Prostate Cancer Induces Bone Metastasis through Wnt-Induced Bone Morphogenetic Protein-Dependent and Independent Mechanisms

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Abstract

Prostate cancer (PCa) is frequently accompanied by osteosclerotic (i.e., excessive bone production) bone metastases. Although bone morphogenetic proteins (BMP) and Wnts are mediators of PCa-induced osteoblastic activity, the relation between them in PCa bone metastases is unknown. The goal of this study was to define this relationship. Wnt3a and Wnt5a administration or knockdown of DKK-1, a Wnt inhibitor, induced BMP-4 and 6 expression and promoter activation in PCa cells. DKK-1 blocked Wnt activation of the BMP promoters. Transfection of C4-2B cells with anaxin, an inhibitor of canonical Wnt signaling, blocked Wnt3a but not Wnt5a induction of the BMP promoters. In contrast, Jnk inhibitor I blocked Wnt5a but not Wnt3a induction of the BMP promoters. Wnt3a, Wnt5a, and conditioned medium (CM) from C4-2B or LuCaP23.1 cells induced osteoblast differentiation in vitro. The addition of DKK-1 and Noggin, a BMP inhibitor, to CM diminished PCa CM–induced osteoblast differentiation in a synergistic fashion. However, pretreatment of PCa cells with DKK-1 before collecting CM blocked osteoblast differentiation, whereas pretreatment with Noggin only partially reduced osteoblast differentiation, and pretreatment with both DKK-1 and Noggin had no greater effect than pretreatment with DKK-1 alone. Additionally, knockdown of BMP expression in C4-2B cells inhibited Wnt-induced osteoblastic activity. These results show that PCa promotes osteoblast differentiation through canonical and noncanonical Wnt signaling pathways that stimulate both BMP-dependent and BMP-independent osteoblast differentiation. These results show a clear link between Wnts and BMPs in PCa-induced osteoblast differentiation and provide novel targets, including the noncanonical Wnt pathway, for therapy of PCa. [Cancer Res 2008;68(14):5785–94]

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and the second leading cause of cancer death among men in the United States (1). The most common site of PCa metastasis is the bone, with skeletal metastases identified at autopsy in up to 90% of patients dying from PCa (2). Skeletal metastasis in PCa patients result in significant complications that diminish the quality of life in affected patients. These complications include bone pain, impaired mobility, pathologic fracture, spinal cord compression, and symptomatic hypercalcemia.

At the metastatic bone site, growth of PCa promotes localized bone remodeling that results in primarily osteosclerotic lesions (also known as osteoblastic lesions characterized by increased production of mineralized bone matrix resulting in increased bone mineral density) with underlying osteolytic lesions (i.e., osteoclast-mediated resorption of mineralized bone matrix resulting in low bone mineral density; reviewed in ref. 3). Although mechanisms contributing to the osteopenic component of PCa-mediated bone lesions have been elucidated (4, 5), the mechanisms responsible for the osteoblastic component of PCa bone lesions are not well-defined, although a variety of factors such as bone morphogenetic proteins (BMP; ref. 6), endothelium-1 (ET-1; ref. 7), vascular endothelial growth factor (VEGF; ref. 8), and Wnts (9) have been implicated as contributing to osteoblastic metastasis. Understanding the mechanisms that promote PCa-induced osteosclerosis may help identify targets to diminish the progression of these lesions.

BMPs, members of the transforming growth factor β superfamily, were originally discovered because of their ability to induce new bone formation (reviewed in ref. 10). BMPs bind to two different types of serine-threonine kinase transmembrane receptors, type I and type II. BMPs initially bind to BMPR-II, which then phosphorylates BMPR-I, which subsequently phosphorylates SMAD proteins. Target genes of BMPs include osteoblast proteins such as OPG (11) and the osteoblast-specific transcription factor Cbfα-1 (12). Many in vitro studies have showed that BMPs induce osteogenic differentiation (13, 14). A number of studies have shown that BMP expression increases with progression of PCa (15–18) and that BMP-6 induces osteoblastic activity in a murine model of PCa (6), although BMP-7 does not seem to do this (19).

Wnt proteins are soluble glycoproteins that mediate homeostatic and developmental activity (reviewed in ref. 20). The canonical Wnts bind to receptor complexes composed of Lrp5/6 and Frizzled proteins and ultimately induced nuclear translocation of β-catenin. Noncanonical Wnts bind to a variety of receptors, including receptor tyrosine kinases, and mediate many different activities, some of which oppose canonical Wnt signaling. Wnt-mediated signaling promotes postnatal bone accrual (21) and is essential for skeletal outgrowth (22). In concordance with the role of Wnts in bone development and growth, PCa-produced Wnts were identified to induce osteoblastic activity in PCa bone metastases (9). However, it is not clear if Wnts mediate this activity through direct action on osteoblasts or through activation of other pro-osteoblastic factors, such as BMPs. As both Wnts and BMPs have been shown to play a role in PCa osteoblastic metastases and Wnts
have been shown to promote BMP expression and modulate the osteogenic action of BMPs in the embryo development (23), we tested if BMPs contribute to the ability of Wnts to regulate osteoblastic activity in PCA bone metastases.

