Bone Morphogenetic Protein-6 Promotes Osteoblastic Prostate Cancer Bone Metastases through a Dual Mechanism

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Abstract
Prostate cancer frequently metastasizes to bone where it forms osteoblastic lesions through unknown mechanisms. Bone morphogenetic proteins (BMP) are mediators of skeletal formation. Prostate cancer produces a variety of BMPs, including BMP-6. We tested the hypothesis that BMP-6 contributes to prostate cancer–induced osteosclerosis at bone metastatic sites. Prostate cancer cells and clinical tissues produced BMP-6 that increased with aggressiveness of the tumor. Prostate cancer-conditioned medium induced SMAD phosphorylation in the preosteoblast MC3T3 cells, and phosphorylation was diminished by anti–BMP-6 antibody. Prostate cancer-conditioned medium induced mineralization of MC3T3 cells, which was blocked by both the BMP inhibitor noggin and anti–BMP-6. Human fetal bones were implanted in severe combined immunodeficient mice and after 4 weeks, LuCaP 23.1 prostate cancer cells were injected both s.c. and into the bone implants. Anti–BMP-6 or isotype antibody administration was then initiated. Anti–BMP-6 reduced LuCaP 23.1–induced osteoblastic activity, but had no effect on its osteolytic activity. This was associated with increased osteoblast numbers and osteoblast activity based on bone histomorphometric evaluation. As endothelin-1 has been implicated in bone metastases, we measured serum endothelin-1 levels but found they were not different among the treatment groups. In addition to decreased bone production, anti–BMP-6 reduced intraosseous, but not s.c., tumor size. We found that BMP-2, BMP-4, BMP-6, and BMP-7 had no direct effect on prostate cancer cell growth, but BMP-2 and BMP-6 increased the in vitro invasive ability of prostate cancer cell. These data show that prostate cancer promotes osteoblastic activity through BMP-6 and that, in addition to its bone effects, suggest that BMPs promote the ability of the prostate cancer cells to invade the bone microenvironment. (Cancer Res 2005; 65(18): 8274-85)

Introduction
Prostate cancer 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 prostate cancer metastasis is the bone, with skeletal metastases identified at autopsy in up to 90% of patients dying from prostate cancer (2–4). Skeletal metastasis in prostate cancer patients results 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 (5–7).

Prostate cancer skeletal metastases are most often radiographically characterized as osteoblastic (i.e., increased mineral density at the site of the lesion) as opposed to osteolytic. However, it is now recognized that osteolytic lesions are a significant component of prostate cancer skeletal metastases (reviewed in ref. 8). In addition to the weakening of bone due osteoclastic bone resorption, prostate cancer–induced osteoblastic lesions result in overall weakening of the bone for the following reasons; Mature, healthy bone is formed of lamellar bone, which allows for tight packing of collagen bundles and optimum bone strength. In contrast, prostate cancer induces production of woven bone, which is composed of loosely packed, randomly oriented collagen bundles that produce bone with suboptimal strength (9, 10). Thus, the combination of underlying osteolysis and production of weak bone leads to a predisposition to fracture. The mechanisms through which prostate cancer cells promote bone mineralization remain poorly understood and although many candidate proteins exist, to date, none have been shown to contribute to prostate cancer–induced osteoblastic activity in vivo in the context of prostate cancer.

Bone morphogenetic proteins (BMP), members of the transforming growth factor (TGF)-β superfamily (11), were originally discovered because of their ability to induce new bone formation (reviewed in refs. 12–14). BMPs bind to two different types of serine-threonine kinase transmembrane receptors, type I and type II (15). BMPs initially bind to BMPR-II, which then phosphorylates BMPR-I, which subsequently phosphorylates SMAD proteins (16). Target genes of BMPs include osteoblast proteins, such as OPG (17) and the osteoblast-specific transcription factor Cbfa-1 (18, 19). Many in vitro studies have shown that BMPs induce osteogenic differentiation, including the ability of BMP-7 (also called osteogenic protein-1) to induce osteogenic differentiation of newborn rat calvarial cells and rat osteosarcoma cells (20–22). Thus, dysregulation of BMP expression in the bone microenvironment would most likely impact bone remodeling.

A number of studies have shown that BMP expression increases with progression of prostate cancer (23). Although the expressions of several BMPs have been evaluated in prostate cancer, BMP-6 seems to be most consistently increased with increasing progression of prostate cancer (24, 25). This observation, taken together with the knowledge that BMPs are pro-osteoblastic and induce bone formation, led us to hypothesize that up-regulation of BMP-6 expression in prostate cancer cells localized in the bone contributes to the development of osteoblastic lesions at prostate cancer metastatic sites in bone. To test this hypothesis, we have done in vitro and in vivo experiments to determine if blocking BMP-6 inhibits the pro-osteoblastic activity of prostate cancer cells.

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Materials and Methods

Animals. Six-week-old male severe combined immunodeficient (SCID) mice (Charles River, Wilmington, MA) were housed under pathogen-free conditions in accordance with the NIH guidelines using an animal protocol approved by the University of Michigan Animal Care and Use Committee.

Immunohistochemistry. Nonneoplastic prostate, primary prostate tumors, and metastatic prostate tumors were obtained from the University of Michigan Department of Pathology tissue bank. Written informed consent was obtained from all patients or their families (for autopsy specimens) and all tissue procurement was approved by the University of Michigan Institutional Review Board. When harvested, tissues were frozen in liquid nitrogen and maintained frozen until used. Frozen tissue blocks were sectioned on a cryostat and frozen sections were placed on slides, which were immersed in acetone at 4°C for 15 minutes and air dried for 30 seconds, then stored in PBS until immunostaining was done. BMP-6 protein expression was determined using standard immunoperoxidase immunohistochemistry. Polyclonal antibody against BMP-6 (R&D Systems, Minneapolis, MN) was used as the primary antibody at a concentration of 1:400 dilutions. The bound antibody was visualized by the streptavidin–biotin horseradish peroxidase (HRP) and diaminobenzidine chromogen (Histostain kit, Zymed, San Francisco, CA). Staining intensity was scored as a semiquantitative evaluation of overall staining of the tissue section independently by two investigators. To accomplish this, each investigator looked at all the slides to develop an appreciation for the range of staining intensity. Then, based on this, a staining intensity grading scale was developed that was composed as no detectable signal (0), weak signal seen only at intermediate to high power (1), moderate signal seen at low to intermediate power (2), and strong signal seen at low power (3), as previously described (26). To score each slide, six ×200 fields were each given a staining intensity. The six fields were designed to represent the upper right end, middle, and left end and the lower right end, middle, and left end for each slide. The overall staining intensity was then determined as the average of the six scores for each slide. The overall scores of each investigator were then averaged to give a final score for the slide. There was >90% interobserver agreement in staining intensity among the two investigators.

