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Overexpression of noggin inhibits BMP-mediated growth of osteolytic prostate cancer lesions

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Abstract

Introduction: Although a majority of metastatic prostate cancer lesions are osteoblastic in nature, some are mixed or lytic; and, osteoblastic lesions require osteolytic activity in order to progress. The role of BMPs in the formation of prostate cancer metastases to bone remains unknown. We hypothesized that BMPs influence the development and progression of osteolytic prostate cancer lesions.
Methods: RT-PCR and Western blot analysis were used to determine BMP receptor expression on the osteolytic prostate cancer cell line PC-3. Migration, invasion, and cellular proliferation assays were performed on PC-3 cells to quantify the effects of BMP-2, -4, and -7. In vivo, PC-3 cells were injected alone, with an empty retroviral vector, or with a retroviral vector overexpressing noggin, into the tibias of SCID mice. The animals were followed for 8 weeks, and histologic and radiographic analysis were performed at 2, 4, 6, and 8 weeks.
Results: BMP receptors are expressed on PC-3 cells, suggesting that they would be responsive to host BMP secretion. BMP-2, and to a lesser extent, BMP-4, stimulated PC-3 cell migration and invasion in a dose-dependent fashion. Noggin inhibited cellular migration and invasion of BMP-2 and -4 stimulated PC-3 cells. BMP-2 alone stimulated PC-3 cell proliferation, but BMP-4 had no effect. BMP-7 had no effect on proliferation, migration, or invasion. PC-3 cells implanted into SCID mouse tibias formed osteolytic lesions as early as 2 weeks and completely destroyed the proximal tibia by 8 weeks. Overexpression of noggin in PC-3 cells inhibited the expansion of the lesion in vivo.
Conclusions: BMPs influence the formation of the osteolytic prostate cancer metastases, and treatment modalities that inhibit BMP activity may limit the progression of the lytic component of prostate cancer metastases.
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Keywords: Osteolytic; Prostate cancer; Bone morphogenetic proteins

Introduction

Prostate adenocarcinoma is the most common malignancy in men, and it is currently the second leading cause of cancer death among men in the United States [23]. Prostate cancer metastases clearly have a propensity for bone as 80% of prostate cancer patients will develop bony lesions [21]. These bony lesions are a considerable source of morbidity, resulting in various clinical complications such as pathologic fractures, spinal cord compression, and intractable pain [11]. In addition, the survival rate of patients with metastatic disease is as low as 16% at 10 years, and the presence of bony lesions decreases the rate of survival [38,46]. Although the vast majority of prostate cancer lesions exhibit an osteoblastic phenotype, a small percentage of lesions exhibit a mixed lytic/blastic phenotype or a purely lytic phenotype

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[21,22,24]. In our laboratory, we have demonstrated that prostate cancer cell lines that exhibit these different phenotypes have considerably different cytokine profiles [26]. The pathophysiology of the development of prostate cancer metastases remains poorly understood, and therefore it has been difficult to identify appropriate treatment modalities for specific lesion types.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily [28]. BMP-2, -4, and -7 initiate cellular signaling through a serine/threonine kinase receptor complex [41]. There are currently three characterized BMP receptors: BMP-R Ia, Ib, and II [29,36,39,48]. Upon BMP-induced heteromeric complex formation, the Type II receptor phosphorylates specific serine and threonine residues, activating Type I receptors to initiate intracellular signaling through phosphorylation of Smad proteins [19,30]. Once phosphorylated, the Smad protein complex can bind to Smad4, which allows translocation into the nucleus. The Smad protein complex can alter gene expression patterns of BMP targets and influence the differentiation, proliferation, and migration of multiple cell types [47]. The release of BMPs from remodeling bone may signal tumor cells with BMP receptors to preferentially migrate to these metastatic sites.

The interaction between host bone and cytokines released from prostate cancer cells may be a key factor in determining the phenotype of a metastatic lesion. We had previously examined the cytokine profiles of prostate cancer cells involved in the formation of osteolytic and osteoblastic lesions [26]. Osteoblastic cell lines secreted BMP-2, BMP-4, osteoprotegrin, and TGF-β, factors that increase bone formation. Osteolytic cell lines produce receptor activator for NF-κB ligand (RANKL), IL-1, and TNF-α, which are associated with osteoclastogenesis, as well as BMP-4 and -7. Osteoblastic cells did not secrete RANKL. Although the expression profiles between osteoblastic and osteolytic cell lines differ, the molecular mechanisms that determine whether a lesion becomes osteolytic or osteoblastic are not known.

Noggin is a potent antagonist to BMPs that exerts its biologic effects by inhibiting the ability of BMPs to bind to their receptors [12,13]. During embryological development, noggin is expressed in conjunction with BMPs and has a key role in defining the final shape and size of BMP-derived structures such as bones and joints [5]. Heterotopic ossification can be inhibited by overexpression of noggin with a retroviral vector in a murine model [14]. In addition, recombinant noggin has been shown to be able to inhibit the oncogenic properties of non-small-cell lung cancer cells in vitro and decrease tumor size in vivo [25]. Thus, noggin has a critical role in regulating BMP activity in normal tissues, and inhibition of BMP activity in oncogenic cell lines that overexpress BMPs may be effective in inhibiting oncogenic properties displayed by these cell lines.

The purpose of this study was to determine the influence of BMPs on the development and progression of purely lytic prostate cancer lesions. We first examined the ability of osteolytic prostate cancer cells to respond to host BMPs by determining their BMP receptor expression, and the effect of BMP-2, -4, and -7 on tumor cell migration and invasion. We selected these particular BMPs because they have been noted in prior studies to modulate tumor cell biology [4,6,15,25,43,44]. We next examined the influence of BMP-2 on the rate of osteolytic prostate cancer cell growth in vivo. We used an SCID mouse intratibial injection model of prostate cancer metastasis to characterize the development of a purely osteolytic prostate cancer lesion [8]. Finally, inhibition of BMPs with noggin was performed both in vitro and in vivo to establish the role of BMPs in the formation and proliferation of osteolytic lesions.

Materials and methods

Cell lines

The human prostate cancer cell line PC-3 was used in this study. PC-3 cells were chosen because they are a very aggressive cell line that produces purely osteolytic lesions when introduced into bone [26,27]. LNCaP is a prostate cancer cell line that was used as a control for the cell proliferation assay. A549 is a non-small-cell lung cancer cell line that was used as a positive control as it has been found to express BMP receptors [25]. 293T cells were used as negative controls. Cell culture reagents were obtained from Gibco (Gibco BRL, Rockville, MD). PC-3 cells were maintained in tissue culture in RPMI medium with 10% fetal bovine serum (FBS). A549 and LNCaP cells were maintained in RPMI with 10% FBS. 293T cells were cultured in DMEM with 10% FBS.