Materials and Methods

Materials. Wnt-3a and -5a, sFRP-1, DKK-1, and Noggin recombinant proteins were obtained from R&D Systems. c-Jun-NH2-kinase (JNK) inhibitor I and JNK inhibitor I–Negative Control were obtained from EMD Biosciences. This inhibitor blocks the interaction of JNK and its substrates (24) and were not toxic at doses used in the studies based on dose response proliferation and cell death (trypan blue staining) assays (data not shown).

Cells and cell culture. LNCaP and PC-3 cells were obtained from American Tissue Culture Collection. PC-3 Clone 8 are cells that have been stably transfected with shRNA-targeting DKK-1 that resulted in a knockdown of DKK-1 by >80% (9). These PCa cells were maintained in RPMI (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Life Technologies, Inc.). The C-28B cell line, a kind gift from Dr. Leland Chang (Emory University, Atlanta, GA), is an osteogenic derivative cell line of LNCaP cells (25) and was maintained in T medium (80% DMEM (Life Technologies, Inc.), 20% F12 (irving Scientific), 3 grams/liter NaCO3, 100 units/liter penicillin G, 100 µg/ml streptomycin, 5 µg/ml insulin, 13.6 pg/ml triiodothyronine, 5 µg/ml transferrin, 0.25 µg/ml biotin, and 25 µg/ml adenine) supplemented with 5% FBS. LuCaP 23.1, kindly provided by Dr. Robert Vessella (University of Washington, Seattle, WA), is an androgen-sensitive, PSA-producing human PCA xenograph derived from the lymph nodes of a patient that had failed androgen deprivation therapy (26). Single cell suspensions of LuCaP 35 were prepared by resecting the s.c. xenografts and cutting them into small pieces in HBSS with 1% FBS as we have previously described (6), then using the Cancer Cell Isolation kit (Panomics) as directed by the manufacturer. Briefly, the small pieces were digested in Tumor Cell Digestion Solution, then the tumor cell suspension was purified by Tumor Cell Purification Solution and the LuCaP 23.1 cells were maintained in RPMI 1640 with 10% FBS. Cell viability was determined by trypan blue counting, and only preparations with >95% viability were used for in vitro injection. The MC3T3-E1 (clone MC-4; kindly provided by Dr. Renny Franceschi, University of Michigan, Ann Arbor, MI) is a murine preosteoblast cell line that was maintained in -MEM and 10% FBS. All cells are checked for Mycoplasma contamination every 3 mo using PCR methods.

For Wnt administration in vitro, recombinant Wnts were added at the indicated doses to culture medium. The dose range was designed to encompass the effective dose 50% for these proteins based on previous studies (27, 28) and the manufacturer (R and D Systems). For inhibitor studies using DKK-1 or noggin, the inhibitory compound was added to the medium at the same time the culture treatments were initiated. Typically, 50% of the medium was replaced every 3 d with fresh conditioned medium (CM) containing FBS and treatment compounds at the original concentrations. This was continued until the end of each study as indicated in the figure legends.

Plasmids and transfections. Flag-tagged human pcdNA3-Axin2 was kindly provided by Dr Eric R. Fearon (Department of Internal Medicine, Human Genetics, and Pathology, University of Michigan, Ann Arbor, MI). The pGL3-BMP4-2443 promoter construct containing 2,443 bp of the proximal BMP-4 promoter was kindly provided by Dr. L. Helvering (Lilly Pharmaceutical, Indianapolis, IN; ref. 29), and the pGL3-BMP6-1168 promoter that contains 1,168 bp of the BMP-6 promoter was kindly provided by Dr. S. Kitazawa (Kobe University School of Medicine, Kobe, Japan; ref. 30). Cells were transfected using Nucleofection as recommended by the manufacturer (Axama, Inc.). Luciferase activity was measured and normalized between transfections using the dual luciferase assay (Promega). Human BMP-4 and BMP-6–shRNA and control scrambled shRNA were obtained from Open Biosystems. The shRNAs were provided in a lentiviral expression vector and were packaged in packaging cell line, TLA-HEK293T, using the Trans-Lentiviral Packaging System (Open Biosystems). C-42B cells were then transduced using lentiviral supernatant as directed by the manufacturer. Stably transfected clones were selected using 2 µg/ml Puromycin (Invitrogen) selection and used as a polyclonal population.

Obtaining CM. CM was obtained from cells as previously described (28). Briefly, 5 × 10^5 cells were plated in 10-cm tissue culture dishes for 12 h in RPMI 1640 with 10% FBS. Cells were allowed to grow to confluence and then the medium was changed to 10 mL of RPMI plus 0.5% FBS, and supernatants were collected 24 h later. To normalize for differences in cell density due to proliferation during the culture period, cells from each plate were collected and total DNA content/plate was determined (spectrophotometric absorbance at 260 nm). CM was then normalized for DNA content between samples by adding RPMI. In some instances, as indicated in the results, cells were pretreated with either DKK-1 or Noggin or both for 12 h, followed by replacing the medium with fresh medium and then collecting CM 24 h later.

