Bone Morphogenetic Protein 7 Is Expressed in Prostate Cancer Metastases and Its Effects on Prostate Tumor Cells Depend on Cell Phenotype and the Tumor Microenvironment

Abstract
Bone morphogenetic protein (BMP) signaling is important in prostate development and prostate cancer (PCa) progression. However, because of the multiple effects of different BMPs, no final conclusions have been made as to the role of BMPs in PCa. In our studies, we have focused on BMP-7 because it is involved in prostate morphogenesis, and its expression is regulated by androgens. The objective of our study was to determine BMP-7 expression in PCa metastases and investigate the effects of BMP-7 on PCa cells. Our results show that BMP-7 is expressed in metastatic PCa and its levels are increased in castration-resistant PCa versus androgen-dependent PCa, whereas the expression of BMP-7 is decreased in primary PCa versus normal prostate. Our in vitro results show that BMP-7 inhibits proliferation of androgen-sensitive LNCaP cells, stimulates androgen receptor signaling, increases the expression of differentiation-associated genes, and decreases the levels of some wingless-regulated transcripts. Interestingly, these effects were not detected in C4-2 castration-resistant PCa cells. In vivo expression of BMP-7 in castration-resistant C4-2 cells did not alter proliferation when these cells were grown subcutaneously, but their growth was inhibited in the bone environment. In summary, our results show that BMP-7 is expressed at the highest level in advanced castration-resistant PCa cells and that the inhibitory effects of BMP-7 are dependent on the differentiation status of PCa cells and the tumor microenvironment. Further studies are needed to identify changes in BMP-7 signaling that lead to the loss of its control of proliferation during PCa progression.

Neoplasia (2010) 12, 192–205

Introduction
A major challenge in prostate cancer (PCa) biology is elucidating the mechanisms of development of PCa and its progression to bone metastases. Bone morphogenetic proteins (BMPs) were originally characterized as inducers of bone formation and later studies demonstrated their roles in the regulation of cell growth and differentiation [1]. Multiple BMPs and BMP receptors (BMPR) are expressed in normal prostate and PCa [2–5]. Therefore, it is not surprising that BMPs are involved in the control of proliferation and differentiation of these cells. Alterations in BMPs and BMPRs have been detected during prostatic development and PCa progression, and numerous reports have been published, demonstrating the effects of BMPs on PCa cells, including effects on proliferation and invasiveness as well as growth in the bone in vivo [6–8]. We have shown that the effects of BMP-2 and BMP-4 on the growth of PCa cells depend on the cells' differentiation status [5]. However, at present, no final conclusions can be made about the effects of specific BMPs because the published results are inconsistent [6,8–11].

In the present study, we have focused our investigations on the expression and effects of BMP-7 in PCa because BMP-7 is critical...
for prostate morphogenesis, is regulated by androgens in normal prostate [12] and because high levels of BMP-7 are expressed in normal prostate epithelium [4]. Furthermore, BMP-7 levels were reported to be downregulated in primary PCa versus normal prostate epithelium [4]. Although BMP-7 messages have been detected in PCa bone metastases [3], BMP-7 expression has been shown to increase in a PTEN-knockout murine model of PCa [10], and increased copy numbers of BMP-7 were detected in PCa tumors and PCa skeletal metastases [3,13]. Studies demonstrating the effects of BMP-7 on proliferation and invasiveness of PCa cells were published, but similarly to other BMPs, opposing effects were reported. BMP-7 was reported to increase the invasive potential of PCa cells [14] and protect PCa cells from stress-induced apoptosis [10], but in contrast, administration of BMP-7 was also shown to inhibit growth of PC-3 bone metastases and inhibit epithelial-mesenchymal transition (EMT) [9]. Furthermore, BMP-7 has also been shown to be instrumental in altering the tumor-stromal microenvironment. BMP-7 increased the expression of SDF1 in prostatic stroma in a PTEN-knockout model of PCa [15], and hepatocyte growth factor increased the expression of BMP-7 in PCa cells [16]. This indicates a role for BMP-7 in the interactions that occur between stroma and PCa cells and illustrates the effects of environment on BMP-7 expression.

Similar to PCa, BMP-7 is thought to play a role in breast cancer. BMP-7 has been detected in breast cancer tissues and it was associated with bone metastases [17,18]; it influences proliferation, migration, and invasion of breast cancer cells [19]. BMP-7 also inhibits estradiol-induced proliferation of breast cancer cells [20]. Despite the discordant results defining the specific effects of BMP-7 in PCa and breast cancers, published data underscore the important role that BMP-7 plays in cancer development and metastasis.

Recent studies show that multiple signaling pathways are altered during cancer progression and support the view that these alterations involve the integration of cross talk between signaling pathways. It has been well established that activities of nuclear hormone receptors are SMAD-dependent. There are several reports demonstrating a cross talk between transforming growth factor β and androgen receptor (AR) signaling pathways [21,22]. Although there is only limited information regarding cross talk between the BMP and AR signaling pathways, BMP-7 is regulated by androgens [12], and recently, it has been shown that SMAD1 interacts with AR and decreases AR transcriptional activity in LNCaP cells [23].

Another signaling pathway, the wingless pathway (Wnt), interacts with SMAD and AR signaling, and is important in the regulation of proliferation and differentiation as well as in cancer progression [24,25]. β-Catenin (β-cat) is an important coactivator of AR [26,27], a regulator of SMAD signaling, and also a major player in Wnt signaling that contributes to PCa cell growth and survival [28–30]. AR-directed transcription has been shown to be affected by Wnt activity [31,32], and ligand-dependent inhibition of the Wnt pathway by AR has also been observed [33,34]. Furthermore, cross talk between BMP and Wnt pathways has been documented [35,36].