RT-PCR

Total RNA for use in reverse transcriptase-polymerase chain reaction (RT-PCR) was harvested from cells in culture using Qiagen Rneasy Kit (Qiagen, Valencia, CA). Concentrations of RNA samples were determined measuring absorption in a light spectrometer at a wavelength of 260 nm. To obtain cDNA, 1 μg of total RNA was incubated for 10 min at 25°C with 1 μl (10 μM) random hexamers, 2 μl (5 mM) dNTP, 4 μl (25 mM $MgCl_2$), 1 μl (0.1 mM) DTT, 2 μl reverse transcriptase buffer, 1μl of RNA inhibitor, and 1 μl of superscript reverse transcriptase. Contaminating DNA was removed with DNase. The mixture was then incubated at 42°C for 50 min followed by 15 min at 70°C. 1 μl of RNAse H was added, and the RNA was degraded for 20 min at 37°C. PCR was performed with the primers and annealing temperatures as shown in Table 1. Reactions were performed with 2 μl of 10× buffer, 0.2 μl of 25 mM $MgCl_2$, 0.4 μl of 10 mM dNTP, 1 μl of forward and reverse primer, 0.4 μl of Taq polymerase, and 2 μl of cDNA. Samples were incubated at 95°C for 10 min followed by 30 cycles at 95°C for 30 s, 55°C for 1 min, 72°C for 1 min, and extension at 72°C for 10 min

Table 1
PCR primers

Gene	Upstream primer 5′–3′	Downstream primer 5′–3′	Base pairs	Anneal temperature
BMP-R-Ia	TAAAGGTGACAGTACACAGGAACA	TCTATGATGGCAAAGCAATGTCC	298	55°C
BMP-R-Ib	TACAAGCCTGCCATAAGTGAAGAAGC	ATCATCGTGAAACAATATCCGTCTG	211	55°C
BMP-R II	TCCTCTATCAGCCATTTGTCCTTC	AGTTACTACACATTCTTCATAG	457	55°C

in a Perkin-Elmer DNA thermal-cycler (Foster City, CA). GAPDH was used as a control for the quantity and integrity of the mRNA and cDNA in each sample. Samples were analyzed on a 1% agarose gel with 1% ethidium bromide. GAPDH served as a positive control.

Western blot analysis

Expression of the BMP-2 receptors (BMP-R Ia, BMP-R Ib, BMP-R II) on PC-3 cells was analyzed with Western blot analysis. Cells were grown in vitro and harvested with a single detergent cell lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.02% Na Azide, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% Triton X-100). The cell lysis buffer was added to the cells in culture plates and allowed to incubate for 20 min at 4°C. The cells were then scraped off of the bottom of the cell culture dish. The supernatant was transferred to a microfuge tube and spun at 12,000 rpm for 2 min at 4°C. Protein concentration was determined by spectrometry at 562 nm.

Cell lysate solutions were electrophoresed on a 15% SDS-PAGE gel and transferred to nitrocellulose at 34 V for 16 h at 4°C. The blots were then incubated overnight with the appropriate primary antibody (BMP-R Ia, BMP-R Ib, BMP-R II, R&D Systems, Minneapolis, MN) in Tris-buffered saline and 1% Tween and 5% nonfat dried milk. BMP receptor proteins were analyzed using an enhanced chemiluminescence system (Amersham, Arlington Heights, IL). An actin protein sample served as a control.

Migration assay

To determine the effects of BMPs on the migration of prostate cancer cells, 1×10^5 cells (PC-3 or 293T) were plated on the upper well of an 8 μm transwell migration chamber (Becton Dickinson, Bedford, MA) in 500 μl of the appropriate serum-free media. Recombinant BMP-2, -4, or -7 (R&D systems, Minneanapolis, MN) at the appropriate concentration (0, 1, 10, 100, or 500 ng/ml) was added to 500 μl of serum-free media in the bottom well. The cells were placed at 37°C for 48 h. To inhibit BMP-2, 100 ng/ml or 500 ng/ml of the BMP-2 was incubated with 10 or 50 μg/ml of the BMP-2 inhibitor noggin (R&D systems) in serum-free media at 37°C for 1 h prior to placing in the lower well. Cell count was determined at 48 h. The cells were washed with 500 μl 1% PBS and trypsinized with 50 μl of trypsin. The cells were then removed from the culture plate and placed in a 1.5 cm^3 centrifuge tube and spun at 3000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in 1 μl 1% PBS. The cells were spun again at 3000 rpm at room temperature for 5 min. The supernatant was removed again, and the cells were resuspended in a 1:1 mix of trypan blue and 1% PBS. A hematocytometer was used to count cells; cell count was expressed as cells/ml.

Invasion assay

An invasion assay was used to determine whether BMPs were able to enhance the ability of prostate cancer cells to invade through an extracellular matrix. The BD Biocoat Invasion Chamber with 8 μm pores (Becton Dickinson) was used for this experiment. 1×10^5 PC-3 cells were placed in 500 μl of serum-free media and placed in the upper well. 293T cells were used as negative controls. BMP-2, -4, or -7 at the appropriate concentration (0, 1, 10, 100, or 500 ng/ml) was added to 500 μl of serum-free media in the bottom well. The cells were incubated to 37°C for 48 h. To determine the effect of the BMP-2 inhibitor noggin, either 100 or 500 ng/ml BMP-2 was mixed with 10 or 50 μg/ml noggin for 1 h prior to addition to the bottom well of the invasion chamber. Cell counts were determined in the same manner as for the migration assay.

Cell proliferation assay

PC-3 (10,000 cells/well) or LNCaP (10,000 cells/well) cells were plated in wells and allowed to attach for 24 h in serum-free media. LNCaP was used as a positive control as previous studies have shown that BMP-2 and -4 can inhibit LNCaP cell proliferation [16,49]. Recombinant BMP-2, -4, or -7 was added to fresh serum-free media at a concentration of 0, 1, 10, 100, or 500 ng/ml and allowed to incubate for 48 h. Cell proliferation was determined at that time using Quick Cell Proliferation Assay (BioVision, Inc., Mountain View, CA). Results were reported as a percentage compared to untreated cells.

In vitro bone wafer pit assay

In vitro bone wafer pit assays were performed as previously described [7]. Briefly, cortical bone wafers ($n = 3$ per group) were cut from bovine femurs, sterilized by ultrasonication, and cultured in osteoclast media containing: minimal essential media with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, and 1% non-essential amino acids (Gibco). Homogenized bone marrow cell preps were isolated from 4- to 6-day-old rat pups by

mincing the femurs and tibias in the osteoclast media described above supplemented with 20 mM HEPES. Equal volumes of this cell suspension were plated with the wafers and incubated at 37°C for 20 min. Wafers were rinsed in warm sterile PBS then incubated with osteoclast media, changing the media every 3 days. As a positive control, wafers were treated for 10 days in the presence of parathyroid hormone 10^{-8} M. For the treatment groups, 20,000 PC-3 or PC-3 + RetroNog cells were added to each well and cultured for 10 days. Afterwards, a digital camera was used to capture an image of the wafer, and pits on the surface of the wafer were traced and the enclosed area determined using Osteometrics^{R} software (Osteometrics, Inc., Atlanta, GA).