Alkaline phosphatase and osteocalcin assay. Alkaline phosphatase (ALP) activity was measured in the cells using a colorimetric assay based on the conversion of P-nitrophenylphosphate as directed by the manufacturer (ALP assay; Sigma). Briefly, MC3T3-E1 cells in 12-well plates were washed with PBS and sonicated in 10 mmol/L of Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100. ALP activity in the lysate was assayed by the hydrolysis of p-nitrophenyl phosphate (Sigma) to p-nitrophenol. Absorbance was determined at 405 nm and compared with a p-nitrophenol (Sigma) standard titration curve. ALP activity was normalized to total protein content (Bio-Rad Protein Assay). Osteocalcin was measured in supernatants using a mouse-specific ELISA as recommended by the manufacturer (Biomedical Technologies, Inc.).

Coculture experiments. MC3T3-E1 cells were plated in a 12-well culture plate at a density of 2 × 10^5 cells per well and grown in α-MEM medium with 10% FBS for 48 h (8–80% confluent). At the same time, C-42B cells were plated at a density of 2 × 10^5 cells per well in 12-well culture plates on 10-mm Anopore tissue culture plate inserts (pore size, 0.2 µm; Nalge Nunc International) and grown in T medium plus 10% FBS plus vehicle or recombinant Wnt 3a or Wnt5a at 50 ng/ml for 48 h. At 48 h after the initiation of cultures, the plates were gently washed with PBS, the culture plate inserts containing C-42B cells were inserted into the culture plates containing the MC3T3-E1 cells, and the medium was replaced with fresh α-MEM medium plus 10% FBS. The cocultures were maintained for 10 d at which time supernatants were subjected to measurement of ALP and osteocalcin.

Immunoblot analysis. For identification of BMP proteins, whole cell lysates were prepared by incubating cells in ice-cold radioimmunoprecipitation assay lysis buffer (Sigma). Lysates were precollected and the protein concentration was determined by the bicinchoninic acid assay (Pierce Biochemicals). For electrophoresis, lysates were supplemented with SDS loading buffer and separated on SDS-12% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. The blots were incubated in TBS containing 0.1% Tween 20 and 5% nonfat dry milk during the blocking and the antibody incubation steps. Human anti–BMP-4 and BMP-6 antibodies (R&D Systems) were used at 1:500 to 1:2,000 dilutions, and rabbit anti-actin (Sigma) antibody was used at 1:1,000, and the horseradish peroxidase (HRP)-conjugated sheep anti-mouse (Amersham International) and the HRP-conjugated donkey anti-rabbit (Amersham Life Science) were used at 1:5,000 dilution. Antibody complexes were detected by enhanced chemiluminescence (Amersham Life Science) and exposure to X-Omat film (Kodak). Band densities were quantified using NIH Scion Image.

Reverse transcription PCR analysis of BMPs mRNA expression. Total RNA was extracted using RNeasy kit (Qiagen) and synthesized to cDNA using First Strand cDNA Synthesis kit (SuperArray Bioscience Co.). The cDNA was amplified by GeneAmp PCR System 9700 (PE Applied Biosystems) using HotStart Sweet PCR master mix (SuperArray) at 95°C for 15 min, 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s for 30 to 40 cycles, and β-Actin was used as an internal control. The PCR primer set used for BMP-4, BMP-6, and β-actin were manufactured by SuperArray Bioscience Co. All products were evaluated by electrophoresis on 2% agarose gels.
Real-time PCR quantitative analysis of BMP mRNA expression. Total RNA was extracted using RNeasy kit (Qiagen) and synthesized to cDNA using Reverse Transcription System (Promega). The cDNA was amplified and quantified by LightCycler (Roche Diagnostics) using the Real-time SYBR Green PCR master mix (SuperArray Bioscience Co.) at 95°C for 15 min, 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 35 to 55 cycles. β-Actin was used as an internal control. The PCR primers used for BMP-4, BMP-6, and β-actin were manufactured by SuperArray Bioscience Co. The size of the PCR products was confirmed using gel electrophoresis after real-time PCR (data not shown).

Data analysis. Statistical significance was determined for multivariate comparisons using ANOVA and Fisher's probable least significant difference for post hoc analysis. Student's t test was used for bivariate analyses. Statistical significance was determined as having a P value of 0.05. Statistical calculations were performed using Statview software (Abacus Concepts).