For evaluation of prostate-specific antigen (PSA) expression, bone implants from a subset of mice (n = 5/group of 10) were decalcified and stained for PSA as described previously (27).

BMP-6 measurement. A human-specific ELISA was established using a pair of anti–BMP-6 antibodies as recommended by the manufacturer ( Duo-Set ELISA Development kit; R&D Systems). The sensitivity of the ELISA was 12 ± 2 pg/mL. It did not cross-react with human BMP-2, BMP-3, BMP-4, or BMP-7 or TGFβ-1 or TGFβ-2.

Immunoblot analysis. For identification of BMP and BMP receptor proteins, whole cell lysates were prepared by incubating cells in ice-cold lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 50 mmol/L NaF, 1% Triton X-100, 5 μg/mL leupeptin, 5 μg/mL pepstatin, and 0.5 mmol/L phenylmethylsulfonyl fluoride]. Cells were sonicated for 8 seconds and then placed on ice for 15 minutes. Lysates were precleared and the protein concentration was determined by the bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). 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. Mouse anti–BMP-2, BMP-4, BMP-6, and BMP-7, BMP receptor IA, IB, and II antibodies (R&D Systems) were used at 1:500 to 1,200 dilutions and rabbit antiactin (Sigma, St. Louis, MO) antibody was used at 1:1,000, and the HRP-conjugated donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-rabbit immunoglobulin (Amersham International, Buckinghamshire, United Kingdom) were used at 1:5,000 dilution. Antibody complexes were detected by enhanced chemiluminescence (Amerbach, Buckinghamshire, United Kingdom) were used at 1:5,000 dilution. Anti-SMAD5/SMAD8 antibody (Cell Signaling Technology, Inc., Beverly, MA) at a 1:1,000 dilution and rabbit antiactin (Sigma) antibody was used at 1:1,000. This antibody stains for the phosphorylated forms of SMAD1, SMAD5, and SMAD8, which are the BMP-induced SMADs. Protein bands were identified by chemiluminescence as described above. Band density was quantified using densitometry with NIH Image.

Obtaining conditioned medium. Conditioned medium was obtained from cells as previously described (28). Briefly, 5 × 10^6 cells were plated in 10 cm tissue culture dishes for 12 hours in RPMI 1640 with 10% fetal bovine serum (FBS). The medium was then changed to 10 mL of RPMI plus 0.5% FBS and supernatants were collected 24 hours 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). Conditioned medium was then normalized for DNA content between samples by adding RPMI.

In vitro mineralization. To show mineralization in cell cultures, we did the alizarin red-S assay as previously described (29). Briefly, cells were cultured in mineralization medium for 8 days in the presence and absence of recombinant noggin (a kind gift from Dr. Richard Harland, University of California Berkeley, Berkeley, CA). Then, culture medium was removed and the cells were air dried, fixed in 50% ethanol thrice for 10 minutes each, then stained with alizarin red (100 mg/mL in 0.01% NaOH; Sigma) for 5 minutes. The bright red color indicates calcium mineralization in the cultures and was photographed. Then, to quantify the amount of alizarin red-S staining, the cells were washed with PBS and retained dye was extracted with 500 μL of a solution of 20% methanol and 10% acetic acid, then measured on a spectrophotometer at A560.

Preparation of single-cell suspension. LuCaP 23.1, kindly provided by Dr. Robert Vessella (Department of Urology, University of Washington, Seattle, WA), is an androgen-sensitive, PSA-producing human prostate cancer xenograft derived from the lymph nodes of a patient that had failed androgen deprivation therapy (30, 31). Single-cell suspensions of LuCaP 35 were prepared by resuspending the s.c. xenografts and cutting them into small pieces in HBSS with 1% FBS. The small pieces were then gently rubbed between frosted glass slides to obtain single-cell suspensions in HBSS containing 1% FBS. RBCs were lysed with ammonium chloride solution (StemCell Technologies, Inc., Vancouver, BC) and the tumor cells were centrifuged at 300 × g for 10 minutes in HBSS with 1% FBS and the cell pellet was resuspended in RPMI 1640 with 10% FBS. Cell viability was determined by trypsin blue counting and only preparations with over 90% viability were used for in vivo injection. Animal experiment. SCID mice (n = 30) had half-sections of human fetal bone (cut in half longitudinally) implanted s.c. as previously described (32). Four weeks later, LuCaP 23.1 prostate cancer cells (3 × 10^6 in 50 μL of RPMI 1640 with 10% FBS) were injected into both the marrow space of the implanted bone and the s.c. tissue (1 × 10^6 in 100 mL of RPMI 1640 with 10% FBS) of two thirds of the animals (n = 20). Intramuscular tumors were allowed to develop for 4 weeks, at which point mice were randomized into isotype or mouse anti-human BMP-6 monoclonal antibody (R&D Systems) treatment groups (10 per group). Anti–BMP-6 was administered twice weekly at 500 μg/mouse i.p. S.c. tumors were measured by caliper every 2 weeks using two perpendicular measurements and the formula as previously described (33): volume = length × width^2 / 2. Mice were sacrificed after 6 weeks of anti–BMP-6 administration (a total of 10 weeks with tumor). One group of mice (n = 10) served as a no tumor control group that received bone implants and 4 weeks after implant (at the same time as the treatment groups received tumor) 30 μL of RPMI with 10% FBS was injected into the marrow cavity of the implants at the same time as tumor cells were injected into the mice receiving tumors. These mice were sacrificed in parallel with the other groups. Serum, urine, and bones were collected for evaluation. Before sacrifice, the animals were anesthetized and magnified flat radiographs were taken with a Faxitron (Faxitron X-ray Corp., Wheeling, IL).

