EB1089 inhibits the parathyroid hormone–related protein–enhanced bone metastasis and xenograft growth of human prostate cancer cells

Vandanajay Bhatia,1 Manjit K. Saini,1 Xiaoli Shen,1 Lian X. Bi,2 Suimin Qiu,3 Nancy L. Weigel,5 and Miriam Falzon1,4

Departments of 1Pharmacology and Toxicology, 2Surgery, and 3Pathology, 5Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, Galveston, Texas; and 4Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas

Abstract
Parathyroid hormone–related protein (PTHrP) plays a major role in prostate carcinoma progression and bone metastasis. Once prostate cancers become androgen-independent, treatment options become limited. Vitamin D analogues represent a potentially valuable class of agents in this clinical context. Using the prostate cancer cell line C4-2 as a model, we studied the effects of PTHrP and the noncalcemic vitamin D analogue EB1089 on markers of prostate cancer cell progression in vitro and in vivo. C4-2 is a second-generation androgen-independent LNCaP subline that metastasizes to the lymph nodes and bone when injected into nude mice and produces mixed lytic/osteonlastic lesions, mimicking the in vivo situation. We report that PTHrP increases cell migration and invasion, and that a pathway via which EB1089 inhibits these processes is through down-regulation of PTHrP expression. PTHrP also increases anchorage-independent cell growth in vitro and xenograft growth in vivo; EB1089 reverses these effects. The in vivo PTHrP effects are accompanied by increased tumor cell proliferation and survival. Treatment with EB1089 reverses the proliferative but not the antiapoptotic effects of PTHrP. PTHrP also increases intratumor vessel density and vascular endothelial growth factor expression; EB1089 reverses these effects. Intracardially injected C4-2 cells produce predominantly osteoblastic lesions; PTHrP overexpression decreases the latency, increases the severity and alters the bone lesion profile to predominantly osteolytic. EB1089 largely reverses these PTHrP effects. A direct correlation between PTHrP immunoreactivity and increasing tumor grade is observed in human prostate cancer specimens. Thus, decreasing PTHrP production by treatment with vitamin D analogues may prove therapeutically beneficial for prostate cancer.

[ perceived effect of PTHrP. ]

Introduction
Prostate cancer is the second-leading cause of cancer-related death in men in the United States (1, 2). Because prostate cancer incidence increases with advancing age, this malignancy is likely to increase in frequency as worldwide life expectancy improves. The most common site of prostate cancer metastasis is the bone (3, 4). Although prostate cancer bone metastases are most often characterized radiographically as predominantly osteoblastic lesions (5), histologic evidence shows that these metastases form a heterogeneous mixture of osteolytic and osteoblastic lesions (6–9).

The prostate is strongly dependent on androgens for normal development and physiologic functions. However, additional factors, including growth factors, neuroendocrine peptides, and cytokines also play important roles in the organ (10); one of these factors is parathyroid hormone-related protein (PTHrP). Both normal and neoplastic prostatic epithelial cells express PTHrP (11, 12), and the protein increases prostate cancer cell proliferation and survival (13–15). PTHrP also plays a role in the progression of prostate carcinoma and its preference to metastasize to bone (16), and is involved in both the initial osteolytic phase and the ensuing osteoblastic phase of the metastases (16). These studies underline the importance of suppressing PTHrP expression in prostate cancer.

