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Prostate Cancer Cells and Bone Stromal Cells Mutually Interact with Each Other through Bone Morphogenetic Protein-mediated Signals*[S]

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Background: Prostate cancer cells interact with bone microenvironment during the process of metastasis.
Results: Bone morphogenetic proteins induce the production of Sonic hedgehog in prostate cancer cells, and this amplifies BMP signals in stromal cells.
Conclusion: Prostate cancer cells accelerate the osteoblastic differentiation of stromal cells.
Significance: Prostate cancer cells and stromal cells interact with each other through BMP-mediated signals.

Functional interactions between cancer cells and the bone microenvironment contribute to the development of bone metastasis. Although the bone metastasis of prostate cancer is characterized by increased ossification, the molecular mechanisms involved in this process are not fully understood. Here, the roles of bone morphogenetic proteins (BMPs) in the interactions between prostate cancer cells and bone stromal cells were investigated. In human prostate cancer LNCaP cells, BMP-4 induced the production of Sonic hedgehog (SHH) through a Smad-dependent pathway. In mouse stromal MC3T3-E1 cells, SHH up-regulated the expression of activin receptor IIB (ActR-IIB) and Smad1, which in turn enhanced BMP-responsive reporter activities in these cells. The combined stimulation with BMP-4 and SHH of MC3T3-E1 cells cooperatively induced the expression of osteoblastic markers, including alkaline phosphatase, bone sialoprotein, collagen type II α1, and osteocalcin. When MC3T3-E1 cells and LNCaP cells were co-cultured, the osteoblastic differentiation of MC3T3-E1 cells, which was induced by BMP-4, was accelerated by SHH from LNCaP cells. Furthermore, LNCaP cells and BMP-4 cooperatively induced the production of growth factors, including fibroblast growth factor (FGF)-2 and epidermal growth factor (EGF) in MC3T3-E1 cells, and these may promote the proliferation of LNCaP cells. Taken together, our findings suggest that BMPs provide favorable circumstances for the survival of prostate cancer cells and the differentiation of bone stromal cells in the bone microenvironment, possibly leading to the osteoblastic metastasis of prostate cancer.

Prostate cancer is one of the most common causes of cancer-related deaths in males. More than 80% of the patients who die of prostate cancer have bone metastases. In contrast to the bone metastasis of breast and other cancers, which form osteolytic lesions, bone metastasis of prostate cancer is characterized by osteoblastic appearance, which is caused by the dysregulation of bone resorption and bone formation (1). Bone metastases cause a number of complications, including severe bone pain, impaired mobility, compression of the spinal cord, and hypercalcemia, resulting in a poor quality of life in individuals with prostate cancer.

Cancer cells and bone microenvironments interact with each other through various growth factors (2, 3). Cancer cells produce angiogenic factors and bone-resorbing factors that enhance the proliferation of cancer cells themselves within the bone microenvironment. Moreover, bone is supposed to act as a repository for growth factors, including transforming growth factor (TGF)-β and bone morphogenetic proteins (BMPs).[3] These growth factors provide fertile ground where cancer cells can efficiently grow. In breast cancer, parathyroid hormone-related protein and interleukin (IL)-11, which are produced by cancer cells, activate osteoclasts by the receptor activator of nuclear factor-κB (RANK)-RANK ligand (RANKL) system. Activated osteoclasts in turn induce the release of bone-derived growth factors. This "vicious cycle" model can be regarded as an explanation for the bone metastasis of breast cancer cells. However, the molecular mechanisms responsible for the bone metastasis of prostate cancer remain to be identified.

BMPs, members of TGF-β family ligands, are multifunctional cytokines. BMPs transmit signals by binding to two types of serine-threonine kinase receptors, the type I and type II

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[3] The abbreviations used are: BMP, bone morphogenetic protein; HH, hedgehog; SHH, Sonic hedgehog; IHH, Indian hedgehog; DHH, Desert hedgehog; Gli1, GLI-Kruppel family member GLI1; Alp, alkaline phosphatase; siRNA, small interfering RNA.

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receptors (4). Activin receptor-like kinase (ALK)-1, ALK-2, ALK-3, and ALK-6 function as BMP type I receptors, and activin receptor (ActR)-IIA, ActR-IIB, and BMP receptor type 2 (BMPR-II) serve as BMP type II receptors. Upon ligand binding, two type I receptors and two type II receptors form a heteromeric complex, which transduces intracellular signals by phosphorylating BMP-specific receptor-regulated Smads (R-Smads), Smad1/5/8. Phosphorylated Smad1/5/8 form a heteromeric Smad complex with the common-partner Smad (Co-Smad), Smad4. This Smad complex translocates into the nucleus, regulates the transcription of various target genes, and exerts a broad range of biological activities (5).

BMPs have an essential role in normal skeletal development (6). BMPs induce the expression of many factors in bone stromal cells, such as Runx2 (7). BMPs are also detected in primary tumors and metastatic bone tumors in prostate cancer (8, 9). Thus, similar BMP-mediated regulatory mechanisms are assumed to be involved in the osteoblastic metastasis of prostate cancer. In the present study, the roles of BMPs in the interactions between prostate cancer cells and bone stromal cells were investigated.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human prostate cancer LNCaP cells, human prostate cancer CWR22 cells (gift of Dr. Wu Lily, University of California at Los Angeles, Los Angeles, CA), human breast cancer MDA-MB-231 cells, and human myeloma U266 cells were cultured, as previously described (10–13). Mouse stromal MC3T3-E1 cells were cultured in α-modified minimum essential medium (αMEM; Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 50 units/ml streptomycin (Invitrogen), and 50 μg/ml penicillin G (Invitrogen). All cells were grown in a 5% CO_2 atmosphere at 37 °C. BMP-4, BMP-6, BMP-9, TGF-β3, activin A, and N-terminal Sonic hedgehog (SHH) were obtained from R&D Systems, Inc. (Minneapolis, MN). BMP-4 and SHH were used at concentrations of 10 ng/ml and 500 ng/ml, respectively, unless otherwise mentioned. Cycloheximide (CHX) and SANT-1 were obtained from Sigma-Aldrich Co.

Separate Co-culture and Cell Proliferation Assays—In separate co-culture experiments, LNCaP cells were seeded at a density of 5×10^5 cells in cell culture inserts (0.4 μm pore size; BD Biosciences, San Jose, CA) in αMEM containing 1% FBS for 1 day (top layer). MC3T3-E1 cells were seeded at a density of 2×10^5 cells/well in 6-well plates in αMEM containing 1% FBS for 1 day (bottom layer). Cell culture inserts with LNCaP cells were transferred into 6-well plates with MC3T3-E1 cells and maintained in αMEM containing 1% FBS in the presence of BMP-4 for 7 days with media changes every 3–4 days. Then, MC3T3-E1 cells were trypsinized, and the cell number was counted.

