Diverse Biological Effect and Smad Signaling of Bone Morphogenetic Protein 7 in Prostate Tumor Cells

Shangxin Yang,1 Chen Zhong,2 Baruch Frenkel,1 A. Hari Reddi,3 and Pradip Roy-Burman1,2

Abstract

We found that bone morphogenetic protein (BMP) 7, a member of the BMP family, was strikingly up-regulated during the development of primary prostatic adenocarcinoma in the conditional Pten deletion mouse model. To determine the relevance of this finding to human prostate cancer, we examined the expression of BMPs and BMP receptors (BMPR) as well as the responsiveness to recombinant human BMP7 in a series of human prostate tumor cell lines. All prostate cell lines tested expressed variable levels of BMP2, BMP4, and BMP7 and at least two of each type I and II BMPRs. In all cases, BMP7 induced Smad phosphorylation in a dose-dependent manner, with Smad5 activation clearly demonstrable. However, the biological responses to BMP7 were cell type specific. BPH-1, a cell line representing benign prostatic epithelial hyperplasia, was growth arrested at G1. In the bone metastasis-derived PC-3 prostate cancer cells, BMP7 induced epithelial-mesenchymal transdifferentiation with classic changes in morphology, motility, invasiveness, and molecular markers. Finally, BMP7 inhibited serum starvation–induced apoptosis in the LNCaP prostate cancer cell line and more remarkably in its bone metastatic variant C4-2B line. Each of the cell lines influenced by BMP7 was also responsive to BMP2 in a corresponding manner. The antiapoptotic activity of BMP7 in the LNCaP and C4-2B cell lines was not associated with a significant alteration in the levels of the proapoptotic protein Bax or the antiapoptotic proteins Bcl-2, Bcl-xl, and X-linked inhibitor of apoptosis. However, in C4-2B cells but not in LNCaP cells, a starvation-induced decrease in the level of survivin was counteracted by BMP7. Taken together, these findings suggest that BMPs are able to modulate the biological behavior of prostate tumor cells in diverse and cell type–specific manner and point to certain mechanisms by which these secreted signaling molecules may contribute to prostate cancer growth and metastasis. (Cancer Res 2005; 65(13): 5769-77)

Introduction

Bone morphogenetic proteins (BMPs) are secreted signaling molecules belonging to the transforming growth factor-β (TGF-β) superfamily. They were originally isolated from bone matrix (1). Although BMPs function as osteogenic factors, they also have pleiotropic roles in cell growth, differentiation, migration, and apoptosis and are critical in embryogenesis and organogenesis (1, 2). They are unique among osteogenic factors, as they can induce osteoblast differentiation and bone formation by uncommitted progenitors in vitro and in vivo (3–5). BMP7 (also called osteogenic protein-1) can induce osteogenic differentiation of newborn rat calvarial cells and rat osteosarcoma cells (6–8). It is also reported that BMP7 could induce osteoblastic cell differentiation of the pluripotent mesenchymal cell line C2C12 (9). Expression of several BMPs (BMP2, BMP3, BMP4, and BMP6) was detected in normal and malignant prostate tissues, and a role of BMP4 and BMP7 was shown in prostate development (10–12). Moreover, BMP4, BMP6, and BMP7 were detected in prostate skeletal metastases (13, 14), implying that these BMPs could play a role in the formation of osteoblastic lesions. It is noteworthy that BMP7 mRNA levels were found to be higher in prostate cancer skeletal metastases than in bone itself (15). The demonstration of BMP receptors (BMPR) in prostate tissues and cell lines further supports the possibility that BMPs expressed by tumor or bone cells affect the behavior of the prostate cancer cells (16). However, only a limited amount of information is available on the effect of specific BMPs on prostate cell lines, although it seems that their activity may vary from one cell line to the other. For example, although BMP2 did not have any effect on the proliferation of androgen-insensitive PC-3 and DU145 prostate cancer cell lines, it inhibited the growth of the androgen-sensitive LNCaP cells (17). Similarly, BMP4 inhibited LNCaP but not PC-3, whereas BMP2 treatment of PC-3 cells resulted in an increase in osteoprotegerin, a factor that inhibits osteoclastogenesis (16). It was also reported recently that vascular endothelial growth factor might act downstream of BMPs in prostate cancer cells to promote osteoblastic activity (18). Of the three type I BMPRs (BMPRIA, BMPRIB, and ActRI) and three type II BMPRs (BMPRII, ActRII, and ActRIB), expression of BMPRIA, BMPRIB, and BMPRII in human prostate cancer tissues was examined in relation to tumor grade. The work based on mostly immunohistochemistry and partly Western blot analysis described frequent loss of expression of these three receptors in high-grade prostate cancer, although only loss of expression of BMPRII correlated with poor prognosis in prostate cancer patients (19, 20).

