A Novel Bone Morphogenetic Protein Signaling in Heterotypic Cell Interactions in Prostate Cancer

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Abstract
We examined the effect of the extracellular bone morphogenetic protein (BMP) 2 and 7, which are up-regulated in the prostate adenocarcinomas of the conditional Pten deletion mouse model, on primary cultures of cancer-associated fibroblasts (CAF) derived from these tumors. In the CAF, we show that BMP2 or BMP7, but not transforming growth factor β-1, can strikingly stimulate secretion of stromal cell–derived factor-1 (SDF-1), also known as CXCL12. The CAF cells express type I and type II BMP receptors as well as the receptor for SDF-1, CXCR4. SDF-1 activation is associated with BMP-induced Smad phosphorylation, and the stimulatory effect is blocked by BMP antagonist, noggin. The findings that BMP treatment can increase SDF-1 pre-mRNA levels in a time-dependent manner and actinomycin D treatment can abolish stimulatory effect of BMP suggest a transcriptional modulation of SDF-1 by BMP signaling. Using a human microvascular endothelial cell line, we show that SDF-1 present in the conditioned medium from the stimulated CAF can significantly induce tube formation, an effect relating to angiogenic function. Furthermore, we found that BMP2 can also protect the CAF from serum starvation–induced apoptosis independent of SDF-1, implying that BMP may induce other factors to sustain the survival of these cells. In short, this report establishes a novel BMP-SDF-1 axis in the prostate tumor along with a new prosurvival effect of BMP that when considered together with our previously described oncogenic properties of BMP indicate a circuitry for heterotypic cell interactions potentially critical in prostate cancer.


Introduction
The stromal microenvironment of the tumor plays a critical role in support of the growth, survival, and dissemination of tumor cells. The majority of the stromal cells are fibroblasts and among them, the myofibroblasts or so-called “activated fibroblasts”, which are characterized by expression of α-smooth muscle actin (α-SMA), are most prominent in prostate cancer. The enriched population of myofibroblasts in the tumor stroma is associated with the production of increased levels of stromal cell–derived factor-1 (SDF-1), also called CXCL12 ligand (1, 2). SDF-1 is widely expressed by stromal cells from various tissues including dendritic cells, endothelial cells, pericytes, fibroblasts, and vascular smooth muscle cells from the skin; osteoblasts and endothelial cells from the bone marrow; and astrocytes and neurons from the brain (3–6). Two SDF-1 isoforms, SDF-1α and SDF-1β, have been identified in human and mouse. These two isoforms arise from a single gene through alternative splicing and the only difference is that SDF-1β contains a four amino acid COOH-terminal extension (7). SDF-1 is highly conserved between species. Human and mouse SDF-1α and SDF-1β are 99% and 97% identical in amino acid sequences, respectively. No differences have been reported between SDF-1α– and SDF-1β–regulated expression and biological activity.

SDF-1 binds primarily to CXCR4 cytokine receptor and the SDF-1-CXCR4 axis has a prominent role in regulating leukocyte and hematopoietic progenitor cell functions, including the survival and migration of hematopoietic progenitor cells to the bone during embryonic development (8, 9). Recent research has shown that SDF-1-CXCR4 axis also plays an important role in tumorigenesis by regulating tumor cell proliferation, survival, migration, and invasion (1, 10, 11). It has been shown that CXCR4 protein expression correlates with tumor grade in human prostate cancer and SDF-1 mRNA expression is elevated in metastatic prostate tumors although not in benign or localized tumors (12). Particular attention has been paid to the SDF-1-CXCR4 axis in prostate cancer bone metastasis, as SDF-1 is able to significantly increase the adhesion of human prostate cancer cells to the osteoblast and endothelial cell culture models, and to enhance their migration and invasion (13). In vivo, administration of a CXCR4-neutralizing antibody or blocking peptides significantly reduces the ability of PC-3 prostate cancer cells to metastasize and grow in bone (14). SDF-1 is also important in angiogenesis where it promotes vascular endothelial cell migration and induces capillary tube formation (15, 16).