Dual-energy X-ray absorptiometry measurement and X-ray. Bone mineral density (BMD) of the excised bone implants was measured using dual-energy X-ray absorptiometry on an Eclipse peripheral DEXA Scanner using pDEXA Sabre software, version 3.9.4 in research mode (Norland Dual-Energy X-ray Absorptiometry).
Medical Systems, Fort Atkinson, WI). Excised implants were scanned at 2 mm/s with a resolution of 0.1 mm. Three 0.5 cm regions of interest were randomly selected for each fragment to determine BMD. Short-term BMD precision (percentage coefficient of variation) was < 3% for this technique. Radiographs were taken after excision of bone implants by using a Faxitron (Faxitron X-ray Corporation).

**Bone histomorphometry.** Bone implants from five animals from each group were used for bone histomorphometry and from five animals from each group for immunohistochemistry. To label bones for dynamic histomorphometry, the mice were injected i.p. 21 and 7 days before sacrifice with 100 μL of 15 μg/kg of calcine (Sigma) dissolved in PBS containing 2% sodium bicarbonate. Bone implants were excised and fixed in 10% neutral-buffered formalin at 4°C for 24 hours then dehydrated in ethanol (70%, 90%, and 100%) for 24, 24, and 48 hours, respectively. The bone samples were then immersed in embedding medium (15 mL methylmethacrylate, 3 mL nonylphenyl-polyethyleneglycol acetate, benzoyl peroxide, and 5% methyl benzoate) at 4°C in 1 volume bone to 9 volume solution. Polymerization was done in polymerizing solution (15 mL methylmethacrylate, 3 mL nonylphenyl-polyethyleneglycol acetate, 75 mg benzoyl peroxide, and 50 mL X,N-dimethylaniline) at 24°C. Sections of bone were cut using a Leica SM 2500 heavy-duty large-surface sectioning machine. The bone sections were cut in 1 volume bone to 1 volume solution of 24°C. Sections were stained with tartrate-resistant acid phosphatase (TRAP; Sigma Diagnostics) to identify osteoclasts. Osteoclasts were determined as TRAP-positive staining multinuclear (>3 nuclei) cells by light microscopy. The mineral apposition rate was calculated by dividing the distance between the two calcine markers by the time interval between their administration. The terminology used was that recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (36). Tumor area was determined as the proportion of tumor in the non-mineralized portion of the bone as previously described (27).

**Prostate-specific antigen measurement.** Total PSA levels in serum were determined using the Accucyte Human PSA assay (Cytimmune Sciences, Inc., College Park, MA). The sensitivity of this assay is 0.488 mg/mL.

**Serum osteocalcin measurement.** Serum human osteocalcin was measured by competitive immunoassay kit (Metra Osteocalcin, Santa Clara, CA). The assay does not cross-react with murine osteocalcin. This antibody is conformationally dependent and recognizes only intact (de novo) osteocalcin and not fragments from resorbed bone tissues. The sensitivity of this assay is 0.05 ng/mL.

**Serum bone-specific alkaline phosphatase measurement.** Serum human bone-specific alkaline phosphatase (BAP) was measured by immunoassay kit (Metra BAP ELA, Santa Clara, CA). This assay does not cross-react with murine BAP. The sensitivity of this assay is 0.7 units/L.

**Urine N-telopeptide and creatinine measurements.** Urine N-telopeptide (NTX) was measured using human-specific ELISA, as recommended by the manufacturer (Osteomark NTx, OsteX, Inc., Seattle, WA). Results were reported as a ratio of NTX to bone collagen equivalents (nmol/L bone collagen equivalent) to urine creatinine (mmol creatinine). Urine creatinine was measured using a creatinine kit (Metra Biosystems, Mountain View, CA) as directed by the manufacturer.

**Serum tartrate-resistant acid phosphatase.** Serum tartrate-resistant acid phosphatase (TRACP) 5b, a specific indicator of bone resorption, was measured using a human-specific ELISA (BoneTRAP assay, IDS, Ltd., Newcastle, United Kingdom) as directed by the manufacturer. Sensitivity of this assay is 0.45 mg/mL.

**Endothelin-1 measurement.** Serum levels of endothelin-1 were measured using ELISA (Human Endothelin-1 Quantiglo ELISA kit; R&D Systems) as directed by the manufacturer. The sensitivity of this assay is 0.064 pg/mL.

**Cell viability.** Single-cell suspensions of LuCaP cells were plated at 2 × 10^4/plate in 60 mm plates in triplicates with RPMI and 10% FBS. After 12 hours of culture, the medium was changed to RPMI plus 0.5% FBS and anti–BMP-6 was added at different concentrations. Twenty-four hours after an additional 24 hours, cells were collected and viability was examined by trypan blue exclusion.

**Cell proliferation.** Cell proliferation was measured using the CellTiter 96 AQ non-radioactive cell proliferation assay (Promega, Madison, WI). To test the effect of anti–BMP-6 antibody on LuCaP proliferation, single-cell suspension of LuCaP cells in RPMI plus 10% FBS were added to the wells of a 96-well plate at 5,000/well in triplicates. After 12 hours of culture, different concentrations of anti–BMP-6 were added. Cells were allowed to grow for 48 hours, then 20 μL/well of combined 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium (MTS)/phena-zine methosulfate (PMS) solution was added. After incubation of 1 hour at 37°C in a humidified 5% CO2 atmosphere, the absorbance at 490 nm was recorded by using an ELISA plate reader. To test the effect of BMPs on C4-2B and LuCaP 23.1 proliferation, cells in RPMI plus 10% FBS were added to the wells of a 96-well plate at 5,000/well in triplicates. After 12 hours of culture, different concentrations of BMPs (1, 10, and 100 ng/mL) were added to the wells. Cells were allowed to grow for 48 hours, then 20 μL/well of combined MTS/PMS solution was added. After incubation of 1 hour at 37°C in a humidified 5% CO2 atmosphere, the absorbance at 490 nm was recorded by using an ELISA plate reader.