Mixed Co-culture and Cell Proliferation Assays—Green fluorescent protein (GFP) was introduced into LNCaP cells (LNCaP-GFP cells) with defective lentiviral vectors, as previously described (14). LNCaP cells (8×10^5 cells/ml) or LNCaP-GFP cells (8×10^5 cells/ml) and MC3T3-E1 cells (2×10^5 cells/ml) were mixed in αMEM containing 1% FBS. Resuspended cells (2 ml) were seeded in 6-well plates in duplicate. Twenty-four hours after seeding, cells were stimulated with BMP-4 for another 72 h in the absence or presence of SANT-1. Cells were trypsinized, and the number of GFP-positive cells was counted.

Quantitative Real-time Reverse Transcription-PCR (RT-PCR)—Extraction of total RNAs, synthesis of first-strand cDNAs, and quantitative real-time RT-PCR were performed as previously described (15). The primer sequences are listed in supplemental Table S1. All samples were run in duplicate for each experiment. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. Data are presented as means ± S.D.

Immunoblotting—Immunoblotting was performed as previously described (16). Anti-Smad1 antibody and anti-Smad1/5/8 antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-phospho-Smad1/5 antibody (Ser463/465) was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Anti-α-tubulin antibody was purchased from Sigma-Aldrich.

Enzyme-linked Immunosorbent Assays (ELISA)—The amounts of the hedgehog (HH) proteins in cell culture supernatants were determined by ELISA with a SHH ELISA kit (Abcam plc, Cambridge, MA), an Indian hedgehog (IHH) ELISA kit (Uscn Lifescience, Inc., Wuhan, China), and a Desert hedgehog (DHH) ELISA kit (Uscn Lifescience, Inc.). All samples were examined in duplicate for each experiment.

Transfection and Luciferase Reporter Assays—MC3T3-E1 cells were seeded in duplicate at a density of 7×10^4 cells/well in 12-well plates and transfected with BMP-responsive elements containing a luciferase reporter construct (BRE-Luc) with a FuGENE HD transfection reagent (Roche Diagnostics). Twenty-four hours after transfection, cells were stimulated with SHH for another 36 h. Then, the cells were stimulated with BMP-4 for another 12 h, and luciferase activities were measured as previously described (17). Values were normalized with *Renilla* luciferase activity under the control of a cytomegalovirus promoter.

Small Interfering RNA (siRNA)—The transfection of siRNA targeting Smad4 was performed as previously described (18). siRNA duplex oligoribonucleotides against human *SMAD4* (Stealth RNAi VHS41700; siSMAD4) or control siRNA (Stealth RNAi 46–2002; siNTC) were synthesized by Invitrogen. LNCaP cells were transfected with 100 pmol of either siSMAD4 or siNTC with 3 μl of Lipofectamine 2000 (Invitrogen) per well of 6-well plates.

Osteoblastic Differentiation and Alkaline Phosphatase (ALP) Staining—The osteoblastic differentiation of MC3T3-E1 cells was induced by BMP-4 in αMEM containing 1% FBS. The activity of ALP in MC3T3-E1 cells was estimated by an ALP Staining kit (Sigma-Aldrich) 7 days after differentiation.

RESULTS

BMP-4 Induces the Production of SHH in Prostate Cancer Cells—Numerous BMP target genes have been identified in many types of cells (4, 5). Among them, HH family genes, which are targets of BMPs in certain types of cells, play a crucial role in skeletal development (19–21). In this study, we first examined

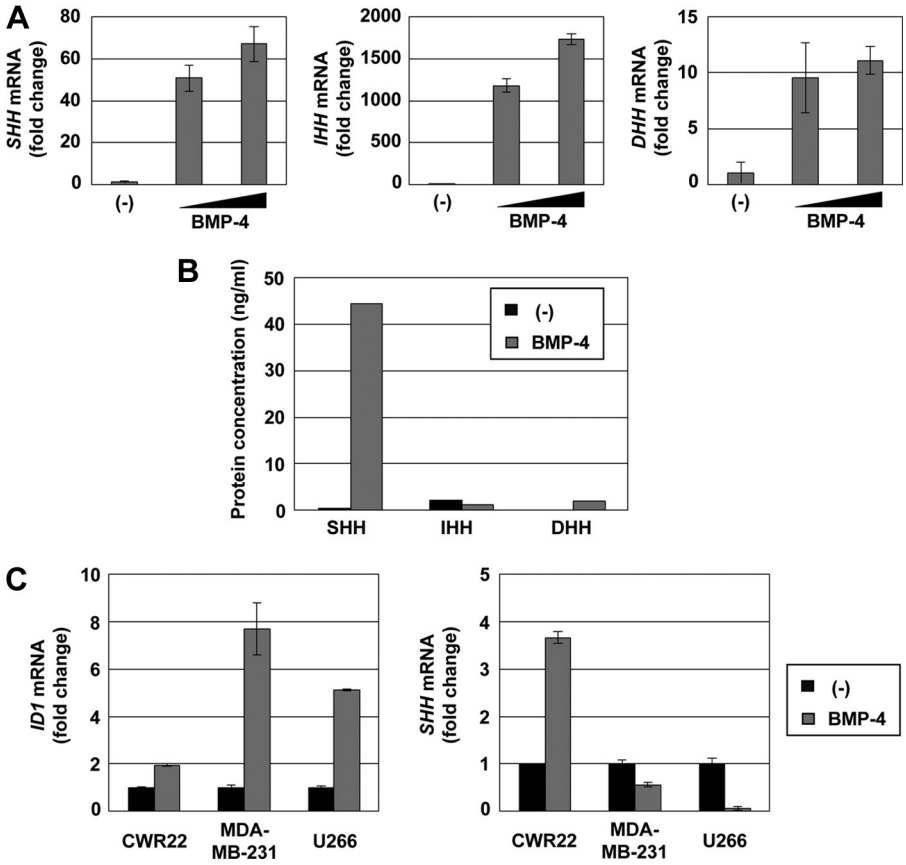

FIGURE 1. **BMP-4 induces the production of SHH in prostate cancer cells.** *A*, LNCaP cells were treated with BMP-4 (10 or 100 ng/ml) for 4 days. Expression levels of HH mRNAs were determined by quantitative real-time RT-PCR. *B*, LNCaP cells were treated with BMP-4 for 7 days. Supernatants were harvested and subjected to ELISA. Protein concentrations of HHs are indicated in absolute concentrations. *C*, each type of cell was treated with BMP-4 for 4 days. Expression levels of *ID1* mRNA and *SHH* mRNA were determined with quantitative real-time RT-PCR. Data are presented as fold changes on a cell-by-cell basis with BMP-4 stimulation.

