BMP4 Promotes Prostate Tumor Growth in Bone through Osteogenesis

Yu-Chen Lee¹, Chien-Jui Cheng⁵,⁶, Mehmet A. Bilen¹, Jing-Fang Lu², Robert L. Satcher², Li-Yuan Yu-Lee⁴, Gary E. Gallick⁵, Sankar N. Maity⁵, and Sue-Hwa Lin¹,²

Abstract

Induction of new bone formation is frequently seen in the bone lesions from prostate cancer. However, whether osteogenesis is necessary for prostate tumor growth in bone is unknown. Recently, 2 xenografts, MDA-PCa-118b and MDA-PCa-133, were generated from prostate cancer bone metastases. When implanted subcutaneously in severe combined immunodeficient (SCID) mice, MDA-PCa-118b induced strong ectopic bone formation while MDA-PCa-133 did not. To identify the factors that are involved in bone formation, we compared the expression of secreted factors (secretome) from MDA-PCa-118b and MDA-PCa-133 by cytokine array. We found that the osteogenic MDA-PCa-118b xenograft expressed higher levels of bone morphogenetic protein BMP4 and several cytokines including interleukin-8, growth-related protein (GRO), and CCL2. We showed that BMP4 secreted from MDA-PCa-118b contributed to about a third of the osteogenic differentiation seen in MDA-PCa-118b tumors. The conditioned media from MDA-PCa-118b induced a higher level of osteoblast differentiation, which was significantly reduced by treatment with BMP4 neutralizing antibody or the small molecule BMP receptor 1 inhibitor LDN-193189. BMP4 did not elicit an autocrine effect on MDA-PCa-118b, which expressed low to undetectable levels of BMP receptors. Treatment of SCID mice bearing MDA-PCa-118b tumors with LDN-193189 significantly reduced tumor growth. Thus, these studies support a role of BMP4-mediated osteogenesis in the progression of prostate cancer in bone. Cancer Res; 71(15): 5194–203. ©2011 AACR.

Introduction

Prostate cancer has a propensity to metastasize to bone. Development of bone metastases substantially shortens survival time of men with prostate cancer and remains a major challenge in the treatment of prostate cancer. The mechanisms that lead to the preferential growth of prostate cancer in bone are not known. Bone metastasis from prostate cancer typically exhibits osteoblastic lesions (1). In the histopathologic analysis of prostate cancer bone metastases, a substantial increase in the numbers of osteoblasts and bone matrix adjacent to prostate cancer cells are often seen. Whereas metastases have an overall bone-forming phenotype, the clinical presentation of prostate cancer bone metastasis suggests that it is a heterogeneous disease that also involves osteoclasts (2, 3). The complex interactions of tumor cells with osteoblasts, osteoclasts, and other cells suggest that prostate cancer cells secrete multiple factors that alter the bone microenvironment and stimulate osteoblast proliferation and/or differentiation at the sites of skeletal metastasis (4). Identification of factors that are involved in the osteoblastic bone lesions will uncover targets for therapy. However, this has been difficult due to anatomic inaccessibility, and the small amount of tumor in biopsies from bone metastasis is not sufficient for biochemical analysis.

Recently, 2 bone metastasis–derived xenografts, MDA-PCa-118b and MDA-PCa-133, have been generated, and they provide sufficient material for the analysis of the paracrine factors that play a role in prostate cancer progression in bone. MDA-PCa-118b (PCa-118b) xenograft exhibits characteristics seen in patients with prostate cancer in bone, for example a strong osteogenic phenotype when implanted into mouse femurs (5). Interestingly, PCa-118b induced new bone formation even when implanted s.c. (5). On the other hand, the MDA-PCa-133 (PCa-133) xenograft proliferates when implanted s.c. but does not induce bone formation. The difference in the bone-forming activity of these 2 xenografts reflects the heterogeneous nature of prostate cancer bone metastases (2).

In this study, we identified the factors secreted by these 2 xenografts using an antibody array that detects cytokines in the conditioned medium. We found that the osteogenic PCa-118b xenograft not only expresses higher levels of osteogenic factors, such as bone morphogenetic protein (BMP4) but also osteoclastogenic factors, such as monocyte chemoattractant protein 1 (MCP1) and macrophage colony-stimulating factor (M-CSF), compared with the PCa-133 xenograft. In addition, BMP4...
secreted from PCa-118b induced osteoblast differentiation in a paracrine fashion. Inhibition of the BMP receptor on osteoblasts by a small molecule inhibitor LDN-193189 reduces PCa-118b tumor growth. These studies provide a biochemical basis for the osteogenic phenotype of PCa-118b and support a role of osteogenesis in the progression of prostate cancer in bone.