There are limited options for the treatment of metastatic prostate cancer. Although these cancers initially respond to androgen ablation therapy, they eventually become androgen-independent. There is thus a need to develop well-tolerated alternative treatments to slow prostate cancer progression. Biological response modifiers such as vitamin D analogues represent a potentially valuable class of agents in this clinical context. 1,25-Dihydroxyvitamin D3 [1,25(OH)2D3], the hormonally active form of vitamin D, regulates cell proliferation, differentiation, apoptosis, immune responses, and angiogenesis in many cancer cell types (17–19). Vitamin D deficiency has been linked with increased prostate cancer incidence (reviewed in ref. 20). The clinical usefulness of 1,25(OH)2D3 is limited by the associated risk of hypercalcemia and soft tissue calcifications. Previous studies have shown that 1,25(OH)2D3 and nonhypercalcemic analogues, such as EB1089,
down-regulate PTHrP expression and attenuate the proliferative effects of PTHrP (21–23). The effect of EB1089 on calcium metabolism in vitro is ~50% lower than that of 1,25(OH)₂D₃ (24, 25). Therefore, this compound offers an appropriate model to study the effects of 1,25(OH)₂D₃ analogues in vitro.

In this study, we investigated the effects of PTHrP on cell apoptosis, migration, and invasion, variables which play major roles in cancer cell progression in vitro and in the effects of EB1089 on these PTHrP-mediated effects. We also asked whether PTHrP enhances prostate cancer cell growth and metastasis in vivo, and whether EB1089 exerts a protective effect. The C4-2 cell line, an androgen-independent, second-generation LNCaP subline that metastasizes to lymph nodes and bone when injected into nude mice (26), was used as a model system. C4-2 cells produce mixed lytic/blastic lesions (16), thereby mimicking the in vivo situation. PTHrP exerts a positive effect on C4-2 cell proliferation (13), whereas 1,25(OH)₂D₃ and EB1089 decrease C4-2 proliferation (21).

Materials and Methods

Materials

1,25(OH)₂D₃ was provided by Dr. M. Uskokovic (Hoffmann La-Roche, Nutley, NJ). EB1089 was provided by Dr. Lise Binderup (Leo Pharmaceuticals, Ballerup, Denmark). Antibodies for immunohistochemistry were obtained from Santa Cruz Biotechnology and Cell Signaling Technologies.

Plasmid Constructs

A cDNA encoding human PTHrP (Genentech, Inc.) was cloned in-frame 5’ to the green fluorescent protein (GFP) reporter in the vector pEGFP-1 (Clontech). This construct has been described previously (13). Control cells were transfected with the empty vector.

Cell Culture and Transfection with PTHrP Constructs

C4-2 cells purchased from UroCor were grown at 37°C in humidified 95% air/5% CO₂ in RPMI 1640 containing 10% fetal bovine serum (FBS) and L-glutamine, and were stably transfected by electroporation. Individual clones were isolated as described (13), and were tested for PTHrP mRNA levels by reverse transcription/real-time PCR (13) and for PTHrP secretion using an immunoradiometric assay (Diagnostic Systems Laboratories; ref. 14). These clones have been described previously (13).

Cell Migration, Invasion, and Apoptosis

Cells were maintained in medium containing 10% dialedyzed FBS for 4 days, then treated with 1,25(OH)₂D₃ or EB1089 (10⁻⁹ to 10⁻⁷ mol/L). Ethanol (0.01% final concentration) was used as vehicle control. After 48 or 72 h, the cells were trypsinized, and 0.5 × 10⁶ cells were loaded with Calcein-AM (10⁻⁹ mol/L) for 24, 48, or 72 h. Pictures were taken before wounding, and at 48 and 72 h after wounding. The extent of migration was analyzed using the NIH Image software.

Soft Agar Growth

After treating with 1,25(OH)₂D₃ or EB1089 (10⁻⁹ to 10⁻⁷ mol/L) for 48 h, cells (1 × 10⁶) were suspended in 2× medium/20% dialyzed FBS. Forty-eight hours after plating, the cells were treated with 1,25(OH)₂D₃ or EB1089 (10⁻⁷ to 10⁻⁹ mol/L) for 24, 48, or 72 h. Pictures were taken before wounding, and at 48 and 72 h after wounding. The extent of migration was analyzed using the NIH Image software.