**Invasion assay.** *In vitro* invasion was assessed as previously described with minor modifications (37). Transwell *in vitro* invasion 24-well chambers (Costar, Cambridge, MA) were used as directed by the manufacturer. Briefly, the upper and lower culture compartments of each well are separated by polycarbonate membranes with 8 μm pore size. The membranes in some wells were precoated with 100 μg/cm² collagen matrix (Matrigel, Collaborative Biomedical Products, Bedford, MA), which was reconstituted by adding 0.5 mL serum-free medium to the well for 2 hours. To assess the ability of the cells to cross the polycarbonate membrane (i.e., baseline migration), 2.5 × 10^5 cells in 0.5 mL medium containing 5% FBS were placed into upper compartment of wells that did not contain collagen matrix and 0.75 mL of medium containing 10% FBS was placed in the lower compartment. In parallel, we assessed the ability of the same cells to penetrate through the collagen matrix by placing 2.5 × 10^5 cells in 0.5 mL of medium containing 5% FBS in the upper compartment of wells that were coated with the reconstituted matrix and 0.75 mL T medium containing 10% FBS in the lower compartment. The transwell chambers were incubated for 24 hours at 37°C in 95% air and 5% CO2. Cell penetration through the membrane was detected by staining the cells on the porous membrane with a Diff-Quik stain kit (Dade Behring, Inc., Newark, DE) and quantified by counting the numbers of cells that penetrated through the membrane in five microscopic fields (at ×200 magnification) per filter. Invasive ability was defined as the proportion of cells that penetrated through the matrix-coated membrane divided by the number of cells that migrated through the uncoated membrane (baseline migration). The results are reported as the mean of triplicate assays as previously reported (38).

To test the effect of BMPs on the invasiveness of C4-2B or LuCaP cells, the *in vitro* invasion assay was done on single-cell suspensions of the prostate cancer cells in the presence and absence of the indicated BMPs (obtained from R&D Systems) in the upper chambers.

**Statistical analysis.** Statistical analysis was done using Statview Software (Abacus Concepts, Berkeley, CA). For comparison among two groups, Student's *t* test was used. For multiple comparisons, ANOVA was used for initial analyses followed by Fisher's protected least significant difference for *post hoc* analyses. Differences with *P* < 0.05 were determined as statistically significant.

**Results.** To ensure that BMP-6 was detectable in clinical prostate cancer and in the prostate cancer cell lines and xenografts we were using for the present studies, we subjected tissues to immunohistochemistry, ELISA, and Western blot analysis for various BMPs. We identified that BMP-6 was expressed in nonneoplastic prostate (n = 9), in primary tumors (n = 15; Gleason scores 6 and 7), and in...
metastases \((n = 11,\ \text{consisting of liver metastases} = 3; \text{bone metastases} = 4; \text{adrenal metastases} = 1 \text{and dural metastases} = 3; \text{Fig. 1A})\). On a scale of 0 (no staining) to 3 (strong staining), non-neoplastic prostate had the lowest staining intensity (mean staining \(\pm SD = 0.78 \pm 0.67\)) compared with primary tumors (mean staining \(\pm SD = 1.92 \pm 0.80; P < 0.001\)) and metastases (mean staining \(\pm SD = 2.64 \pm 0.50; P < 0.001\)). Metastases had more intense staining than primary tumors \((P = 0.01)\). To quantify levels of BMP-6 produced by various prostate cancer cell lines, we subjected culture supernatants to ELISA for BMP-6 and determined that BMP-6 expression increased as the osteoblastic nature of the prostate cancer cell line/xenograft increased (Fig. 1B). To show the repertoire of BMPs expressed in prostate cancer cells, we did immunoblots for several BMPs and identified that LNCaP and C4-2B cell lines produced BMP-2, BMP-4, BMP-6, and BMP-7 (Fig. 1C). These data confirmed that prostate cancer expresses BMPs and suggest that BMP expression may have true clinical relevance because it is expressed in clinical prostate cancer tissue.

Prostate cancer cells have been shown to induce mineralization, although the exact mechanisms through which they achieve this are unknown. To determine if BMPs play a role in prostate cancer–induced mineralization in vitro, we grew MC3T3 osteoblast precursor cells in C4-2B-conditioned medium in the presence or absence of the BMP inhibitor noggin, which inhibits several BMPs, including BMP-2, BMP-4, BMP-6, and BMP-7 \((39–45)\). Prostate cancer-conditioned medium induced an \(\sim 100\%\) increase in mineralization of MC3T3 cells (Fig. 2A and B). Noggin abrogated the increase by \(\sim 80\%\) (Fig. 2B) in a dose-responsive fashion (Fig. 2C). These results indicate that prostate cancer cells mediated their osteogenic effects, in part, through BMPs. To determine which BMPs noggin mediates its inhibitory effect through, we incubated MC3T3 cells with C4-2B- or LuCaP 23.1-conditioned medium in the presence of various anti-BMP antibodies or isotype control antibodies. We used neutralizing antibodies for only BMP-4, BMP-6, and BMP-7 as one was not available for BMP-2. At equal concentrations, the antibodies inhibited C4-2B conditioned