The objective of our studies was to examine BMP-7 expression in advanced PCa and to investigate the effects of BMP-7 on cross talk among the SMAD, AR, and Wnt signaling pathways in PCa cells. Our results demonstrate that BMP-7 expression is increased in castration-resistant (CR) versus androgen-dependent (AD) PCa cells and that BMP-7 is expressed in PCa metastases. Our results also show that BMP-7 elicits differential effects on CR and AD PCa cells. BMP-7 inhibited the growth of LNCaP cells, stimulated AR-mediated transcription in these cells (that represent an earlier stage of PCa progression), and induced differentiation of LNCaP cells. These effects were not observed in the C4-2 CR PCa cells. Furthermore, our data demonstrate that the mechanisms intrinsic to these effects involve alterations in cross talk between SMAD/AR and Wnt signaling pathways. Finally, our in vivo results show that despite no detectable effects of BMP-7 on proliferation of C4-2 cells in vitro, BMP-7 decreased growth of these cells and increased AR-mediated transcription when grown in the bone environment. Illustrating biologic activities in vitro may not predict those in vivo [37].

Materials and Methods

Cell Culture and Supplements

The LNCaP PCa cell line (ATCC, Manassas, VA), its CR sublines C4-2 and C4-2B (UroCor, Oklahoma City, OK), CR PCa cells DU 145, PC-3 (ATCC), MDA PCa 2b (gift from Dr. Nora Navone), and VCaP (gift from Dr. Kenneth Pienta) were maintained under standard tissue culture conditions in RPMI 1640 with 10% fetal bovine serum (FBS). Tissue culture medium and supplements were obtained from Invitrogen (Carlsbad, CA). Recombinant human BMP-7 was purchased from R&D Systems (Minneapolis, MN).

Real-time Polymerase Chain Reaction

Total RNA was extracted from primary PCa and normal prostate tissues, PCa cell lines (LNCaP, C4-2, C4-2B, PC-3, DU145, MDA PCa 2b; control cells, and cells treated with BMP-7 for 3 days), and from paired AD and CR PCa LuCaP xenografts (LuCaP 23.1, LuCaP 35, LuCaP 77, LuCaP 96), using STAT-60 (Tel-Test, Friendswood, TX). First-strand complementary DNA (cDNA) was prepared using the Advantage RT-for-PCR kit (BD Biosciences, Palo Alto, CA). Real-time polymerase chain reaction (PCR) was performed on a Rotor-Gene RG-3000 (Corbett Research, Sydney, Australia) using Platinum Quantitative PCR SuperMix-UDG reagent (Invitrogen) and SYBR Green 1 (Molecular Probes, Eugene, OR). PCR primers (Table 1) were designed to span intron-exon boundaries. The PCR parameters were as we have described previously [38]. Reactions were carried out in duplicate, and expression levels were calculated from standard curves that were generated from a four-fold dilution of LNCaP cDNA (all standard curves had $R^2$ values >0.99).

Immunohistochemistry

Forty-four fixed paraffin-embedded PCa metastases from the University of Washington “Tissue Acquisition Necropsy Program” were used for immunohistochemical analyses. This program has been previously described and has received local institutional review board approval [39]. All tissues were processed by fixation in 10% neutral-buffered formalin (bone metastases were decalcified in 10% formic acid) and embedded in paraffin. Five-micrometer sections of tissue were used, and staining was performed with antigen retrieval as previously described [40] using a rabbit polyclonal anti-BMP-7 antibody (ab56023; Abcam, Inc, Cambridge, MA) at a concentration of 4 μg/ml. Negative control slides were incubated with rabbit immunoglobulin G (Vector Laboratories, Inc, Burlingame, CA) at the same concentration. Immunostaining was assessed by a pathologist Dr. Xiaotun Zhang, using a quasi-continuous BMP-7 score, created by multiplying each intensity level (0 for no stain, 1 for faint stain, 2 for strong, and 3 for intense stain) by the corresponding percentage of cells in the slide staining at that intensity, and then summing the results. The response variable for the analysis is the BMP-7 score.
Immunocytochemistry

LNCaP and C4-2 cells were cultured on Lab-Tek Chamber Slides (Nalge Nunc Naperville, IL). After treatment in phenol red–free RPMI with 5% charcoal-stripped serum or without BMP-7 (500 ng) for 48 hours, cells were fixed and incubated with mouse monoclonal anti-human AR antibody (1:60; BioGenex, San Ramon, CA) or rabbit polyclonal anti-human β-catenin antibody (5 μg/ml; Santa Cruz Biotechnology, Inc, Santa Cruz, CA) and then with a goat–antimouse Alexa Fluor 546 and goat–anti-rabbit Alexa Fluor 488 both at a dilution of 1:400, respectively, and mounted with ProlongGold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen).

Proliferation Studies

LNCaP (4000 cells per well) and C4-2 (3000 cells per well) were seeded in 96-well plates in 100 μl of phenol red–free RPMI 1640 medium with 5% FBS. The following day, BMP-7 (R&D Systems) was added (0, 100, 250, or 500 ng/ml). After 3 days, the effects on cell proliferation were determined using the Quick Cell Proliferation assay (BioRad DC Protein Assay Kit, BioRad Laboratories, Hercules, CA). Analysis with specified antibodies: mouse monoclonal anti–human AR antibody (3:1000; BioGenex); rabbit polyclonal anti–human phospho-SMAD1, 5, and 8 antibody (1:1000; Cell Signaling Technology, Inc, Danvers, MA); mouse monoclonal anti–human β-catenin (E-5, 1:5000); rabbit polyclonal anti–human AR (C-19, 3:1000); and E-cadherin (G-10, 3:1000) from Santa Cruz Biotechnology, Inc. To determine BMP-7 expression in BMP-7–C4-2 cells, we used mouse monoclonal anti–human BMP-7 antibody (MAB3541, 1:5000; R&D Systems). To determine BMP-7 expression in PCa metastases, xenografts, and cell lines, we used rabbit polyclonal anti–BMP-7 antibody (ab56023; Abcam, Inc). To confirm uniform loading of protein, blots were reprobed with rabbit polyclonal anti-GAPDH antibody (Ab9385, 1:1000; Abcam, Inc), rabbit polyclonal antiaactin antibody (1:1000; Sigma Chemical Co, St. Louis, MO) for cytosolic extracts or rabbit polyclonal anti–human histone 4 (H-97, 3:1000; Santa Cruz Biotechnology, Inc) for nuclear extracts.