Materials and Methods

Materials
Recombinant human proteins were used: basic fibroblast growth factors (FGF; FGF-2), BMP2, tissue inhibitor of metalloproteinase 2 (TIMP2), and insulin-like growth factor binding protein 2 (IGFBP-2; R&D), BMP4 (Sigma-Aldrich), growth-related protein (GRO)-α, GRO-β, GRO-γ, interleukin (IL)-8 stem cell factor (SCF) and brain-derived neutrophic factor (BDNF; Cell Science). LDN-193189 was from Axon Medchem (Netherlands). Prostate cancer xenograft tumors PCa-118b (5) and PCa-133 were derived from bone lesions of patients with castration-resistant prostate cancer. Fingerprinting of cells isolated from PCa-118b and PCa-133 xenografts showed that their profiles are unique as expected. The PC3 cell line was confirmed by fingerprinting.

Xenograft tumors and conditioned medium
Subcutaneous grafts of PCa-118b or PCa-133 were generated by implanting fragments (less than 1 mL) of tumors s.c. into severe combined immunodeficient (SCID) mice. The tumors were allowed to grow until they reached the size of 500 mL, dissected, weighed, cut into small pieces, and suspended (250 mg wet weight per mL medium) in BGJb medium (Invitrogen) with antibiotics. The conditioned media were collected every 24 hours for 2 days, spun to clarify, and stored at −80°C.

Assessment of chemokines in conditioned medium
Chemokines and cytokines in the conditioned media were assessed by using RayBio Human Cytokine Antibody Array C (RayBiotech; Membranes VI and VII) according to the manufacturer’s instructions. The signal intensity of each spot was quantified by ImageJ. The signal intensity of the negative control was subtracted from each spot. The experiments were carried out 3 times using conditioned medium from 3 different batches of xenograft tumors. BMP4 or BMP6 levels were measured using ELISA kits (RayBiotech).

Reverse transcription and quantitative PCR analysis
Total RNA was extracted from PCa-118b or PCa-133 using RNeasy Mini Kit (Qiagen). The relative mRNA level for each gene was analyzed by quantitative real-time RT-PCR (qRT-PCR) with SYBR Green (Applied Biosystems), using GAPDH as a control.

Measurement of luciferase and alkaline phosphatase activities in calvarial osteoblasts
The transgenic mice harboring luciferase transgene (Col-luc mice) were described previously (6). Calvarial osteoblasts from Col-luc mice or CD1 mice were incubated with conditioned medium from PCa-118b or PCa-133 with or without neutralizing antibody or LDN-193189 for 3 days. Cells were lysed and the luciferase activity assessed (Promega, Cat. No. E1501). Alkaline phosphatase activity was measured using p-nitrophenyl phosphate liquid substrate system (Sigma–Aldrich).

Measurement of bone morphogenetic protein reporter activity
C2C12 cells stably expressing a BMP-responsive Id1 promoter fused to a luciferase reporter gene (C2C12/BRA) were kindly provided by Dr. Daniel B. Rifkin (7). C2C12/BRA cells were incubated with conditioned medium from PCa-118b or BMP4 with or without 100 nmol/L LDN-193189 for 12 hours and the luciferase activity assessed as above.

Western blotting for Smad 5 phosphorylation
Cells in 6-well plates were treated with or without 100 nmol/L LDN-193189 and/or 0.68 ng/mL BMP4 for 16 hours. LDN-193189 was added 15 minutes before BMP4 addition. Cell extracts were resolved by SDS-PAGE followed by Western blotting analysis, using phospho-SMAD-1/5 and SMAD-5 antibodies (Cell Signaling).

Effect of LDN-193189 on tumor growth in vivo
In the first experiment, SCID mice were implanted with MDA-PCa-118b tumors. After 7 days when tumors reached measurable sizes, mice were injected with LDN-193189 (3 mg/kg) or with vehicle intraperitoneally twice a day. Tumor sizes and body weights were measured weekly. Mice were injected with calcein at 3 days and 1 day prior to sacrifice. Blood was collected and tumors were weighed. A portion of the tumors were fixed in formaldehyde for micro-computed tomography (microCT), using FVS CT (General Electric), or further decalcified for bone histomorphometric analysis, using OsteoMeasure Analysis System (Osteometrics, Inc.), or flash frozen for RNA preparation. Osteocalcin in the mouse serum was determined by ELISA (Biomedical Technologies). In the second experiment, PCa-118b tumors were first digested with Accumax (eBioscience), and the isolated cells were plated overnight, digested by Accutase (eBioscience), resuspended in Matrigel in 1:1 ratio, and injected into SCID mice (1 × 10⁶ cells/mouse) s.c. Mice were treated with LDN-193189 5 days post-injection.