Virus production and transfection

Noggin cDNA was amplified with PCR and cloned into a murine leukemia virus-based retroviral vector pCLX at the *Not*I and *Bgl*II sites resulting in CLNog. PCLX had been previously created from pLXSN (obtained originally from A. Dusty Miller of Fred Hutchinson Cancer Research Center, Seattle, Washington) by removing the SV40 promoter and the neomycin resistance gene and replacing the U3 in the 5′ LTR with the human CMV promoter as described by Peng et al. [34,35].

The CLNog vector DNA was converted into replication defective retrovirus by contransfection with calcium phosphate precipitation into the GP-293 cell line (Clontech, Palo Alto, CA) with a plasmid, pVSVG, that expressed vesicular stomatitis virus glycoprotein as the viral envelope [34,35]. Conditioned medium containing the retrovirus (RetroNog) was centrifuged at 3000 rpm for 5 min to remove cellular debris and then was stored at −80°C for future use.

PC-3 cells were transfected with retrovirus containing noggin cDNA (RetroNog). 1×10^5 PC-3 cells were plated on a sterile culture dish in RPMI medium with 10% FBS. The cells were washed once with 1% PBS, and 2 ml fresh medium containing the retrovirus at an MOI of 100 was added to the cells. The virus was allowed to incubate with the cells for 2 h, after which the virus containing media was removed. The cells were incubated at 37°C overnight and harvested for tibial injection 24 h after viral infection. The supernatants were preserved to assay for noggin production by Western blot. After transduction, the PC-3 cells were tested for the ability to express Noggin with use of an in vitro BMP-4 inhibition alkaline phosphatase assay as described by Hannallah et al. [14]. Serial dilutions of conditioned media, taken from flasks containing Noggin-expressing PC-3 cells, were used to inhibit the ability of BMP-4 to activate alkaline phosphatase in C2C12 cells (CRL-1772; American Type Culture Collection, Manassas, Virginia), a mouse myoblast cell line. Each BMP-4 inhibition alkaline phosphatase assay was performed in triplicate, and the average number of positive cells was used to calculate the concentration of Noggin expressed by the PC-3 cells. Known amounts of recombinant mouse Noggin (R&D Systems, Minneapolis, Minnesota) were used as a control.

Animals

Eight-week-old male severe-combined immunodeficient (SCID) mice were housed under pathogen-free conditions in accordance with the protocol approved by the Chancellor's Animal Research Committee at the author's institution.

Tibial implantation

1×10^5 cells of each cell type were prepared in 15 μl of 1% PBS with 15 μl of matrigel for each tibial injection. 30 μl of the PC3- or PC-3 + RetroNog matrigel mix was injected into the left tibia of 8- to 10-week-old SCID mice. The mice were anesthetized (100 mg ketamine/kg body weight, 10 mg xylazine/kg body weight). The overlying skin was prepped in sterile fashion with 70% ethanol and betadine. A 3-mm longitudinal incision was made over the patellar ligament with a no. 12 scalpel blade, and then a 2-mm longitudinal incision was made along the medial border of the patellar ligament to the tibial plateau. A 27 ½ ga. needle was introduced through the proximal tibial plateau and into the proximal tibia. 30 μl of the desired matrigel mixture was injected into the cavity. The wound was closed with a single 5-0 Vicryl suture (Ethicon Inc., Somerville, NJ). Animals were sacrificed at 8 weeks or earlier if the tumor became too large, in accordance with university protocol. At sacrifice, both hindlimbs were harvested for histological analysis.

Treatment groups

In this study, SCID mice underwent tibial implantation of either PC-3 cells alone, PC-3 cells transduced with an empty retroviral vector (PC-3 + EV), or PC-3 cells overexpressing noggin (PC-3 + RetroNog). There were a total of 25 mice in each group; 5 of each were sacrificed at 2, 4, and 6 weeks; and 10 were sacrificed at 8 weeks, or earlier if tumor size became greater than 1.5 cm or ulceration occurred.

Radiographic analysis

Prior to their sacrifice, animals were anesthetized, and radiographs were obtained using a Faxitron (Field Emission Corp., McMinnville, OR). Two independent reviewers blinded to the treatment groups analyzed the radiographs for the presence of osteolytic lesions according to a previously established protocol [8,30]. Radiographs were scored as follows: 0—normal; 1—lytic lesion present within the medullary canal only; 2—obliteration of one cortex; 3—obliteration of two cortices [26].

Histologic analysis

The tibias were fixed in 10% buffered formalin followed by decalcification in 10% EDTA solution for 2 weeks at room temperature with gentle stirring. Sections were cut and

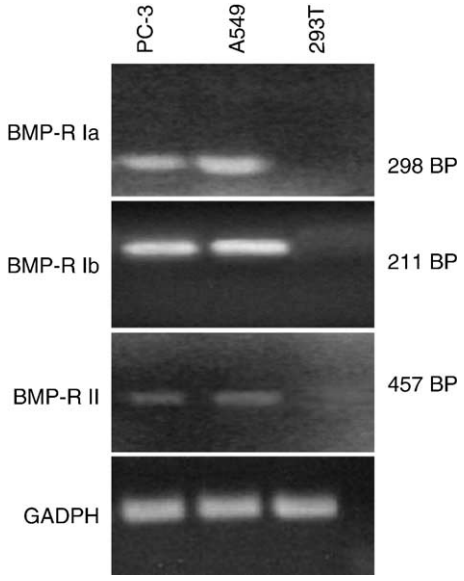

Fig. 1. RT-PCR analysis of BMP receptor expression. BMP receptor (BMPR-I, BMPR-IIA, and BMPR-IIB) mRNA was found in sufficient quantities to be amplified by RT-PCR in PC-3 cells. BMP receptor mRNA was also found in the non-small-cell lung cancer cell line A549. 293T cells did not express BMP receptors. GADPH was used as a control.

stained with hematoxylin and eosin and for OG for histologic and histomorphometric analysis. Tartrate-resistant acid phosphate (TRAP) staining was also performed to detect osteoclast number. Histomorphometric analysis was performed on an Olympus system (Olympus, Melville, NY). Bone area in the proximal tibia was calculated as a percentage of total area using BIOQUANT software (BIOQUANT Image Analysis Corp., Nashville, TN). The number of osteoclasts per millimeter of bone was determined by examination of TRAP-stained sections at 10× magnification. Tibias from every animal were analyzed in this manner.

Statistical analysis

P values less than or equal to 0.05 were considered to be statistically significant. The mean hindlimb tumor diameter values were assessed using one-way ANOVA. The radiographic data were assessed using χ^2 testing, with a kappa statistic calculated to determine interobserver agreement. The histomorphometric results were evaluated using one-way ANOVA.

Results

Expression of BMP receptors on PC-3 cells

BMP receptor Ia, Ib, and II expression was found in the PC-3 cells. 293T cells were used as a negative control, and A549 cells were used as a positive control as they had previously been shown to express BMP receptors [25]. All 3 subtypes of BMP receptor mRNA were demonstrated to be present in PC-3 cells as well as A549 cells (Fig. 1). GADPH was used as a positive control.

Protein expression of the BMP receptors Ia, Ib and II was confirmed with Western blot analysis. All three subtypes of BMP receptors were present in PC-3 cells and corresponded with previously published sizes for the BMP receptors (data not shown). There was no BMP receptor protein expression in 293T cells.