We initiated the studies described in this report to better understand the mechanisms of BMP action in the context of prostate tumor cells. Of ~20 BMP family members, which have been characterized to date, we were motivated to place emphasis on BMP7 because of two major findings: (a) as stated above, its association with skeletal metastases of human prostate cancer, and (b), our own observation, which is also described here, that BMP7 protein expression is strikingly increased with the growth of the prostatic adenocarcinoma in the Pten conditional deletion mouse model of prostate cancer (21–23).

Materials and Methods

Cell lines and bone morphogenetic protein treatment. The immortalized human prostate epithelial cell line MLC SV40 (24) and the

References

prostate cancer cell lines PC-3, DU145, LAPC-4, LNCaP, and CWR22R were cultured as described previously (25, 26). The benign prostate epithelial hyperplasia cell line (BPH-1; ref. 27) and two prostatic stromal cell lines, HTS-4OC and HPS-4OF (28), were cultured in the RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. C4-2B, a variant LNCaP with propensity for bone metastasis (29, 30), was cultured in DMEM/Ham’s F-12 (4:1) supplemented with 10% FBS, 1% penicillin/streptomycin antibiotics, 5.0 µg/mL insulin, 13.7 µg/mL triiodothyronine, 4.4 µg/mL apotransferrin, 0.2 µg/mL t-nicotin, and 12.5 µg/mL adenine. Human recombinant BMP2 protein (BMP2) and TGF-β1 protein were purchased from R&D Systems, Inc. (Minneapolis, MN), and human recombinant BMP7 protein (BMP7) was provided by A. Hari Reddi. For determining the effect of BMP7, BMP2, or TGF-β1 on cell proliferation, BPH-1, PC-3, and DU145 were grown in 0.5% serum medium. In wound healing assay, Matrigel invasion chamber assay, and all other treatments for BPH-1 or PC-3, 0.5% serum medium was used. For serum starvation analysis BPH-1, PC-3, and DU145 were grown in 0.5% serum medium. For serum starvation analysis BPH-1, PC-3, and DU145 were grown in 0.5% serum medium. For serum starvation analysis BPH-1, PC-3, and DU145 were grown in 0.5% serum medium. For serum starvation analysis BPH-1, PC-3, and DU145 were grown in 0.5% serum medium. For serum starvation analysis BPH-1, PC-3, and DU145 were grown in 0.5% serum medium.

Cell proliferation assay. Cells (0.5 × 10^5) were plated in each well of the six-well plate in triplicates in the absence or presence of 50 ng/mL BMP7. Cells were counted using a Coulter counter (Beckman Coulter, Inc., Miami, FL) every 2 days. The culture medium was changed and BMP7 replenished every 2 days.

RNA preparation and semiquantitative reverse transcription-PCR. Total RNAs were extracted by TRIzol reagent (Invitrogen, San Diego, CA) following the protocol recommended by the manufacturer. The RNA (5 µg) was reverse transcribed by oligo(dT)20 and SuperScript III reverse transcriptase (Invitrogen) in a volume of 21 µL. The synthesized cDNA was subjected to PCR with the primers described in Table 1. The optimal cycle that can reflect the amount of original template was determined and used in the semiquantitative PCR experiment.

Cell cycle assay. Cells were suspended in 1% (w/v) paraformaldehyde in PBS on ice for 30 minutes and then fixed in 70% ethanol at −20°C overnight. After cells were stained with propidium iodide/RNase A solution (Phoenix Flow Systems, Inc., San Diego, CA) in the dark for 30 minutes at room temperature, samples were then analyzed by a flow cytometry system.