Statistical analysis
Data are expressed as the mean ± SD unless otherwise stated. Statistical analyses were carried out using Student t-test (2-tailed, paired). P values less than 0.05 were considered significant.

Results
PCa-118b and PCa-133 xenografts
To delineate pathways that lead to osteoblastic phenotype of prostate cancer bone metastasis, we selected prostate cancer xenografts derived from bone metastasis with high- or low-osteogenic activity. PCa-118b and PCa-133 xenografts were established by implanting biopsy specimens from bone...
lesions into SCID mice s.c. Interestingly, PCa-118b induced ectopic new bone formation as shown by radiography, microCT, histology, and serum osteocalcin (Fig. 1A). MicroCT analysis showed heterogeneous mineralization with various densities within the tumor (Fig. 1A). In contrast, the femur/tibia bone exhibits uniform and much higher density than those in PCA-118b. Calcein labeling shows that the newly formed bone is irregular. In contrast, the PCA-133 xenograft did not induce bone formation (Fig. 1B).

Cytokine array analysis of conditioned medium from xenografts

The strong bone-forming activity of PCA-118b may be due to the secretion of paracrine factors that stimulate osteoblast proliferation and/or differentiation. To identify these factors, we compared the expression of cytokines in the conditioned medium from PCA-118b and PCA-133 xenografts, using the Human Cytokine Antibody Array that detects 120 cytokines and growth factors. BMPs and FGFs are the 2 major families of protein factors that have effects on osteoblast proliferation and differentiation (8). The cytokine arrays indicate that PCA-118b expressed higher levels of BMP4 and BMP6 than PCA-133, whereas PCA-133 expressed a higher level of FGF2, but not FGF4 and FGF9, than PCA-118b (Fig. 2B). Both xenografts expressed very low levels of FGF6 and FGF7 (Fig. 2B).

Other factors that are highly expressed in PCA-118b compared with PCA-133 include GRO (including α, β, and γ), IL-8, TIMP-2, SCF, IGFBP-2, migration inhibitory factor (MIF) MCP1, M-CSF, soluble tumor necrosis factor receptor 1 (sTNF1), and sTNFRII (Fig. 2C). On the other hand, PCA-133 expressed a higher level of BDNF compared with PCA-118b (Fig. 2C).

BMP4 expression in PCA-118b

To confirm the cytokine array analysis, the levels of BMP RNA transcripts were determined by qRT-PCR. Of BMP1 to BMP9 examined, BMP4 was the most highly expressed in PCA-118b xenograft. Low levels of BMP3 and BMP6 were also detected (Fig. 2D). None of these BMP messages were detected in PCA-133 (data not shown). Further, ELISA assays of the conditioned medium showed that BMP4 in PCA-118b was 13-fold higher than that found in PCA-133 conditioned medium, with the levels at 624.8 ± 17.3 pg/mL and 47.2 ± 4.3 pg/mL, respectively (Fig. 2D). BMP6 in PCA-118b was at 62.4 ± 2.6 pg/mL but too low to be detected in the PCA-133 conditioned medium (Fig. 2D).
Figure 2. Cytokine expression profile of conditioned media from PCa-118b and PCa-133 xenografts. A, conditioned media from PCa-118b or PCa-133 were incubated with human antibody arrays that detected 120 cytokines. Cytokines that were differentially expressed are boxed. B, the relative density of the array signals for BMPs and FGFs in the conditioned media of PCa-118b and PCa-133 xenografts was determined by ImageJ and expressed as arbitrary units. *, P < 0.05. C, cytokines upregulated in PCa-118b versus PCa-133 conditioned media were determined by ImageJ and expressed as fold increase. Similar results were obtained using conditioned medium prepared from 3 different batches of xenograft tumors. D, BMP RNA transcripts in PCa-118b or PCa-133 tumors were determined by qRT-PCR using primers specific to human BMPs (see primer list in Supplementary Table S1). Levels of human BMP4 and BMP6 proteins in PCa-118b and MDA-PCa-133 conditioned media were determined by ELISA.
Effect of cytokines and conditioned medium on osteoblast proliferation and differentiation

Next, we examined the effects of these cytokines on osteoblast proliferation and/or differentiation. To improve the specificity of the measurement, primary calvarial osteoblasts isolated from a 2.3-kb collagen promoter-driven luciferase reporter transgenic mice (Col-luc mice) were used (6, 9). Previous studies have established that the luciferase reporter activity of the Col-luc osteoblasts correlated with osteoblast proliferation (9). Treatment with 10 ng/mL FGF2 induces an elongated spindle shape morphology, an increase in osteoblast cell number, an increase in luciferase activity, and a small but significant inhibition of alkaline phosphatase activity (Fig. 3A), showing that FGF2 stimulates Col-luc osteoblast proliferation. In contrast, treatment with 100 ng/mL BMP2 induces a significant increase in alkaline phosphatase activity without affecting the luciferase activity (Fig. 3A), suggesting that BMP2 stimulates Col-luc osteoblast differentiation.