Effect of BMPs on PC-3 cell migration

We performed a migration assay to determine whether different BMPs stimulated the migration of the PC-3 cells in vitro. At lower concentrations (1 ng/ml, 10 ng/ml), recombinant BMP-2 had no significant effect on PC-3 cell migration compared to control 293T cells. At higher concentrations (100 ng/ml, 500 ng/ml), BMP-2 stimulated the migration of PC-3 cells significantly compared to control ($P < 0.001$; Fig. 2A). BMP-4 stimulated PC-3 cell migration only at 500 ng/ml compared to controls ($P < 0.01$ vs. 293T). BMP-7 had no effect on PC-3 cell migration at any concentration (P = NS vs. 293T). To determine if the inhibitor of BMPs, noggin, could inhibit PC-3 cell migration,

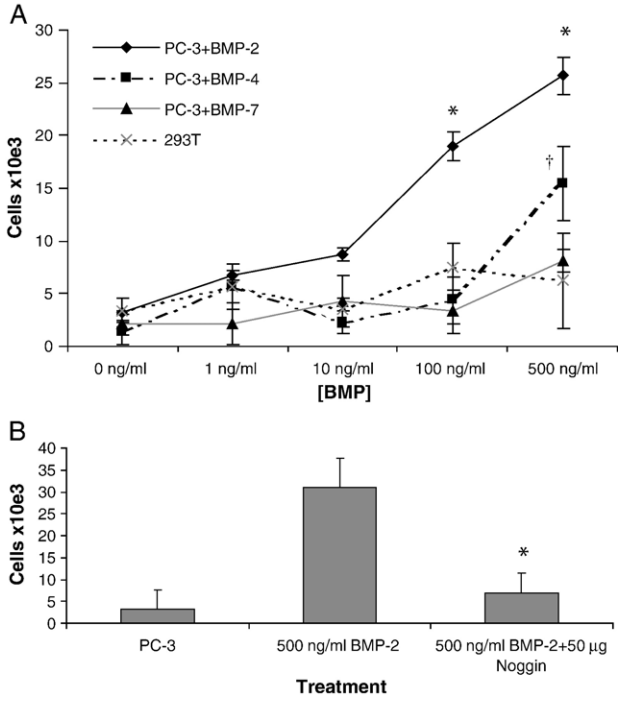

Fig. 2. Effects of BMPs on cellular migration. A migration assay was performed to determine the effects of BMPs on tumor cell migration on multiple cell lines (A). BMP-2 stimulated PC-3 cells to migrate in a dose-dependent fashion. This was statistically significant compared to control cells (293T) at 100 ng/ml and 500 ng/ml (*$P < 0.01$ vs. 293T). BMP-4 had a significant effect at 100 ng/ml and 500 ng/ml (†$P < 0.05$ vs. 293T cells), but BMP-7 had no effect at any concentration. (B) A migration assay was then repeated after BMP-2 was incubated with its inhibitor, noggin. PC-3 cell migration was significantly reduced after BMP-2 was treated with 50 µg/ml noggin at 500 ng/ml (*$P < 0.001$ vs. BMP-2 alone).

10 μg/ml and 50 μg/ml recombinant noggin was incubated with 1 ng/ml, 10 ng/ml, 100 ng/ml, and 500 ng/ml BMP-2 for the migration assay. At both doses, noggin significantly limited PC-3 cell migration at BMP-2 concentrations of 100 ng/ml and 500 ng/ml compared to BMP-2 alone ($P < 0.005$ at both concentrations; Fig. 2B). Both 10 μg/ml and 50 μg/ml recombinant noggin inhibited the effects of BMP-4 on PC-3 cell migration (data not shown).

Effect of BMPs on PC-3 cell invasion

An invasion assay was next performed to determine if BMPs were able to stimulate the invasion of PC-3 prostate cancer cells through extracellular tumor matrix. In the presence of BMP-2, PC-3 cells invaded through the extracellular tumor matrix in a dose-dependent fashion (Fig. 3A). This was statistically significant compared to negative control 293T cells at 100 ng/ml and 500 ng/ml of BMP-2 ($P < 0.05$ at 100 ng/ml; $P < 0.01$ at 500 ng/ml). BMP-2-mediated stimulation of cellular invasion was inhibited with 10 μg/ml and 50 μg/ml of its inhibitor noggin at BMP-2 concentrations of 100 ng/ml and 500 ng/ml ($P < 0.05$ at both concentrations; Fig. 3B). BMP-4 was also

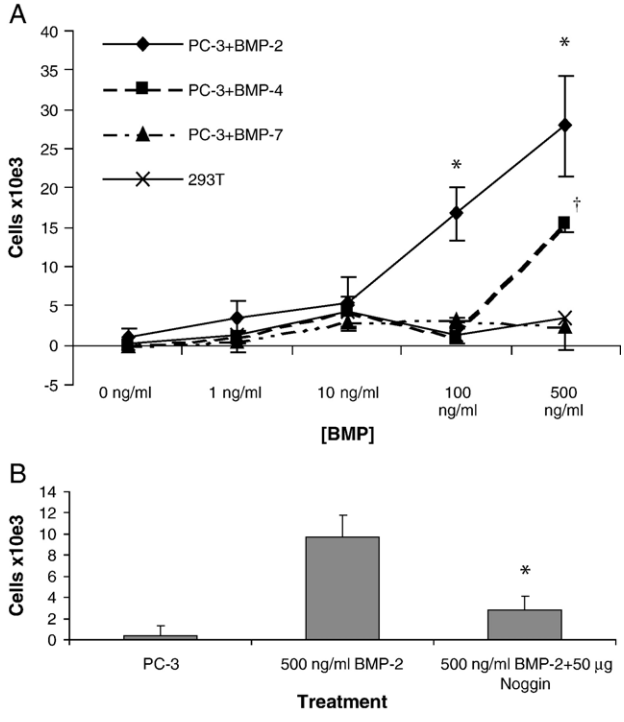

Fig. 3. Effects of BMPs on cellular invasion. (A) An invasion assay was performed to determine the effects of BMPs on the ability of PC-3 cells to invade through a tumor extracellular matrix. BMP-2 stimulated invasion of PC-3 cells at concentrations of 10 ng/ml, 100 ng/ml, and 500 ng/ml. This was statistically significant compared to control cells (*$P < 0.05$ vs. 293T cells). BMP-4 stimulated invasion of PC-3 cells only at 500 ng/ml (†$P < 0.05$ vs. 293T cells), and BMP-7 had no significant effect. (B) An invasion assay was repeated after noggin was incubated with BMP-2. 50 μg/ml noggin significantly reduced PC-3 cell invasion at 500 ng/ml of BMP-2 (*$P < 0.05$ vs. BMP-2 alone).

able to stimulate PC-3 cell invasion at 500 ng/ml, but significantly less than BMP-2 ($P < 0.05$ vs. BMP-2; $P < 0.01$ vs. 293T). BMP-7 had no significant effect on PC-3 cell invasion. 10 μg/ml and 50 μg/ml of noggin inhibited invasion of PC-3 cells at 500 ng/ml of BMP-4 ($P < 0.05$ vs. BMP-4 alone).