Results

Wnt3a and Wnt5a promote BMP-4 and BMP-6 expression in C4-2B cells. To investigate whether Wnt3a, a canonical Wnt, and Wnt5a, a noncanonical Wnt, regulate the expression of BMP-4 and BMP-6 in PCa cells, the LNCaP derivative C4-2B cells (1 × 10⁵ cells per well) were plated in 12-well plates in T medium plus 10% FBS. The cells were treated, respectively, with 0, 25, 50, and 100 ng/mL Wnt3a or Wnt5a or vehicle for 8 hours, followed by real-time reverse transcription-PCR (RT-PCR) to quantify mRNA levels. Wnt3a increased BMP-4 mRNA expression but had no effect on BMP-6 mRNA expression (Fig. 1A), whereas Wnt5a increased BMP mRNA expression but had no effect on BMP-4 mRNA expression in C4-2B cells (Fig. 1B). To determine if protein levels reflected changes in mRNA levels, C4-2B cells were treated with Wnt3a (50 ng/mL), Wnt5a (50 ng/mL), or vehicle for 24 hours followed by Western blot. Similar to the changes in mRNA levels, Wnt3a increased BMP-4 but not BMP-6 protein expression, whereas Wnt5a increased BMP-6 but not BMP-4 protein expression in C4-2B cells (quantification shown in Supplementary Fig. S1A; Fig. 1C). These findings show that Wnts regulate BMPs expression in C4-2B cells and suggest that BMPs are differentially regulated by canonical versus noncanonical Wnts. To determine if this pathway is also active in a PSA-negative, AR-negative cell line, we used PC-3 cells. We have previously reported that PC-3 cells, which are osteolytic, express high levels of DKK-1 (9). In a previous study to determine the activity of Wnts in PC-3 cells, we had a made PC-3 cell line that was stably transfected with shDKK-1 (PC-3 clone 8), resulting in >80% reduction of DKK-1 protein levels (9). We identified in that study that knockdown of DKK-1 conferred pro-osteoblastic activity on PC-3 cells including the ability of PC-3 cells to promote both AP activity and mineralization of MC-3T3 cells in vitro. To determine if this activity was associated with changes in BMP expression, we measured BMP-4 and BMP-6 protein and mRNA levels in control-transfected or PC-3 cells stably transfected with shDKK-1 (PC-3 clone 8). Knockdown of DKK-1 induced both BMP-4 and BMP-6 mRNA and protein expression (quantification shown in Supplementary Fig. S1B; Fig. 1D). These data show that inhibition a Wnt signaling repressor promotes BMP expression, indicating that the relation between Wnts and BMP expression is functional in the PSA-negative, AR-negative PC-3 cell line. This finding also suggests that loss of the osteoblastic activity in the PC-3 cell line may be due to repression of Wnt-mediated BMP expression secondary to DKK-1 repression of Wnt activity.

Figure 1. Wnts increase BMP mRNA and protein expression in PCa cells. C4-2B cells (1 × 10⁵ cells per well) were plated in 12-well plates in T medium plus 10% FBS. A and B, the cells were treated with 0, 25, 50, and 100 ng/mL of the indicated Wnt3a and Wnt5a for 8 h, then total RNA was collected and subjected to real-time RT-PCR for (A) BMP-4 and (B) BMP-6. C, cells were treated with Wnt3a (50 ng/mL) or Wnt5a (50 ng/mL) or vehicle for 24 h. Total protein was collected and subjected to Western blot for BMP-4 and BMP-6. β-Actin was used as loading control. Representative Western blot is shown. D, total mRNA or protein from control shRNA–transfected or shDKK-1–transfected PC-3 cells (PC-3 Clone 8) were collected and subjected to PCR or Western blot for BMP-4, BMP-6, and β-actin. Columns, mean from two to three experiments; bars, SD. *, P < 0.05 versus 0 ng/mL.
Wnt3a and Wnt5a activate BMP-4 and BMP-6 promoter activity in C4-2B cells through Wnt canonical pathway and noncanonical JNK-dependent signaling pathway. The observation that Wnts increased steady-state BMP mRNA and protein expression gave rise to the possibility that Wnts activate BMP promoters. To test this possibility, C4-2B cells were transfected with a luciferase reporter vector driven by the BMP-4 promoter (pGL3-BMP4), the BMP-6 promoter (pGL3-BMP6), or empty control vector (pGL3-basic) and treated with Wnt3a or Wnt5a in the absence or presence of two different inhibitors of canonical Wnt signaling: (a) DKK-1 (1 ug/mL), which inhibits the Wnt receptor LRP-6 and (b) sFRP-1 (5 ug/mL), which sequesters Wnts from the membrane-bound Frizzled, which trimerizes with LRP-6 and Wnt (reviewed in ref. 31). After 8 hours of treatment, cells were harvested, and total cell lysates were collected for measurement of luciferase levels. Wnt3a induced BMP-4 but not BMP-6 promoter activity, whereas Wnt5a induced BMP-6 but not BMP-4 promoter activity. Axin-2, a negative regulator of the intracellular component of the canonical Wnt pathway, or were pretreated with a cell permeable JNK inhibitor (30 μmol/L; JNK inhibitor I) for 1 hour, then cells were treated with either vehicle, Wnt3a (50 ng/mL), or Wnt5a (50 ng/mL). Eight hours later, cells were harvested, and total cell lysates were assayed for luciferase levels. Axin-2 partially diminished Wnt3a-induced BMP-4 promoter activity (Fig. 3A), whereas JNK inhibitor I had no effect on the Wnt3a-induced BMP-4 promoter activity (Fig. 3B). In contrast, Axin-2 had no effect on Wnt5a-induced BMP-6 promoter activity (Fig. 3C), whereas JNK inhibitor I partially diminished Wnt5a-induced BMP-6 promoter activity (Fig. 3D). These results indicate that Wnt3a and Wnt5a induce activation of the BMP-4 and BMP-6 promoter, respectively, through a LRP-6–dependent mechanism.