Apoptosis analysis (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling). Cellular apoptosis was assessed using APO-BRDU kit (Phoenix Flow Systems) following the instructions of the manufacturer. The nuclear extracts of the cells were prepared using Active Motif Nuclear Extraction kit (Dr. Dario Altieri (University of Massachusetts, Worcester, MA). The antibodies used in the Western blot experiments were goat anti-BMP7 (R&D Systems), rabbit anti-Smad1, rabbit anti-Smad5, rabbit anti-phospho-Smad1,5,8, rabbit anti-X-linked inhibitor of apoptosis (XIAP), rabbit anti-Bcl-2, rabbit anti-Bcl-xL, rabbit anti-Bax (Cell Signaling Technology, Beverly, MA), and goat anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-survivin antibody was a generous gift from Dr. Dario Altieri (University of Massachusetts, Worcester, MA).

Immunostaining. Cells cultured on the 24-well plate were washed by PBS twice, fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes, and permeabilized by 0.2% Triton X-100 for 2 minutes. Endogenous peroxidase was quenched by 1% H2O2 (v/v) in methanol for 30 minutes. After blocking in the TBS buffer containing 0.1% gelatin, 5% normal horse serum, and 0.5% Tween 20 for 30 minutes, the membranes were incubated with the primary antibody for 1 hour at 4°C overnight. After washing the membranes with TBS, the membranes were incubated with secondary antibody for 60 minutes before reacting with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) for 45 minutes. Color was developed by using DAKO liquid 3,3'-diaminobenzidine substrate-chromogen system (DAKO Co., Carpinteria, CA). The antibodies used in the experiment were mouse anti-smooth muscle actin (SMA; DAKO) and mouse anti-E-cadherin (Zymed Laboratories, Inc., South San Francisco, CA).

Table 1. Primer sequences for PCR

<table>
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<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR product (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Origin</th>
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<td>CCTACATGCTTAGCTTGATCGGAGC</td>
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<td>hBMP7</td>
<td>ACGTCTTGGTTAAGAGGTGTTCT</td>
<td>TGGGTTTGTAGGAGGTAGTCAAG</td>
<td>572</td>
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<td>Parallel et al. (52)</td>
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<tr>
<td>hBMP8a</td>
<td>GCATCTACAGAGCACCAATG</td>
<td>TAGAGTTTCTCTCCGATGG</td>
<td>1401</td>
<td>60</td>
<td>Ide et al. (17)</td>
</tr>
<tr>
<td>hBMP8b</td>
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<td>TTTCTACCCCTCTACACACT</td>
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<td>60</td>
<td>Ide et al. (53)</td>
</tr>
<tr>
<td>hActRI</td>
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<td>CTGTAGCTCTGGGATGTA</td>
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<td>58</td>
<td>Vanttinen et al. (54)</td>
</tr>
<tr>
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<td>This study</td>
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<td>59</td>
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<td>AACCATTAGGTAAGGCTTGGAGGAGG</td>
<td>355</td>
<td>59</td>
<td>Jester et al. (55)</td>
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<tr>
<td>hE-cadherin</td>
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<td>CTCGTTGAGGCTGGTGGAGGAGAGA</td>
<td>745</td>
<td>60</td>
<td>This study</td>
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Immunoprecipitation. Whole cell lysates (600 μg) were precleared by incubating with 0.25 μg normal goat IgG and 20 μL resuspended volume of the Protein A/G Plus-Agarose (Santa Cruz Biotechnology) at 4°C for 30 minutes. The beads were pelleted by centrifugation and the supernatant fluid was transferred into two fresh 1.5 mL microcentrifuge tubes in equal parts. To one of the tubes, 1 μg normal goat IgG was added and to the other tube 1 μg goat anti-Phospho antibody (Santa Cruz Biotechnology) was added. After 1 hour of incubation at 4°C, suspended volume (20 μL) of the Protein A/G Plus-Agarose was added into each sample. The tubes were incubated at 4°C overnight. The pellet was collected by centrifugation at 3,000 × g for 4 minutes and the supernatant was removed. After the pellet was washed with PBS for 4 times, 1× electrophoresis loading buffer (40 μL) was added to release the bound protein from the pellet. Each sample (10 μL) was boiled for 2 to 3 minutes and subjected to Western blot by using rabbit anti–Phospho antibody (Cell Signaling Technology).

Statistical analysis. All experiments were done in triplicates and repeated at least twice. Statistical comparisons were made using an unpaired, two-tailed t test.

