Prostate Cancer Cells Promote Osteoblastic Bone Metastases through Wnts

Christopher L. Hall, Anna Bafico, Jinlu Dai, Stuart A. Aaronson, and Evan T. Keller

Abstract
Prostate cancer produces painful osteoblastic bone metastases. Although prostate cancer cells produce numerous osteogenic factors, to date, none have been shown to mediate osteoblastic bone metastases in an in vivo model of prostate cancer. Wnts are a large family of proteins that promote bone growth. Wnt activity is antagonized by endogenous proteins including dickkopf-1 (DKK-1). We explored if prostate cancer cells mediate osteoblastic activity through Wnts using DKK-1 as a tool to modify Wnt activity. A variety of Wnt mRNAs were found to be expressed in prostate cancer cell lines and Wnt mRNA expression was increased in primary prostate cancer compared with nonosteoplastic prostate tissue. In addition to expressing Wnts, PC-3 prostate cancer cells expressed the Wnt inhibitor DKK-1. To determine if DKK-1 masked Wnt-mediated osteoblastic activity in osteolytic PC-3 cells, the cells were stably transfected with DKK-1 short hairpin RNA. Decreasing DKK-1 enabled PC-3 cells to induce osteoblastic activity, including alkaline phosphatase production and mineralization, in murine bone marrow stromal cells indicating that DKK-1 blocked Wnt-mediated osteoblastic activity in PC-3 cells. Another prostate cancer cell line, C4-2B, induces mixed osteoblastic/osteolytic lesions. To determine if Wnts contribute to C4-2B's ability to induce mixed osteoblastic/osteolytic lesions, C4-2B cells were stably transfected with either empty vector or DKK-1 expression vector to block Wnt activity. The cells were then injected in the tibiae of mice and allowed to grow for 12 weeks. Blocking Wnt activity converted the C4-2B cells to a highly osteolytic tumor. Taken together, these data show that Wnts contribute to the mechanism through which prostate cancer induces osteoblastic activity. (Cancer Res 2005; 65(17): 7554-60)

Introduction
Prostate cancer is the second leading cause of cancer-related deaths in men resulting in over 30,000 deaths annually (reviewed in ref. 1). More than 80% of all men who die of prostate cancer have metastatic disease within the bone (2). Growth of prostate cancer within the bone promotes localized bone turnover that results in primarily osteoblastic (increased bone density) lesions with underlying osteopenic (low bone density) lesions (3). Although mechanisms contributing to the osteoblastic component of prostate cancer-mediated bone lesions have been elucidated (4), the mechanisms responsible for the osteoblastic component of prostate cancer bone lesions are unknown. Several proteins including endothelins and bone morphogenetic proteins have been hypothesized to play roles in osteoblastic lesions (5); however, there are no published data showing that they mediate prostate cancer-induced osteoblastic lesions in vivo.

Wnt proteins are soluble glycoproteins that bind to receptor complexes composed of Lrp5/6 and Frizzled proteins. Wnt-mediated signaling promotes postnatal bone accrual (6). Additionally, analysis of both chick and mouse limb development has shown that expression of Wnt proteins is essential for skeletal outgrowth (7–10). The activity of the Wnt family is antagonized by several secreted factors including dickkopf (DKK), Wnt inducible factor-1, secreted frizzled-related proteins, and cerberus. DKK-1 controls Wnt signaling by binding to the Lrp coreceptor and sterically blocking Wnt binding to the receptor complex (11, 12). DKK-1 modulation of Wnt signals is also required to achieve normal limb development in vertebrates. Recently, the expression of DKK-1 was found in osteolytic foci of multiple myeloma (13) suggesting that cancer-mediated modulation of Wnt activity influences bone remodeling. In the present study, we tested whether the balance between Wnts and a Wnt antagonist influences the osteoblastic phenotype of prostate cancer–induced bone lesions.