To elucidate further the roles of the Wnt canonical pathway and noncanonical JNK-dependent signaling pathways in Wnt-induced BMP promoter activity, C4-2B cells transfected with either the BMP-4 or BMP-6 promoter were cotransfected with Axin-2, a negative regulator of the intracellular component of the canonical Wnt pathway, or were pretreated with a cell permeable JNK inhibitor (30 μmol/L; JNK inhibitor I) for 1 hour, then cells were treated with either vehicle, Wnt3a (50 ng/mL), or Wnt5a (50 ng/mL). Eight hours later, cells were harvested, and total cell lysates were collected for measurement of luciferase levels. Columns, mean from three experiments; bars, SD. *, P < 0.05 versus vehicle or Control or Vehicle; #, P < 0.05 versus Wnt3a or Wnt5a alone.

Wnt3a and Wnt5a activate BMP-4 and BMP-6 promoter activity in C4-2B cells through Wnt canonical pathway and noncanonical JNK-dependent signaling pathway. The observation that Wnts increased steady-state BMP mRNA and protein expression gave rise to the possibility that Wnts activate BMP promoters. To test this possibility, C4-2B cells were transfected with a luciferase reporter vector driven by the BMP-4 promoter (pGL3-BMP4), the BMP-6 promoter (pGL3-BMP6), or empty control vector (pGL3-basic) and treated with Wnt3a or Wnt5a in the absence or presence of two different inhibitors of canonical Wnt signaling: (a) DKK-1 (1 ug/mL), which inhibits the Wnt receptor LRP-6 and (b) sFRP-1 (5 ug/mL), which sequesters Wnts from the membrane-bound Frizzled, which trimerizes with LRP-6 and Wnt (reviewed in ref. 31). After 8 hours of treatment, cells were harvested, and total cell lysates were subjected to assay for luciferase (lux) levels. Wnt3a induced BMP-4 but not BMP-6 promoter activity, whereas Wnt5a induced BMP-6 but not BMP-4 promoter activity. Axin-2, a negative regulator of the intracellular component of the canonical Wnt pathway, or were pretreated with a cell permeable JNK inhibitor (30 μmol/L; JNK inhibitor I) for 1 hour, then cells were treated with either vehicle, Wnt3a (50 ng/mL), or Wnt5a (50 ng/mL). Eight hours later, cells were harvested, and total cell lysates were assayed for luciferase levels. Axin-2 partially diminished Wnt3a-induced BMP-4 promoter activity (Fig. 3A), whereas JNK inhibitor I had no effect on the Wnt3a-induced BMP-4 promoter activity (Fig. 3B). In contrast, Axin-2 had no effect on Wnt5a-induced BMP-6 promoter activity (Fig. 3C), whereas JNK inhibitor I partially diminished Wnt5a-induced BMP-6 promoter activity (Fig. 3D). These results indicate that Wnt3a induces BMP-4 promoter activity, through the canonical pathway and Wnt5a, activates the BMP-6 promoter activity through a JNK-dependent noncanonical Wnt pathway.
**BMPs contribute, in part, to the pro-osteoblastic activity of Wnt.** As PCa produces a variety of pro-osteoblastic factors, including Wnts and BMPs, we wanted to define the relationship between Wnt- and BMP-mediated osteoblast activities in our *in vitro* system. Mouse osteoblast precursor cells, MC3T3-E1 cells, were treated with Wnt3a (50 ng/mL), or Wnt5a (50 ng/mL) and either Noggin, an inhibitor of BMP activity, or DKK-1 or the combination of both. Noggin is a soluble protein has been shown to inhibit a variety of BMPs including BMP-2, BMP-4, and BMP-6 by binding them and sequestering them from the BMP receptor (32–35). Neither Noggin nor DKK-1 alone or in combination had an effect on basal ALP or osteocalcin levels (data not shown). Wnt3a increased ALP activity and osteocalcin levels, indicators of osteoblast maturation, in MC3T3-E1 cells (Fig. 4A and B). Noggin (1 µg/mL) partially blocked Wnt3a-mediated osteoblast activity, whereas DKK-1 (1 µg/mL) completely blocked Wnt3a-mediated osteoblast activity, and both together did not show further inhibition (Fig. 4A and B). Similar to Wnt3a, Wnt5a increased ALP activity and osteocalcin levels (Fig. 4C and D). However, DKK-1 and Noggin only partially blocked Wnt5a-induced ALP activity, and together, they had an additive effect on ALP inhibition (Fig. 4C and D). Additionally, both DKK-1 and Noggin inhibited Wnt5a-induced osteocalcin activity, without any additional inhibition when added together. The observation that Noggin only partially blocked Wnt3a-induced osteoblast differentiation indicates that Wnt 3a induces osteoblast differentiation both through BMP-dependent and BMP-independent paths. However, the observation that DKK-1 completely blocked Wnt3a-induced osteoblast differentiation indicates that Noggin-repressible BMP induced activity is dependent on Wnt3a. In contrast, the observation that Noggin and DKK-1 had an additive inhibitory effect on Wnt5a-induced osteoblast differentiation, yet Noggin alone had no effect on basal levels (data not shown), indicates that DKK-1 only partially blocked the Wnt5a induced activity, such that Wnt5a was still able to induce BMP activity in the face of DKK-1.