whether HH family genes were induced by BMP-4 in human prostate cancer LNCaP cells, which exhibited histological features of osteoblastic tumors within human bone implants (22). Quantitative real-time RT-PCR demonstrated that BMP-4 increased the transcription of all the members of the HH family, including SHH, IHH, and DHH, in LNCaP cells (Fig. 1*A*). Although increased levels of IHH protein and DHH protein in response to BMP-4 were not observed by ELISA, SHH protein levels were increased up to 44.4 ng/ml by BMP-4 in LNCaP cells (Fig. 1*B*). The transcriptional regulation of *SHH* mRNA by BMP-4 was also examined with other cancer cells that are able to metastasize to bone (Fig. 1*C*). *ID1* mRNA, one of the best characterized direct BMP target genes, was up-regulated by BMP-4 in the tested cells, indicating that BMP signals were activated in these cells. Similarly to LNCaP cells, expression of *SHH* mRNA was increased by BMP-4 in human prostate cancer CWR22 cells, which were reported to cause osteoblastic lesions when injected into immunodeficient rat tibia (23). In addition, we have examined breast cancer MDA-MB-231 cells and multiple myeloma U266 cells, the bone metastatic lesions of which are known to show osteolytic characteristics. Unlike LNCaP cells and CWR22 cells, the induction of *SHH* by BMP-4 was not observed in these cells. We thus postulated that the BMP-4-induced SHH production is specific to certain prostate cancer cells, which is an important characteristic for the development of osteoblastic metastasis.

BMPs Up-regulate the Transcription of SHH mRNA in LNCaP Cells in a Smad-dependent Manner—Next, we examined which types of TGF-β family ligands regulate the expression of *SHH* in LNCaP cells (Fig. 2*A*). BMPs can be classified into several subgroups, including the BMP-2/4 group, the osteogenic protein (OP)-1 group, and the BMP-9/10 group, each of which show differential binding properties to BMP type I and type II receptors (4). BMP-4 (BMP-2/4 group), BMP-6 (OP-1 group), and BMP-9 (BMP-9/10 group) induced the expression of *SHH* mRNA in LNCaP cells in a dose-dependent manner, although BMP-9 was less potent than BMP-4 and BMP-6. Other members of the TGF-β family, such as TGF-β3 and activin A, were unable to up-regulate the expression of *SHH* mRNA. To confirm the involvement of Smad-dependent signal transduction in the induction of *SHH*, endogenous Smad4 was knocked down in LNCaP cells. The expression of *SMAD4* mRNA was successfully silenced by siRNA targeting *SMAD4* (Fig. 2*B*), and the BMP-4-induced SHH production was attenuated in this condition. Time-course experiments revealed that expression of *SHH* mRNA was augmented after 24 h of treatment with BMP-4 in LNCaP cells (Fig. 2*C*). The induction of *SHH* mRNA by BMP-4 was abolished by the suppression of *de novo* protein synthesis by treatment with CHX, whereas the induction of *ID1* mRNA, a direct target of BMPs, was not (Fig. 2*D* and data not shown). These findings suggested that BMPs indi-

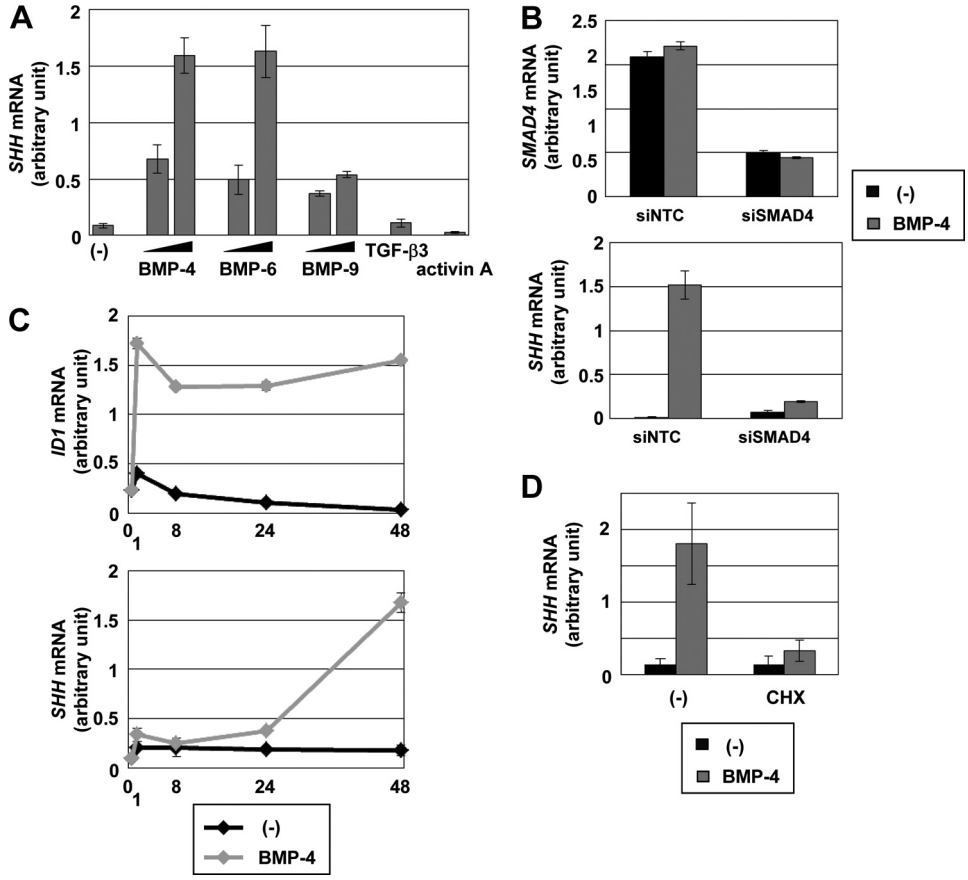

FIGURE 2. **BMPs up-regulate the transcription of *SHH* mRNA in LNCaP cells in a Smad-dependent manner.** *A*, LNCaP cells were treated with BMP-4, BMP-6, BMP-9 (10 or 100 ng/ml), TGF-β3 (1 ng/ml), or activin A (30 ng/ml) for 4 days. Expression levels of *SHH* mRNA were determined by quantitative real-time RT-PCR. *B*, LNCaP cells were transfected with either siNTC or siSMAD4. Then, the cells were treated with BMP-4 for 72 h. The expression levels of *SMAD4* mRNA and *SHH* mRNA were determined by quantitative real-time RT-PCR. *C*, LNCaP cells were treated with BMP-4 for 1 to 48 h. Expression levels of *SHH* mRNA were determined by quantitative real-time RT-PCR. *D*, LNCaP cells were pretreated with CHX (0.3 μg/ml) for 30 min. Then, the cells were treated with BMP-4 for 48 h. Expression levels of *SHH* mRNA were determined by quantitative real-time RT-PCR.

rectly regulate the transcription of *SHH* mRNA through the Smad-dependent pathway in LNCaP cells.