Previously, we and other groups have shown that the aberrant expression of bone morphogenetic protein (BMP) is linked to prostate cancer progression and bone metastasis, and that BMP can promote prostate cancer cell survival and invasion, and stimulate angiogenesis (17–21). Thus far, however, these studies have been limited to the tumor cells only. In this study, we focus on the BMP signaling in the cancer-associated fibroblasts (CAF) and their heterotypic interactions in prostate cancer where BMP is found to be highly up-regulated and seem to facilitate both paracrine and autocrine circuits.

Materials and Methods
Isolation of CAF. The conditional Pten deletion/luciferase reporter activation (cPten–/−L) mice were monitored by bioluminescence imaging for the tumor growth (22) and then euthanized at desired time points to collect tumors. The tumor tissues were carefully dissected, minced with
crossed scalpels (size 11 blades), and cultured in B6s medium: DMEM (Invitrogen) supplemented with 5% fetal bovine serum, 5% Nu serum (BD Biosciences), 0.5 μg/mL R1881 (PerkinElmer), 5 μg/mL insulin (Sigma-Aldrich), and 1% penicillin/streptomycin. After 1 week of culturing, cells that migrated from the tissue clumps were trypsinized, transferred to new culture dishes, and allowed for surface attachment for a short period of time, varying from 1 to 10 min. Cells that were not attached were then removed, and the dishes with firmly attached cells were washed with PBS to further eliminate loosely attached cells. Based on the observations of the cell morphology, the cultures that contained most homogenous fibroblastic-like cells were expanded and used for experiments within 12 passages. The dorsolateral lobes of the prostate from littermate controls were similarly processed to obtain normal prostate fibroblast cultures.

Cell culture, recombinant proteins, SDF-1 neutralizing antibody, and conditioned medium. CAF cells were cultured in the same B6s medium that was used to isolate them. Human microvascular endothelial cells (HMVEC) were cultured in 131 Medium with Microvascular Growth Supplement (Cascade Biologics) in 0.1% gelatin coated dishes. The human cells (HMVEC) were cultured in Medium 131 with Microvascular Growth and conditioned medium.

fibroblast cultures.

littermate controls were similarly processed to obtain normal prostate secretions within 12 passages. The dorsolateral lobes of the prostate were then removed, and the dishes with firmly attached cells were washed with PBS and incubated with the serum-free medium (SFM) with 0.1% bovine serum albumin, 5 μg/mL insulin, and 0.5 μg/mL R1881 for 24 h. The debris in the conditioned medium was separated by centrifuge, and the aliquots of the supernatant fluid were stored at –80 °C. The SFM with the same supplements indicated above was used to serum starve the CAF for the apoptosis experiments.

Immunofluorescence analysis. Cells cultured on 4-well chamber slide (Nalge Nunc International) were washed with PBS twice, fixed with 4% (weight/volume) paraformaldehyde in PBS for 30 min, and permeabilized by 0.05% Triton X-100 in PBS for 10 min. After washing with PBS twice, slides were incubated in the TBS buffer containing 0.05% Tween and 3% normal horse serum for 30 min. Monoclonal SMA-Cy3 antibody (Sigma) was added at 1:200 dilution and allowed to bind at room temperature for 1.5 h. The chamber slides were then washed in PBS thrice and mounted with Vectashield mounting medium containing nucleus staining solution 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Western blot analysis. The tissue lysates and whole cell lysates were prepared as described before (18). The antibodies used in the Western blot experiments were goat anti-BMP2/4 (R&D Systems), rabbit anti-Smad5, anti–phospho-Smad1,5,8 (Cell Signaling Technology), and goat anti-actin (Santa Cruz Biotechnology).

SDF-1 ELISA. The mouse SDF-1α concentration in the conditioned medium from CAF was measured using a commercially available SDF-1 ELISA kit (R&D Systems). Because the number of the CAF cells and the volume of SFM used for conditioned medium preparation varied in different experiments, the ELISA results were further normalized by cell number and presented as total amount of SDF-1α secretion per one million cells (ng/million cells).