**PCa cells increase osteoblast activity through both Wnt-induced BMPs and BMP-independent paths in MC3T3-E1 cells.** We have previously shown that both clinical PCa tissues and PCa cell lines and xenografts produce Wnts and BMPs (6, 8, 9). Thus, it is plausible that PCa can mediate osteoblastic activity through production of Wnts and BMPs similar to the recombinant proteins described above. Thus, to determine the relevance of these observations to PCa-mediated osteoblast activity, it was next examined if secreted proteins from PCa cells mediate osteoblast activity *in vitro*. MC3T3-E1 preosteoblast cells were treated with C4-2B cell or LuCaP23.1 cell CM. Both C4-2B and LuCaP23.1 CM increased ALP activity and osteocalcin levels (Supplementary Fig. S2A and B for LuCaP23.1; Fig. 5A and B for C4-2B). Both DKK-1 and Noggin partially blocked CM-mediated pro-osteoblast activity and together had an additive inhibitory effect (Supplementary

![Figure 3](image-url)
Fig. S2A and B for LuCaP23.1; Fig. 5A and B for C4-2B). These results show that C4-2B and LuCaP23.1 cells promote an osteoblastic phenotype through both Wnts and BMPs. We previously published that knockdown of DKK-1 in PC-3 cells confers on them pro-osteoblastic ability (9). We also have now described above that knockdown of DKK-1 promotes BMP-4 and BMP-6 expression in PC-3 cell lines (Fig. 1D). To determine if the pro-osteoblastic ability gained by PC-3 cells secondary to knockdown of DKK-1 depends, in part, on BMP expression, we determined if Noggin could inhibit this ability. To evaluate this, MC3T3-E1 cells were incubated with CM from either shControl-transfected PC-3 cells or shDKK-1–transfected cells (PC-3 clone 8) followed by measurement of ALP and osteocalcin levels. The shControl-transfected CM had no effect on either ALP or osteocalcin levels (Fig. 5C and D, compare second and third bars in each figure). Both DKK-1 and Noggin partially blocked PCa CM–induced ALP and osteocalcin production (Fig. 6A and B). In contrast, pretreatment of PCa cells with Noggin only partially blocked PCa CM–induced ALP and osteocalcin production (Fig. 6A and B). Pretreatment with the combination of DKK-1 and Noggin had no further inhibition than pretreatment with DKK-1 alone. These data show that DKK-1 was sufficient to block the CM-induced pro-osteoblastic activity and that inhibition of BMP with Noggin did not provide any additional inhibition of PCa CM–induced osteoblastic activity. In contrast, blocking BMPs with Noggin only had partial reduction of pro-osteoblastic activity, which was further blocked by the addition of DKK-1. Taken together, these results indicate that C4-2B cells and LuCaP23.1 cells increase osteoblast activity through Wnt-mediated BMPs in MC3T3-E1 cells. To explore further if Wnts mediate their effects through BMPs, we performed coculture experiments using MC3T3-E1 cells and C4-2B cells in which BMP-4 and/or BMP-6 expression was decreased.
through shRNA knockdown. Transduction of C4-2B cells with lentiviral shRNA vectors targeted to BMP-4 and BMP-6 resulted in >80% decrease of their respective mRNA and protein levels (Supplementary Fig. S3). C4-2B cells were grown on culture inserts and treated with either Wnt3a or Wnt5a for the C4-2B with knockdown of BMP-4 or BMP-6, respectively, for 48 hours. Then the inserts with the C4-2B cells were transferred to wells containing MC3T3-E1 cells. After 10 days, ALP and osteocalcin expression were then quantified. Wnt3a and Wnt5a induced both ALP and osteocalcin in wild-type (labeled "No shRNA") and shControl-transduced C4-2B cells (for Wnt5a, Supplementary Fig. S4A and B; for Wnt3a, Fig. 6C and D). Knockdown of BMP-4 and BMP-6, respectively, inhibited Wnt3a and 5a-mediated induction of ALP and osteocalcin (for BMP-6, Supplementary Fig. S4A and B; for BMP-4, Fig. 6C and D). The inhibition was similar to that observed by Noggin-mediated inhibition of Wnt-induced osteoblastic activity. These results indicate that Wnts mediate osteoblastic activity both through BMPs and independently of BMPs.

