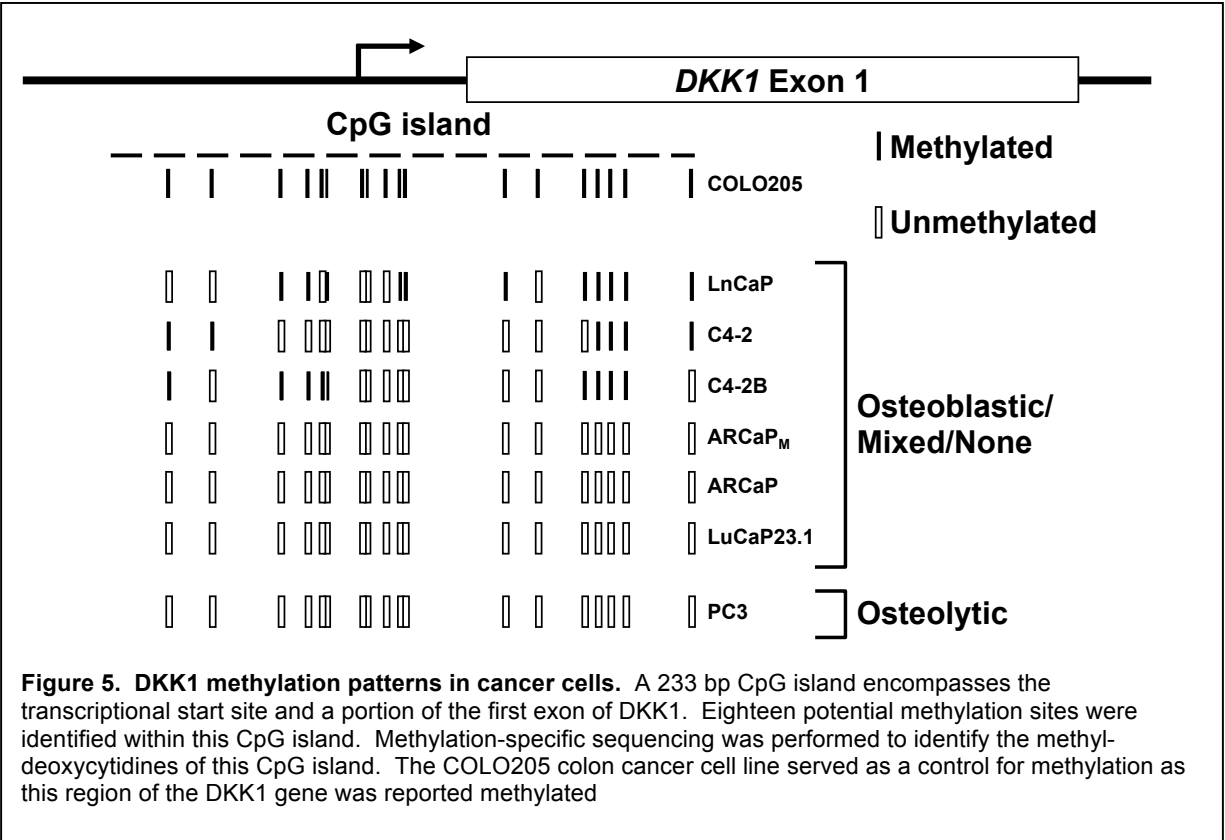
#### Cancer cell DKK1 expression predicts bone phenotype

DKK1 mRNA expression and protein secretion into the surrounding medium was surveyed in selected prostate cancer cells lines and correlated with the phenotypic response of the cancer cells in bone (**Figure 4**). The androgen-dependent prostate cancer cell line LNCaP and the subline derivatives C4-2 and C4-2B (17) expressed nearly undetectable DKK1. Of these, only C4-2B elicits a skeletal response after inoculation with mixed osteoblastic and osteoclastic characteristics. The human prostate cancer xenograft LuCaP23.1 produces osteoblastic bone lesions with intratibial inoculation (18), and little DKK1 expression was detected. The prostate cancer cell line ARCaP and the bone avid osteoblastic subline ARCaP<sub>M</sub> (17) expressed more DKK1. ARCaP<sub>M</sub> is a reliable model of osteoblastic bone metastasis. Among the cancer cells lines tested, mRNA concentration correlated well with absolute protein secreted into the surrounding medium.