SHH Enhances BMP Signals in MC3T3-E1 Cells through the Induction of ActR-IIB and Smad1—Because BMPs are involved in the osteoblastic differentiation of stromal cells, we next studied the effects of SHH on BMP signals in bone stromal cells. As shown in Fig. 3*A*, treatment with SHH in stromal MC3T3-E1 cells enhanced the BMP-responsive reporter activity induced by BMP-4. Then, to assess the mechanisms by which SHH enhanced BMP signaling in MC3T3-E1 cells, expression levels of BMP signal components in MC3T3-E1 cells were examined by quantitative real-time RT-PCR (Fig. 3*B*). Among the BMP type I and type II receptors, the expression of ActR-IIB (*Acvr2b*) was up-regulated by SHH. Among the BMP-specific R-Smads and Co-Smad, the expression of *Smad1* was up-regulated by SHH. Immunoblotting also revealed an increase in Smad1 protein levels by SHH in MC3T3-E1 cells (Fig. 3*C*). Moreover, the BMP-4-induced phosphorylation of the C-terminal region of Smad1 (pSmad1) was enhanced in SHH-treated MC3T3-E1 cells (Fig. 3*C*). Thus, SHH seemed to amplify BMP signals in MC3T3-E1 cells through the induction of the BMP signaling components, ActR-IIB and Smad1.

LNCaP Cells Promote the Osteoblastic Differentiation of MC3T3-E1 Cells through BMP-induced SHH—To examine the role of SHH in osteoblastic differentiation, MC3T3-E1 cells were co-stimulated with BMP-4 and SHH. Quantitative real-time RT-PCR demonstrated that expression of the GLI-Kruppel family member (*Gli1*) mRNA, a well-known target of SHH, was increased by SHH (Fig. 4*A*). The up-regulation of *Id1* mRNA by BMP-4 was enhanced in SHH-treated MC3T3-E1 cells. Neither SHH nor BMP-4 affected the proliferation of MC3T3-E1 cells (Fig. 4*B*). However, several types of osteoblastic markers, such as *Alp*, bone sialoprotein (integrin binding sialoprotein, *Ibsp*), collagen type II α1 (*Col2a1*), and osteocalcin (bone γ carboxyglutamate protein, *Bglap*) were highly induced by co-stimulation with BMP-4 and SHH. ALP staining demonstrated a synergistic effect of BMP-4 and SHH (Fig. 4*C*), suggesting that MC3T3-E1 cells that were co-stimulated by BMP-4 and SHH further acquired osteoblastic phenotypes compared with those stimulated with BMP-4 or SHH.

To examine the role of SHH that is derived from prostate cancer cells, MC3T3-E1 cells were monocultured or co-cultured with LNCaP cells separately through the use of cell culture inserts (Fig. 5*A*). In the separate co-culture system, LNCaP cells in the top layer expressed high levels of *SHH* mRNA and

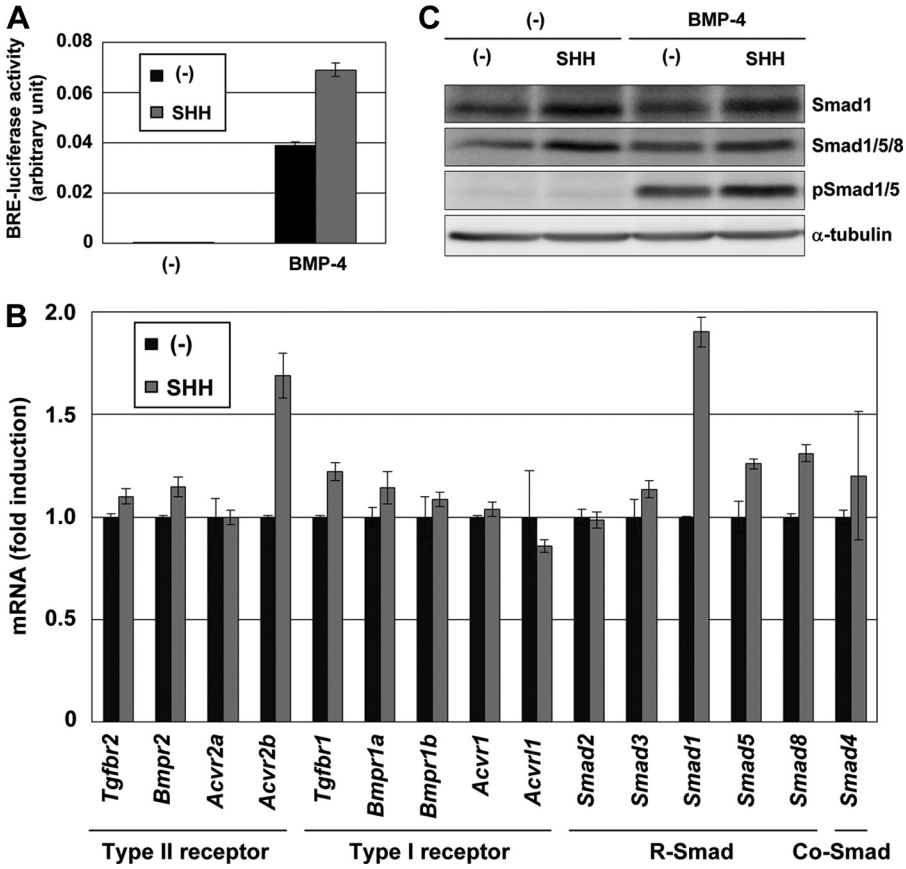

FIGURE 3. **SHH enhances the BMP signals in MC3T3-E1 cells through the induction of ActR-IIB and Smad1.** *A*, MC3T3-E1 cells were transfected with the luciferase reporter construct BRE-Luc. Then, the cells were treated with SHH for 36 h and treated with BMP-4 for another 12 h. Cell lysates were subjected to a luciferase reporter assay. *B*, MC3T3-E1 cells were treated with SHH for 48 h. Expression levels of TGF-β family signal components were determined by quantitative real-time RT-PCR. *C*, MC3T3-E1 cells were treated with SHH for 48 h. Then, the cells were treated with BMP-4 for 1 h. Cell lysates were subjected to immunoblotting with the indicated antibodies.