RNA preparation, semiquantitative, and real-time reverse transcription-PCR. Total RNA was extracted by TRIzol Reagent (Invitrogen, Inc.) or RNeasy Mini kit (Qiagen) after the protocols recommended by the manufacturers. DNasel (Ambion) was used to remove contaminants of genomic DNA in the RNA samples. The RNA (2 μg) was reverse transcribed by using iScript DNA Synthesis kit (BioRad). The synthesized cDNA was then subjected to PCR with the primers described in Supplementary Table S1. The optimal cycle that can reflect the amount of original template was determined and used in the semiquantitative PCR experiment at the indicated annealing temperatures. Real-time PCR was carried out using 13 μL of 2× Brilliant SYBER Green QPCR Master Mix (Stratagene) with 1 μL of cDNA in a total volume of 25 μL. The PCR conditions were as follows: 1 cycle of 95°C for 10 min; 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s; and 1 cycle of 95°C for 1 min and 55°C for 30 s. Reactions were carried out using the Stratagene MX3000P PCR machine, and the cycle thresholds were determined with its accompanying software. Actin was used for each sample as control. Real-time PCR primers are also listed in the Supplementary Table S1.

Tube formation assay. Forty-eight–well plates were coated with 150 μL Matrigel (BD Biosciences) at 4°C and were incubated for 1 h at 37°C. HMVEC cells (2 × 10^4) were added to the Matrigel-coated wells in 0.5 mL SFM or conditioned medium from fibroblasts. After 24-h incubation, cells were photographed under phase-contrast microscopy, and tubes were counted at low-power magnification from five randomly chosen fields in each well.

Apoptosis analysis (TUNEL). Cellular apoptosis was assayed using APO-BRDU kit (Phoenix Flow Systems) following the instructions of the manufacturer.

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

Results

BMP2 is overexpressed in prostate adenocarcinomas. Previously, we reported that BMP7 was strikingly up-regulated with the growth of primary prostate tumors (18) in the conditional Pten deletion (cPten−/−) mouse model (23, 24). Here, we analyzed the expression levels of two other BMPs, BMP2 and BMP4. We extracted proteins from the anterior lobe, the ventral lobe, and the dorsolateral lobes of tumor-harboring cPten−/− or cPten−/−L mice and the corresponding normal tissues of their littermate controls. In preliminary experiments, we found that the presence of the
reporter gene in \textit{cPten}^{-/-}L model did not significantly affect any of the expression analyses we described for the \textit{cPten}^{-/-} model. Considering this matter, we interchangeably used tissues from either of these models for this work. Western blot analysis of the dorsolateral lobes with antibodies that recognize both BMP2 and BMP4 revealed progressive overexpression of these proteins in parallel to tumor development. As shown in Fig. 1A, at age 1.5 months, when tumors are yet to form (18), there was no significant difference in the BMP2/4 protein level between the prostates from the experimental mice and their littermate controls. At age 3 months, BMP2/4 protein levels increased in tumors as illustrated for dorsolateral lobes. In a 12.5-month-old mouse, BMP2/4 protein expression was strongly up-regulated in tumors of all lobes (Fig. 1B). Because the antibody used in the Western blot was not able to distinguish between BMP2 and BMP4 protein, we used semiquantitative reverse transcription-PCR (RT-PCR) to detect the specific transcripts for BMP2 and BMP4 individually in a 13-month-old mouse model and its littermate control. As shown in Fig. 1C, BMP2 mRNA expression was consistently higher in every lobe of the tumor model compared with the normal prostate. No increase in BMP4 mRNA levels was detected in any of the tumor-bearing lobes of the prostate. Together, the results support a pattern of BMP2 overexpression, similar to that of BMP7, with the growth of primary prostate tumors.

**CAF cells express \alpha-SMA, BMPs, and BMP receptors.** We derived two primary cultures of CAF, CAF-1 and CAF-2, from tumors of \textit{cPten}^{-/-}L mice of two different ages, 4.5 and 7.5 months, respectively. The cultured CAF cells, exhibited fibroblastic cell morphology, and most cells were strongly positive for \alpha-SMA (Fig. 2A). In addition to the expression of BMP members (BMP2, BMP4, and BMP7), the expression of the three type I and three type II receptors was also examined by RT-PCR assays. A representative pattern of expression is shown in Fig. 2B. In both CAF-1 and CAF-2, mRNAs corresponding to BMP2, BMP4, and BMP7, and all six BMP receptors, were readily detected, although CAF-1 and CAF-2 varied in the expression levels of specific receptors. Although CAF-1