#### Methylation of the DKK1 CpG island downregulates DKK1 expression

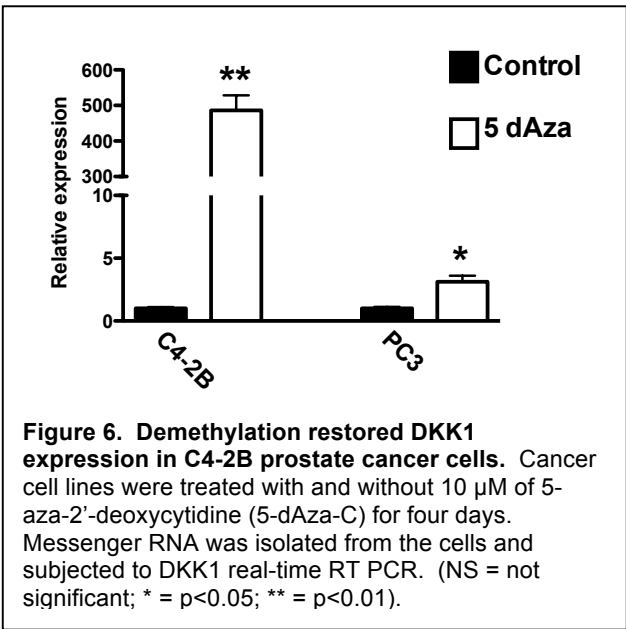
The cancer cell lines selected for study demonstrated wide variation in DKK1 expression. Alteration in DNA methylation pattern is one mechanism that promotes tumorigenesis. The DKK1 gene contains a 233 bp CpG island surrounding the transcriptional start site and a portion of the first exon (19) (**Figure 5**). This area has a GC content of 68% containing 18 potential cytosine methylation points where a cytosine precedes a guanine. This CpG island is a focus of methylation previously reported to regulate DKK1 transcription in colon cancer (19,20), acute myeloid leukemia (21), malignant glioma (22) and multiple myeloma (23). The extent to which a similar epigenetic mechanism of gene regulation





regulates DKK1 expression in prostate and breast cancer cells was tested. Using a methylation-specific sequencing approach, the cell lines with negligible expression (LNCaP, C4-2, and C4-2B) had some degree of methylation of the CpG island indicating a putative mechanism of transcriptional repression (**Figure 5**). Methylation of the DKK1 CpG island was not detected in the remaining cell lines or xenograft.

The impact of DNA methylation on DKK1 transcriptional repression was examined in C4-2B cells, a cell line with detectable DKK1 CpG island methylation that reliably produce osteoblastic lesions in animal models. The unmethylated PC3 cell line served as a control. Treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine resulted in a nearly 500-fold increase in DKK1 mRNA in the C4-2B cell line, strongly indicating that methylation is responsible for transcriptional repression (**Figure 6**). Interestingly, PC3 cancer cell lines exhibited a marginal increase in DKK1 mRNA despite being unmethylated at the DKK1 promoter. This result was likely due to indirect phenomena, such as the presence of epigenetically controlled transcriptional activators that regulate DKK1 transcription.



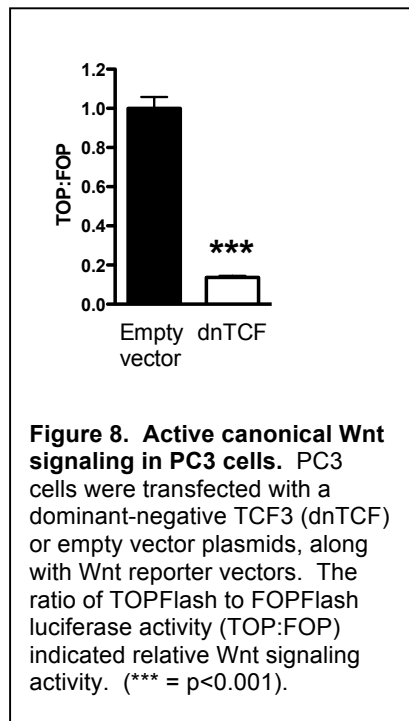
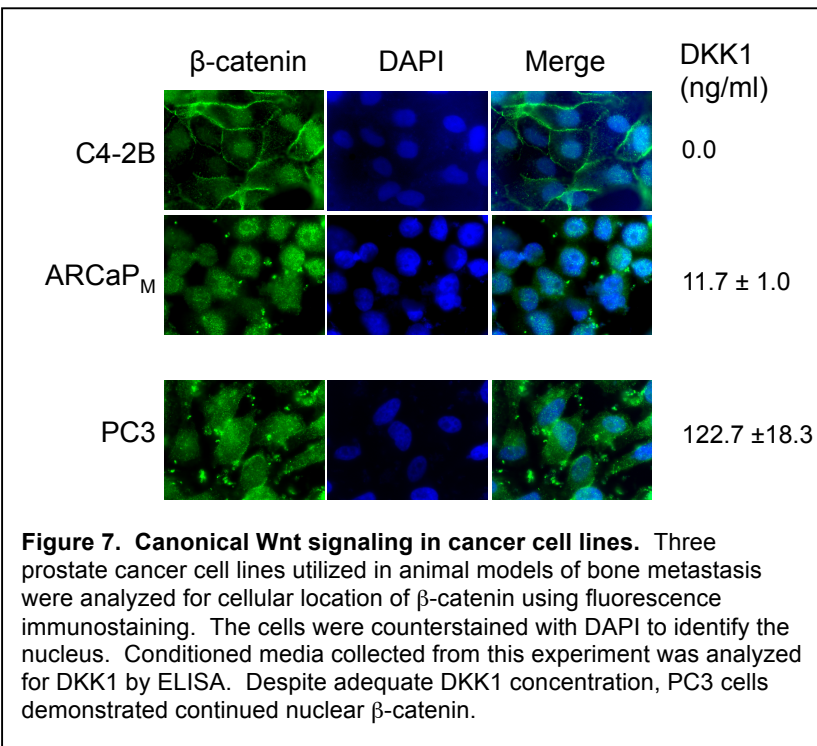