secreted SHH protein in response to BMP-4 (Fig. 5, *B* and *C*). Then, the SHH ligand may translocate through the pore (Fig. 5*C*) and act on MC3T3-E1 cells seeded on the bottom layer. As shown in Fig. 5*D*, the growth of MC3T3-E1 cells did not differ between the monoculture condition and the co-culture condition. However, the levels of *Gli1* mRNA in MC3T3-E1 cells were increased by BMP-4 when they were co-cultured with LNCaP cells (Fig. 5*E*). Co-cultured MC3T3-E1 cells with BMP-4 expressed a number of osteoblastic markers, including *Alp*, *Ibsp*, *Col2a1*, and *Bglap* at higher levels than cells monocultured with BMP-4. A cooperative effect of BMP-4 and LNCaP cells in the osteoblastic differentiation of MC3T3-E1 cells was also visualized by ALP staining (Fig. 5*F*). Next, to directly confirm the involvement of SHH during osteoblastic differentiation, LNCaP cells and MC3T3-E1 cells were treated with SHH inhibitors. LNCaP cells and MC3T3-E1 cells were mixed and co-cultured in single dishes in the presence of SHH inhibitors (Fig. 6*A*), and quantitative real-time RT-PCR using mouse specific primers was performed. Although the expression of *Gli1* mRNA, *Alp* mRNA, and *Ibsp* mRNA in MC3T3-E1 cells was apparently induced by BMP-4, this induction was attenuated by treatment with the SHH inhibitors, SANT-1 and itraconazol (Fig. 6*B* and supplemental Fig. S1). SANT-1 also diminished ALP activities in MC3T3-E1 cells that were separately co-cultured with LNCaP cells in the presence of BMP-4 (supplemental Fig. S2). These findings suggested that LNCaP cells produce the SHH ligand in response to BMPs and that BMPs and BMP-induced SHH cooperatively facilitate the osteoblastic differentiation of MC3T3-E1 cells. However, the induction of *Col2a1* and *Bglap* mRNA by LNCaP in the presence of BMP-4 was not suppressed by SANT-1, suggesting that the stimulation of the osteoblastic differentiation of MC3T3-E1 cells by LNCaP may be induced by SHH as well as by factor(s) other than the HH family proteins.

Proliferation of LNCaP Cells May Be Promoted by BMP-4-induced Growth Factors—Finally, we explored the cooperative role of bone stromal cells and BMPs in the growth and survival of prostate cancer cells with a mixed co-culture system (Fig. 7*A*). LNCaP-GFP cells were monocultured or co-cultured with MC3T3-E1 cells in the presence of BMP-4, and the proliferation of GFP-expressing cells was evaluated. BMP-4 inhibited the growth of LNCaP cells in the monoculture condition (Fig. 7, *B* and *C*). However, when LNCaP cells were co-cultured with MC3T3-E1 cells, the growth inhibitory effect of BMP-4 was abolished, and BMP-4 promoted the growth of LNCaP cells, suggesting that MC3T3-E1 cells support the growth and/or survival of LNCaP cells. Since the growth and survival of prostate cancer cells may be strongly dependent on many growth factors (24–26), we postulated that MC3T3-E1 cells provided certain types of growth factors for prostate cancer cells in

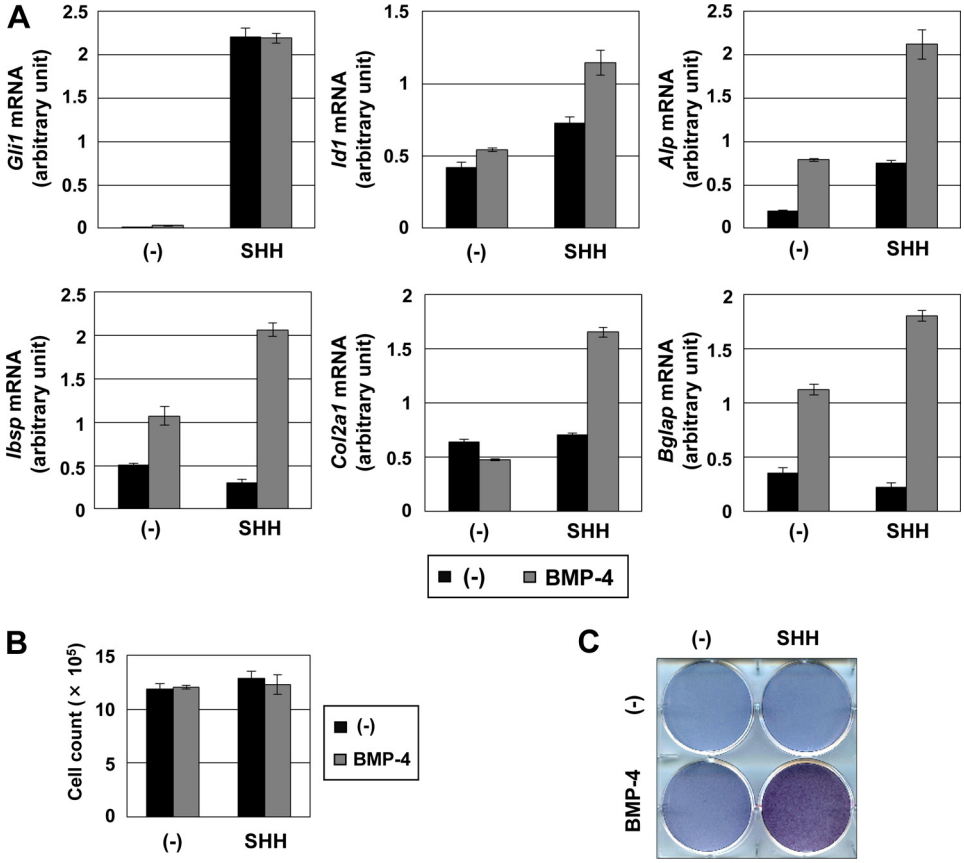

FIGURE 4. **Combined stimulation with BMP-4 and SHH cooperatively induces the expression of the osteoblastic differentiation of MC3T3-E1 cells.** *A*, MC3T3-E1 cells were treated with BMP-4 and SHH for 72 h. Expression levels of each mRNA were determined by quantitative real-time RT-PCR. *B*, MC3T3-E1 cells were treated with BMP-4 and SHH for 72 h. Cell number was counted and presented as means ± S.D. *C*, MC3T3-E1 cells were treated with BMP-4 and SHH for 7 days. ALP activities in the cells were determined by ALP staining.

response to BMPs. To identify these growth factors, expression levels of mRNAs for several growth factors were examined (Fig. 7*D*). Among the mRNAs we examined, expression levels of the mRNA for fibroblast growth factor-2 (*Fgf2*) and epidermal growth factor (*Egf*) in MC3T3-E1 cells were up-regulated by BMP-4 only when co-cultured with LNCaP cells. Furthermore, the cooperative effect of BMP-4 and MC3T3-E1 cells on the growth of LNCaP cells was abolished by treatment with an inhibitor of FGF or EGF signals (supplemental Fig. S3). These findings suggested that BMPs stimulate the release of growth factors from bone stromal cells, and these contribute to the growth and/or survival of prostate cancer cells in bone microenvironments.