Materials and Methods
Cells. PC-3 human prostate cancer cells were obtained from the American Type Culture Collection (Rockville, MD). PC-3M and highly metastatic PC-3M-LN4 cells are isogenic clones selected in vivo for enhanced metastatic potential and were kind gifts from Dr. Curtis Pettaway (University of Texas M.D. Anderson Cancer Center, Houston, TX; ref. 14). Each cell line was maintained on plastic in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1 mmol/L sodium pyruvate, 1% penicillin-streptomycin, 0.1 mmol/L nonessential amino acids, 2 mmol/L L-glutamine, and 1% vitamin solution (Life Technologies, Grand Island, NY), at 37°C in 5% CO2-95% air. C4-2B, an isogenic LNCaP variant capable of spontaneous metastasis to the bone following intraprostatic injection, was obtained from UroCor (Oklahoma City, OK). C4-2B cells were maintained in T-medium [80% DMEM/20% Ham's F12 (Life Technologies), 5 μg/mL insulin, 13.6 μg/mL triiodothyronine, 5 μg/mL transferrin, 0.25 μg/mL biotin, 25 μg/mL adenine (Sigma, St. Louis, MO), 1× penicillin/streptomycin, and 5% FBS]. C4-2B cells were infected with a retrovirus-expressing human DKK-1 or vector control containing the puromycin marker, and selected mass populations were maintained in T-medium supplemented with 1 μg/mL puromycin (Sigma). Murine bone marrow stromal ST-2 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan) and maintained in α-MEM supplemented with 10% FBS, 1 mmol/L sodium pyruvate, 1% penicillin-streptomycin, and 2 mmol/L L-glutamine. All cells were shown free of Mycoplasma by PCR ELISA (Roche Diagnostics, Indianapolis, IN).

Reverse transcription-PCR analysis of Wnt gene expression. The expression of Wnt family members was evaluated in both the PC-3M and LNCaP prostate cancer cell systems. Unique primer sets for 19 Wnts, four Frizzled, two Lrp, DKK-1, SFRP-5, and glyceraldehyde-3-phosphate dehydrogenase were designed using Primer3 (15) and blasted against the human genome.
Prostate Cancer Induces Osteoblastic Lesions through Wnts

To do PCR, total RNA was isolated from subconfluent cells using TRIzol Reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA/primer pair was then amplified using the access reverse transcription-PCR (RT-PCR) system per manufacturer's instructions (Promega, Madison, WI). PCR was done in a Perkin-Elmer GeneAmp 9700 as follows: 94°C, 60 seconds; 94°C, 5 minutes followed by 30 cycles at 94°C for 25 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. This was not designed to be in the linear range of the amplification process; thus, we can only make semiquantitative statements when differences are large. All products were in the linear range of the amplification process; thus, we can only make multiple comparisons among different studies, similar to a meta-analysis, as of differences was done using ONCOMINE algorithms to account for the

**Quantitative real-time PCR.** DKK-1 RNA levels in prostate cancer cell lines were evaluated by quantitative real-time RT-PCR using the primers for DKK-1 indicated in Table 1. Briefly, 100-ng RNA was amplified using the LightCycler SYBR Green kit according to the manufacturer's instructions (Roche Diagnostics) on a LightCycler. Briefly, amplification was done at 94°C for 5 seconds, 62°C for 5 seconds, and 72°C for 5 seconds for 45 cycles. RT-PCR of ß-actin was used as an internal control to normalize for loading differences between samples.

**cDNA microarray analysis.** The ONCOMINE database and gene microarray analysis tool, a repository for published cDNA microarray data (http://141.214.6.50/oncomine/main/index.jsp; ref. 16), was explored for mRNA expression of Wnt pathway mediators in nonneoplastic prostate, primary prostate cancer, and prostate cancer metastases. Statistical analysis of differences was done using ONCOMINE algorithms to account for the multiple comparisons among different studies, similar to a meta-analysis, as previously described (16).

**Design of DKK-1 short hairpin RNAs.** The Block-it RNAi designer (Invitrogen) was used to design a short hairpin RNA molecules (shRNA) specific to human DKK-1 (accession no. NM_012242; position 351-371; 5'-CAATGGTCTGGATATTCTTCAGGAAAGGAATAAGTACCAAGACATTGGAGACACCGT-3'). A DKK-1 shRNA control was generated by inverting the bases at position 9-13 within the DKK-1 351 siRNA. Resulting sequences were cloned into the RNA expression vector pENTR/H1/TO (Invitrogen) and sequence confirmed. DKK-1 shRNAs and control were transfected into human PC-3 prostate cancer cells using a CaPO4 method and individual clones selected using 100 μg/mL Zeocin (Invitrogen).