### Regulation of DKK1 expression by Wnt signaling

The absence of DKK1 promoter methylation within the LuCaP23.1, ARCaP, ARCaP<sub>M</sub>, and PC3 cells indicated that other mechanisms regulated DKK1 expression. Wnt signaling itself is one candidate. DKK1 expression is regulated, at least partly, by TCF/LEF Wnt signaling

responsive elements located within the DKK1 promoter, and thus fits with DKK1 operating in a negative feedback loop regulating Wnt signaling (24). A unifying mechanism of DKK1 regulation by Wnt signaling in the studied cancer cell lines was investigated by assessing the degree of nuclear localization of  $\beta$ -catenin, a marker for active Wnt signaling. The cell lines that reproducibly form bone lesions in animal models (C4-2B, ARCaP<sub>M</sub> and PC3) were selected for examination and all demonstrated nuclear  $\beta$ -catenin staining (**Figure 7**). Controls using secondary antibody without primary antibody showed no staining (data not shown). The C4-2B cell line demonstrated additional staining of the cell membrane. This staining pattern may indicate  $\beta$ -catenin reserve and a lower level of Wnt signaling, and/or the presence of maturity adherens junction complexes that associate with  $\beta$ -catenin.

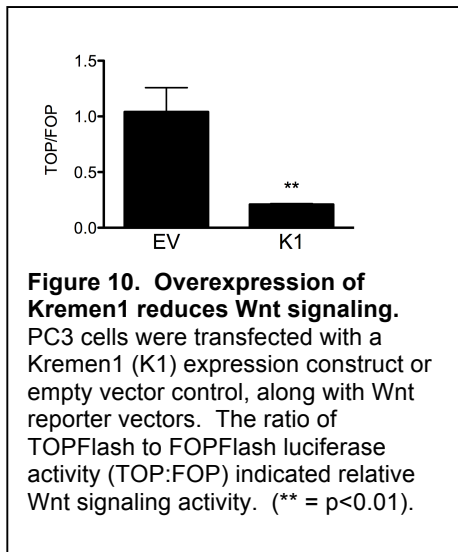
A puzzling aspect of the data is that DKK1 itself is a potent inhibitor of Wnt signaling. In most cells, 10-50 ng/ml of DKK1 is sufficient to block Wnt signaling (11,12,25). DKK1 was once again assayed from conditioned media of the cells that underwent immunofluorescent analysis (**Figure 7**). PC3 cells secreted more than sufficient quantities of DKK1 (>50 ng/ml) to block Wnt signaling. To confirm active Wnt signaling in these cell line, a dominant-negative expression construct for the mutant form of TCF3 lacking the  $\beta$ -catenin binding site was co-transfected along with Wnt signaling reporter vectors into the PC3 cell line. The strategy efficiently downregulated Wnt signaling, again suggesting that Wnt signaling is active in these cells (**Figure 8**). These data support that the PC3 cell line is insensitive to the Wnt-suppressive actions of DKK1.