Figure 1. Prostate cancer cells express BMPs. A, prostate tissues were subjected to BMP-6 immunohistochemistry. Normal prostate, primary prostate cancer, and bone metastasis at \(\times 100\) (top row) and \(\times 400\) (bottom row). The brown color indicates the presence of BMP-6. The staining occurs mostly on the cytoplasm of epithelial cells. Arrows, epithelial cells and area that is magnified to \(\times 400\). B, ELISA for BMP-6 in indicated prostate cancer cell lines. C, Immunoblot for indicated BMPs in LNCaP and C4-2B prostate cancer cell lines and SaOS2 cell line that is a human osteosarcoma cell line known to express BMPs. *\(P < 0.05\) versus PC-3 and LNCaP; #\(P < 0.05\) versus PC-3, LNCaP, and C4-2B. ANOVA and Fisher’s protected least significant difference for post hoc analysis.
medium–induced mineralization from greatest to least in the following order: BMP-6 (51.4%) > BMP-7 (41.1%) > BMP-4 (37.9%; Fig. 2D). Similarly, at equal concentrations, the antibodies inhibited LuCaP conditioned medium–induced mineralization from greatest to least in the following order: BMP-6 (46.1%) > BMP-7 (32.2%) > BMP-4 (23.2%; Fig. 2D). These data show that prostate cancer cells promote mineralization through multiple noggin-sensitive BMPs and suggest that as an initial step in dissecting the role of BMPs in prostate cancer–induced osteoblastic activity that blocking BMP-6 in vivo may provide a greater antosteoblastic effect than blocking individually blocking BMP-7 or BMP-2. However, it should be noted that these data do not rule out that BMP-4 or BMP-7 contributes to effects of prostate cancer cells and do not exclude other noggin-sensitive BMPs as also contributing to prominer-

Figure 2. Inhibition of BMPs diminishes prostate cancer–induced mineralization in vitro. MC3T3-E1 cells were plated at 25,000 cells/well in 24-well plates and grown at 37°C in α-MEM for 2 days. Medium with ascorbic acid (50 μg/mL) was added and compounds were tested as indicated. A and B, PBS vehicle (V), 1 mg/mL noggin (N), 50% conditioned medium (CM), or conditioned medium + noggin (CM + N) were added; γ-glycerophosphate (10 mmol/L) was added at day 8 and cells were stained for alizarin red, which was solubilized at day 10; then, absorbance was measured. C, all wells received conditioned medium and the indicated dose of noggin and cultured as described above. D, conditioned medium was added as indicated with the addition of either control IgG or neutralizing anti-BMP antibodies as indicated at 1 μg/mL. Results are shown from three experiments (B and D) and triplicates (C). B, *P < 0.01 versus vehicle or noggin; #P < 0.01 versus conditioned medium; C, *P < 0.01 versus no noggin. D, *P < 0.01 versus no conditioned medium IgG; #P < 0.01 versus conditioned medium IgG. Analyzed using ANOVA and Fisher’s protected least significant difference for post hoc analysis.

BMPs act through their receptors to phosphorylate SMAD1, SMAD4, and SMAD5 transcription factors (reviewed in ref. 46). To determine if the BMP produced by the prostate cancer cells can functionally induce intracellular signaling in osteoblast-like cells, we collected conditioned media from the C4-2B and LuCaP prostate cancer cells and incubated the MC3T3 preosteoblast line with the prostate cancer-conditioned medium to determine its ability to induce phosphorylation of SMADs. Conditioned medium from both cell lines induced phosphorylation of the SMADs (Fig. 3). Because anti–BMP-6 had the greatest antiminer-
levels by ~47.7%. LuCaP 23.1 increased serum BAP levels by 134% and anti–BMP-6 antibody decreased the LuCaP 23.1–induced increase by 58.4%. LuCaP tumors induced over an 18-fold increase in urinary NTx levels and anti–BMP-6 antibody had no effect on this. Serum TRACP 5b, another measure of bone resorption, paralleled the NTx results (data not shown). Taken together, these data indicate that LuCaP 23.1 induced both osteoblastic and osteoclastic bone remodeling. Furthermore, the data indicate that BMP-6 contributes to the osteoblastic component, but does not seem to play a role in the osteoclastic component of the LuCaP 23.1–induced bone remodeling activity.

To determine the cellular mechanisms through which LuCaP 23.1 promotes osteoblastic activity in bone, the bones were subjected to static and dynamic histomorphometry. LuCaP 23.1 cells induced an increase in overall bone volume, which was accounted for by an increased trabecular thickness (Fig. 6, arrows; Table 1). The increased trabecular thickness was associated with increased osteoid surface, osteoid thickness, and mineral apposition rate (Fig. 6, calcein labeling; Table 1) indicating that LuCaP 23.1 cells induced increased production of mineralized bone. The increased mineralization of bone was associated with an increased osteoblast surface (percentage of trabecular bone covered by osteoblasts) and an increased number of osteoblasts (number of osteoblasts per millimeter of trabecular bone). Taken together, these data indicate that LuCaP 23.1 induces production of active osteoblasts. Anti–BMP-6 antibody reduced the LuCaP 23.1–induced increased of all these osteoblastic parameters (Fig. 6; Table 1). These observations indicate that BMP-6 contributes to the osteoblastic activity of LuCaP 23.1–induced bone lesions through inducing osteoblastogenesis. In addition to its osteoblastic effects, LuCaP 23.1 increased the osteoclast surface (percentage of trabecular bone covered by osteoclasts) by 87% and the osteoclast number by 6.9-fold (number of osteoclasts per mm of trabecular bone). Anti–BMP-6 had no effect on LuCaP 23.1–induced osteoclastogenesis (Table 1). Taken together, these results show that prostate cancer cells induce an increased rate of bone deposition through increasing the number of osteoblasts and,
Administration of anti–BMP-6 antibody decreased LuCaP 23.1–induced serum PSA levels by 48.1% (Fig. 7). Furthermore, show that BMP-6 contributes to the osteogenic activity of prostate cancer in this model.