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Characterization of the CAF cells. A, photographs of live CAF cells were taken under a regular light microscope (left). Right, CAF cells were fixed and detected for the expression of \alpha-SMA by immunofluorescence. The Cy3-labeled \alpha-SMA antibody gives rise to red fluorescence in the cytoplasm, and the DAPI to blue fluorescence in the cell nuclei. B, RT-PCR assays for BMP2, BMP4, and BMP7, and each of the six known BMP receptors (BMPR), including three type I (BMPRIA, BMPRIB, and ActRII) and three type II (BMPRII, ActRIII, and ActRIIB) in the CAF. C, RT-PCR assays for SDF-1 and CXCR4 expression in CAF and HMVEC cells.
displayed a relatively higher level of BMPRIB, CAF-2 expressed more ActRI and ActRIIB.

**BMP2 and BMP7 stimulate SDF-1 secretion in CAF.** We examined the RNA expression of SDF-1 and CXCR4 in the CAF and in HMVEC and found that all of these cell cultures express SDF-1 and CXCR4 constitutively (Fig. 2C). To test a possible relationship between BMP and SDF-1 signaling in CAF, we measured SDF-1 secretion in our two CAF cultures after treatment with BMP2 or BMP7 or other extracellular signaling molecules that may potentially influence SDF-1 secretion, namely TGFβ-1, interleukin 8 (IL-8), and osteopontin. BMP2 or BMP7 was found to strongly up-regulate SDF-1 secretion in both CAF-1 and CAF-2 in a dose-dependent manner (Fig. 3A, a and C, a). The effect of BMP2 was stronger, with 6-fold up-regulation observed at the 200 ng/mL concentration (Fig. 3A, a). This was paralleled by phosphorylation of BMP-Smads, where BMP2 again was more effective than BMP7 (Fig. 3A, b and C, b), TGFβ-1 (Fig. 3A, a and C, a), IL-8, and osteopontin (data not shown), which were tested at various concentrations, could not significantly affect SDF-1 secretion. In the presence of noggin, which abolished the ability of BMP2 and BMP7 to cause Smad phosphorylation (Fig. 3B, b and C, b), the stimulatory effect on SDF-1 secretion was also similarly nullified (Fig. 3B, a and C, a). Although CAF-1 and CAF-2 seemed to respond similarly to BMP treatment, the basal level of SDF-1 was, however, determined to be 3- to 4-fold higher in CAF-1 compared with CAF-2. To test whether SDF-1 can also be up-regulated in the fibroblasts from normal prostate tissue, we tested a primary fibroblast culture from a normal control mouse and observed a very similar effect of BMP2 and BMP7 on these fibroblasts. The SDF-1 secretion is increased by BMP2 or BMP7 treatment in a dose-dependent manner, accompanied by phosphorylation of Smad that could also be blocked by noggin (Supplementary Fig. S1).

**BMP2 transcriptionally up-regulates SDF-1 expression.** When we measured the levels of SDF-1 mRNA by quantitative RT-PCR (qRT-PCR), BMP2 treatment for 24 h was found to increase the level by 1.9-fold in CAF-1 and 1.7-fold in CAF-2 (Fig. 4A). This indicated a transcriptional regulation. However, an alternative could be that the increased SDF-1 expression was due to mRNA stabilization. We addressed this issue by measuring the effect of BMP2 on SDF-1 pre-mRNA, which provides a close estimate of the transcription rate of the gene. Therefore, the SDF-1 transcripts were analyzed by qRT-PCR using primers that spanned an exon-intron boundary of this gene. DNaseI treatment and appropriate