Effect of BMPs on PC-3 cell proliferation

BMPs have been shown to stimulate cell proliferation of multiple oncogenic and normal cell lines [20,21,25,32,42]. To determine the effects of BMPs on PC-3 cell growth, we performed a cell proliferation analysis with increasing concentrations of BMPs. At 100 ng/ml and 500 ng/ml of BMP-2, PC-3 cell proliferation was significantly stimulated compared to addition of no BMP-2 ($P < 0.001$) (Fig. 4). Either 10 μg/ml or 50 μg/ml of noggin was added to PC-3 cells with 100 ng/ml and 500 ng/ml BMP-2. At both concentrations of noggin, there was a significant decrease in cell growth compared to BMP-2 alone (Fig. 4). Noggin itself was not cytotoxic as addition of 100 μg/ml noggin alone did not significantly alter PC-3 cell number. In addition, a trypan blue stain showed no difference in numbers of cell death with addition of 100 μg/ml recombinant noggin to PC-3 cells (data not shown). BMP-4 and BMP-7 showed no significant effects on PC-3 cell proliferation at any concentration. BMP-2 and -4 inhibited LNCaP cell proliferation by 31% and 17%, respectively ($P < 0.05$ vs. LNCaP alone), results similar to previous studies [16,49]. BMP-7 had no effect on LNCaP cells.

Osteoclast activity in vitro

In order to determine if osteolysis was affected by transduction of the noggin transgene, we investigated the effects of PC-3 cells alone compared to PC-3 cells transduced with the retrovirus containing the noggin virus (PC-3 + RetroNog) on osteoclastic bone resorption in vitro. Primary rat bone cells were placed on bovine cortical wafers and cultured in sterile media. PC-3 cells or PC-3 + RetroNog cells were added to the wafers, and osteoclast activity was analyzed 10 days later by tracing the pitted areas of the wafers and measuring the percent of the total area that was resorbed. PTH was used as a positive control. Wafers treated with PC-3 cells showed 28.9% resorption at 10 days, but wafers treated with PC-3 + RetroNog showed no resorption over the same time point, suggesting that noggin was able to inhibit osteoclast activity (Fig. 5). Wafers treated with PTH exhibited 100% resorption.

Effect of noggin overexpression on tumor formation in vivo

We had previously demonstrated that PC-3 cells formed a purely lytic lesion when injected into the proximal tibia of SCID mice [26]. For this study, we injected either PC-3 cells alone, PC-3 cells treated with an empty retroviral

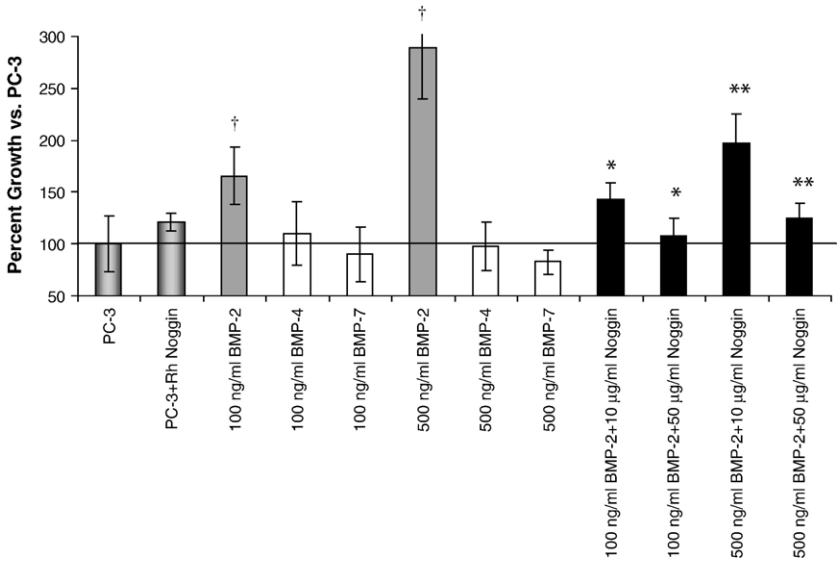

Fig. 4. Effects of BMP-2 on PC-3 cell proliferation. To determine the effects of BMPs on PC-3 cell proliferation, 100 ng/ml or 500 ng/ml recombinant BMP-2, -4, or -7 was incubated with PC-3 cells in serum-free media. At both concentrations, PC-3 cell proliferation was significantly enhanced with BMP-2 at 48 h compared to controls (†$P < 0.05$ vs. PC-3 cells alone). Addition of 10 μg/ml recombinant noggin did not significantly change cell proliferation (PC-3 + Rh Noggin, P = NS vs. PC-3 alone). Addition of 50 μg/ml of recombinant noggin significantly decreased PC-3 cell proliferation with both 100 ng/ml BMP-2 and 500 ng/ml of BMP-2, and 10 μg/ml recombinant noggin decreased cell proliferation of PC-3 cells stimulated by 500 ng/ml BMP-2 (*$P < 0.01$ vs. 100 ng/ml BMP-2; **$P < 0.01$ vs. 500 ng/ml BMP-2). Treatment with BMP-4 and BMP-7 led to no significant changes in PC-3 cell proliferation.

vector (PC-3 + EV), or PC-3 cells overexpressing noggin (PC-3 + RetroNog) with a retroviral vector. Noggin expression was confirmed with Western blot analysis (data not shown). In addition, an in vitro BMP inhibition assay demonstrated that Noggin was expressed by PC-3 cells at a level of 55 ng per million cells per 24 h. At 2, 4, 6, and 8 weeks, radiographs demonstrated smaller lytic lesions for tibias implanted with PC-3 + RetroNog compared to tibias implanted with PC-3 cells alone and PC-3 + EV (Fig. 6A). In the PC-3 alone group, lesions were consistently seen as early as 2 weeks, compared to 4 weeks in the PC-3 + RetroNog group. Cortical disruption occurred earlier in the PC-3 group as well. Complete destruction of the proximal tibia was seen by 6 weeks in most PC-3 alone animals, whereas it was rarely seen in the PC-3 + RetroNog group by 8 weeks. Treatment with PC-3 + EV had no effect on the tumor progression as

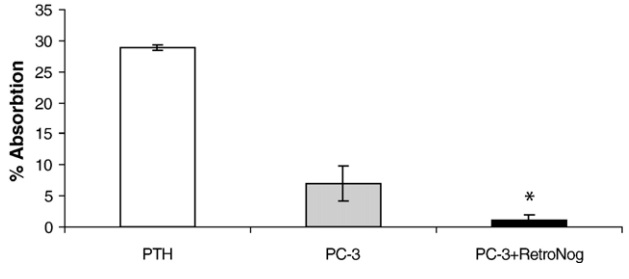

Fig. 5. In vitro osteoclast activity of PC-3 and PC-3 + RetroNog. Bone wafers were incubated in the presence of media with PTH (positive control), PC-3 cells alone, or PC-3 cells overexpressing noggin via retroviral transduction (PC-3 + RetroNog). The percent pitted area was determined after 10 days. Data are presented as the mean ± SEM for each group of three wafers (*$P < 0.05$ vs. PC-3).

these animals had tumors similar to PC-3 cells alone. The radiographic score was significantly less at all time points for PC-3 + RetroNog as well ($P < 0.05$ at 2 weeks; $P < 0.01$ at 4, 6, and 8 weeks). The kappa statistic was 0.79, suggesting reproducible intraobserver reliability. There was no difference in radiographic score between the PC-3 cells and the PC-3 + EV group, suggesting that transfection with a retroviral vector did not alter tumor progression.