In addition to effects on bone remodeling, we evaluated the effects of BMP-6 on tumor growth. Serum PSA levels, which were undetectable in mice with bone implants alone (data not shown), were detected in mice with LuCaP 23.1 tumors (Fig. 7). Administration of anti–BMP-6 antibody decreased LuCaP 23.1–induced serum PSA levels by 48.1% (Fig. 7A). Histology revealed PSA-positive tumor cells replaced the bulk of the bone marrow (Fig. 6). Additionally, bone histomorphometry revealed that administration of anti–BMP-6 antibody decreased the tumor area (TuV/BV) by 20.2% (Fig. 7B). In contrast to intratibial tumors, anti–BMP-6 had no effect on growth rate or final size of s.c. tumors (Fig. 7C). These data indicate that BMP-6 contributes to intraosseous tumor growth; however, the observation that blocking BMP-6 had no effect on s.c. tumor growth suggests that the effect of BMP-6 on prostate cancer growth in bone is indirectly mediated through modulating the bone microenvironment.

Because our data indicated the possibility that the effects of BMP-6 are due to an indirect effect on the cancer cells through modulation of the bone microenvironment, we sought to determine if BMP-6 had a direct effect on prostate cancer cells. To explore this possibility, we first examined for BMP receptors on prostate cancer cells. We identified that several prostate cancer cell lines express BMP receptors BMPR-IA, BMPR-1B, and BMPR-2 (Fig. 8A,B), indicating that prostate cancer cells may be able to respond to BMPs. To test if BMPs could modulate growth of prostate cancer cells directly, we incubated prostate cancer cells with several BMPs, BMP-2, BMP-4, BMP-6, and BMP-7 did not alter C4-2B or LuCaP 23.1 cell proliferation (data not shown). The observations that BMPs did not alter proliferation of the cells and that inhibiting BMP-6 did not alter s.c. growth provide strong evidence that the BMPs do not directly affect tumor growth. However, it is possible that BMPs may affect other aspects of metastatic biology that could account for the ability of prostate cancer cells to successfully establish metastases in bone. A previous report indicated that SMADs, which are activated by BMPs, may modulate invasive ability of mammary cancer cells (47). Accordingly, we assessed if BMPs modified invasion of prostate cancer cells. BMP-2 and BMP-6, but not BMP-4 or BMP-7, increased in vitro invasiveness of the C4-2B and LuCaP 23.1 cells (Fig. 8B and C). To determine if endogenous BMP-6 expressed in prostate cancer cells influenced invasion, we incubated C4-2B and single-cell suspensions of LuCaP 23.1 with either anti–BMP-6 or control IgG isotype antibody. Inhibition of BMP-6 decreased the in vitro invasive ability of both cell lines (Fig. 8D). These data indicate that BMP-6, in addition to modulating the bone microenvironment, may promote the ability of prostate cancer cells to enter the bone microenvironment.

Discussion

The osteoblastic nature of prostate cancer bone metastases has been recognized for decades. However, the mechanisms through which prostate cancer induces osteoblast activity have not been previously elucidated using in vivo models of prostate cancer. In vitro studies have identified that prostate cancer produces a variety of pro-osteoblastic factors that may play a role in prostate cancer–induced osteoblastic activity. In the current study, we identified that one of these factors, BMP-6, contributes to the osteoblastic activity of prostate cancer in vivo. These results are consistent with the many reports that BMPs are present in prostate cancer and the well-known osteoinductive activity of BMPs. Furthermore, our results provide evidence, at the cellular level, that prostate cancer induces increased bone formation rate through increasing osteoblast numbers.

BMP expression has been reported to increase with prostate cancer progression. Several studies have examined the expression of BMPs in normal and neoplastic prostate tissues. Using Northern blot analysis, Harris et al. (23) examined for BMP-2, BMP-3, BMP-4, and BMP-6 mRNA expression in human normal prostate and
prostate cancer cell lines. They found that normal human prostate predominantly expressed BMP-4. The androgen-dependent non-metastatic LNCaP human prostate cancer cell line produced very low to undetectable levels of BMPs. The aggressive androgen-independent PC-3 cell line expressed very high levels of BMP-3 and slightly lower levels of BMP-2, BMP-4, and BMP-6 compared with normal cells, but much higher than LNCaP cells. In support of these results, Weber et al. (48), using PCR analysis, identified that 16 (73%) of 22 prostate cancer samples were positive for BMP-7 mRNA compared with 8 (57%) of 14 normal prostate tissue samples. In another PCR-based analysis, Bentley et al. (49) found that several BMPs were expressed in both benign and malignant prostate tissue and in the PC3 and DU145 prostate cancer cell lines. BMP-6 expression was detected in the prostate tissue of over 50% of patients with clinically defined metastatic prostate cancer, but was not detected in nonmetastatic or benign prostate samples. In another study focused on BMP-6 mRNA and protein expression, Barnes et al. (24) 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 prostate cancer 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 et al. (25), 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. In addition to BMP, there have been several reports on prostate cancer expression of BMPR; it seems that as prostate cancer progress, the cells down-regulate their own expression of BMPRs (50, 51), which may be a protective mechanism as it has been shown that BMP-2 can inhibit prostate cancer cell proliferation (52). Taken together, these observations show that prostate cancer cells produce increasing levels of BMPs as they progress to a more aggressive phenotype. Our results are consistent with these observations and further show that increased BMP-6 expression is not specific to bone metastases, but can be identified in soft tissue metastases too. This observation suggests that BMPs may have a general effect on prostate cancer progression and metastasis and that the prostate cancer–mediated expression of BMPRs in the bone microenvironment promotes osteoblastogenesis as a “side effect” of the expression of BMP. Alternatively, the BMP-induced osteoblastogenesis may make the bone microenvironment favorable for the establishment of prostate cancer metastases, which would account for the predisposition of prostate cancer metastases to bone (53). Osteoblasts produce a variety of factors such as transforming growth factor-β and interleukin-6 that may promote prostate cancer progression (54–60). Thus, if BMPs promote osteoblastogenesis,
the resulting osteoblasts may then cross-talk with the prostate cancer cells through these factors to further promote prostate cancer growth.