Results

Bone morphogenetic protein 7 expression increases with the growth of primary prostate tumors. Prostate-specific knockout of Pten was found to cause prostatic adenocarcinoma in all of the lobes of the mouse prostate (21–23). Because the tumors in the anterior lobe (AP) are generally most prominent and visible, we first compared BMP7 expression levels in the AP lobe between Pten knockout mice and littermate controls of different ages. As shown by Western blot analysis, the development of primary prostate tumors in the Pten null prostate mice was associated with progressively increased expression of BMP7 over an age range of 1.5 to 7.5 months (Fig. 1A). From an 11-month-old Pten knockout mouse and its littermate control, we extracted proteins from different lobes, including AP, ventral lobe (VP), and dorsolateral lobe (DLP). The Western blot analysis showed that in all these lobes there was overexpression of BMP7 in the prostate tumor compared with the normal tissue (Fig. 1B).

Prostatic cell lines constitutively express bone morphogenetic proteins and bone morphogenetic protein receptors. Expression of three BMP family members (BMP2, BMP4, and BMP7) and that of the three type I and three type II receptor subtypes was determined by using reverse transcription-PCR (RT-PCR). A representative pattern of expression is shown in Fig. 1C. Of the three BMPs tested, BMP4 exhibited a relatively consistent expression profile in all of the cell lines, including nonneoplastic prostatic epithelial cell lines (MLC and BPH-1), prostate cancer cell lines (PC-3, LAPC-4, DU145, LNCaP, C4-2B, and CWR22R), and prostatic stromal cell lines (HPS-40F and HTS-40C). BMP2 and BMP7 expression was variable, although all of the cell lines tested positive (Fig. 1C). Accumulation of BMP7 protein was evaluated in five of the cell lines by Western analysis (Fig. 1D). The relative levels of secreted BMP7 generally followed the respective mRNA results, with PC-3 and DU145 cells displaying the lowest and the highest levels, respectively. The variant cell line C4-2B, derived from LNCaP, exhibited a pattern of expression very similar to LNCaP cells in terms of both RT-PCR and Western blot assays (data not shown). By RT-PCR, most of the cell lines tested were positive for the expression of both type I and II BMPRs. There were, however, two

![Figure 1](https://www.aacrjournals.org/doi/10.1158/0008-5472.CAN-05-0746-s1)

**Figure 1.** Detection of BMP7 expression in mouse prostate adenocarcinoma and analysis of expression of BMPs and BMPRs in human prostatic cell lines. A, the mouse urogenital system was surgically isolated, and the individual prostatic lobes were dissected out under a dissecting microscope (50, 51). Protein was extracted for Western blot in which the top band (55 kDa) corresponded to the size of BMP7. B, protein from individual lobes, AP, VP, and DLP, from an 11-month-old Pten conditional knockout mouse and its littermate control was used for Western blot. A or B, c, littermate control, homozygous for the floxed Pten allele; p<sub>−/−</sub>, homozygous deletion of the floxed Pten allele. C, RT-PCR assays for BMP2, BMP4, and BMP7, and each of the six known BMPRs, including three type I (BMPRIA, BMPRIB, and AcRi) and three type II (BMPRII, ActRII, and ActRIB) in the cell lines. D, conditioned medium from cells serum starved for 24 hours was concentrated and protein (25 μg) was subject to Western blot to examine the level of secreted BMP7 protein in five selected cell lines.
exceptions. Although BPH-1 cells produced detectable levels of two of the type I receptors (BMPRIA and BMPRIIB) and two of the type II receptors (BMPRII and ActRIIB), they had undetectable levels for the remaining type I (ActRI) and type II (ActRII) receptors. PC-3 cells, which were tested positive for all three type I and two type II receptors, had, however, the relatively lowest level of expression for BMPRII (Fig. 1C), although its presence could be detected by increasing the PCR cycle from 30 to 35 (data not shown). Overall, all prostatic cell lines of nontumorigenic epithelial or tumorigenic epithelial or stromal origin displayed two or more of each of the type I and II receptors. This is noteworthy because type I and II receptors are both indispensable for BMP signal transduction. Although not shown, the RT-PCR receptor expression pattern in C4-2B cells was comparable with LNCaP cells. In this work, we did not study BMPR protein expression. A previous report, however, described detection of BMPRIA, BMPRIIB, and BMPRIII by Western blot analysis in all four human prostate cancer cell lines (LNCaP, DU145, PC-3, and PC-3M) that were tested (20).