DISCUSSION

In the present study, we presented a novel model for the BMP-mediated interactions between prostate cancer cells and bone stromal cells. In LNCaP cells, BMPs induce the production of SHH through the Smad pathway (Fig. 8*A*). SHH acts on MC3T3-E1 cells and enhances BMP signals, possibly through the induction of ActR-IIB and Smad1, which accelerate their differentiation into osteoblasts. BMP-4 also induces the production of growth factors in MC3T3-E1 cells, such as FGF-2 and EGF, which may enhance the proliferation of LNCaP cells (Fig. 8*B*). Thus, prostate cancer cells and bone stromal cells provide favorable circumstances for exhibiting their biological activities through BMP-mediated signaling (Fig. 8*C*).

Role of Prostate Cancer Cells on the Differentiation of Bone Stromal Cells—Increased serum levels of osteoblast proliferation markers, such as bone-specific ALP, has been reported in prostate cancer patients with bone metastasis (27). This finding strongly supports the possibility that prostate cancer cells secrete many factors that activate stromal cells in bone microenvironments (1). Prostate cancer cells produce endothelin-1, urokinase-type plasminogen activator, and prostate-specific antigen, and these stimulate the biological activity of osteoblasts (28). In addition, SHH, which is produced by prostate cancer cells, is known to promote the differentiation of osteoblasts (29). Here, we showed the important role of BMPs and BMP-induced SHH as inducers of the osteoblastic differentiation of bone stromal cells (Fig. 2, *A* and *C*). Our co-culture experiments demonstrated that BMPs and BMP-induced SHH cooperatively enhanced the differentiation of bone stromal cells (Fig. 5, *E* and *F*). BMPs and HH families have been reported to be co-expressed in various tissues, suggesting that signal crosstalk between BMP signaling and HH signaling may exist in certain physiological and pathological conditions (30). SHH initiates the expression of *Bmp2* in the mesoderm (31). During osteogenesis and chondrogenesis, SHH alters the response of cells to BMPs (32, 33). SHH has been reported to facilitate BMP-2-mediated Smad1 activation in osteoblasts (34). In addition to this signaling crosstalk, our findings suggested that SHH up-regulates the

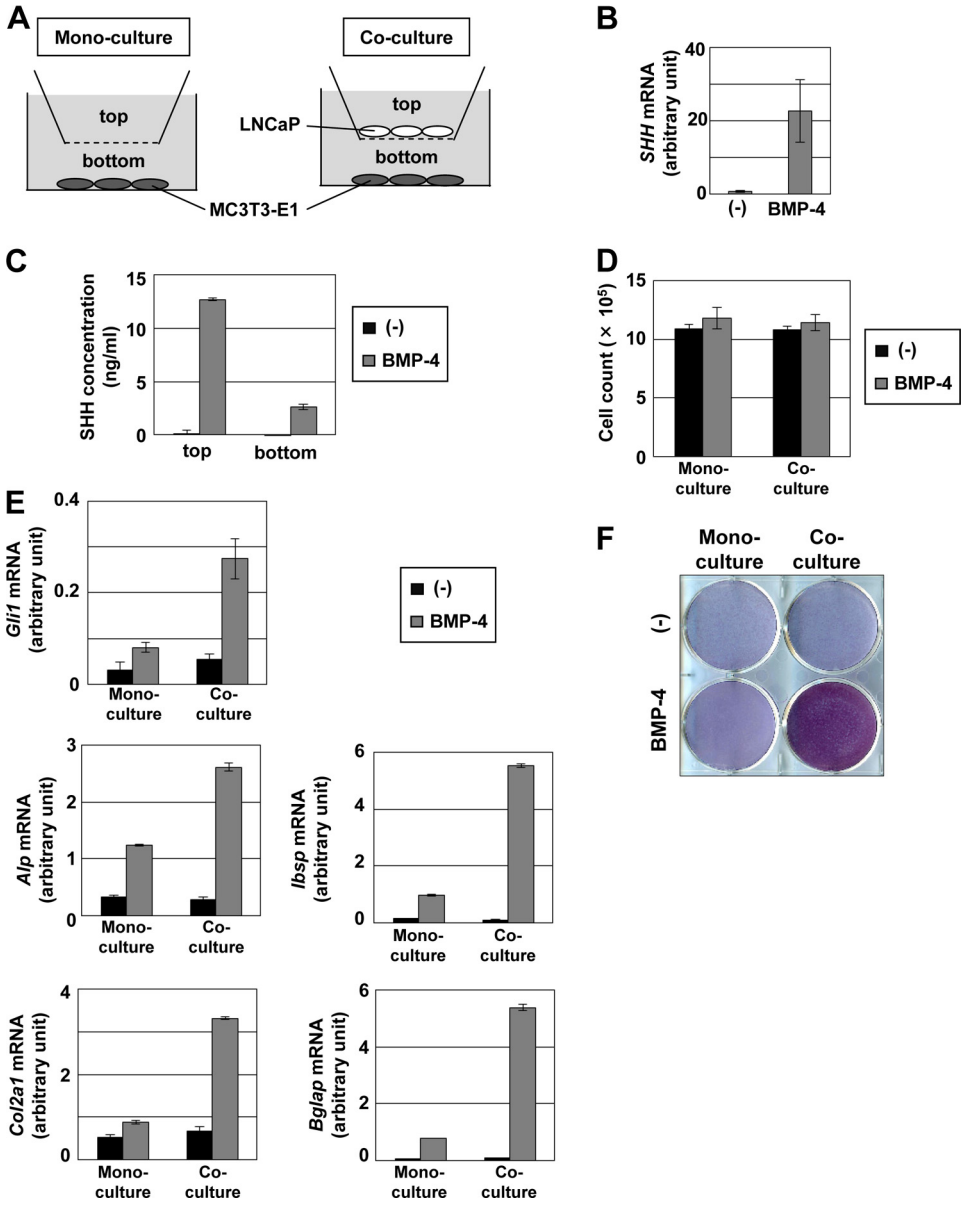

FIGURE 5. **Osteoblastic differentiation of MC3T3-E1 cells is enhanced by SHH induced by LNCaP cells in response to BMP-4.** *A*, schematic representation of the monoculture and the separated co-culture with cell culture inserts. *B*, LNCaP cells were treated with BMP-4 for 7 days on culture inserts in the presence of MC3T3-E1 cells. Expression levels of *SHH* mRNA in LNCaP cells were determined by quantitative real-time RT-PCR. *C*, LNCaP cells were treated with BMP-4 for 7 days on culture inserts in the presence of MC3T3-E1 cells. Supernatants of the top layers and the bottom layers were harvested and subjected to ELISA. The concentrations of the SHH protein are indicated in absolute concentrations and presented as means ± S.D. *D*, MC3T3-E1 cells were treated with BMP-4 for 7 days in the presence of culture inserts containing LNCaP cells. The number of MC3T3-E1 cells was counted and presented as means ± S.D. *E*, expression levels of each mRNA in the MC3T3-E1 cells in *D* were determined by quantitative real-time RT-PCR with mouse-specific primers. *F*, ALP activities in the MC3T3-E1 cells in *D* were determined by ALP staining.