Next, when the Col-luc osteoblasts were treated with recombinant BMP2, BMP4, or BMP6, a significant increase (about 300% compared with the control, n = 3) of alkaline phosphatase activity was observed, with no effect on luciferase activity (Fig. 3B). In contrast, treatment with 10 ng/mL of FGF2 or FGF9 for 3 days led to an increase in luciferase activity, but a decrease in alkaline phosphatase activity (Fig. 3B). These observations suggest that BMP2, BMP4, and BMP6 exert similar effects on osteoblast differentiation, whereas FGF2 and FGF9 exert similar effects on osteoblast proliferation.

Cytokines, including MIF (Fig. 3B), IGFBP2, BDNF, and SCF, did not have a significant effect on luciferase or alkaline phosphatase activity of Col-luc osteoblasts (Table 1). GRO (including GRO-α, β, and γ), IL-8, and TIMP-2, induced small but significant increases in alkaline phosphatase (Table 1) but not luciferase activity (data not shown). These observations suggest that these cytokines may be involved, but to a lesser extent than BMP4, in the osteogenesis of the PCa-118b xenograft.

Osteoblast regulatory activities in the conditioned media of PCa-118b and PCa-133 xenografts

Next, the conditioned media from PCa-118b or -133 were examined for their effects on osteoblast differentiation or proliferation. Treatment of Col-luc calvarial osteoblasts with conditioned media from PCa-133 led to a 5-fold increase in luciferase activity, while PCa-118b xenograft only resulted in an average of 30% increase (Fig. 3C). In contrast, conditioned medium from PCa-118b induced about a 2-fold increase in alkaline phosphate activity compared with that from PCa-133, which induced an average of 15% increase (Fig. 3C).

We further used a neutralizing antibody against BMP4 to determine if BMP4 might be involved in the increase in alkaline phosphate activity seen in the CM of PCa-118b xenograft. The BMP4 antibody inhibited about 40% of PCa-118b-induced increase in alkaline phosphate activity (Fig. 3D), whereas control IgG or BMP6 antibody (data not shown) did not show an inhibitory effect. These observations suggest that BMP4 plays a significant role in PCa-118b-induced osteoblast differentiation.

BMP4 signals through bone morphogenetic protein receptor in osteoblasts

To examine whether PCa-118b conditioned medium activates BMP signaling in osteoblasts, we first examined its effect on Id1 promoter activity. Id1 is a direct target of the BMP pathway, and its promoter contains multiple BMP-specific SMAD-binding elements (10). The C2C12 cell line expresses BMP receptors (7) and can be induced to become osteoblasts by BMP2. C2C12/BRE was generated by transfecting the Id1 promoter reporter into C2C12 cells (7). When the C2C12/BRE cells were incubated with MDA-PCa-118b conditioned medium, luciferase reporter activity was increased by 5-fold, which was accompanied by a 3-fold increase in endogenous Id1 message (Fig. 4A). In contrast, conditioned medium from PCa-133 did not stimulate Id1 promoter activity (Fig. 4B, bottom). LDN-193189, a small molecule inhibitor of BMP type I receptor (11), was used to specifically inhibit BMP signaling. In osteoblasts, LDN-193189 inhibited osteoblast differentiation (Fig. 4), but did not induce osteoblast cell death or inhibit osteoblast proliferation [data not shown and (11)]. Treatment of C2C12/BRE cells with 100-nmol/L LDN-193189 led to an inhibition of BMP4-induced Id1 promoter activity at various BMP4 concentrations (Fig. 4B, upper panel), suggesting that LDN-193189 blocks BMP4-induced signaling. Further, LDN-193189 also inhibited BMP signaling to the Id1 promoter from PCa-118b conditioned medium (Fig. 4B, lower panel).

Next, we examined the effect of PCa-118b conditioned medium on the activation of Smad5, a BMP receptor signaling molecule. In C2C12/BRE cells, conditioned medium from PCa-118b but not PCa-133 induced Smad5 phosphorylation, and this activity was inhibited by LDN-193189 (Fig. 4C). Similar results were observed using calvarial mouse osteoblasts (Fig. 4C). These observations suggest that PCa-118b conditioned medium activated Smad5 signaling through the BMP receptors.