The effects of BMPs on prostate cancer cells have not been well delineated. In a previous study, BMP-6 was shown to decrease proliferation of androgen-independent DU145 and androgen-dependent LNCaP prostate cancer cells in vitro (45). Our results differ from these as we did not observe any effect of BMP-6 on proliferation of androgen-dependent LuCaP 23.1 or androgen-independent C4-2B cells in vitro. The difference for this may be due simply to the difference in cell lines or methodology, e.g., we evaluated cell proliferation at 48 hours as opposed to 7 days as did the previous study. Our in vitro findings were supported by our observation that anti–BMP-6 did not alter growth of s.c. LuCaP 23.1 tumors in mice, which indicates that BMP-6 does not have a direct effect on tumor cell growth as we observed in vitro. In contrast to no effect on s.c. tumor growth, anti–BMP-6 reduced the amount of tumor in the tibia. This observation, when combined with a lack of direct effect on tumor growth in vivo or s.c., suggests that BMP-6 promotes intraosseous tumor growth indirectly, perhaps through modulation of the bone microenvironment. This type of phenomenon has been reported previously. Specifically, we and others have reported that inhibition of bone remodeling through inhibiting osteoclast activity, either with a RANKL inhibitor (28) or a bisphosphonate (61), reduced tumor growth in bone in the absence of a direct effect on s.c. tumor itself. Furthermore, parathyroid hormone–mediated induction of bone remodeling increased intraosseous prostate cancer tumor growth in mice (62). Taken together, these findings suggest that the process of bone remodeling itself may contribute to the ability of prostate cancer cells to become established metastases in bone.

BMPs are known to induce a variety of their own inhibitors, such as noggin, sclerostin, chordin, gremlin, and others, in osteoblasts, presumably as a negative feedback (reviewed in ref. 63). Noggin is known to inhibit activity of several BMPs, including BMP-2, BMP-4, BMP-6, and BMP-7 (39–45). BMP-6 was shown to induce noggin expression in androgen-independent DU-145 cells and androgen-dependent LNCaP cells (45). However, in these cells, BMP-6 also induced SMAD phosphorylation, which indicates that the noggin produced by the prostate cancer cells did not effectively block BMP-6 activity. Similar to these findings, we observed that BMP-6 induced SMAD phosphorylation in the osteoblasts (as opposed to examining the prostate cancer cells). This finding is also consistent with a previous report that BMP-6 induces phosphorylation of SMAD1 and SMAD5 in osteoblasts (64). However, we did not evaluate if the prostate cancer cells or osteoblasts produced noggin in response to the prostate cancer–produced BMP-6. The observation that prostate cancer induced phosphorylation of SMADs in osteoblasts suggests that if noggin was expressed, it was not at levels that significantly inhibited BMP-6 activity. Thus, although we cannot rule out that BMP-6 activity was diminished to some extent by noggin, these data indicate that prostate cancer cells produced functional BMPs that can activate SMAD signaling in osteoblasts.

BMPs are in the TGF-β superfamily of proteins that are characterized by a dual-receptor system and intracellular signaling through SMADs. Although they stimulate different receptor-associated SMADs than TGF-β, BMPs and TGF-β both mediate their signaling through a common co-SMAD (i.e., SMAD4 that binds the activated receptor SMADs). Whereas not described previously for BMPs, TGF-β has been shown to promote metastasis. For example, TGF-β activates invasion of mammary tumors (65, 66). This effect seems to be dependent on the stage of the cancer cell (47). When a TGF-β receptor that is mutated so it cannot bind SMADs (i.e., inhibits endogenous SMAD signaling) is transfected into well-differentiated mammary tumors, it promotes tumor growth; however, when it is transfected into an aggressive mammary tumor cell line, it inhibits metastasis. Because it is inhibiting metastasis in conjunction with its ability to inhibit SMAD signaling, this shows that SMADs contribute to metastasis.

### Table 1. Effect of anti–BMP-6 on LuCaP 23.1–induced changes of histomorphometric indices of bones implanted in SCID mice

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>Measurement (unit)</th>
<th>Treatment group</th>
<th>No tumor</th>
<th>Isotype</th>
<th>Anti–BMP-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume</td>
<td>BV/TV (%)</td>
<td></td>
<td>13.0 ± 1.7$^a$</td>
<td>30.4 ± 3.9$^a$</td>
<td>23.3 ± 1.7$^b$</td>
</tr>
<tr>
<td>Trabecular thickness</td>
<td>Tb. Th. (µm)</td>
<td>0.19 ± 0.03</td>
<td>0.30 ± 0.04$^a$</td>
<td>0.23 ± 0.04$^a$</td>
<td></td>
</tr>
<tr>
<td>Osteoid surface</td>
<td>OS/BS (%)</td>
<td>10.2 ± 1.1</td>
<td>20.4 ± 1.7$^a$</td>
<td>15.7 ± 1.9$^a$</td>
<td></td>
</tr>
<tr>
<td>Osteoid thickness</td>
<td>O.Th. (µm)</td>
<td>10.06 ± 0.54</td>
<td>30.8 ± 3.57$^a$</td>
<td>18.38 ± 2.47$^a$</td>
<td></td>
</tr>
<tr>
<td>Osteoblast surface</td>
<td>OBs/BS (%)</td>
<td>3.6 ± 0.7</td>
<td>5.8 ± 0.9$^{b,a}$</td>
<td>3.9 ± 0.6$^{b,a}$</td>
<td></td>
</tr>
<tr>
<td>No. osteoblasts</td>
<td>N.Os/BS (/mm)</td>
<td>1.7 ± 0.9</td>
<td>4.7 ± 1.7$^a$</td>
<td>3.2 ± 1.4$^{b,a}$</td>
<td></td>
</tr>
<tr>
<td>Marginal apposition rate</td>
<td>MAR (µm/day)</td>
<td>0.69 ± 0.09</td>
<td>2.24 ± 0.42$^a$</td>
<td>1.57 ± 0.30$^{b,a}$</td>
<td></td>
</tr>
<tr>
<td>Osteoclast surface</td>
<td>Ocs/BS (%)</td>
<td>1.6 ± 0.2</td>
<td>2.9 ± 0.6$^{b,a}$</td>
<td>3.0 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>No. osteoclasts</td>
<td>N.Oc/BS (/mm)</td>
<td>0.11 ± 0.01</td>
<td>0.87 ± 0.10$^{b,a}$</td>
<td>0.78 ± 0.09$^{b,a}$</td>
<td></td>
</tr>
</tbody>
</table>

*Four random sections from each bone implant were analyzed to determine histomorphometric parameters.