**Figure 3.** BMP2 and BMP7 stimulate SDF-1 secretion and induce Smad phosphorylation in CAF-1 and CAF-2 cells. A (a), B (a), and C (a), conditioned medium (CM) from CAF-1 or CAF-2 cells with different 24 h treatments as indicated in the charts were assayed for SDF-1a concentration by ELISA, and the level of secreted SDF-1a, represented as nanogram per one million cells (ng/million cells), was compared among different groups by normalization with conditioned medium volume and cell number. A (b), B (b) and C (b), the same CAF cells that are used for ELISA were lysed and subjected to Western blot analysis to detect Smad phosphorylation and the expression of Smad5. Analysis of actin was used as a loading control.
controls without reverse transcription verified that genomic DNA was excluded from our RNA preparations (data not shown). As shown in Fig. 4B, BMP2 treatment increased the SDF-1 pre-mRNA level in a time-dependent manner, with a 2-fold stimulation observed after a short treatment for a period of 2 h. These results suggest direct effect of BMP2 on SDF-1 at the transcriptional level, we measured the effects of BMP-2 on SDF-1 mRNA and SDF-1 secretion in transcriptionally arrested CAF-1 cells. As shown in Fig. 4C, pretreatment with the transcriptional inhibitor actinomycin D abolished the stimulatory effect of BMP2 on SDF-1 secretion. These results suggest that actinomycin D was able to suppress BMP2 stimulated SDF-1 secretion (data not shown).

**Increased SDF-1 secretion by BMP2 treatment enhances the ability of CAF to stimulate microvascular tube formation.** Using a human dermal microvascular endothelial cell line, HMVEC, recombinant murine SDF-1α, and BMP2 were examined for their ability to stimulate HMVEC tube formation. As shown in Fig. 5A and B, BMP2 could not stimulate more tube formation than the SFM, whereas low concentrations (1 to 10 ng/mL) of SDF-1 increased the tube formation in a dose-dependent manner. At high concentrations (100 ng/mL), SDF-1 however displayed a rather inhibitory effect (data not shown). It would be important to note that the concentrations of SDF-1 (as determined by ELISA) in the conditioned medium from CAF in our experiments ranged from 0.2 to 0.6 ng/mL at basal level and to 1 to 6 ng/mL after BMP2/7 stimulation (data not shown). We then used the conditioned medium from the variously treated CAF-1 for the HMVEC tube formation assay. The conditioned medium from CAF-1 with BMP2 treatment, which contained a higher level of SDF-1, stimulated relatively more tube formation (45%) than the conditioned medium from nontreated or noggin-treated CAF-1.
cells, and this stimulatory effect was reversed when CAF cells were cotreated with BMP2 and noggin (Fig. 5A and C). The stimulatory effect was also abolished, as shown in Fig. 5D, when the HMVEC cells were cotreated with SDF-1–neutralizing antibody (20 μg/mL).

**BMP2 protects CAF from serum starvation induced apoptosis.** To examine whether BMP might confer survival advantage to CAF, we serum starved the CAF-1 and CAF-2 with or without BMP2 treatment and measured the apoptotic rate in these cells. As shown in Fig. 6A, serum starvation for 4 days induced 15% apoptosis in CAF-1, whereas BMP2 treatment significantly reduced this apoptotic rate to only 6%. At days beyond 4 days, most of these cells underwent apoptosis irrespective of the presence or absence of the added BMP in the medium. In CAF-2, which was found to be more resistant to serum starvation compared with CAF-1, the experiment could be extended to 6 days. The reasons for differences in the sensitivity for apoptosis between CAF-1 and CAF-2 are not clear at this time. Under serum starvation for 6 days, the apoptosis was 80% for CAF-2 but reduced to only 29% in the presence of BMP2 (Fig. 6B). This protective effect of BMP2 is independent of SDF-1 up-regulation, as SDF-1 alone could not significantly enhance the survival of CAF cells (Fig. 6A and B).

**Discussion**

Previously, we showed that BMP7 expression increases progressively with the growth of the prostate adenocarcinoma in the conditional Pten deletion mouse model (18). Here, we show that BMP2 expression is also increased during the progression of prostate tumor in this model. This new observation is consistent with the findings that describe elevated expression of BMP2 in human prostate cancer (20, 25–27). The most exciting finding in this report, however, is the observation that BMP can strongly induce in CAF the expression of SDF-1, a chemokine that functions as both chemottractant for cell migration and mitogen for cell proliferation and survival. By using two different CAF primary cultures, CAF-1 and CAF-2 isolated from 4.5- and 7.5-month-old animals, respectively, we have shown a similar response. Both CAF cultures exhibit myofibroblastic traits and a major difference between them is the basal level of SDF-1, which is higher in CAF-1 relative to CAF-2. They also differ in their sensitivity to serum starvation, CAF-1 being more susceptible to apoptosis. In contrast to BMP, we show that TGFβ-1, representing a major member of the superfamily to which BMP belongs, practically lacks the ability to induce SDF-1 in CAF. CAF cells are critical in prostate...
tumorigenesis, a point that was originally shown by the potency of human prostatic CaF, but not normal prostatic fibroblasts, to induce tumor formation from initiated, nontumorigenic human prostatic epithelial cells (28, 29). Of the multiple mechanisms by which CaF may support tumor growth, secretion of elevated levels of proteases, growth regulators, and extracellular matrix proteins have been implicated to date (30). In human breast and prostate cancer, several studies have shown that SDF-1 is overexpressed in CaF and can contribute to both tumor growth and angiogenesis (1, 30, 31).