**Western blot for DKK-1 Expression.** The amount of DKK-1 protein was determined using Western blotting of total cell lysates. Briefly, cells were washed once on ice with ice-cold PBS and scraped into 0.3 mL ice-cold lysis buffer [1% Triton X-100, 20 mmol/L Tris-HCl (pH 8.0), 137 mmol/L NaCl, 10% glycerol (v/v), 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 50 μg/mL trypsin inhibitor, 1 mmol/L sodium orthovanadate]. Cell lysates were then clarified by centrifugation and aliquots of each were removed for protein determination by the bichinchoninic acid protein assay (Pierce, Rockford, IL). Equal amounts of protein (30 μg/sample) were resolved using 10% SDS-PAGE. Separated proteins were transferred onto 0.45-μm polyvinylidene difluoride membranes (Millipore, Bedford, MA). The filters were blocked with 3% bovine serum albumin in TBS and probed with goat anti-human DKK-1 pAb (1:1,000, R&D Systems, Minneapolis, MN). Protein bands were visualized using the enhanced chemiluminescence detection system (Cell Signaling, Beverly, MA). To normalize for differences in loading, the blots were stripped and reprobed with mouse anti-β-actin monoclonal antibody (1:1,000, Sigma).

**Cell proliferation.** Cells were plated in 96-well plates at a density of 1.5 × 10^4 cells per 0.2 mL per well in complete medium in triplicate. The total number of viable cells on one plate was determined every 24 hours by the addition of a final concentration of 1 mmol/L 3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyl-2H-tetrazolium bromide. The formazan product was

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**Table 1. PCR primers used to evaluate Wnt family member and receptor mRNA expression**

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<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<td>5'-GAGCAGCAGGAGGAGGAGG-3'</td>
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A cell/cell in vitro mineralization assay. Cells were trypsinized, washed, and treated in suspension with a cytostatic dose of 15 Gy of γ-radiation using a 137Cs source. To ensure that irradiation did not alter Wnt gene expression RT-PCR was also done on PC-3 parental, shRNA control, and DKK1 shRNA-transfected cells 48 hours after 15-Gy γ-radiation. Irradiation did not affect Wnt expression (data not shown). Irradiated prostate cancer cells (6.0 × 10^6) were plated with or without 6.0 × 10^5ST-2 murine bone marrow stromal cells to 12-well plates. After 24 hours, cells were changed to a mineralization media (maintenance media plus 50 μg/mL ascorbic acid and 5 mmol/L β-glycerophosphate). ST-2 cells in mineralization media or with the addition of rBMP-2 (Peprotech, Rocky Hill, NJ) were used as mineralization negative and positive controls, respectively. Separately, rWnt3a (R&D Systems) was evaluated to show Wnt-mediated mineralization. Following 11 days in culture, conditioned medium was evaluated for alkaline phosphatase activity using the Sigma Diagnostics Alkaline Phosphatase kit (Sigma). The presence of mineral was determined by staining for calcium phosphate using silver nitrate (von Kossa staining) at the experimental end point as previously described (17).

In vivo animal model of bone metastasis. The effect of prostate cancer–derived DKK-1 expression on bone turnover was evaluated following direct injection into the tibia of male C57B6 severe combined immunodeficient mice (intratibial injection) as described previously (4, 18). Tumors were allowed to grow for 12 weeks at which time mice were sacrificed. Evidence of tumor-induced bone change was evaluated at 12 weeks after tumor injection using Faxitron radiography (Faxitron X-ray Systems, Buffalo, NY). Radiographs were digitized and the percent osteolytic area of the total tibial bone area was quantified using Scion Imaging Software (Scion Corp., Frederick, MD). Tumor-injected tibiae and control lateral tibiae without tumors were removed, fixed in 10% normal buffered formalin, and bone mineral density (BMD) measured using dual-energy X-ray absorptiometry (DEXA) with a pDEXA Sabre scanner (Orthometrix, Hampton, NH), paraffin embedded, and histologic sections stained with H&E. The animal protocol was approved by the University of Michigan Institutional Animal Care and Use Committee.