Immunoprecipitation

Five micrograms of rabbit polyclonal anti–human AR (C-19; Santa Cruz Biotechnology, Inc) was prebound to 50 μl of Dynabead Protein A Dynabeads (Invitrogen). The primary antibody–bead complexes were blocked and then incubated with ~200 μg of nuclear extracts from BMP-7–treated and control cells. Proteins were separated on an SDS-PAGE gel and blotted as described previously. β-Catenin was detected using a mouse monoclonal anti–human β-catenin (E-5, 1:5000; Santa Cruz Biotechnology, Inc).

Transient Transfections

An Amaxa Nucleofector II system (Amaxa Biosystems, Inc, Gaithersburg, MD) was used for the transfection of PCa cells using solution V and program T-27 according to the manufacturer’s directions. LNCaP and C4-2 cells were transfected with ARE [42,43], E-cadherin, and TOPflash reporter plasmids (1 μg) and, if specified, with the effector plasmids

### Table 1. Primer Sequences.

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<th>Accession No</th>
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Whole-Cell Lysate, Nuclear, and Cytoplasmic Protein Preparation

Whole-cell lysates and nuclear and cytoplasmic proteins were prepared as we described previously [41]. Protein levels were determined using the BioRad DC Protein Assay Kit (BioRad Laboratories, Hercules, CA).

Western Blot Analysis

Proteins were separated on SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat dried milk in TBST (15 mM Tris-HCl, pH 7.4, 0.9% NaCl, and 0.05% Tween 20, pH 7.4) or with 5% BSA followed by blot
containing the open reading frame for β-cat (S33F), SMAD1, and AR (1 μg or as specified). TK renilla-luciferase plasmid was transfected under the same conditions to allow for normalization of transfection efficiencies. Cells were then plated in phenol red–free RPMI 1640 medium, supplemented with 5% FBS and incubated for 48 hours. Cells were lysed and subjected to a dual-luciferase assay according to the manufacturer’s recommendations (Promega, Madison, WI). Experiments were performed in triplicate and repeated two to three times.

**Stable Transfection of C4-2 Cells with BMP-7**

C4-2 cells were transfected using the Amaxa Nucleofector II with EcoRV linearized pcDNA3.1-V5-His-TOPO containing the ORF of the human BMP-7 gene, which was amplified using forward GAGCCC-GGGGCGTATCGGCCTAA and reverse AGGTCTCACAA-AAGGCAGTTGTC primers then sequenced. Transfected cells were selected using 600 μg/ml G418 and maintained in 300 μg/ml G418 (Invitrogen).

**Alkaline Phosphatase Activity**

Primary cultures of human osteoblasts (ScienCell, Carlsbad, CA) were grown in osteoblast medium (ScienCell) and cocultured in trans-well plates with pcDNA-C4-2, BMP-7-C4-2 cells or with 500 ng/ml recombinant BMP-7 (R&D Systems). Alkaline phosphatase activity in the cell lysate was determined using p-nitrophenyl-phosphate as a substrate (Vector Laboratories, Inc) and calculated as ΔA450/μg protein.

**C4-2 Subcutaneous and Intratibial Tumors**

For subcutaneous tumor formation, 6-week-old male severe combined immunodeficient (SCID) mice (Charles River Laboratories, Tacoma, WA) were injected subcutaneously with 2 × 10^6 PCa cells mixed with Matrigel (BD Biosciences, San Jose, CA). Two groups of five animals each were used: 1) control pcDNA-C4-2 cells and 2) BMP-7-C4-2 cells. Tumor volumes (TuV) were measured twice a week and calculated using the formula \( V = \frac{4}{3} \pi \times H \times W \times 0.5326 \). Blood samples were drawn from animals weekly for determination of serum prostate-specific antigen (PSA) levels starting at day 20 after tumor cell injection (IMX Total PSA Assay; Abbott Laboratories, Abbott Park, IL). Animals were killed when tumors reached 1000 mm^3 or if animals were compromised.

For intratibial tumor formation, 6-week-old male SCID mice were injected with 2 × 10^5 PCa cells into the tibiae as we described previously [44]. Two groups of 10 animals each were used: 1) pcDNA-C4-2 and 2) BMP-7-C4-2. Blood samples were drawn from animals weekly for determination of serum PSA levels starting at week 2 after tumor cell injection to monitor tumor growth. Animals were killed 9 weeks after tumor cell injection. Bone mineral density (BMD) was measured using a PIXImus Lunar densitometer (GE Healthcare, Waukesha, WI), and radiographs were obtained using a Faxitron Specimen Radiography System, Model MX-20 (Faxitron x-ray Corp, Wheeling, IL) just before sacrifice. After sacrifice, the tibiae with tumor were harvested and embedded in methyl methacrylate for bone histomorphometry (BHM).

**Bone Histomorphometry**

Six-micrometer longitudinal sections of undecalcified tibiae embedded in methacrylate and stained with Goldner trichrome were used [45]. BHM analysis was performed on the whole longitudinal section in the middle of the tibia (n = 5 per group). The bone volume (BV), TuV, and tissue volume (TV) were measured using Bioquant Software (Bioquant Image Analysis Corp, Nashville, TN).

**Data Analyses**

Statistical analyses of the results were performed using Prism software (Prism GraphPad, San Diego, CA). Significance of differences was evaluated using paired and unpaired Student’s t tests as appropriate, except for immunohistochemical analysis where we used the Wilcoxon signed-rank test, \( P \) values ≤ .05 indicating statistical significance.