Mean hindlimb diameter was measured at 2, 4, 6, and 8 weeks to compare the rate of growth in vivo between PC-3 cells alone and PC-3 + RetroNog. At 4, 6, and 8 weeks, the tumor size was significantly greater in tibias implanted with PC-3 cells and PC-3 + EV cells compared to tibias implanted with PC-3 + RetroNog cells ($P < 0.05$ at 4 weeks; $P < 0.01$ at 6 and 8 weeks; Fig. 6B).

Histologic sections were taken at each time point to compare treatment groups (Fig. 7). As early as 2 weeks, animals treated with PC-3 cells alone or PC-3 + EV had a much larger lesion that PC-3 + RetroNog, and there was early cortical destruction. Osteolytic lesions were present in both groups, although there was significantly more cortical destruction at 4, 6, and 8 weeks in the PC-3-implanted tibias. Cortical destruction did not occur until 8 weeks with the PC-3 + RetroNog-treated animals, approximately 6 weeks later than with PC-3-treated animals. TRAP stains were performed on similar sections to determine the presence of osteoclasts within the osteolytic lesions (Fig. 8). In both groups, there were abundant osteoclasts at the periphery of the lesion, along the host cortical bone. There was no significant difference in osteoclast number between the three

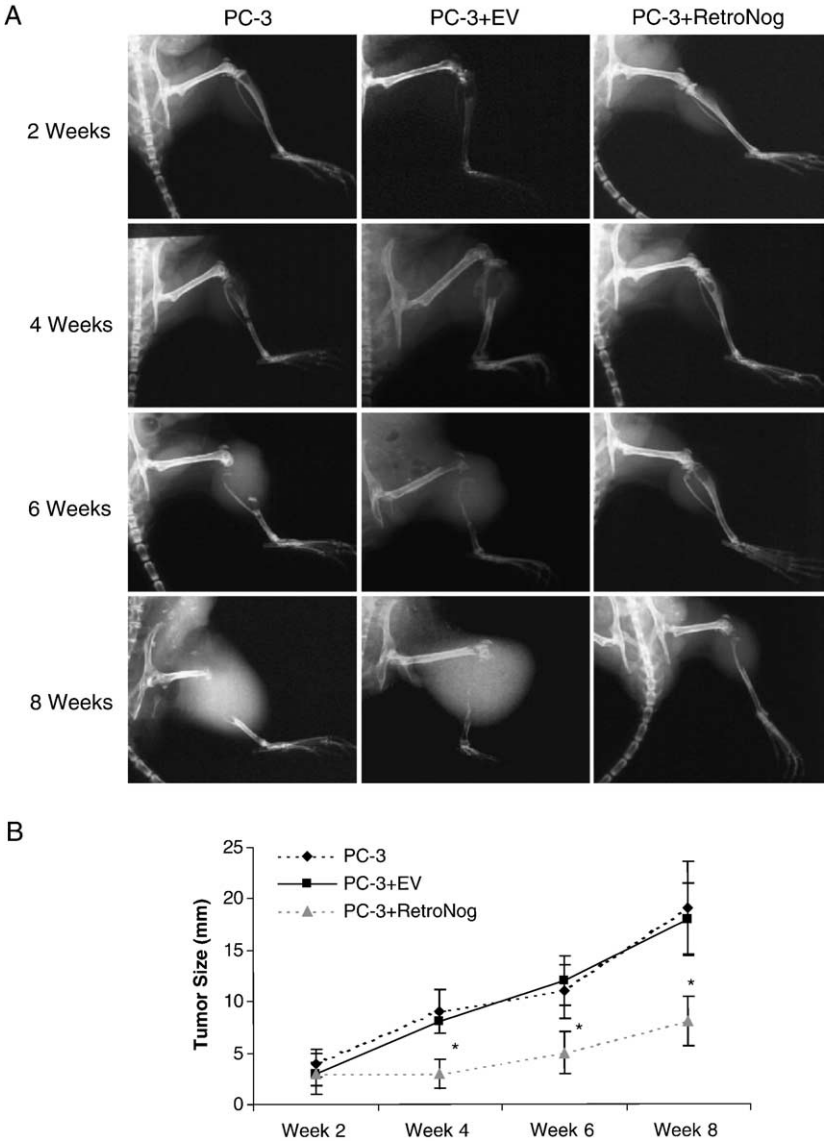

Fig. 6. Radiographic analysis. (A) Radiographs obtained at 2, 4, 6, and 8 weeks demonstrate significant difference between groups in osteolytic tumors present in the proximal tibia of SCID mice. (B) Tumor size was also quantified at 2, 4, 6, and 8 weeks by measuring hindlimb diameter. Tumor size with PC-3 + RetroNog was significantly smaller at 4, 6, and 8 weeks. *$P < 0.05$ vs. PC-3 alone and PC-3 + EV.

groups at any time point (Table 2). Thus, noggin overexpression did not decrease the number of osteoclasts present within the tibia. There was no histologic difference between PC-3 cells alone and PC-3 + EV.

Discussion

In this study, we sought to determine the role of bone morphogenetic proteins in the development and progression of osteolytic prostate cancer lesions. In vitro, BMP-2 stimulated cellular proliferation, migration, and invasion of the osteolytic prostate cancer cell line PC-3; BMP-4 stimulated migration and invasion but did not influence cellular proliferation. The effects of BMP-2 and BMP-4 on PC-3 cells were able to be inhibited by overexpression of noggin. In an established injection model of bony metastasis, overexpression of noggin was able to successfully inhibit the growth of osteolytic prostate cancer lesions, supporting the hypothesis that BMPs exert a significant influence on the progression of osteolytic prostate cancer lesions. These results also suggest that by inhibiting the critical interplay between host bone and tumor cells the tumor growth and bone loss can be limited.