We further examined the effect of LDN-193189 on conditioned medium-mediated osteoblast activity. PCa-118b conditioned medium-induced alkaline phosphate activity was significantly reduced (about 50%) by treating the osteoblasts with 100-nmol/L LDN-193189 (Fig. 4D, consistent with the inhibition observed with BMP4 neutralizing antibody (Fig. 3D). LDN-193189, at a concentration of 100 nmol/L, had no effect on osteoblast cell number (data not shown), indicating that the inhibition of alkaline phosphate activity by LDN-193189 is not due to toxicity. Together, these results suggest that PCa-118b conditioned medium activates BMP receptor signaling in osteoblasts, and this effect is inhibited by the BMP type I receptor inhibitor LDN-193189.

Expression of bone morphogenetic protein receptors in PCa-118b

Whereas the results showed that PCa-118b tumor secretes BMP4 to induce osteoblast differentiation in a paracrine fashion, it is not clear whether BMP4 may have an autocrine effect on PCa-118b tumor cells. To examine this possibility, we digested the PCa-118b tumor and cultured the isolated cells in CnT-52 medium that allows tumor cell but not osteoblast survival (data not shown). Under this condition, we did not...
Figure 3. BMPs, FGFs, and PCa-118b and PCa-133 conditioned media on osteoblast proliferation or differentiation. A, calvarial osteoblasts from Col-luc mice were incubated without or with BMPs (100 ng/mL) or FGF2 (10 ng/mL) for 3 days. Cell morphology (×100), cell numbers, luciferase activity, and alkaline phosphatase activity were measured. B, BMP2, BMP4, BMP6, FGF2, and FGF9 on Col-luc osteoblasts. C, conditioned media from PCa-118b or PCa-133 xenografts on Col-luc osteoblasts. D, neutralizing anti-BMP2/4 antibody on Col-luc osteoblasts. Each experiment was carried out in triplicates and repeated at least three times. The averages of triplicates ± SD were shown. Data in A–C were expressed as a percent of control. *P < 0.05.
detect phosphorylated Smad5 in BMP4-secreting PCa-118b cells, even after further BMP4 addition (Fig. 5A). In addition, LDN-193189 did not affect the expression of BMP4 target gene Id2 as well as BMP4 expression in PCa-118b cells (ref. 12; Supplementary Fig. S1). Consistent with a lack of response to endogenous or exogenous BMP4, PCa-118b cells did not express significant levels of BMP type I receptors, such as ALK2, ALK3, and ALK6 (Fig. 5B) or BMP type II receptors, such as BMPR2 and ACVR2 (Fig. 5C). In contrast, Smad5 was activated in PC-3 cells and this activation was inhibited by LDN-193189 treatment (Fig. 5A). PC-3 cells were also found to express significant levels of BMP type-I and -II receptors (Fig. 5B and 5C). Together, these results suggest that BMP4 secreted from PCa-118b likely has minimal autocrine effects on PCa-118b cells.

Effect of LDN-193189 on PCa-118b tumor growth in vivo

Our studies suggest that BMP4 is one of the principal paracrine factors that increases osteoblast differentiation in PCa-118b tumors. To assess the effect of BMP4-mediated osteoblast differentiation on PCa-118b tumor growth in vivo, we tested the effect of LDN-193189 on PCa-118b xenograft growth in vivo. In the first experiment, LDN-193189 (3 mg/kg) was injected intraperitoneally twice a day after tumors were established.

Table 1. Alkaline phosphatase activity in Col-luc primary mouse osteoblasts treated with cytokines

<table>
<thead>
<tr>
<th>Concentration (ng/mL)</th>
<th>Alkaline phosphatase activity (% increase over control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 0</td>
<td>0 ± 4.7</td>
</tr>
<tr>
<td>GRO-α 10</td>
<td>26.5 ± 9.3*</td>
</tr>
<tr>
<td>GRO-β 10</td>
<td>21.8 ± 2.6*</td>
</tr>
<tr>
<td>GRO-γ 10</td>
<td>20.1 ± 5.4*</td>
</tr>
<tr>
<td>IL-8 750</td>
<td>30.2 ± 2.0*</td>
</tr>
<tr>
<td>TIMP-2 500</td>
<td>18.5 ± 2.9*</td>
</tr>
<tr>
<td>IGFBP-2 900</td>
<td>1.0 ± 4.0</td>
</tr>
<tr>
<td>BDNF 500</td>
<td>0.9 ± 5.6</td>
</tr>
<tr>
<td>SCF 50</td>
<td>−3.6 ± 7.3</td>
</tr>
</tbody>
</table>

Col-Luc primary mouse osteoblasts (PMO) were treated with cytokines for 3 days and alkaline phosphatase activity was measured. PMOs without treatment were used as controls and defined as 100%.

*p < 0.05.