**Results**

**BMP-7 Messenger RNA Levels Are Altered during PCa Progression**

It has been reported that levels of BMP-7 messages and protein are decreased in PCa versus normal prostate [4,46]. However, amplification of the BMP-7 gene was detected in PCa [13] and increased levels of BMP-7 were associated with PCa in PTEN conditional knockout mice [10,47]. Therefore, in our first studies, we set out to examine BMP-7 levels in PCa and normal prostatic epithelium and in PCa metastases. We used matched pairs of microdissected normal epithelium and PCa cells from the same patients, obtained at radical prostatectomy (\( n = 13 \)). Our data showed that levels of BMP-7 messenger RNA (mRNA) were lower in primary PCa versus NP in all pairs examined (−33.1 ± 10.1% [mean ± SEM], \( P = .0001 \)), which is in agreement with published data. Next, we examined the levels of BMP-7 in AD versus CR PCa cells because BMP-7 expression is regulated by androgens in normal prostate epithelial cells [12], and PCa progression is associated with alterations in AR signaling. To do this, we used the LNCaP/C4-2/C4-2B progression model and four pairs of AD and CR PCa xenografts. In the LNCaP/C4-2/C4-2B model of PCa progression, levels of BMP-7 mRNA were lowest in the androgen-sensitive LNCaP cells, and higher levels were detected in the CR C4-2, C4-2B cells (Figure 1A). We then examined the expression of BMP-7 in PCa paired AD and CR xenografts established in our laboratory (LuCaP 77, LuCaP 96, LuCaP 23.1, and LuCaP 35). BMP-7 messages were detected in all of the AD xenografts: in LuCaP 96 that was established from primary PCa as well as in LuCaP 77, LuCaP 23.1, and LuCaP 35 that were established from PCa metastases. When we compared levels of BMP-7 mRNA in the AD and CR sublines, BMP-7 mRNA levels were increased in all CR-PCa xenograft lines (Figure 1B). The LuCaP 23.1 xenograft had the smallest relative increase in BMP-7 mRNA levels. However, it should be noted that although it is not apparent from Figure 1B, LuCaP 23.1 expresses high levels of BMP-7 mRNA in AD cells (data not shown).

**BMP-7 Protein Is Highly Expressed in PCa Metastases**

BMP-7 mRNA levels are decreased in primary PCa versus NP, but our data from the PCa xenografts suggested increased expression in CR versus AD PCa. Therefore, it was important to determine whether BMP-7 is expressed in patient samples of advanced PCa, to establish clinical relevance of this factor to advanced disease. In one report, BMP-7 mRNA was detected in PCa bone metastases [3], but no studies have been published about the levels of BMP-7 protein in PCa bone metastases. In our studies, Western blot results showed that BMP-7 is...
expressed in human samples of NP, primary PCa, and in PCa metastases. The BMP-7 antibody in used in these analyses detects one major band of expected molecular weight, indicating high specificity of this antibody (data not shown). We therefore used this antibody for the immunohistochemical evaluation of BMP-7 expression in NP, primary PCa, and PCa metastases. We used tissues from NP (n = 12; from 12 patients), primary PCa (n = 12; from 12 patients), soft tissue metastases (n = 20; from 15 patients), and PCa bone metastases (n = 14; from 11 patients) from patients who died of advanced PCa. These specimens were acquired under our "Tissue Acquisition Necropsy Program" [39,48]. BMP-7 was detected in all metastatic samples and was predominantly localized in the cytoplasm of the tumor cells with occasional nuclear staining. Nuclear BMP-7 expression has been previously observed in breast tumors [46].

The BMP-7 scores in the epithelial portion of the tissues examined were as follows (mean ± SD): NP = 123.7 ± 50.5, range = 54-230; primary PCa = 123.1 ± 63.4, range = 10-210; soft tissue metastasis = 161.6 ± 93, range = 5-300; and bone metastasis = 191.6 ± 79.7, range = 10-300. The metastases scored higher than NP and primary PCa; however, there was a considerable range of staining within each of the groups. There were no significant differences between the cytoplasmic BMP-7 score between NP and primary PCa (P = 1) or bone and soft tissue metastases (P = .7896). The only significant difference between groups was between NP and bone metastases (P = .0407). Representative examples of BMP-7 immunoreactivity in NP, primary PCa, and PCa metastases are shown in Figure 1C. The large ranges in staining activity in these 58 clinical specimens illustrate yet again the heterogeneity of target protein expression in NP, primary PCa, and PCa metastases [39].

Effect of BMP-7 on PCa Cell Proliferation and AR Transcriptional Activity

BMP-7 regulates growth and differentiation of epithelium, and because our studies showed protein as well as gene expression in normal prostate epithelium, primary PCa, and PCa metastases, we next evaluated the effects of BMP-7 on PCa cell proliferation. We found that BMP-7 inhibits proliferation of the AD, AR-expressing LNCaP cells but not the CR PC-3 cells (Figure 2A). These results are concordant with our previous data showing that BMP-2 and BMP-4 inhibited growth of LNCaP cells, whereas PC-3 cells were not affected [5]. One of the obvious differences between LNCaP and PC-3 cells is the lack of AR expression in PC-3 cells. Thus, we next investigated whether AR is critical for the inhibition of LNCaP proliferation. For these experiments, we used the C4-2 and C4-2B sublines of LNCaP

Figure 1. BMP-7 is expressed in PCa cell lines, xenografts, and metastases. (A) Expression of BMP-7 mRNA in PCa cell lines. (B) Expression of BMP-7 mRNA in four AD and corresponding CR LuCaP xenografts. Results are plotted as mean ± SEM. *CR significant from AD (P < .05; n = 2-6). (C) Representative samples of BMP-7 expression in NP (a and f), PCa (b and g), and bone (c and h), lymph (d and i), and lung (e and j) PCa metastases. Original magnification, ×200.
that express AR but are CR; only LNCaP cells were inhibited by BMP-7 (Figure 2, A and B). In these experiments, proliferation of C4-2 cells was actually slightly increased as determined by the alteration of mitochondrial activity; however, the manual cell count did not confirm this phenomenon. In the next set of experiments, we examined whether AR expression is critical for BMP-7 inhibition of LNCaP proliferation. LNCaP cells were transiently transfected with a control small interfering RNA (siRNA) or AR siRNA and treated with BMP-7. Real-time PCR showed that levels of AR dropped to ~20% after AR siRNA transfection versus control siRNA transfected LNCaP cells. LNCaP cells with significantly decreased levels of AR showed no inhibition of proliferation. This demonstrates that the AR is required for BMP-7 inhibition of LNCaP proliferation. These results together clearly indicate that AR alone is not sufficient to