Discussion

Bone metastases are a frequent complication of advanced PCa. However, the mechanisms through which PCa modulates the bone microenvironment are unclear. A variety of factors have been implicated in the production of osteoblastic lesions in PCa bone metastases including endothelin-1, VEGF, parathyroid hormone-related protein, BMPs, and Wnts. Most likely, no one factor is responsible for these lesions, but rather these factors work in concert to produce the ultimate osteoblastic phenotype. Understanding the interaction between these factors is critical toward defining targets for treatment of bone metastasis. In the current article, we identified that PCa promotes osteoblast activity through both canonical and noncanonical Wnt pathways, which induce osteoblast activity through both stimulating BMP-dependent and BMP-independent mechanisms. This finding may help lead to identifying targets to modulate PCa-induced metastatic bone lesions.

BMPs are widely recognized as mediators of bone production. Thus, many investigators have examined for their expression in PCa. Using Northern analysis, Harris and colleagues (15) examined for BMP-2, -3, -4, and -6 mRNA expression in human normal prostate and CaP cell lines. They found that normal human prostate predominantly expressed BMP-4. The androgen-dependent nonmetastatic LNCaP human PCa cell line produced very low to undetectable levels of BMPs, whereas the aggressive androgen-independent PC-3 cell line expressed very high levels of BMP-3 and slightly lower levels of BMP-2, -4, and -6 compared with normal cells but much higher than LNCaP cells. In support of these results, Weber and colleagues (36), using PCR analysis, identified 16 (73%) of 22 PCa samples were positive for BMP-7 mRNA compared with eight (57%) of 14 normal prostate tissue samples. In another PCR-based analysis, Bentley and colleagues (37) found that several

Figure 5. PCa cells increase osteoblast activity through Wnts and BMPs in MC3T3-E1 cells. CM was obtained from C4-2B cells or the indicated PC-3 cell sublines by plating 5 × 10^5 cells in 10-cm tissue culture dishes in RPMI and 10% FBS and after cells grew to confluence, the medium was changed to RPMI with 0.5% FBS and then supernatants were collected after 24 h. RPMI was added to normalize CM among cell cultures based on cell culture total DNA content. To test effect of CM on osteoblast activity, MC3T3-E1 cells were plated in 12-well plates (1 × 10^3 cells per well) in α-MEM medium plus 10% FBS. After cells were 80% confluent, they were treated with (A and B) C4-2B CM (80%), or (C and D) PC-3 shControl-transfected or PC-3 shDKK-1–transfected CM (80%) and Noggin (1 μg/mL) or DKK-1 (1 μg/mL). One half of the medium was replaced every 3 d with fresh CM containing FBS and treatment compounds. At 10 d (24 h after the previous medium replacement), supernatants were collected and subjected to assays for ALP or osteocalcin. Columns, mean from three experiments; bars, SD *. P < 0.05 versus no CM or PC-3 shControl CM; #, P < 0.05 versus C4-2B CM alone or PC-3 shDKK-1 CM alone; †, P < 0.05 versus CM plus only Noggin or CM plus only DKK.
BMPs were expressed in both benign and malignant prostate tissue and in the PC3 and DU145 PCa cell lines. BMP-6 expression was detected in the prostate tissue of >50% of patients with clinically defined metastatic PCa but was not detected in nonmetastatic or benign prostate samples. In another study focused on BMP-6 mRNA and protein expression, Barnes and colleagues (16) observed that BMP-6 was produced by normal and neoplastic human prostate (radical prostatectomy specimens and human carcinoma cell lines DU145 and PC3). However, BMP-6 mRNA and protein expression was higher in PCa compared with adjacent normal prostate, with higher grade tumors (Gleason score of 6 or more) having greater BMP-6 immunostaining than the lower grade tumors (Gleason score of 4 or less). These results were consistent with a later study by Hamdy and colleagues (17), who reported that BMP-6 mRNA expression was detected exclusively in malignant epithelial cells in 20 of 21 patients (95%) with metastases, in 2 of 11 patients (18%) with localized cancer, and undetectable in 8 benign samples. We identified on tissue microarrays of clinical samples that BMP-6 expression increased with progression in PCa (6). These studies not only suggest that BMP may play a functional role in progressive PCa but also implicate the potential utility of BMP expression as a biomarker for PCa prognosis or diagnosis. However, to determine if this is useful, controlled prospective studies must be performed.

In addition to the observation that BMP expression increases as PCa progress, it seems that the levels of BMPs produced by PCa cells are similar to those that have been shown to induce osteoblast differentiation in vitro. For example, BMP-6 has been shown to induce osteoblast differentiation at 25 ng/mL (38), and we have measured that a variety of PCa cell lines produce BMP-6 in a range of 10 to 60 ng/mL (6). Thus, the levels produced by PCa cells are in the functional range. Taken together, these observations show that PCa cells produce increasing levels of BMPs as they progress to a more aggressive phenotype and that they are functional levels; however, the mechanisms that regulate BMP expression in PCa have not been clearly identified. Our results suggest that Wnt expression may contribute to induction of BMP expression in PCa. Additionally, the observation that reduction of DKK-1 in the osteolytic PC-3 PCa cell line promoted BMP expression suggests that DKK-1–mediated inhibition of Wnt in this cell line confers an osteolytic phenotype through inhibiting BMP, in addition to other Wnt-mediated BMP-independent pro-osteoblastic activity.