Figure 4. PCa-118b conditioned medium on BMP receptor signaling. A, C2C12/BRE cells stably expressing a BMP-responsive Id1 promoter-luciferase reporter were incubated with PCa-118b conditioned medium for 12 hours. Cell lysates were measured directly for luciferase activity. Endogenous Id1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages were analyzed by qRT-PCR and expressed as Id1/GAPDH ratio. B, C2C12/BRE cells were treated with increasing concentrations of either BMP4 (upper) or conditioned media from PCa-118b or PCa-133 (lower) in the presence or absence of 100 nmol/L LDN-193189 for 12 hours, and luciferase activities were analyzed. C, C2C12/BRE cells (upper) or primary osteoblasts (lower) were treated as in B, and cell lysates were immunoblotted for phosphorylated Smad5 followed by reblotting for total Smad5. D, primary mouse calvarial osteoblasts were treated as in C except that alkaline phosphatase activity was determined after 3 days. *, P < 0.05; NS, not significant.
Density were reduced in the tumors of the LDN-193189-treated group compared with that of controls (Fig. 6B). Quantitative determination of the bone volume by microCT, using a threshold of 300 as the cut-off for bone density measurement, showed that LDN-193189 treatment reduced the bone volume in the tumors (Fig. 6B). Serum osteocalcin levels of the PCa-118b tumor-bearing mice were significantly higher than those in the non–tumor-bearing mice (Fig. 6C). The levels of osteocalcin in the LDN-193189-treated group were reduced compared with those without the treatment (Fig. 6C). Together, these observations suggest that treating PCa-118b tumor-bearing mice with LDN-193189 led to reduced bone formation and attenuated tumor growth rate. Quantitative RT-PCR for mouse osterix, Runx2, alkaline phosphatase as a measurement for osteoblast cells in the tumors showed that there were no differences in both control and LDN treated groups (Supplementary Fig. S3), suggesting that LDN-193189 reduces bone formation but does not change the proportion of mature osteoblasts in the tumor. A similar experiment was carried out with PCa-133. We found that LDN-193189 has no effect on PCa-133 tumor growth. There is no evidence of bone formation in the PCa-133 tumors as measured by microCT. Further, LDN-193189 treatment did not affect serum osteocalcin levels (Supplementary Fig. S4).

Discussion

Although prostate cancer bone metastasis is frequently associated with new bone formation, it is not clear whether tumor-induced osteogenesis plays a role in prostate cancer progression in the bone. We postulated that prostate cancer cells secrete paracrine factors that lead to an increase in bone formation, and the newly formed bone in turn secretes factors to enhance prostate cancer progression in bone (4). We have identified several cytokines that are differentially expressed between the osteogenic xenograft PCa-118b and nonosteogenic xenograft PCa-133. We showed that BMP4 is one of the cytokines that play a role in the osteogenesis of the PCa-118b xenograft. Because PCa-118b does not express BMP receptors, this has allowed us to explore whether blocking BMP4-induced osteogenesis affects PCa-118b tumor growth. We showed that BMP4-induced osteoblast differentiation was reduced by the BMP receptor kinase inhibitor LDN-193189, which resulted in a decrease in PCa-118b tumor growth. These studies thus provide evidence that tumor-induced osteogenesis mediated by paracrine BMP signaling plays a role in supporting PCa progression in bone (Fig. 6D), and supports our previously proposed model that osteoblast activation, resulting from prostate cancer paracrine signaling, leads to prostate cancer cell growth in bone (4).

Our observations raised an interesting question about the mechanism of decreased tumor growth upon reduction of osteogenic support by LDN-193189 treatment. We did not find significant differences in tumor-cell proliferation and apoptosis, using Ki67 staining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays, respectively, in the control versus LDN-193189–treated PCa-118b tumors (data not shown). Our interpretation is that tumor-cell growth and osteoblast differentiation are interdependent.

**Figure 5.** Lack of BMP4 signaling on PCa-118b cells. A, PCa-118b cells and PC3 were treated with BMP4 with or without LDN-193189 for 12 hours and analyzed for Smad5 phosphorylation. Levels of BMP type I receptor shown in B (ALK2, 3, 6; panel B) and type II receptor (BMPR2, ACVRs; panel C) transcripts in PCa-118b tumors and PC-3 cells were determined by qRT-PCR (see Supplementary Table S1).