Figure 2. Effect of BMP-7 on cell proliferation and AR activity in LNCaP and C4-2 cells in vitro. (A) Effect of BMP-7 (500 ng) on proliferation in LNCaP, C4-2, C4-2B, and PC-3 cells. (B) Effect of increasing doses of BMP-7 on proliferation in LNCaP and C4-2 cells. (C) Effect of silencing AR on proliferation in LNCaP cells in the presence or absence of BMP-7 (500 ng). (D) Expression of AR mRNA in four AD and corresponding CR LuCaP xenografts. (E) Altered expression of phosphorylated Smad1 (P-Smad1) in LNCaP and C4-2 cells in the presence or absence of BMP-7 (500 ng) for 3 days. Histone H4 (H4) was blotted as a control. (F) Western analysis of nuclear, cytosolic, and total AR (with GAPDH as control for total AR expression) in LNCaP and C4-2 cells with or without BMP-7 (500 ng) for 3 days.
inhibit proliferation in response to BMP-7 of C4-2 and C4-2B cells but that AR is a critical player in the BMP-7 inhibition of proliferation of LNCaP cells (Figure 2C). We hypothesize that alteration in AR signaling in CR PCa makes these cells resistant to inhibition of proliferation by BMP-7. These data are further supported by the fact that the expression of AR in the CR LuCaP xenografts, similar to BMP-7, is significantly increased when compared with their AD counterparts (Figure 2D). To better understand the mechanisms behind the differential effects of BMP-7 on PCa cells and the role of AR in this inhibition, we chose to further examine the effects of BMP-7 on LNCaP and C4-2 cells, as the pair of the most closely related cell lines that express AR but exhibit different responses to BMP-7. Published results indicate that PCa cells express BMP receptors and our PCR data are concordant with these results [47]. We confirmed that LNCaP and C4-2 cells express SMAD1 and that SMAD1 is phosphorylated in both LNCaP and C4-2 cells after treatment with BMP-7, demonstrating the presence of active BMP signaling in both cell lines (Figure 2E).

Effects of BMP-7 on AR Levels and AR-Mediated Transcription

Our results show that AR expression in LNCaP cells is critical for BMP-7 inhibition of proliferation. Because AR is involved in regulating PCa proliferation and changes in AR signaling occur in CR PCa versus AD PCa, we decided to investigate whether BMP-7 exhibits differential effects on AR-mediated signaling. Western blot analysis showed that while BMP-7 did not alter total levels of AR, it resulted in an accumulation of AR in the nucleus and a concomitant decrease in the cytoplasm of LNCaP cells, whereas no alteration of cellular distribution was detected in C4-2 cells where AR was mainly nuclear in the presence or absence of BMP-7 (Figure 2F). Immunofluorescence also showed that AR, which is mainly localized in the cytoplasm in LNCaP cells in the absence of androgens, translocates to the nucleus on BMP-7 treatment, similarly to treatment with dihydrotestosterone (DHT). In contrast, in C4-2 cells, AR is already localized mainly in the nuclei in the absence of androgens, and the addition of BMP-7 or DHT do not cause any visible changes (Figure 3, A and B). Because BMP-7 increased nuclear localization of AR in LNCaP cells, we next evaluated the effects of BMP-7 on AR-mediated transcription using a synthetic ARE reporter construct. BMP-7 increased AR transcriptional activity in LNCaP cells in a concentration-dependent fashion, whereas no increases were detected in C4-2 cells (Figure 3C). To confirm that the lack of AR signaling in response to BMP-7 is not cell line–specific, we examined AR-mediated transcription in two other CR-PCa lines, PC-3 cells transfected to express AR and VCaP cells. BMP-7 did not increase AR-mediated transcription in PC-3 cells transfected to express AR (P = .9145) and VCaP cells (P = .2170), further supporting the hypothesis that BMP-7 does not alter...
AR-mediated transcription in CR-PCa cells. In an effort to further substantiate our assertion that BMP-7 alters AR-mediated transcription in LNCaP cells, we extended our studies to examine the effects of BMP-7 on PSA levels in the medium of LNCaP and C4-2 cells. Levels of PSA (ng/ml/10^5 cells) were increased 3.02 ± 0.08-fold in the medium of BMP-7–treated LNCaP cells versus parental cells, whereas no significant change (1.16 ± 0.39) was observed in C4-2 cells (P = .0013). Additional evidence that BMP-7 alters AR-mediated transcriptional activity was provided by real-time PCR. Increased levels of AR mRNA and endogenous AR-regulated genes including PSA and human kalikrein-2 (KLK2) mRNA were detected in LNCaP cells, but not in C4-2 cells, on BMP-7 treatment (Figure 3D).

SMAD1 Increases AR Activity in LNCaP Cells

It has been reported that SMAD1 associates with AR [23]. To demonstrate that SMAD1 is instrumental in increasing AR-transcriptional activity in LNCaP cells, we overexpressed SMAD1 in these cells. SMAD1 overexpression caused a 5.4 ± 0.7-fold increase in AR transcriptional activity versus control cells (P = .038; Figure 4A).

Figure 4. BMP-7 alters AR interactions with β-cat in LNCaP cells. (A) Luciferase assay to determine the effect of Smad1 and β-cat on ARE binding and promoter activation in LNCaP cells. (B) Expression of β-cat and AR in LNCaP and C4-2 cells with or without BMP-7 (500 ng) for 3 days. GAPDH was blotted as a control (the same blot as in Figure 2F). (C) Immunoprecipitation of AR/β-cat complexes in the nucleus of LNCaP and C4-2 cells with or without BMP-7 (500 ng) for 3 days. Nuclear β-cat was blotted as a control. (D) Expression of E-cadherin in LNCaP and C4-2 cells with or without BMP-7 (500 ng) for 3 days. GAPDH was blotted as a control. (E) Immunohistochemical analysis of β-cat in LNCaP and C4-2 cells cultured in charcoal-stripped serum with or without BMP-7 (500 ng) for 2 days.
**BMP-7 Alters AR Interactions with β-catenin in LNCaP Cells**