expression of *Acvr2b* and *Smad1* and serves as an amplifier of BMP signals in bone stromal cells (Fig. 3).

HH family proteins have been recognized as key mediators in embryonic development and cancer progression (35). GLI1, a target of SHH, was originally identified as a gene that was expressed in human gliomas. Since then, mutations in other HH signaling components have been found in various malignancies. Since HH family members promote cell proliferation through the regulation of cyclin D, cyclin E, and Myc, SHH is regarded as a dominant oncogene in certain types of tumors. In the present study, we demonstrated that the expression of *SHH* mRNA in prostate cancer cells is induced by BMPs in a Smad-dependent manner (Fig. 2, *A* and *B*). The induction of *SHH* mRNA by BMP-4 was relatively slow and thoroughly diminished by CHX treatment (Fig. 2, *C* and *D*), suggesting that SHH is an indirect target of BMPs. Since the forced expression of ID1 did not increase the expression level of *SHH* mRNA in LNCaP cells (data not shown), transcription factor(s) other than ID1 are presumed to be involved. We also found that expression levels of GLI1 in LNCaP cells were not altered by BMP-4 (data not shown), indicating that BMP-4-induced SHH does not act on LNCaP cells autonomously.

Role of Bone Stromal Cells and BMPs on the Growth of Prostate Cancer Cells—Lang *et al.* demonstrated that culture media conditioned by osteoblast-like cells has the potential to stimu-

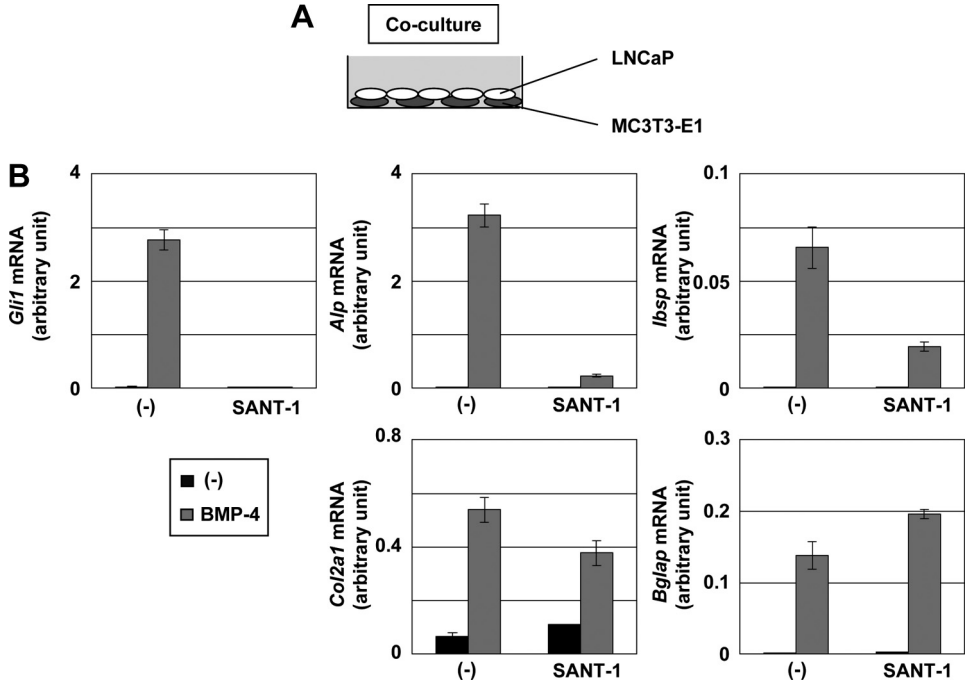

FIGURE 6. **LNCaP cells promote the osteoblastic differentiation of MC3T3-E1 cells through the BMP-induced SHH protein.** *A*, schematic representation of the mixed co-culture. *B*, LNCaP cells were co-cultured with MC3T3-E1 cells and treated with BMP-4 and SANT-1 (1 μM) for 72 h. Expression levels of each mRNA in MC3T3-E1 cells were determined by quantitative real-time RT-PCR.