In normal tissue, BMPs have multiple roles related to growth, differentiation, proliferation, and cell migration [1,9,42,51,52]. The exact response to BMP is mediated by these intracellular events and differs based on the cell type. For example, in the embryonic heart, BMP expression is responsible for the formation of endocardial cushions, but, in

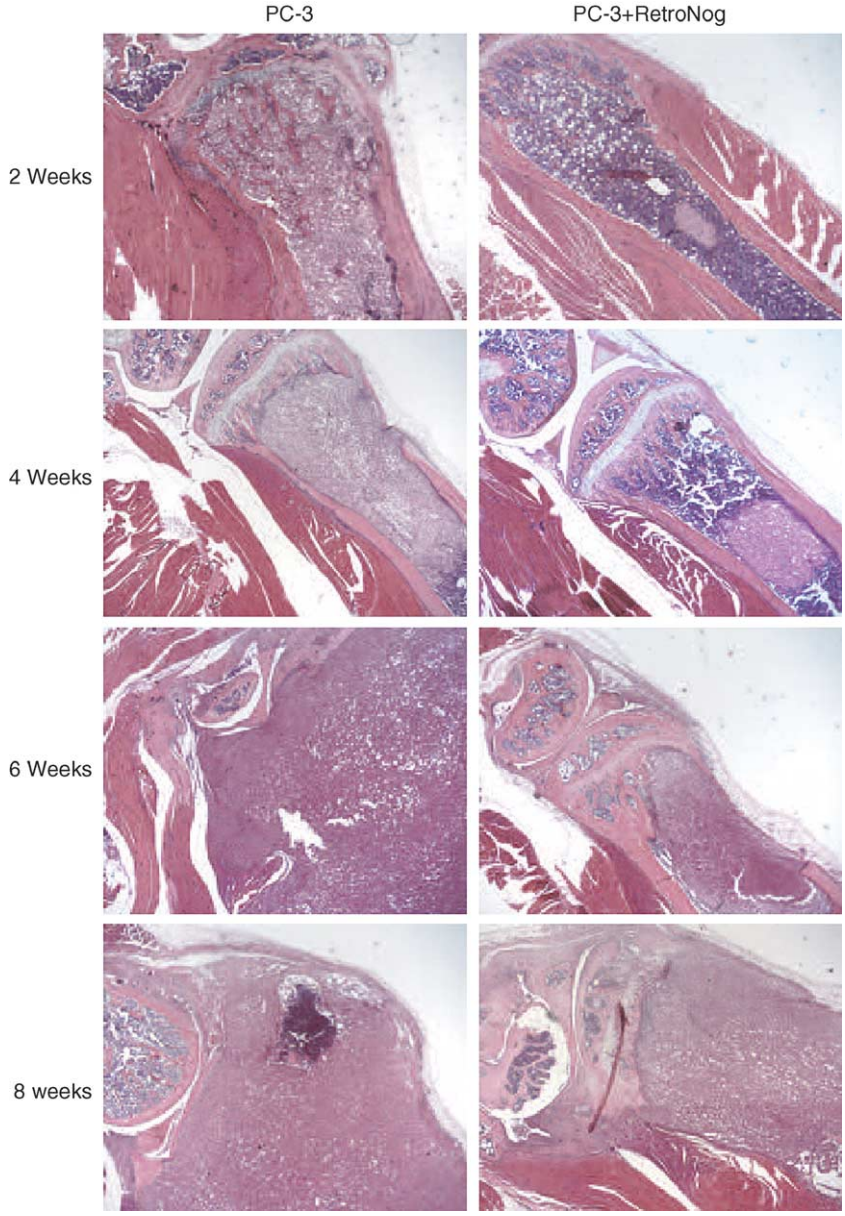

Fig. 7. Histologic analysis. Hematoxylin and eosin stains were performed on representative sections of tibias treated with PC-3 or PC-3 + RetroNog cells at 2, 4, 6, and 8 weeks. Images were obtained at 4× magnification. At 2 weeks, the osteolytic tumor size is significantly greater in the PC-3 alone group compared to the PC-3 + RetroNog group. The tumor cells are shown with an arrow. At 4 weeks, there is cortical disruption in the PC-3 group, whereas the PC-3 + RetroNog group shows only tumor growth. By 6 weeks, there is complete obliteration of the anterior and posterior cortex in the PC-3 group. The invasiveness of the PC-3 + RetroNog tumor remains significantly smaller at 6 and 8 weeks.

bone, it is responsible for the differentiation of osteoblasts and subsequent bone formation [42,47]. BMP receptors have been found to be expressed on multiple oncogenic cell lines, suggesting that BMPs may contribute to the oncogenic properties of multiple cancer cell types [2,16,25,37,43,50]. In our study, we determined that BMP receptors are expressed by PC-3 cells with RT-PCR and Western blot analysis, suggesting that these cells may be able to respond to host cell BMP to affect cellular proliferation, migration, and invasion.

Disordered cell proliferation is a cardinal feature of oncogenic cells. External stimuli such as growth factors may enhance the growth rate of tumors. In our current study, PC-3 cell proliferation was stimulated by recombinant BMP-2 in serum-free media, but BMP-4 and BMP-7 exerted no effect. Addition of recombinant noggin inhibited the effects of BMP-2 on cell proliferation. In addition, high concentrations of noggin were not cytotoxic, confirming that the lower number of cells was due to inhibition of cell proliferation rather than cell death. Our data are consistent with previous studies that have demonstrated that BMP-2 has a significant influence of oncogenic cell proliferation [25]. Langenfeld et al. demonstrated that BMP-2 stimulated the proliferation of the non-small-cell lung cancer cell line A549 in vitro and

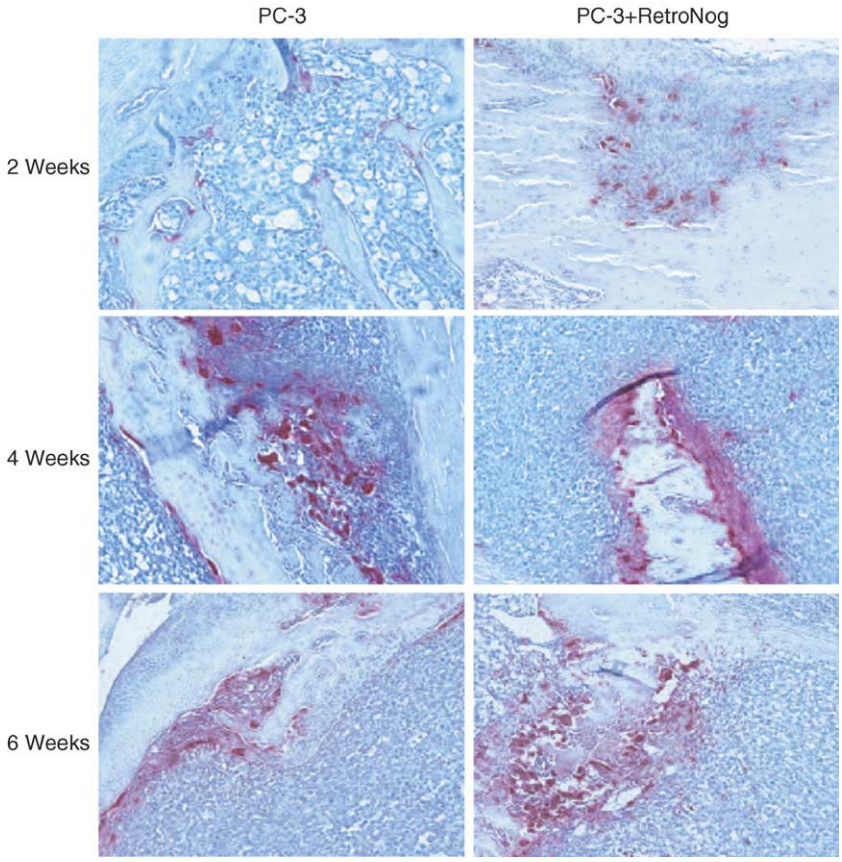

Fig. 8. TRAP staining of proximal tibial lesions. TRAP stains were performed on representative sections to determine osteoclast number at 2, 4, 6, and 8 weeks. Images were obtained at 10× magnification. Osteoclasts were determined by multinuclear cells that stained red after TRAP staining. There was no difference in osteoclast number at any time point between the tibias treated with PC-3 alone and PC-3 + RetroNog cells.