β-catenin is a transcription cofactor that is involved in regulating AR as well as SMAD signaling, SMAD1 overexpression increases AR transcriptional activity in the presence of β-catenin over that of β-catenin alone (Figure 4A). Therefore, we next examined whether the levels of β-catenin or its interaction with AR are altered in LNCaP and C4-2 cells on BMP-7 treatment. Real-time PCR results showed that BMP-7 treatment resulted in significant increases of levels of β-catenin in LNCaP cells 1.00 ± 0.018 (mean ± SEM; Control) versus 1.72 ± 0.025 (BMP-7; P = .0018) but not in C4-2 cells (P = .9) in the absence of androgens. Similarly, Western analysis showed a subtle increase in β-catenin protein levels in LNCaP but not in C4-2 cells after BMP-7 treatment (Figure 4B). In contrast to our expectations, when we examined the association between AR and β-catenin, our data show that BMP-7 treatment decreased the association of AR with β-catenin in LNCaP cells (Figure 4C). Next, we looked at the localization of β-catenin in LNCaP and C4-2 cells after BMP-7 treatment in the absence of androgens. The immunofluorescent staining showed that there is increased membrane-bound β-catenin in the LNCaP cells but not C4-2 cells after BMP-7 treatment (Figure 4E). These results suggest that alterations in protein levels as well as protein–protein associations among AR, SMAD1, and β-catenin may be a significant mechanism whereby biologically relevant cross talk occurs between the SMAD1 and AR pathways.

**Cross talk between the AR and Wnt Pathway in PCa Cells and BMP-7 Effects on Wnt-Regulated Genes**

In a complex signaling network, it is inevitable that an alteration in one pathway affects other pathways. It has been suggested that cross talk between AR and Wnt signaling exists in PCa cells [24,25,33,49]. Activation of the Wnt pathway is associated with cancer proliferation and progression [25], and increased levels of Wnt3a and β-catenin have been detected in clinical samples of PCa [28,29,34,50]. To confirm that there is cross talk between AR and Wnt signaling in LNCaP cells, we used a reporter assay with an ARE-luc and TOPflash reporter. TOPflash activity is below the limit of detection in LNCaP cells; therefore, we transfected these cells with constitutively active β-catenin (S33F) to stimulate Wnt activity. Our results show that DHT, while stimulating AR-mediated transcription, caused inhibition of Wnt signaling (Figure 5A). In addition, AR expression in PC-3 cells inhibited Wnt signaling and abolishing AR using AR siRNA in LNCaP cells stimulated WNT signaling (Figure 5, B and C). These data demonstrate that a competition exists between AR and Wnt signaling in PCa cells. For that reason and because BMP-7 increased AR-mediated transcription in LNCaP cells, we investigated whether BMP-7 also alters Wnt signaling in PCa cells. Because the Wnt transcriptional activity in PCa cells without the overexpression of β-catenin was below the limit of the reporter assay, we used real-time PCR to examine the effects of BMP-7 on WNT-regulated genes c-Myc and survivin. In our real-time studies, described previously, our data show that BMP-7 treatment decreased the levels of c-Myc and survivin transcripts in LNCaP cells, with no significant changes in C4-2 cells (Figure 3D).

**Effect of BMP-7 on E-cadherin and Vimentin Expression in PCa Cells**

The increases in AR-mediated transcription and decreases in the Wnt-regulated genes suggest that BMP-7 may stimulate differentiation of LNCaP cells. It is well documented that BMP-7 plays a role in epithelial differentiation and that alterations in differentiation and EMT are associated with tumor progression and metastasis in cancer. EMT is accompanied by decreases in the expression of E-cadherin, a marker of epithelial cells, and by increases in the expression of vimentin, a marker of mesenchymal cells [51]. To further investigate the mechanisms of effects of BMP-7 on the differentiation status of PCa cells, we showed that there is a trend toward increased levels of E-cadherin mRNA and decreased levels of vimentin mRNA levels in LNCaP cells after BMP-7 treatment but not in C4-2 cells (Figure 3D). To further support these data, we showed that BMP-7 treatment increased the levels of E-cadherin protein in LNCaP cells and decreased E-cadherin protein levels in the CR C4-2 cells (Figure 4D). As described earlier, β-catenin localized to the membrane in BMP-7–treated LNCaP cells, possibly interacting with E-cadherin, promoting a more differentiated epithelial phenotype.

**The Characterization of C4-2 Overexpressing BMP-7 (BMP-7–C4-2) Cells**

The finding that in vitro more differentiated LNCaP cells are inhibited by BMP-7 whereas CR-PCa cells are not affected suggests that there are alterations in the cross talk between BMP and AR signaling during PCa progression. Although the in vitro data are helpful in increasing our understanding of PCa biology, it is important to confirm these findings in vivo and particularly in the bone environment. Therefore, to substantiate our claim that BMP-7 signaling is altered in CR PCa, resulting in loss of growth control, we generated C4-2 cells overexpressing BMP-7 and evaluated the effects of BMP-7 on the growth of CR C4-2 cells in vivo subcutaneously and in the bone environment.

Before growing these cells in vivo, we characterized them in vitro. Characterization of the BMP-7–C4-2 cells showed that these cells expressed ~60-fold more BMP-7 mRNA when compared with control pcDNA–C4-2 cells (Figure 6A) and that BMP-7 protein is secreted into the medium of BMP-7–C4-2 cells, whereas no BMP-7 was detected in the control cell medium (Figure 6B). Overexpression of BMP-7 in BMP-7–C4-2 cells did not significantly alter proliferation versus pcDNA–C4-2 cells (Figure 6C), but lower levels of PSA were detected in the medium of the BMP-7–C4-2 cells (Figure 6D) similarly to treatment with recombinant BMP-7 (Figure 3D). To ensure that the expressed and secreted BMP-7 possessed biologic activity, we examined the effects of conditioned medium from BMP-7–C4-2 and pcDNA–C4-2 cells on alkaline phosphatase activity in human osteoblasts. Conditioned medium from BMP-7–C4-2 cells increased alkaline phosphatase activity in osteoblasts relative to control pcDNA–C4-2 cells, demonstrating that the secreted BMP-7 is biologically active (Figure 6E).