The growth rates between these 2 groups, as measured by tumor size, were significant 6 and 7 weeks after treatment (Fig. 6A). The tumor weights also showed significant differences at the termination of the study at week 7 (Fig. 6A). The X-ray of the tumors showed that the ectopic bone volume and bone density were reduced in the tumors of the LDN-193189-treated group compared with that of controls (Fig. 6B). Quantitative determination of the bone volume by microCT, using a threshold of 300 as the cut-off for bone density measurement, showed that LDN-193189 treatment reduced the bone volume in the tumors (Fig. 6B). Serum osteocalcin levels of the PCa-118b tumor-bearing mice were significantly higher than those in the non–tumor-bearing mice (Fig. 6C). The levels of osteocalcin in the LDN-193189-treated group were reduced compared with those without the treatment (Fig. 6C). Together, these observations suggest that treating PCa-118b tumor-bearing mice with LDN-193189 led to reduced bone formation and attenuated tumor growth rate. Quantitative RT-PCR for mouse osterix, Runx2, alkaline phosphatase as a measurement for osteoblast cells in the tumors showed that there were no differences in both control and LDN treated groups (Supplementary Fig. S3), suggesting that LDN-193189 reduces bone formation but does not change the proportion of mature osteoblasts in the tumor. A similar experiment was carried out with PCa-133. We found that LDN-193189 has no effect on PCa-133 tumor growth. There is no evidence of bone formation in the PCa-133 tumors as measured by microCT. Further, LDN-193189 treatment did not affect serum osteocalcin levels (Supplementary Fig. S4).

Discussion

Although prostate cancer bone metastasis is frequently associated with new bone formation, it is not clear whether tumor-induced osteogenesis plays a role in prostate cancer progression in the bone. We postulated that prostate cancer cells secrete paracrine factors that lead to an increase in bone formation, and the newly formed bone in turn secretes factors to enhance prostate cancer progression in bone (4). We have identified several cytokines that are differentially expressed between the osteogenic xenograft PCa-118b and nonosteogenic xenograft PCa-133. We showed that BMP4 is one of the cytokines that play a role in the osteogenesis of the PCa-118b xenograft. Because PCa-118b does not express BMP receptors, this has allowed us to explore whether blocking BMP4-induced osteogenesis affects PCa-118b tumor growth. We showed that BMP4-induced osteoblast differentiation was reduced by the BMP receptor kinase inhibitor LDN-193189, which resulted in a decrease in PCa-118b tumor growth. These studies thus provide evidence that tumor-induced osteogenesis mediated by paracrine BMP signaling plays a role in supporting PCa progression in bone (Fig. 6D), and supports our previously proposed model that osteoblast activation, resulting from prostate cancer paracrine signaling, leads to prostate cancer cell growth in bone (4).

Our observations raised an interesting question about the mechanism of decreased tumor growth upon reduction of osteogenic support by LDN-193189 treatment. We did not find significant differences in tumor-cell proliferation and apoptosis, using Ki67 staining and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays, respectively, in the control versus LDN-193189–treated PCa-118b tumors (data not shown). Our interpretation is that tumor-cell growth and osteoblast differentiation are interdependent.

**Figure 5.** Lack of BMP4 signaling on PCa-118b cells. A, PCa-118b cells and PC3 were treated with BMP4 with or without LDN-193189 for 12 hours and analyzed for Smad5 phosphorylation. Levels of BMP type I receptor shown in B (ALK2, 3, 6; panel B) and type II receptor (BMPR2, ACVRs; panel C) transcripts in PCa-118b tumors and PC-3 cells were determined by qRT-PCR (see Supplementary Table S1).
during PCa-118b tumor growth. Thus, LDN-193189 treatment resulted in a proportional decrease of both types of cells. We speculate that the interdependency of tumor cells and osteoblasts involves a wide spectrum of secreted factors mediating paracrine/autocrine signaling, and that LDN-193189 partially interrupted such an interdependency. Our studies suggest that LDN-193189 treatment led to a decrease in bone formation, as reflected in serum osteocalcin levels, and this resulted in a decrease in tumor volume due to the tumor/osteoblast interdependency.

Bone metastases of prostate cancer predominantly result in osteoblastic lesions. However, very few cell lines or xenografts generated from human prostate cancer bone metastasis exhibit this phenotype. MDA-PCa-118b is one of the few xenografts that elicit a strong osteogenic phenotype when implanted in SCID mice (5). How MDA-PCa-118b induces ectopic bone formation under the s.c. site is not clear. It is likely that MDA-PCa-118b secretes several factors that are able to stimulate osteoblast proliferation or differentiation. Indeed, in a search for these secreted factors (prostate cancer secretome), we showed that several factors, including BMP4 secreted by MDA-PCa-118b, are able to stimulate osteoblast differentiation. In addition, previous studies by Li and colleagues (5) showed that FGF9, which stimulates osteoblast proliferation, also contributes to the osteoblastic phenotype of MDA-PCa-118b. Other factors yet to be identified may also be involved in the osteoblastic phenotype of MDA-PCa-118b tumor.