Results

Wnts are expressed in prostate cancer. The expression of Wnt family members was evaluated in both the PC-3M, derived from PC-3, and LNCaP prostate cancer cell systems. Both PC-3M and LNCaP were repeatedly passaged in vivo to produce isogenic clones that are highly metastatic and capable of metastasis to the bone, PC-3M-LN4 and C4-2B, respectively. Using RT-PCR, we found that the PC-3M series cells express the mRNAs for Wnt 2, Wnt 3a, Wnt 5b, Wnt 6, and DKK-1, compared with LNCaP cells that did not express these transcripts (Fig. 1A). Additionally, the mixed osteoblastic/osteolytic C4-2B line expressed both Wnt 7a and Wnt 8b, whereas its parental cell line LNCaP did not. Wnt 1, Wnt 6, Wnt 8a, and Wnt 10a were not detected in any prostate cancer cell lines. To ensure that the PCR primer sets used for the Wnt mRNAs that were not detected could amplify these mRNA appropriately, the primers were used to successfully amplify these target mRNA in known positive control cell lines (data not shown). The Oncomine cDNA microarray analysis repository (16) was explored for differences in mRNA expression of Wnt pathway mediators among nonneoplastic prostate, primary prostate cancer, and prostate cancer metastases cDNA microarrays. Shaded box, interquartile range marking the 25th to 75th percentile; whiskers, 10th to 90th percent range; asterisk, minimum and maximum; bar, median. *, P < 2.8 × 10^-6 versus primary prostate cancer; †, P < 1.5 × 10^-4 versus nonneoplastic prostate; ‡, P < 0.004 versus nonneoplastic prostate. C, quantitative real-time PCR was done on total RNA on the indicated cells lines. DKK-1 mRNA levels were normalized to β-actin levels. *, P < 0.01, compared with all other cell lines. D, RT-PCR and Western analysis of indicated prostate cancer cell lines for DKK-1 mRNA and protein expression. Amplonc size and molecular weight markers are indicated for RT-PCR and Western blot, respectively.

Figure 1. Wnts are expressed in human prostate cancer. A, Wnt gene expression was evaluated by RT-PCR in PC-3M and LNCaP as well their highly metastatic variants PC-3M-LN4 and C4-2B, respectively. One microgram of total RNA for each primer pair was amplified and products evaluated by electrophoresis on 1.2% agarose gels. Key in figure indicates lanes 1 through 5 for each mRNA evaluated. B, ONCOMINE gene microarray database was explored for differences in mRNA expression of Wnt pathway mediators among nonneoplastic prostate, primary prostate cancer, and prostate cancer metastases cDNA microarrays. Shaded box, interquartile range marking the 25th to 75th percentile; whiskers, 10th to 90th percent range; asterisk, minimum and maximum; bar, median. *, P < 2.8 × 10^-6 versus primary prostate cancer; †, P < 1.5 × 10^-4 versus nonneoplastic prostate; ‡, P < 0.004 versus nonneoplastic prostate. C, quantitative real-time PCR was done on total RNA on the indicated cells lines. DKK-1 mRNA levels were normalized to β-actin levels. *, P < 0.01, compared with all other cell lines. D, RT-PCR and Western analysis of indicated prostate cancer cell lines for DKK-1 mRNA and protein expression. Amplonc size and molecular weight markers are indicated for RT-PCR and Western blot, respectively.

To further characterize basal DKK-1 expression in the PC-3 and LNCaP series of cell lines, we did quantitative real-time PCR, RT-PCR, and Western analysis. In the osteolytic PC-3 series, DKK-1 tumors. DKK-1 expression was decreased in prostate cancer versus normal prostate tissue (Fig. 1B).
mRNA and protein was most highly expressed in the parental PC-3 cell line and decreased with increasing malignancy (Fig. 1C and D). These results are consistent with the relative decrease of DKK-1 expression levels observed in the clinical specimens using the Oncomine gene array data. In the mixed osteoblastic/osteolytic cell lines LNCaP and C4-2B, DKK-1 mRNA and protein were undetectable (Fig. 1C and D), consistent with the increased osteoblastic nature of these cell lines. Taken together, these results indicate that as prostate cancer progress, DKK-1 expression level decreases and suggest that as the cell line becomes osteoblastic (i.e., C4-2B versus PC-3) that DKK-1 expression is decreased.