**Effect of BMP-7 Overexpression in C4-2 In Vivo**

**Subcutaneous Tumors.** The take rate of subcutaneous C4-2 tumors was not altered by BMP-7 expression. BMP-7–C4-2 cells and control pcDNA–C4-2 cell take rates were both 100%. Similar to in vitro results, BMP-7–C4-2 TuV was not significantly different when compared with the pcDNA–C4-2 tumors (Figure 7A), whereas serum PSA levels in mice with BMP-7–C4-2 tumors were significantly lower versus those with pcDNA–C4-2 (P = .0354; Figure 7B). The PSA index at sacrifice (ng/ml PSA per mm³ of tumor) was also
lower in the BMP-7–C4-2 group (43.9 ± 5.9% of the control, \( P = .0023 \)). Real-time PCR confirmed that high BMP-7 expression was maintained in the BMP-7–C4-2 cells under in vivo conditions (data not shown).

**Tumors in the bone environment.** Expression of BMP-7 did not alter take rates of the tumor cells in the tibiae (100% in both groups).

To investigate the effects of BMP-7 expression on C4-2 tumor growth in the bone, we performed BHM analysis. BMP-7 expression significantly decreased BMP-7–C4-2 TuV versus pcDNA–C4-2 TuV (0.40 ± 0.05 vs 0.61 ± 0.9 mm², \( P = .033 \)), and the tissue volume (TV) of tibiae bearing BMP-7–C4-2 versus pcDNA–C4-2 tibiae (0.56 ± 7.3 vs 0.78 ± 4.8 mm², \( P = .31 \)). In contrast, no significant effects on bone were detected: BVs of the tibiae with tumor were not different.
between the groups, and there were no significant differences in BMD between the BMP-7–C4-2 and pcDNA–C4-2 tibiae (Figure 7D). Representative examples of the intratibial tumors are shown in Figure 7E. To further evaluate the effects of BMP-7 expression on C4-2 cells in the bone microenvironment, we also measured serum PSA levels in animals bearing intratibial tumors. The serum PSA levels were significantly higher in the BMP-7–C4-2 group versus the control pcDNA–C4-2 group (P = .026) by day 56 after implantation (Figure 7C).

Discussion
A better understanding of the mechanisms involved in PCa progression and changes in cellular signaling is important for the development of effective treatments for this disease. A considerable number of studies investigated the role of the BMPs in the prostate, PCa in situ, and PCa metastasis, but still no conclusions can be made about their effects during disease progression.

In our studies, we focused on investigating the effects of BMP-7 during PCa progression. To demonstrate the clinical relevance of BMP-7, we showed decreases in BMP-7 mRNA levels in PCa in comparison to normal prostatic epithelium. These results are in concordance with previously published results [4]. These results together with our results showing inhibition of PCa cell proliferation suggest that BMP-7 is involved in the regulation of proliferation of these cells, and therefore, alteration in BMP-7 signaling is important for disease progression. However, our results have also shown that despite the decreases in BMP-7 mRNA expression in primary PCa versus NP, BMP-7 mRNA levels are increased in CR versus AD PCa xenografts.

In our immunohistochemical analyses of BMP-7 in clinical samples, we observed no difference in BMP-7 protein expression in primary PCa versus NP. The discordance between the mRNA and protein levels of BMP-7 in primary PCa versus NP is not unusual and may relate to alterations in translational mechanisms in the cells [52]. However, similar to our CR models, BMP-7 is expressed at higher levels in CR PCa metastases when compared with primary PCa and NP.

BMP-7 expression is regulated by androgens in normal prostate, and therefore, one would expect decreased levels in PCa cells after castration. However, it has been shown that levels of AR are increased in some CR PCa tumors in comparison to AD tumors and that intratumoral androgens are present at similar levels in AD and CR tumors despite the decreases in serum levels of these hormones [53]. In agreement with these facts, we showed that CR PCa xenografts have higher levels of AR mRNA and the levels of AR correlated with BMP-7 expression. Therefore, we hypothesize that BMP-7 expression is increased in CR tumors in response to higher levels of AR and that cross talk between these two pathways would have potential significance in PCa progression of AD and CR disease.

Our results showed that BMP-7 inhibits proliferation of AD, more differentiated LNCaP cells but not the proliferation of CR PC-3 cells, similar to our previous results with BMP-2 and -4 [5]. We hypothesized that the expression of AR and AR signaling might be the

Figure 6. Characterization of BMP-7 overexpressing C4-2 cells. (A) Expression of BMP-7 mRNA in BMP-7–C4-2 cells. (B) Western analysis of BMP-7 of medium (10× concentrated) from BMP-7–C4-2 and pcDNA–C4-2 cells. (C) Cell count to determine cell growth of pcDNA–C4-2 and BMP-7–C4-2 cells in vitro. (D) PSA secretion in pcDNA–C4-2 and BMP-7–C4-2 cells in vitro. (E) Alkaline phosphatase activity in osteoblasts treated with conditioned medium from pcDNA–C4-2 cells, BMP-7–C4-2 cells, or BMP-7 alone (500 ng). Results are plotted as mean ± SEM. *Significant from pcDNA–C4-2.
cause of the differential response of PCa cells to BMPs. Our data showed that AR expression is needed in androgen-sensitive differentiated LNCaP cells for BMP-7 inhibition of proliferation, whereas AR expression by itself is not sufficient to result in the inhibition of proliferation by BMP-7 in CR C4-2 and C4-2B PCa cells. Therefore, the differential response of the AD and CR PCa cells, which both express AR, to BMP-7 suggests that alteration in AR and BMP signaling and cross talk take place during PCa progression.

In contrast to our results, it has been reported that the expression of AR in PC-3 cells made these cells susceptible to growth inhibition by Smad signaling [23], whereas parental PC-3 cells were not inhibited. We do not have a firm explanation of the discordance between these results, but it is possible that forced expression of AR in PC-3 cells does not recapitulate PCa disease progression in the same fashion as the LNCaP/C4-2/C4-2B progression model.