Bone morphogenetic proteins induce G0-G1 arrest in BPH-1 cells. We examined the effect of BMP7 on the growth of prostate cells in culture. Three cell lines, BPH-1, PC-3, and DU145, were selected for the study because they could sustain growth in medium with reduced amount of serum (0.5%) for a reasonable period of time. BMP7 (50 ng/mL) did not affect the growth of either PC-3 or DU145 cells. In contrast, the growth of BPH-1 cells was significantly inhibited, up to 44% on day 8 of treatment (Fig. 2A). The growth inhibitory activity of BMP7 in BPH-1 cell cultures was dose dependent at the range of 25 to 200 ng/mL (Fig. 2B). Thus, it was not inconsistent to find an effect of exogenous BMP7 when the factor was already produced constitutively in BPH-1 cells. To test whether this growth inhibition reflected attenuation of the cell cycle, BPH-1 cells treated with 50 ng/mL BMP7 for 4 days were subjected to cell cycle analysis. As shown in Fig. 2C, BMP7 significantly reduced the percentage of cells in the S-G2-M phases of the cell cycle, indicating inhibition at the G0-G1 phase. BMP2 inhibited the growth of BPH-1 cells to an extent very similar to BMP7 (data not shown).

Bone morphogenetic proteins induce epithelial-mesenchymal transdifferentiation in PC-3 cells. Although the growth rate of the PC-3 cells was not significantly affected by exposure to BMP7, we observed a remarkable morphologic change in these cells as shown by the micrograph of cells treated with 50 ng/mL BMP7 for 4 days (Fig. 3A). The treated cells appeared to lose their polygonal shape, which is distinctive of epithelial cells, and to acquire an elongated spindle-shaped morphology, a phenotype more similar to fibroblastic cells. This switch was prolonged because the fibroblastic morphology persisted for a few days even after BMP7 withdrawal. Like BMP7, BMP2 (100 ng/mL) could also induce a similar morphologic change in PC-3 cells, but this was not the case for TGF-β1 at a concentration of 5 to 100 ng/mL (Fig. 3A). Furthermore, TGF-β1 did not counteract the morphologic changes induced by either BMP2 or BMP7 (data not shown). We then tested by semiquantitative RT-PCR whether alterations occurred in the expression of SMA and E-cadherin during the BMP7-induced morphologic changes in PC-3 cells. As shown in Fig. 3B, SMA mRNA was strikingly up-regulated as early as the second day of BMP7 treatment, whereas the opposite effect was observed with respect to E-cadherin mRNA. The results of immunohistochemical analyses of SMA and E-cadherin were consistent with the results of the RT-PCR analysis (Fig. 3C). Because SMA is a marker for myofibroblast and E-cadherin is a cell adhesion molecular marker for epithelial cells, the results were indicative of a phenomenon commonly called epithelial-mesenchymal transdifferentiation (EMT). BMP-induced EMT was, however, unique to PC-3 cells, because several other prostate tumor cell lines (BPH-1, DU145, LAPC-4, LNCaP, and C4-2B), which were also treated similarly to PC-3 cells, did not exhibit the morphologic or molecular characteristics of EMT.

The transdifferentiated PC-3 cells were examined for their mobility and invasive ability. PC-3 cells, treated with BMP7 (50 ng/mL) for 6 days, were subjected to wound healing and Matrigel chamber invasion assays. As illustrated in Fig. 3D, movement of the transdifferentiated cells into the wound line was found to be clearly faster than the control PC-3 cells. By 36 hours after wounding, BMP7-treated cells could move in to fill up the space by ~50%,
whereas only a few scattered cells were detected in the wound made in the control cultures. Similarly, in Matrigel chamber assays, penetration of the treated cells through the basement membrane material barrier was found to be 70% more efficient than the control cells during the overnight incubation period (Fig. 3E).