In the current study, we showed that Wnts regulate BMP expression in a pathway-specific manner through activation of the Wnt pathway. We found that BMP-6 expression is regulated by Wnt3a and that the level of BMP-6 expression is increased in PCa cells when exposed to Wnt3a. However, the mechanism by which Wnt3a increases BMP-6 expression is not well understood. Further studies are needed to identify the molecular mechanisms by which Wnts regulate BMP expression in PCa.
BMP promoters. This is the first demonstration of Wnt-mediated regulation of BMP in PCa. To the authors’ knowledge, there is only one previous report on the regulation of BMP expression in prostate tissues. In that report, BMP-7 mRNA levels was significantly decreased after orchietomy and increased by testosterone and dihydrotestosterone in the murine ventral and dorsal prostate (39). Consistent with our results, it has been shown that Wnt-4 induces BMP-4 expression in developing renal strroma (40) and Wnts induce BMP expression in embryonic stem cells (41). Furthermore, activation of β-catenin was shown to be required for BMP-4 expression in cancer cells (42), and LiCl, an inducer of Wnts, was shown to induce BMP-7 in renal tissue (43). Additionally, our results are consistent with the observation that Bmps induced ALP expression in proosteoblast cells through Wnt-mediated activity (44). Although there is little known about BMP regulation in prostate, several studies have shown that many factors regulate BMP mRNA expression including estradiol (45), 1,25(OH)2D3 (46), retinoic acid (47), and even Bmps (39) in nonprostate tissues.

In the current study, we showed that Wnts regulate BMP mRNA expression through promoter activation. The BMP-2, BMP-4, BMP-6, and BMP-7 promoters have been cloned (29, 30, 48–50), and early reports suggested that there were multiple transcription start sites for the human BMP-2 and BMP-4 promoters (48, 51). However, it was recently reported that these promoters were found to have a single transcript initiation site that is conserved across species (29). Retinoic acid compounds and the phorbol ester, phorbol 12-myristate 13-acetate, were found to stimulate several BMP promoters (29). Our study indicates that both Wnt-stimulated β-catenin and a Jnk-dependent pathway also activate BMP promoters. Although the current study shows that Wnts induce BMP promoter activation, it does not rule out that posttranscriptional mRNA regulation, such as altered mRNA degradation rate, also occurs.

The mechanisms through which PCa induces osteoblastic lesions are not well-defined. In addition to ET-1 (52), fibroblast growth factor (53), and VEGF (8), both Wnts and BMPs have been implicated as potential mediators of PCa-induced osteoblastic activity (6, 9). The observation in the current study that inhibition of Wnt activity, using DKK-1, in PCa CM significantly diminished PCa CM–induced osteoblastic activity in vitro suggests that Wnts are a major component of this activity. Similarly, that inhibition of BMP activity, using Noggin, in PCa CM partially diminished PCa CM–induced osteoblastic activity in vitro suggests that Bmps also contribute to the osteoblastic activity. The current study provides several lines of evidence that indicate a large component of BMP activity is dependent on Wnts. Specifically, (1) Wnts up-regulated BMP expression in PCa cells, and (2) when PCa cells were pretreated with DKK-1 before collection of CM, both Wnt and BMP pro-osteoblastic activity were lost (indicating that BMP activity was dependent on Wnt activity); whereas, pretreatment of PCa cells with Noggin resulted in loss of only BMP but not Wnt activity (indicating that Wnt activity was independent of BMP activity). Previous studies have shown that the relationship between Wnts and BMP are very cell context specific. Although Wnts and BMPs have been shown to synergize to promote osteoblast differentiation (54), in some cases, Wnts have been shown to regulate BMP activity (40), whereas in other instances, BMPs have been shown to regulate Wnt activity (55). For example, Wnt 3A was shown to induce BMP-4 expression in osteoblasts (56), and β-catenin signaling has been shown to be required for BMP-2–mediated induction of osteogenesis (57). Additionally, Sclerostin, an osteocyte-derived negative regulator of bone formation, inhibits BMP-stimulated bone formation through antagonizing Wnt signaling in osteoblastic cells (58). Thus, the current study is consistent with these previous reports in other systems as Wnts were shown to regulate BMP activity as a component of the mechanism through which PCa induces osteoblast activity.

In summary, the current study shows that PCa mediates pro-osteoblastic activity through both canonical and noncanonical Wnts, which in turn contribute to osteoblast activity through BMP-dependent and BMP-independent pathways. These results indicate that further exploration of these pathways should lead to further refinement of the mechanism through which PCa induced osteoblastic activity. These studies also suggest that targeting both canonical and noncanonical Wnts and their signaling pathways may be necessary to affect PCa-induced bone remodeling.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 12/11/2007; revised 3/14/2008; accepted 4/25/2008.

Grant support: National Cancer Institute grant P01 CA093900.

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We thank Dr. R. Chung for C4-2B cells, Dr. R. Vessella for the LuCap 23.1 xenograft, Dr. R. Franceschi for MC3T3 cells, Dr. L. Helvering for the BMP-4 promoter, Dr. S. Kitaizawa for the BMP-6 promoter, and Dr. E. Fearon for the Axin construct.

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