The pivotal role of SDF-1/CXCR4 axis in tumor progression and metastasis has been widely recognized. However, the current knowledge on how SDF-1 may be regulated, especially in tumors, is very limited. It is reported that IL-17 induces the production of SDF-1, as well as the expression of SDF-1 mRNA, in cultured Rheumatoid fibroblast-like synovial fibroblasts in a dose-dependent manner (32). Interestingly, in a murine bone marrow stromal cell line, SDF-1 expression is found to be down-regulated by TGFβ-1, both at the mRNA level and at the protein level (33). In dermal wound healing, it has also been shown that IL-1 and tumor necrosis factor-α inhibit the expression of SDF-1 by human fibroblasts in vitro (34). The SDF-1 induction by BMPs that we describe in our study is not confined to only CaF. A similar effect is observed in the fibroblasts derived from normal prostate tissues. These results imply that regardless of the origin of the prostate fibroblasts, and despite the variation of the constitutive levels of SDF-1 in these cell populations, any increase in extracellular BMP is likely to positively induce SDF-1 expression in the prostate tissue microenvironment.

We show that supernatants from CaF cells treated with BMP2 correlated with increased in vitro capillary tube formation by human microvascular endothelial cells. Many previous studies have shown that SDF-1 induces tube-like structure formation in endothelial cells such as human umbilical vein endothelial cells, murine brain capillary endothelial cells, and endothelial progenitor cells (6, 15, 35). In our study, the increased level of SDF-1 secreted by CaF after BMP exposure is indeed shown to be a major contributory factor in tube formation, implying that BMP signaling in the fibroblasts may be an important factor for angiogenesis in the prostate tumor.

Another function of BMP observed in this study is that BMP2 can protect CaF cells from serum starvation-induced apoptosis. This effect seems to be practically independent of SDF-1. We have reported previously that BMP7 is able to inhibit serum starvation-induced apoptosis in the LNCaP prostate cancer cell line and, more remarkably, in its bone metastatic variant C4-2B, through up-regulation of survivin expression and c-Jun-NH2-kinase activation (19). Further investigation will be needed to determine if a similar or a different mechanism is involved in this protection of the CaF cells by BMP.

One important issue arising from this study is whether BMP functions in a paracrine or an autocrine fashion or both for the induction of SDF-1. Based on our results that were reported previously and studies from other groups, it is clear that prostate cancer cells do generally express high levels of BMP. Although we show that CaF cells also express detectable levels of BMP, we did not observe any significant inhibition of the basal level of SDF-1 by noggin treatment, indicating that the endogenous functional levels of BMP in CaF may not be sufficient to enhance SDF-1 expression. However, noggin treatment was found to induce increased apoptosis in CaF under the serum starvation condition (data not shown), which may suggest a possible protective mechanism of the endogenous BMP in relation to antiapoptosis signaling in the CaF. As CXCR4 is found to be present in the CaF, whether SDF-1 may also have autocrine effects on CaF is still another point that remains to be defined.

Taken together, the evidence for strong induction of SDF-1 by BMP in CaF along with the demonstrated expression of BMPR and CXCR4 in both prostate cancer and CaF cells, and expression of CXCR4 in endothelial cells seem to indicate potentially important heterotypic cell-cell interactions driven by both autocrine and paracrine mechanisms in prostate cancer. This newly identified BMP-SDF-1 axis as well as the SDF-1-independent protective function of BMP on the CaF that we describe highlight novel BMP signaling variables in the prostate cancer microenvironment. These results further underscore the contention that the intervention of BMP signaling activity may lead to a potential therapeutic treatment for prostate cancer.

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