Reduction of DKK-1 expression in PC-3 human prostate cancer cells by short hairpin RNA. Prostate cancer lesions are considered osteoblastic; however, they are also accompanied by osteolytic activity (1) as is reflected by the PC-3 prostate cancer cell line. Because PC-3 cells expressed high DKK-1 levels (Fig. 1) and induce osteolytic bone lesions, we tested whether suppression of DKK-1 in PC-3 cells might unmask this prostate cancer cell line’s ability to induce osteoblastic lesions. To decrease DKK expression, PC-3 cells were stably transfected with an shRNA molecule against human DKK-1. Two clones, numbers 3 and 8, exhibited >80% down-regulation of the DKK-1 protein (Fig. 2A), and these clones were used for further studies. To determine if down-regulation of DKK-1 had an effect on cell proliferation, we measured in vitro proliferation over 48 hours. There was no difference in cell proliferation between shRNA control versus DKK-1 shRNA clones (Fig. 2B).

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PC-3 cells induce osteoblastic activity in bone marrow stromal cells through Wnts. To test whether prostate cancer mediate osteoblastic activity through Wnts, we used PC-3 prostate cancer cells expressing DKK-1 shRNA and analyzed their ability to induce osteoblastic lesions. To decrease DKK expression, PC-3 cells were stably transfected with an shRNA molecule against human DKK-1. Two clones, numbers 3 and 8, exhibited >80% down-regulation of the DKK-1 protein (Fig. 2A), and these clones were used for further studies. To determine if down-regulation of DKK-1 had an effect on cell proliferation, we measured in vitro proliferation over 48 hours. There was no difference in cell proliferation between shRNA control versus DKK-1 shRNA clones (Fig. 2B).
that occurs in late osteoblast differentiation) in vitro. In these assays, parental PC-3 cells, shRNA control–transfected cells, or DKK-1 shRNA–transfected cells were treated in suspension with a sublethal dose of radiation to provide an intact, cytostatic population of prostate cancer cells. Irradiated prostate cancer cells were then co-cultured with osteogenic ST-2 murine bone marrow stromal cells. Down-regulation of DKK-1 induced a 27% to 49% increase in alkaline phosphatase activity over PC-3 parental cells (Fig. 3A). In mineralization media, ST-2 cells that receive an osteoinductive stimulus will differentiate and deposit calcium phosphate mineral. PC-3 parental or shRNA control–transfected cells (both which express high levels of endogenous DKK-1) failed to induce mineralization of ST-2 cells (Fig. 3B). In contrast, both of the PC-3 DKK-1 shRNA–transfected clones, in which Wnts are not blocked by endogenous DKK-1, induced strong mineralization of ST-2 cells (Fig. 3B). Taken together, these results show that reducing the expression of the Wnt inhibitor DKK-1 unmasked an osteoinductive effect in osteolytic PC-3 cells. Additionally, recombinant Wnt3a induced alkaline phosphatase activity (Fig. 3A) and mineralization (Fig. 3B), confirming the ability of a Wnt to induce osteoblastic activity.