Bone morphogenetic proteins protect LNCaP and C4-2B cells against serum deprivation–induced apoptosis. Because LNCaP and its variant C-2B are most sensitive to serum starvation compared with BPH-1 and other prostate cancer cell lines, these two cell lines were selected for the analysis of the effect of BMPs on serum deprivation–induced apoptosis. These cells were cultured in medium containing 0.1% serum in the absence or presence of 50 ng/mL BMP7 for 6 days. As illustrated in Fig. 4A, serum-starved C4-2B cells appeared mostly rounded up and feeble. In contrast, the BMP7-treated, serum-starved cells appeared fairly robust (Fig. 4A). The same protective effect was observed with 100 ng/mL BMP2 but not with 10 ng/mL TGF-β1. To test whether the healthy morphology of the BMP7-treated, serum-starved cells reflected protection from apoptosis, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays were done. As shown in Fig. 4B, the survival effect of BMPs was significantly high as determined by the measurement of the cell death from apoptosis. For example, at the sixth day of serum starvation, 34% of LNCaP cells were apoptotic and C4-2B, being more sensitive than LNCaP, displayed 96% of cells being apoptotic. However, in the presence of BMP7, the percentage of apoptotic cells was reduced to 15% for LNCaP and 21% for C4-2B. C4-2B cells were also analyzed for the expression of a panel of proapoptotic and antiapoptotic factors. Of the Bcl-2 family members, we examined Bcl-2, Bcl-xl, and Bax, and from the IAP family, XIAP and survivin. Whereas Bcl-2 protein expression was not detected in the C4-2B cells, Bcl-xl, Bax, and XIAP, which were expressed, appeared not to undergo any major changes in expression levels when cultured in the absence or presence of BMP7 (Fig. 4C). In contrast, whereas there was a progressive decline in the level of survivin in the serum-starved control C4-2B cells to a value of 20% at the sixth day, the level was sustained to 60% at this end point in the presence of BMP7 (Fig. 4C and D). When serum-starved LNCaP cells were subjected to such analyses, no remarkable changes in the levels of survivin, XIAP, Bcl-xl, or Bax were observed in serum-starved cells with or without the presence of BMP7 (Fig. 4E and F). Like C4-2B, LNCaP cells did not produce detectable Bcl-2.

Bone morphogenetic protein treatment affects Smad signaling in prostate tumor cell lines. The kinetic and quality of Smad activation were determined in BPH-1, PC-3, LNCaP, and C4-2B, the prostate tumor cell lines in which we showed a definitive but variable biological effect of BMP7. Because BMP signaling is largely mediated by three specific regulatory Smads (i.e., Smad1, Smad5, and Smad8), we limited the analysis of Smad activation in prostate tumor cell lines only to these Smads. In BPH-1 cells, the analysis was further simplified because BPH-1 did not have detectable levels of Smad1 expression. Using an antibody

Figure 3. Changes in morphology and behavior of PC-3 cells by BMPs. A, light microscopy pictures illustrate the morphology of PC-3 cells after treatment with BMP7, BMP2, or TGF-β1 for 6 days at the indicated concentrations in medium containing 0.5% serum. B, semiquantitative RT-PCR was used to compare the RNA levels of SMA and E-cadherin between the control (−) PC-3 cells and those (+) treated with 50 ng/mL BMP7 for different time periods from 2 to 6 days. C, PC-3 cells were immunostained for SMA and E-cadherin after culturing for 6 days in the absence (Control) or presence of 50 ng/mL BMP7. D, representative light microscopy pictures to indicate increased motility into a wound line of PC-3 cells, which were exposed to 50 ng/mL BMP7 relative to the control cells. E, in Matrigel chamber invasive assay, PC-3 cells grown in the absence (Control) or presence of 50 ng/mL BMP7 for 6 days were seeded into the inserts coated with a layer of Matrigel basement membrane matrix. After 24 hours, the cells on the top chamber were removed and the cells that invaded to the bottom chamber were stained and counted.
that could react with phospho-Smad1 (Ser463/Ser465)/phospho-Smad5 (Ser463/Ser465)/phospho-Smad8 (Ser426/Ser468), we showed Smad activation by BMP7 in each of the cell lines tested. Two characteristics were obvious: (a) Smad phosphorylation, although variable in strength (strong in BPH-1 and PC-3 cells and weaker in LNCaP or C4-2B cells), was dependent on the dose of BMP7, and (b) whereas Smad phosphorylation induced by BMP7 in BPH-1, LNCaP, or C4-2B persisted for at least 24 hours after exposure, the strong induction noted at 1 hour in PC-3 cells was followed by a fairly rapid decline in signal intensity (Fig. 5A–D).