$^a$Data are presented as mean ± SD of the combined values from five animals per group.

$^b$P ≤ 0.001 versus no tumor.

$^c$P ≤ 0.001 versus isotype.

$^d$P ≤ 0.01 versus isotype.

$^e$P < 0.01 versus no tumor.

$^{b,a}$P < 0.05 versus isotype.
Further evidence for a role of SMADs in metastasis is provided by the observation that a high frequency of SMAD4 mutations is associated with an increased prevalence of distant metastasis in colorectal cancers (67). Our observation that several BMPs stimulate *in vitro* invasion is consistent with the observation that SMAD are associated with invasive ability. This could account for the ability of prostate cancer cells to invade into the bone marrow microenvironment and thus, when BMP-6 activity was blocked with anti–BMP-6, activation of SMADs was decreased (as shown by our *in vitro* study), and invasion was decreased resulting in decreased establishment of intraosseous tumor.

A variety of factors have been suggested to contribute to the prostate cancer–induced osteoblastic activity (reviewed in ref. 68). Most recently, endothelin-1 has been shown to play a role in a murine model of osteoblastic lesions in breast cancer (69). Furthermore, it has been shown that endothelin-1 is expressed in prostate cancer (70) and that administration of atrasentan, an endothelin receptor A antagonist, to men with bone metastatic prostate cancer disease maintained serum BAP levels compared with the increase in BAP observed in men on placebo (71). Taken together, these data suggest that endothelin-1 may contribute to the osteoblastic lesions induced by prostate cancer. In our model, we could measure serum human endothelin-1 in the presence of tumor, but could not identify changes in endothelin-1 serum levels between isotype and anti–BMP-6–treated mice (data not shown). This suggests that in this model, the decreased osteosclerosis in the anti–BMP-6–treated animals occurred independently of modulating endothelin-1. This finding is supported by the observation that blocking endothelin-1, using an endothelin-1 receptor A inhibitor, did not diminish the ability of prostate cancer to induce primary murine osteoblasts to produce alkaline phosphatase and mineralize (72). Furthermore, in a clinical trial in which atrasentan administration maintained BAP levels (71), it also diminished increases in several systemic markers of osteoclast activity, indicating a generalized inhibition of bone remodeling or a direct antitumor effect that may have resulted in less tumor burden and thus less tumor-induced bone remodeling. Our study contrasts this observation in that although anti–BMP-6 decreased multiple indices of osteoblast activity, there was no effect on osteoclast activity as either (a) measured systemically by both urinary NTx and serum TRACP 5b or (b) observed histomorphometrically as osteoclast number. These data show that anti–BMP-6 targeted only the pro-osteoblastic activity of LuCaP 23.1. This finding is consistent with the clinical observations that even in men with osteoblastic prostate cancer bone metastases, systemic indices of osteoclastic activity are elevated (73) and that inhibiting osteoclastic activity using bisphosphonates, such as zoledronic acid, decreases bone fracture and bone pain (74).

Based on the currently reported data and those previously published, we propose the following mechanism through which prostate cancer induces osteoblastic bone metastases: As prostate cancer progresses, BMP-6 expression is up-regulated resulting in increased invasive ability that enhances its ability to invade through the bone microenvironment. Upon entering the bone microenvironment, prostate cancer stimulates osteoclast activity (28); however, eventually, BMP-6, through binding to the BMP receptors, stimulates phosphorylation of SMADs in osteoblast precursors, which promotes osteoblastogenesis and osteoblast differentiation (along with other as yet to be shown osteoblastic factors from the prostate cancer) resulting in increased mineralization bone production, which outpaces the prostate cancer–induced osteoclastic activity. Thus, essentially, instead of an ongoing vicious cycle of osteoclastic activity, the prostate cancer tumor switches from an osteolytic tumor to an osteoblastic tumor. This model accounts for the clinical
observations that men with prostate cancer have both systemic indices of osteoblastic activity (i.e., increased osteocalcin and BAP) as well as osteolytic activity (i.e., increased deoxypyridinoline and NTx of collagen). It is important to note that this model does not preclude other prostate cancer–produced osteoblastic factors, which most certainly exist. However, our model shows that BMP-6 is one of these important factors and plays a dual role on the development of prostate cancer bone metastases, including directly promoting invasion and indirectly through modifying the bone microenvironment. These findings suggest that targeting BMPs or SMADs may provide clinical utility for the prevention and treatment of osteoblastic prostate cancer metastases. Most likely, this type of therapy would need to be used in conjunction with an antiosteoclastic agent to ensure an overall balanced decrease of bone remodeling. Ultimately, the decreased bone remodeling would make the bone microenvironment unfertile soil for the prostate cancer cells.

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Figure 8. BMP-2 and BMP-6 promote invasive ability of prostate cancer cells. A, total cell lysate was collected from the indicated prostate cancer cells and subjected to immunoblot for the indicated BMP receptors. Shown are two different immunoblots: one for LuCaP cells and another for LNCaP, C4-2B, and PC-3 cells. C4-2B cells (B) or single-cell suspension of LuCaP 23.1 cells (C) were subjected to in vitro invasion assay over 24 hours with the addition of increasing doses of the indicated BMP. D, C4-2B cells or single-cell suspension of LuCaP 23.1 cells were incubated with either IgG isotype antibody or anti–BMP-6 antibody (1 μg/mL) during an in vitro invasion assay that was done over 24 hours. *P < 0.01 versus BMP (0 μg/mL; Student’s t test); †P < 0.01 versus IgG isotype (Student’s t test).

References
Bone Morphogenetic Protein-6 Promotes Osteoblastic Prostate Cancer Bone Metastases through a Dual Mechanism

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