increased subcutaneous tumor size in vivo [25]. Other studies have demonstrated that BMPs inhibit the proliferation of oncogenic cell lines, suggesting that the influence of BMPs on cell growth varies with the individual cell phenotype [4]. Although the proliferation of multiple oncogenic cell lines is stimulated by BMP-2, this does not suggest that BMP-2 is primarily responsible for the disordered cell growth that characterizes oncogenic cells. It is more likely that the cells respond to BMP-2 in a normal manner, but downstream regulators of cell growth are no longer inhibited, allowing for the disordered cell growth seen in cancer cell lines. Elucidating these mechanisms may provide a means to slow the growth rate of osteolytic lesions.

Table 2
Osteoclasts/mm

	2 weeks	4 weeks	6 weeks	8 weeks
PC-3	5.48 ± 1.4	7.76 ± 2.3	3.02 ± 1.8	2.00 ± 1.5
PC-3 + EV	4.18 ± 2.3	5.39 ± 3.9	2.62 ± 0.9	3.29 ± 3.2
PC-3 + RetroNog	4.34 ± 0.9	6.01 ± 1.1	2.23 ± 1.0	2.21 ± 1.0

Comparison of osteoclast number in TRAP-stained histologic sections. There was no difference in the number of osteoclasts between PC-3-, PC-3 + EV-, and PC-3 + RetroNog-treated tibias at any time point.

BMP-2 stimulated the migration and invasion of osteolytic PC-3 cancer cells in vitro. BMP-4 showed moderate effects on migration and invasion, and BMP-7 had no influence on PC-3 cell migration and invasion, suggesting that BMP-2 and, to a lesser extent, BMP-4 are important cytokines stimulating osteolytic prostate cancer cell chemotaxis. The molecular mechanisms responsible for tumor cells to migrate and invade into specific tissues are key factors in the development in metastatic lesions. The presence of BMP receptors on PC-3 cells suggests that these cells may be able to respond to the BMPs that are constitutively expressed in the normal host bone and stored in large concentrations in the extracellular matrix [45], guiding metastatic migration and invasion primarily into bone. In addition, the BMPs that are present in the marrow may stimulate cell migration and invasion of these metastatic cells to bony sites. PC-3 cells express BMPs [26], which may allow for local differentiation of osteoblast and osteoclast precursors and stimulation of increased bone turnover with release of multiple growth factors. Thus, BMPs may act in an autocrine and paracrine fashion to stimulate tumor growth in osteolytic prostate cancer lesions.

Noggin is a cysteine knot protein that is constitutively expressed in areas of BMP expression and has been shown to

be an essential regulator of BMP activity in vivo [5,12,13,32]. The results from the wafer assay suggest that noggin decreases osteoclast activity, either by inhibiting osteoclast number or by inhibiting osteoclast function. We found similar numbers of osteoclasts in groups with PC-3 cells alone and with PC-3 cells overexpressing noggin, suggesting that overexpression of noggin did not effect osteoclast number. Thus, it appears that noggin decreases osteolysis primarily by inhibiting osteoclast activity in our model, leading to a decrease in the overall size of the lytic lesion in vivo. Our results are consistent with in vitro studies examining the role of noggin on osteoclast activity. Abe et al. found that noggin inhibited osteoclast activity in a culture containing stromal cells and osteoblasts but had no effect on osteoclastogenesis in a model without stromal cells and osteoblasts. They concluded that the effects of noggin on osteoclastogenesis were indirect, secondary to decreasing stromal/osteoblastic cell differentiation toward a state capable of supporting osteoclast development [1]. They also determined that noggin decreased the production of key osteoclast proteins, including RANK [1]. Kaneko et al. found that BMP-2 and BMP-4 increased messenger RNA expression of cathepsin K and carbonic anhydrase II, key enzymes for the degradation of organic and inorganic bone matrices, in osteoclasts [18]. Taken together, these results suggest that, in PC-3 cells transduced with RetroNog, there was a decrease in the size of the lytic lesion partially due to a direct effect of noggin on osteoclast protein synthesis and also due to an inhibition of BMP-mediated stimulation of mesenchymal precursors. Therefore, clinical strategies that utilize the direct and indirect effects of noggin on BMPs and mesenchymal cell differentiation may be a method to limit tumor cell proliferation, migration and invasion into host bone, and osteoclast activity, thus limiting the morbidity associated with metastatic disease.

It remains unclear why BMP-2 and -4 appeared to have more potent activity in stimulating PC-3 cell migration and invasion, compared to BMP-7, despite the fact they act through the same signal transduction pathway. Previous studies have also shown different responses to BMP-2, -4, and -7 in other oncogenic cell lines [3,40]. BMP-2 has been shown to have a higher binding affinity to the BMP receptor complex, which may allow for more potent activity in certain cell lines [31]. In addition, two recent studies have focused on a more complicated BMP ligand–receptor interaction [10,33]. Prior to ligand binding, heterocomplexes of BMPR II with BMPR I are most common. After BMP-2 binding, there is an elevation in the amount of heteromeric BMP receptor complexes, as well as increased levels of homomeric BMPR I complexes. The homomeric complexes triggered the p38 map kinase pathway, an alternative from the Smad pathway. This flexible oligomerization pattern allows for flexibility in response to similar BMPs. Activation of multiple downstream signaling pathways could explain why BMP-2, -4, and -7 had different effects on PC-3 cell migration and invasion.

PC-3 cells were chosen for this study because they represent a very aggressive prostate cancer cell line that has been shown to produce purely osteolytic lesions when introduced into bone [26,27]. They are a highly aneuploidic cell line that was derived from a grade IV prostatic adenocarcinoma [17]. Although other cell lines such as LNCaP may represent a typical metastatic prostate cancer lesion in vivo, they do not produce a purely osteolytic lesion [53]. Furthermore, the ability of noggin to inhibit the growth of aggressive PC-3 lesions in vivo highlights the importance of BMPs in the progression of aggressive metastatic prostate cancer lesions.

In summary, we demonstrated that BMPs play a key role in the progression of osteolytic lesions by prostate cancer cells. Inhibition of BMPs with noggin effectively limited BMP-mediated cell proliferation, migration, and invasion in vitro, as well as tumor growth and osteolysis in vivo. These results suggest that local inhibition of BMP activity in patients with prostate cancer may allow for new therapeutic modalities that decrease the morbidity and mortality associated with metastatic disease.

Acknowledgments

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