**Decreasing Wnt activity in C4-2B prostate cancer cells decreases their osteoblastic activity in vivo.** Our PCR data indicated that C4-2B cells express Wnts but not DKK-1. To investigate whether C4-2B cells mediate the osteoblastic component of their bone remodeling activity through Wnts, we diminished Wnt activity in C4-2B cells through stable retroviral-mediated transduction of DKK-1. To determine if up-regulation of DKK-1 had an effect on cell proliferation, we measured in vitro proliferation over 48 hours. There was no difference in cell proliferation between control empty vector–transfected versus DKK-1 expression vector clones (Fig. 2C). The cells were next injected into the tibia of mice, and tumors were allowed to develop for 12 weeks. Radiography revealed that control vector–transfected C4-2B cells produced mixed osteolytic/osteoblastic lesions (Fig. 4A, X-ray and X-ray Mag). In contrast, DKK-1–transfected C4-2B cells produced highly osteolytic lesions (Fig. 4A, X-ray and X-ray Mag) associated with marked increase in the bone density. Normal tibiae were scanned. #, P < 0.01, compared with tibia of normal mice. * represents statistically significant difference at P < 0.01 among all groups. ** indicates low bone mineral density) in the empty vector–transfected cell-injected tibia compared to the amount of blue (indicating low bone mineral density) in the DKK-1-transfected cell-injected tibia. Fourth row, H&E histology of tibiae showing normal trabeculae (arrow) in uninjected tibia; thickened trabeculae (arrow) in the C4-2B vector–transfected cells and nearly complete destruction of the trabeculae (arrow) in the tibiae injected with the DKK-1-transfected C4-2B cells. Note the replacement of marrow (M) by tumor (T) in the tumor cell–injected tibia. Arrowhead indicates growth plate. Original magnification, 40×. B, % osteolytic area of total tibial area. Radiographs from of normal tibiae without tumor or from the tumor-injected tibiae (n = 5 per group) were digitized and % osteolytic area of the total tibial area was quantified. *, P < 0.013, versus C4-2B vector cells. C, bones were subjected to DEXA to quantify bone mineral density. Normal tibiae (n = 7), tibiae injected with C4-2B vector–transfected cells (n = 5), or C4-2B DKK-1-transfected cells (n = 5) were scanned. #, P < 0.01, compared with normal tibiae or C4-2B vector–transfected tibiae.
Prostate Cancer Induces Osteoblastic Lesions through Wnts

Prostate cancer-induced osteolysis is mediated, in part, through Wnt signaling with DKK-1 allowed for the osteolytic phenotype to be expressed. However, we cannot rule out that in addition to unmasking an endogenous osteolytic effect that DKK-1 also induces osteolytic activity by also inhibiting normal Wnt signaling in the bone microenvironment.

A variety of factors have been postulated to mediate the osteoblastic nature of prostate cancer cells. Most likely, not one factor is the sufficient for this activity, but rather several factors acting in concert promote the overall osteoblastic activity. Endothelin-1 (ET-1), which has been shown to be pro-osteoblastic in murine models of breast cancer (22), may be an osteoblastic factor in prostate cancer. Specifically, inhibition of ET-1 with administration of an endothelin-A receptor antagonist in hormone-refractory prostate cancer patients suppressed indices of bone remodeling (23). Another class of factors, the bone morphogenetic proteins, is expressed by prostate cancer and mediates osteoblastic activity by working directly on osteoblasts (24) or through induction of vascular endothelial growth factor (25). Whereas these factors may play a role in prostate cancer–mediated osteoblastic activity, the demonstration of Wnt-mediated activity in prostate cancer–mediated osteoblastic lesions in the current article is the first in vivo demonstration for a prostate cancer pro-osteoblastic factor.

There were several challenges in deciding how to modulate Wnt activity in the prostate cancer cells. Overexpression of Wnt to promote osteoblastic activity in the PC-3 cells would most likely not be informative as this would not define a role of endogenously expressed Wnts. Additionally, inhibition of a specific Wnt in C4-2B cells would be challenging as it is not clear which Wnt participates in the osteoblastic response. Accordingly, we initially chose to alter Wnt activity through modulation of DKK-1, a specific inhibitor of the canonical Wnts (26). This strategy allowed us to evaluate modulation of the activity of several canonical Wnts at one time to initially identify if Wnts play a role in prostate cancer–induced osteoblastic activity.

In conclusion, we provide evidence that Wnts contribute to prostate cancer–mediated osteoblastic activity. Furthermore, as prostate cancer bone metastases have both an osteolytic and osteoblastic components, these data suggest that as opposed to an ongoing vicious cycle in prostate cancer bone lesions, as observed in breast cancer (27), that there is a shift in the balance from osteolytic to osteoblastic activity as prostate cancer progresses. Our results indicate that the balance between DKK-1 and Wnt expression may contribute to determining which type of bone lesion is predominant at any particular time point in the development of prostate cancer bone metastatic lesions.

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