Many prostate cancer–derived cell lines express BMP receptors and BMPs. Several reports have shown that BMPs have autocrine effects on prostate cancer cells. Feeley and colleagues (13, 14) showed that BMP2 stimulated PC-3 and LAPC4 cell migration and invasion. Similarly, BMPs increased the in vitro invasive ability of LuCaP23.1 and C4-2B cells (15, 16). Dai and colleagues (16) also showed that BMPs induced VEGF protein and mRNA expression in C4-2B cells. MDA-PCa-118b is unique in that it expresses low to undetectable BMP receptors and does not respond to BMP4. Thus, the effect of LDN-198183 on tumor growth is most likely due to the blocking of BMP4 paracrine effects on tumor stromal cells (Fig. 6D). This property allowed us to examine, for the first time, the effect of tumor-induced osteogenesis on prostate tumor growth.

The ectopic bone formation of MDA-PCa-118b xenograft under the s.c. site suggests that host stromal cells/osteoblasts are recruited to the s.c. tumor implants. Indeed, a previous study by Li and colleagues (5) showed that FGF9, which stimulates osteoblast proliferation, also contributes to the osteoblastic phenotype of MDA-PCa-118b. Other factors yet to be identified may also be involved in the osteoblastic phenotype of MDA-PCa-118b tumor.

Figure 6. LDN-193189 treatment attenuates PCa-118b tumor growth in vivo. A, cells were isolated from PCa-118b tumors, mixed 1:1 with Matrigel, and injected into SCID mice subcutaneously (1 × 10^6 cells per mouse). LDN-193189 or vehicle treatment was started 5 days post-tumor cell injection. Tumor sizes were measured weekly. At week 7, tumors were removed and weighed. B, tumors from vehicle and LDN-193189–treated groups were X-rayed. Bone volumes were determined by microCT (μCT) using a 300 value as the cut-off. C, serum osteocalcin levels were determined for non–tumor-bearing (n = 5), PCa-118b tumor bearing (n = 10), and PCa-118b tumor bearing but LDN-193189–treated (n = 10) mice. D, model of bidirectional effect between PCa-118b osteogenic tumor and osteoblasts. PCa-118b secretes BMP4 that induces osteoblast differentiation. Inhibition of the paracrine BMP4 effect on osteoblasts leads to the attenuation of PCa-118b tumor growth, suggesting that osteoblasts play a role in supporting PCa-118b tumor growth.
study using in situ hybridization with mouse-specific probes indicated that the osteoblasts present in the xenograft are of mouse origin (5). How the stromal cells/osteoblasts are recruited to the ectopically implanted tumor remains to be determined.

Despite the bone-forming phenotype in prostate cancer bone metastasis, pathologic analysis of the metastatic lesions in bone showed that both osteoblastic and osteolytic lesions are present in the same loci (17, 18). Histomorphometric quantification also consistently shows changes in both bone formation and resorption within metastatic foci in iliac crest biopsy samples (19). Consistent with the clinical observations, osteoclasts were also present in MDA-PCa-118b xenograft tumors (5). Thus, tumor-induced osteoblastic or osteolytic responses likely reflect the overall effects from factors that regulate osteoblast proliferation, differentiation, and osteoclast activation in vivo. Among the factors secreted from MDA-PCa-118b xenografts, MCP-1, M-CSF, and IL-8 have been shown to have effects on osteoclast activation (20–22). It is likely that other factors yet to be identified are also involved in the osteoclastic component in MDA-PCa-118b xenografts.

In conclusion, MDA-PCa-118b exhibits the unique properties that allow examination of the contribution of paracrine signaling in osteoblastic lesions of prostate cancer. We show that BMP4, an osteogenic factor, acts as one of the important paracrine factors. By modulating BMP4-mediated paracrine signaling, we found evidence that supports a role of osteogenesis in the progression of prostate cancer in bone. Further studies on the factors that induce osteogenesis in prostate cancer bone metastasis are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Nora Navone for providing MDA-PCa-133 xenograft for this study. This work was supported by grants from NIH including CA111479, P50 CA183388 and CA16672, the Prostate Cancer Foundation, the U.S. Department of Defense (PC091332), Cancer Prevention and Research Institute of Texas (CPRIT RP10327), and the Robert Wood Johnson Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 3, 2010; revised May 3, 2011; accepted May 30, 2011; published OnlineFirst June 13, 2011.

References

BMP4 Promotes Prostate Tumor Growth in Bone through Osteogenesis

Yu-Chen Lee, Chien-Jui Cheng, Mehmet A. Bilen, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-4374

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/06/10/0008-5472.CAN-10-4374.DC1

Cited articles
This article cites 22 articles, 8 of which you can access for free at:
http://cancerres.aacrjournals.org/content/71/15/5194.full#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/71/15/5194.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.