A network of interactions between growth factors, Wnt, and AR signaling pathways may be responsible for the onset of the aggressive tumor phenotype [54]. To understand the potential mechanistic differences in

Figure 7. Growth response of BMP-7 overexpressing subcutaneous and intratibial tumors in vivo. (A) Subcutaneous TVs of pcDNA–C4-2 and BMP-7–C4-2 tumors in SCID mice (n = 5 per group). (B) Serum PSA in animals with subcutaneous pcDNA–C4-2 and BMP-7–C4-2 tumors in SCID mice (n = 5 per group). (C) Serum PSA in animals with intratibial pcDNA–C4-2 and BMP-7–C4-2 tumors in SCID mice (n = 10 per group). (D) BMD of control and tibiae with tumor of BMP-7–C4-2 and pcDNA–C4-2 tumors in SCID mice (n = 10 per group). (E) Control (pcDNA–C4-2) and BMP-7–C4-2 intratibial tumors. Results are plotted as mean ± SEM. *Significance, P < .05.
the action of BMP-7 in PCa progression from the AD to a CR phenotype, we examined the interactions between the BMP, Wnt, and AR signaling pathways.

BMP-7 increased the recruitment of nuclear AR and increased AR-mediated transcriptional activity in the more differentiated LNCaP cells, whereas these effects were not observed in C4-2 cells. Again, this demonstrates alteration in the cross talk between AR and BMP signaling during PCa progression. These differential effects are not due to differential BMP-7 signaling through SMAD1 because SMAD1 is phosphorylated in both cell lines in the presence of BMP-7. Whereas BMP-7 and SMAD1 can clearly increase AR activity and the expression of AR-regulated genes, BMP treatment decreased the association between β-catenin/AR with a concomitant increase in E-cadherin and membrane-associated β-catenin in androgen-sensitive LNCaP cells. This was not observed in the CR C4-2 cells, suggesting that the loss of nuclear β-catenin complexes in the more differentiated LNCaP cells may be the crucial step that determines the differential response of LNCaP cells to BMP-7 treatment.

Further exploring the relation between the AR and Wnt signaling pathways, we found a reciprocal relation between the AR and Wnt (β-catenin) signaling pathways in PCa cells with increases in ARE activity and a reciprocal decrease in β-catenin activity in response to androgens. This underscores our preliminary experiments that show BMP-7 increases AR activity and the expression of androgen-regulated genes while decreasing Wnt-regulated genes in LNCaP cells. Therefore, our results suggest that BMP-7 promotes AR activity and inhibits β-catenin activity in differentiated PCa cells.

We observed an increase in AR expression and androgen-regulated differentiation-associated genes PSA and human kallikrein 2, a loss of Wnt-regulated genes c-Myb and survivin, and the alteration of EMT-associated genes E-cadherin and vimentin in BMP-7-stimulated LNCaP cells. This demonstrates that BMP-7 is promoting the epithelial phenotype in the LNCaP cells, promoting the secretory phenotype, decreasing proliferation, and inhibiting EMT. The more dedifferentiated C4-2 cells did not exhibit these changes. These results suggest that interactions among BMP-7, AR, and Wnt pathways can promote the epithelial phenotype and that the cross talk between these signaling pathways is altered in advanced PCa cells resulting in loss of BMP-7 control of proliferation and differentiation of these cells. Identifying the events that are altered in response to BMP-7 signaling in LNCaP and C4-2 cells may provide putative targets that determine epithelial cell fate in prostate tumorigenesis.

The tumor microenvironment has considerable impact on tumor growth and development. Therefore, to determine if the events we observed in vitro take place in vivo, we investigated whether C4-2 cells are resistant to BMP-7 growth inhibition in vivo. Overexpression of BMP-7 did not significantly alter the growth of subcutaneous C4-2 tumors, although accompanied by slight decreases in serum PSA levels. In contrast to subcutaneous tumors, when BMP-7–C4-2 and pcDNA–C4-2 cells were grown in the bone environment, expression of BMP-7 resulted in decreased tumor area and interestingly, increases in serum PSA levels in animals bearing BMP-7–C4-2 tumors when compared with pcDNA–C4-2 intratibial tumors. These results are similar to those of Buijs et al. [9], who reported that administration of recombinant BMP-7 did not affect the growth of orthotopically implanted PC-3M–Pro4/luc+ PCa cells in athymic mice. However, the growth of micrometastatic deposits from human PC-3M–Pro4/luc+ PCa cells in bone marrow was inhibited significantly. Collectively, these results demonstrate that the bone/microenvironment can alter responses of tumor cells to BMP-7. Whether this is due to the effect of BMP-7 on the stroma with a reciprocal response from the stroma on the tumor cells or the direct effect of the stroma in concert with BMP-7 on the tumor cells remains to be elucidated.

We expected that the expression of BMP-7 would increase new bone formation in the intratibial tumors, yet there were no significant differences observed in BMD or BV. One possible explanation is that the osteolytic factors produced by C4-2 cells may be sufficient to override BMP-7 osteogenic activity in this tumor type in vivo [30,55].

In conclusion, we have shown that BMP-7 protein is expressed at higher levels in PCa bone and soft tissue metastasis when compared with primary PCa and NP suggesting our hypothesis that BMP-7 signaling is relevant to clinical disease progression. Furthermore, our data suggest that interactions among the BMPs, Wnt, and AR signaling pathways are altered during PCa progression. Moreover, we have demonstrated in vivo that the tumor microenvironment can impact tumor growth in bone in response to BMP-7. Further investigation of the effects of BMPs on PCa cells is warranted to delineate the signal transduction network alterations in PCa cells. These results demonstrate the critical need to consider the different tumor/stromal microenvironments when investigating pathways relevant to PCa metastatic progression.

Acknowledgments

The authors thank Kristen Brubaker, Kathy Emami, and Holly Nguyen for helpful discussions; Xiaorun Zhang for pathology; Richard Pestell who kindly provided the β-catenin S33F mutant construct; Stephen Plymate for the ARE and wild-type AR constructs; and Randall Moon for the TOPFlash reporter plasmid. The authors thank the patients and their families who were willing to take part in the PCa rapid autopsy series.

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