By comparing phospho-Smad reactivity in the Western blots of whole cell lysates and nuclear extracts of BMP7-treated BPH-1 or PC-3 cells, we determined that phospho-Smad was efficiently transported to the nucleus in these cells (Fig. 5F). As Western analyses for the two cell types were done on the same blot, it was curious to find each time that the electrophoretic mobility of Smad activation, we conducted immunoprecipitation assays. Immuno-precipitates collected with anti-Smad5 antibody, when Western blotted for phospho-Smad reactivity, showed that Smad5 was indeed phosphorylated in response to BMP7 exposure in both BPH-1 and PC-3 cells. To confirm the speculation of Smad5 being the major target of activation, we conducted immunoprecipitation assays. Immuno-precipitates collected with anti-Smad5 antibody, when Western blotted for phospho-Smad5 reactivity, showed that Smad5 was indeed phosphorylated in response to BMP7 exposure in both BPH-1 and PC-3 cells. Smad5 might also be activated in PC-3 cells could not be ruled out.

Discussion

Although much remains to be understood about the complexity of BMP signaling in cancer, there is, however, emerging evidence from a variety of tumor systems that the effects of BMPs are cell specific and could be either protumorigenic or antitumorigenic (31, 32). Consequently, results derived from the study of a cell system frequently could not be applied to other systems of the same cancer type. This contention is further affirmed by the results of our study. Although it is clearly documented that the expression of BMP7 increases with the growth of prostate cancer in the Pten conditional null model and that BMP7 is widely expressed in human prostatic cells, including epithelial, stromal, and cancer, the biological effect induced by BMP7 is varied and both dose dependent and cell type dependent. In addition to validating the
biological relevance for the study of BMP7 or other BMPs in the context of prostate tumorigenesis, the data presented here describe three different types of response that can be manifested in prostate tumor cells from exposure to BMP7 or BMP2.

The first type of response is growth inhibition noted in BPH-1, a benign prostatic hyperplasia cell line. This inhibition of proliferation was not seen on two prostate tumor cell lines, PC-3 and DU145, which were also tested. A previous report, however, described BMP7-induced inhibition of PC-3 and DU145 growth in a medium containing 1% serum (33). Besides the level of serum, which was 0.5% in our study, that study also used a 10-fold increased concentration of BMP7 (500 ng/mL) compared with what we tested. The degree of inhibition of BPH-1 growth was BMP dose dependent and was determined to be largely from G1 cell cycle arrest. In the absence of detection of Smad1 and Smad8, and with direct evidence from the immunoprecipitation experiment, we suggest that Smad5 phosphorylation may be responsible, at least partially, for the observed effect on BPH-1 cells. However, there are two anomalies in BPH-1 cells that remain to be addressed. First, although BPH-1 expresses two type I and two type II BMPRs, it lacks expression of one major BMP type I receptor (ActRI or Alk-2) and a type II receptor (ActRII). Second, the electrophoretic mobility of either Smad5 or phospho-Smad is different from the counterparts in several other prostate cancer cell lines examined. These two properties, whether separate or connected, may be a contributing factor in cell cycle arrest of the BPH-1 cells.

Antiproliferative effect of TGF-β on nonmalignant prostate epithelial cells but not prostate cancer cells has been reported

![Figure 5. Time course and the dose-dependent Smad activation by BMP7 in different prostate tumor cell lines (A) PC-3, (B) BPH-1, (C) C4-2B, and (D) LNCaP. Cells were treated with 50 ng/mL BMP7 for different time periods from 1 to 24 hours as indicated or treated for a fixed time (1 hour) at variable BMP7 concentrations up to 100 ng/mL. Cell lysates were subjected to Western blot analysis for phospho-Smad1,5,8 (p-Smad1,5,8), Smad1, and Smad5. In BPH-1 cells, Smad1 was not detected. E, nuclear translocation of phosphorylated Smad in BPH-1 and PC-3 cells. Cells were treated with 50 ng/mL BMP7 for 1 hour and the whole cell lysates (WCL) or nuclear extracts (NE) were obtained for Western blot analysis for phospho-Smad1,5,8. The absence (−) or presence (+) of BMP7 is indicated. F, electrophoretic mobility of both phospho-Smad1,5,8 and Smad5 bands was different between BPH-1 and PC-3 cells. The Western blot analysis was done in the same blot from a 4% to 12% gradient gel, so that the exact position can be compared by overlapping the films. G, Smad5 was identified as a Smad that is phosphorylated in BMP1 and PC-3 treated with BMP7 by immunoprecipitation. For the negative control, the normal goat IgG was added instead of goat anti-Smad5 antibody that was used to pull down the Smad5 protein.]