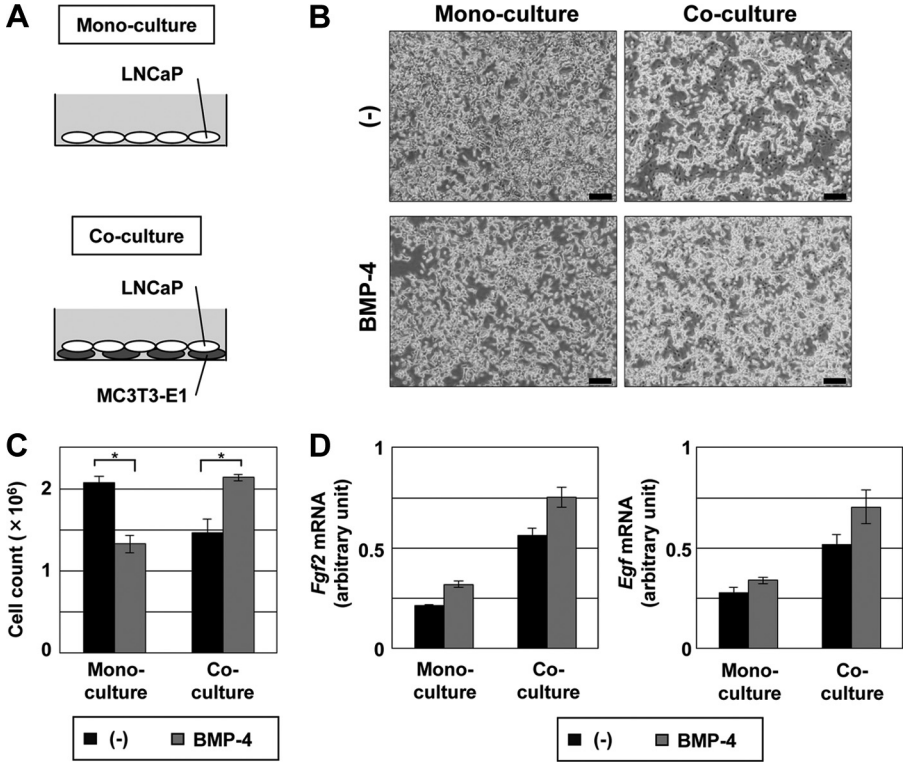

FIGURE 7. **Proliferation of LNCaP cells may be promoted through BMP-4-induced growth factors.** *A*, schematic representation of the monoculture and mixed co-culture. *B*, LNCaP-GFP cells were monocultured or co-cultured with MC3T3-E1 cells and treated with BMP-4 for 72 h. LNCaP-GFP cells were observed by phase-contrast microscopy. Scale bar, 200 μm. *C*, number of GFP-positive cells in *B* was counted and presented as means ± S.D. *, $p < 0.05$. *D*, expression levels of *Fgf2* mRNA and *Egf* mRNA in MC3T3-E1 cells in *B* were determined by quantitative real-time RT-PCR.

late the growth of prostate cancer cells, but not other types of cancer cells (36). Moreover, stromal cell-derived factors might determine the metastatic potential of prostate cancer cells (1, 37). These reports indicate that the proliferation of prostate cancer cells in bone microenvironments might be dependent on bone stromal cell-derived factors. In the present study, the functional roles of bone stromal cells on prostate cancer cells were studied using a co-culture system. Since BMPs are abun-

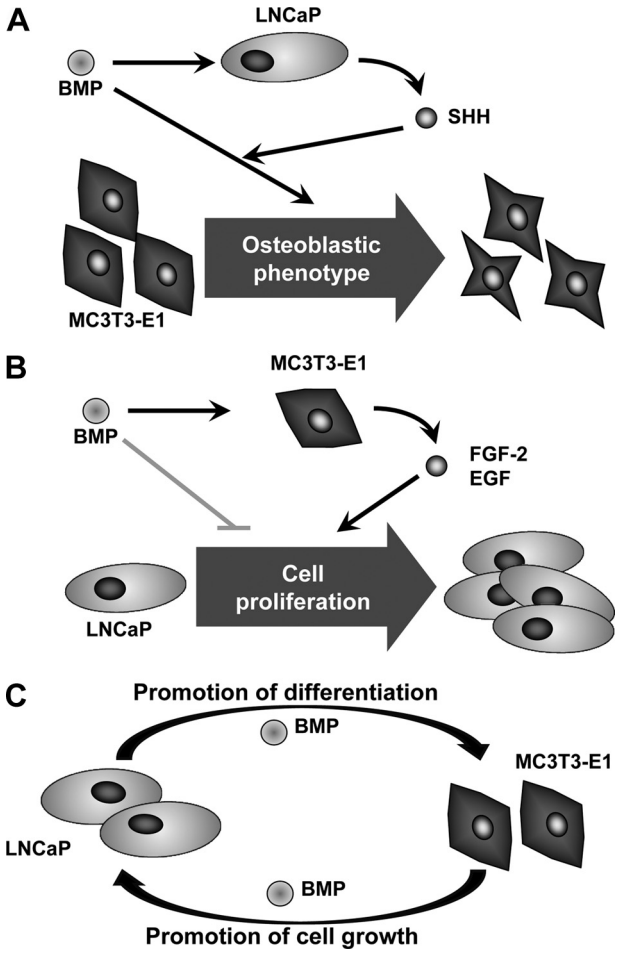

FIGURE 8. **BMPs and SHH cooperatively provide favorable circumstances for the growth and/or survival of prostate cancer cells and the differentiation of stromal cells.** *A*, BMPs induce the production of SHH through a Smad-dependent pathway. SHH enhances the BMP signals in MC3T3-E1 cells, possibly through the induction of ActR-IIB and Smad1. The osteoblastic differentiation of MC3T3-E1 cells was facilitated by SHH that was induced by LNCaP cells in response to BMPs. *B*, BMPs exhibit a growth-inhibitory action in LNCaP cells, whereas they promote the proliferation of LNCaP cells in the presence of MC3T3-E1 cells. *C*, BMPs provide favorable circumstances for the growth and/or survival of prostate cancer cells and the differentiation of bone stromal cells.

dant in bone microenvironments, a mixed co-culture system was utilized in the presence of BMPs. The proliferation of LNCaP cells was promoted by BMP-4 in the presence of MC3T3-E1 cells (Fig. 7, *B* and *C*). SANT-1 did not have an influence on the BMP4-mediated growth regulation of LNCaP cells, regardless of the presence or absence of MC3T3-E1 cells (data not shown), suggesting that SHH is not involved in these processes. FGF-2 and EGF have been identified as candidates that may account for the BMP-mediated growth promotion of prostate cancer cells (Fig. 7*D* and supplemental Fig. S3). However, our experiments also revealed that the co-stimulation of BMP-4 and SHH failed to promote the expression of FGF-2 and EGF (data not shown), indicating that LNCaP-derived factors other than SHH were required for the induction of FGF-2 and EGF in MC3T3-E1 cells by BMPs.

Recently, the roles of BMPs during the progression of prostate cancer have been documented. BMPs are regarded as promoters of prostate cancer metastases to bone tissues (38–42). High levels of expression of *BMP4* mRNA were not commonly observed in LNCaP cells and CWR22 cells (data not shown), suggesting that BMPs are derived mainly from bone microenvironments. The overexpression of the BMP antagonist noggin in prostate cancer cells inhibited bone metastasis in a mouse model (43). In the present study, BMP-4 indeed promoted the proliferation of LNCaP cells in the presence of MC3T3-E1 cells (Fig. 7, *B* and *C*). However, it must also be noted that BMP-4 inhibited the proliferation of LNCaP cells in the absence of bone stromal cells (Fig. 7*C*). Yang *et al.* reported diverse biological effects of BMP-7 on prostate cancer cells (44). BMP-2 is also known to elicit anti-proliferative effects in androgen-insensitive prostate cancer cells at low concentrations of FBS (10). These findings implicate the bidirectional roles of BMPs in the progression of prostate cancer. However, our present findings revealed that, in the presence of stromal cells, BMPs inhibited the proliferation of prostate cancer cells, suggesting that the inhibition of BMP signaling may have a therapeutic benefit in the treatment of bone metastasis of prostate cancer. The effects of BMPs on cancer cells might be context-dependent, and further studies are required to elucidate the roles of BMPs in the progression of prostate cancer.

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