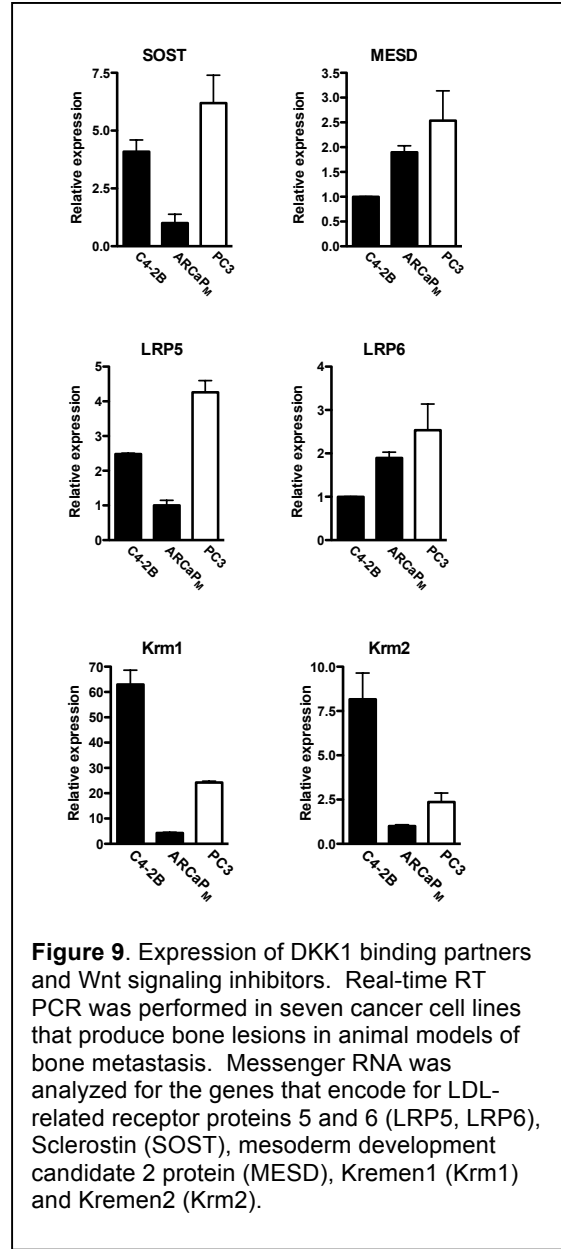
Kremen downregulation causes DKK1 resistance

DKK1 action is dependent on other Wnt signaling components and dysregulation of these members could result in DKK1 resistance. DKK1 binds to the high-affinity transmembrane Kremen1 and Kremen2 receptors. The DKK1-Kremen receptor complex sequesters LRP5 and LRP6 away from the Wnt ligand and Frizzled receptor, leading to LRP removal from the cell membrane and downregulation of Wnt signaling (26,27) (**Figure 1**). Downregulation of LRPs and/or Kremen receptors could render DKK1 inactive. Two other Wnt inhibitors, Sclerostin (encoded by the gene SOST) and mesoderm development candidate 2 protein (MESD), compete with DKK1 for LRP binding (28,29). Excessive expression of these Wnt antagonists could mask DKK1-mediated Wnt inhibition. Expression of these genes was assessed in the three prostate cancer cell lines that produce bone lesions in animal models of bone metastasis (**Figure 9**). A consistent pattern of low Kremen1 and Kremen2 expression in PC3 cells suggested that DKK1 is unable to transmit Wnt inhibitory effects and supports a mechanism of DKK1 resistance.

To test the extent to which expression of Kremen could restore DKK1-mediated Wnt signaling inhibition, Kremen1 was overexpressed in PC3 cells and Wnt signaling was assessed. Kremen1 substantially reduced Wnt signaling in this cancer cell line suggesting that downregulation of Kremen membrane receptors is in part responsible for DKK1 resistance (**Figure 10**).



**Figure 10. Overexpression of Kremen1 reduces Wnt signaling.** PC3 cells were transfected with a Kremen1 (K1) expression construct or empty vector control, along with Wnt reporter vectors. The ratio of TOPFlash to FOPFlash luciferase activity (TOP:FOP) indicated relative Wnt signaling activity. (\*\* = p<0.01).



**Figure 9.** Expression of DKK1 binding partners and Wnt signaling inhibitors. Real-time RT PCR was performed in seven cancer cell lines that produce bone lesions in animal models of bone metastasis. Messenger RNA was analyzed for the genes that encode for LDL-related receptor proteins 5 and 6 (LRP5, LRP6), Sclerostin (SOST), mesoderm development candidate 2 protein (MESD), Kremen1 (Krm1) and Kremen2 (Krm2).

*The data encompassing this task has been submitted to the journal Experimental and Clinical Metastasis. This report is currently being revised based on the Reviewers' suggestions.*

A portion of Task 3 proposed to examine DKK1 methylation in human prostate cancer bone metastasis samples. We received bone metastasis frozen sections from Dr. Robert Vessella, a collaborator on this grant. Unfortunately, the amount of DNA harvested from these samples was inadequate for methylation-specific sequencing using the bisulfite conversion strategy. We

now know that significantly more DNA is required for this strategy than was available from the samples provided.

## KEY RESEARCH ACCOMPLISHMENTS

- The 5 alpha-reductase gene is positioned within an interval of chromosome 13 that may modulate the host response to prostate cancer bone metastasis.
- 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
- Kremen overexpression in PC3 prostate cancer cells partially restores DKK1 inhibition of canonical Wnt signaling.

## REPORTABLE OUTCOMES

The manuscript entitled “DKK1 and Kremen Expression Predicts the Osteoblastic Response to Bone Metastasis” has been submitted to *Clinical and Experimental Metastasis*. This manuscript has been reviewed and revisions are currently in process

## 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. 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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