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earlier (34, 35), and in this regard, the similarity between BMPs and TGF-β is noteworthy, although limited to a single cell line at this time.

The second response relates to induction of EMT to PC-3 cells by BMP7 or BMP2. EMT was originally defined as a morphologic conversion occurring at specific sites in embryonic epithelia to give rise to individual migratory cells (36). Subsequently, it was shown that EMT could contribute to tumor progression, such as that of skin carcinoma and breast cancer (37). During EMT, epithelial cells lose their polygonal morphology and adhesive cell contact molecules (e.g., E-cadherin) and acquire fibroblastic-like characteristics, including elongated shape, expression of mesenchymal markers (e.g., SMA), and increased motility and invasiveness (38).

TGF-β-1 is a well-characterized inducer of EMT in renal tubular and mammary ductal epithelial cells (39, 40), and it is reported that BMP7 can counteract TGF-β-induced EMT in mouse skin injury, implying a potential cross-talk between BMP7 and TGF-β in the regulation of EMT (41). In the present work, we show that EMT can also be induced by BMPs in prostate cancer cells, at least in the PC-3 cell line with all of the expected phenotypic and biological attributes. It is also shown, however, that TGF-β1, which could not induce EMT to PC-3, could neither counteract BMP-induced EMT in these cells. In terms of BMP signaling, we detected a strong activation of Smad3, but the possibility of Smad1 activation as well could not be ruled out. Because BMP7 and BMP2 both could induce EMT, the ligand specificity for EMT in PC-3 is not strictly limited.

Finally, by using LNCaP and its variant C4-2B, we show another distinctive effect of BMPs, which is to render protection to stress-induced apoptosis. The C4-2B is an aggressive androgen-insensitive subline selected for propensity for metastases to the bone (42–44).

We used serum starvation to induce apoptosis, which was extensive at day 6 of culture. The antiapoptotic effect of BMPs (BMP7 or BMP2) was more pronounced in C4-2B cells (96% in controls versus 21% with BMP) than in LNCaP cells (34% versus 15%). Besides confirming BMP-induced activation of Smad, we also attempted to detect the expression pattern of a series of proapoptotic and antiapoptotic proteins as may be altered by exposure to BMPs. Among the factors tested, survivin, a unique member of the IAP family (45, 46), could be a player in the protection. Although the protein expression levels for Bcl-xl, Bax, or XIAP remained comparatively even at corresponding time points between serum starved with or without BMP C4-2B or LNCaP cultures, there was a remarkable conservation of survivin level at the end point (day 6) in the presence of BMP7 in the C4-2B cells but not in the LNCaP cells. Recognizing that survivin is cell cycle regulated and mostly expressed at mitosis (47, 48), the decline of survivin level in the control cultures was likely to be caused by G1 arrest from serum starvation. Although the underlying mechanism for the rescue of survivin in C4-2B by BMP7 is currently unknown, there are two possibilities. First, BMP7 signaling may activate some pathways, Smad dependent or Smad independent, which can transcriptionally up-regulate survivin expression despite the cell cycle arrest. Second, because survivin is a relatively short-lived protein (1/2 = 30 minutes) and phosphorylation on Thr51 has been reported to increase protein stability (49), a post-translational stabilization of survivin by BMP7 signaling is also possible.

However, survivin may only explain a part of the variables, because expression of survivin is not sustained in LNCaP cells, which are also protected from serum deprivation–related apoptosis by BMPs.

In summary, our results suggest that BMPs, specifically BMP7 and BMP2, may be critical molecules in determining the fate of prostate cancer cells, thereby playing a significant role in prostate cancer progression. BMP signaling in prostate epithelial cells is found to be associated with Smad activation, although it is likely that Smad-independent BMP signaling may also be operative. Furthermore, findings with bone-derived prostate cancer cell lines, such as EMT induction in PC-3 cells and strong inhibition of stress-induced apoptosis in C4-2B cells, suggest that up-regulation of BMPs in prostate cancer cells or, by extension, presence of BMPs in the bone microenvironment may be significant contributors for the homing and survival of the cancer cells in the bone tissues.

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References


Bone Morphogenetic Proteins and Prostate Cancer


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