Nude Mouse Tumor Xenograft Studies

C4-2 cells were cultured in medium containing 10% FBS. At 70% confluency, the cells were trypsinized and the pellet was washed once with PBS-containing medium and then resuspended in PBS. The cells were then resuspended in a small volume of PBS. Matrigel was then added to the PBS/cell suspension such that the final Matrigel/cell ratio was 5:1, at a concentration of 3 × 10⁶ cells/100 μL. Male athymic nude mice (Harlan Sprague-Dawley), ~6 weeks of age, were anesthetized with a ketamine/xylazine mix, then injected s.c. on the dorsal surface with 100 μL of the cell suspension. The following C4-2 cell clones were used (six mice/group): three independent PTHrP-overexpressing clones, two independent empty vector–transfected clones (control), and parental cells. Body weight and tumor volumes were monitored twice weekly (28). Mice were sacrificed on day 45 after injection, and the tumors were excised and weighed.

Intracardiac Injection of C4-2 Cells

PTHRp-overexpressing and control cells were cultured and processed as described above. Cells (1 × 10⁵) were then
suspended in 0.1 mL PBS and injected into the left ventricle of mice anesthetized as described above. The following C4-2 cell clones were used (10 mice/group): three independent PTHrP-overexpressing clones, two independent empty vector-transfected clones (control), and parental cells. Tumor metastases were monitored by whole-body imaging, using a fluorescence light box illuminated by fiber optic lighting (Lightools Research).

**Immunohistochemistry**

Portions of the dissected mouse tumors were fixed immediately in 10% neutral buffered formalin for 24 h at room temperature after harvesting, then placed in 70% ethanol. Bone samples were similarly fixed and decalcified using EDTA. Formalin-fixed tissues were embedded in paraffin, and sections (5 μm) were cut from the paraffin blocks. The sections were deparaffinized in xylene, and rehydrated in a descending ethanol series. Protein staining was done using the DAKO EnVision kit (Dako Corporation), as described (28). Apoptosis was measured using the terminal nucleotidyl transferase (TdT)-mediated nick end labeling (TUNEL) cell death detection kit (Roche Applied Science). All sections were counterstained with hematoxylin and observed by light microscopy. For negative controls, sections were incubated with rabbit IgG (Santa Cruz Biotechnology) in place of primary antibody. Images were recorded using a Nikon microscope.

**Tissue Procurement**

Prostate adenocarcinoma and normal prostate patient specimens were obtained from prostate biopsy samples over a 4-year period from 2001 to 2005 at the University of Texas Medical Branch, Galveston, TX, and were provided to us by the Department of Surgical Pathology. Biopsy tissues were fixed in 10% neutral buffered formalin. Tumor stage and differentiation grade were assessed using the Gleason score. The following specimens were used (Gleason score in parentheses): normal prostate, 18 samples; well-differentiated adenocarcinoma (Gleason score, 5–6), 14 samples; moderately differentiated adenocarcinoma (Gleason score, 6–7), 15 samples; and poorly differentiated adenocarcinoma (Gleason score, 8–9), 12 samples. Tissue processing and immunohistochemistry were done as described above. Tissue acquisition and subsequent use were approved by the University of Texas Medical Branch Institutional Review Board.

**Statistics**

Numerical data are presented as the mean ± SE. The data were analyzed by ANOVA, followed by a Bonferroni post test to determine the statistical significance of differences. All statistical analyses were done using InStat Software (GraphPad Software, Inc.). *P < 0.01* was considered significant.

**Results**

**Down-Regulation of PTHrP Expression by EB1089 Plays a Role in the Antimigratory and Anti-Invasive Effects of EB1089 In vitro**

We previously showed that transfecting C4-2 cells with the construct expressing wild-type PTHrP resulted in significant (20-fold to 25-fold) increases in PTHrP mRNA levels and secreted PTHrP levels, compared with control cells (13, 30). PTHrP overexpression significantly increased cell migration (∼1.8-fold) and invasion (∼1.5-fold; Fig. 1A and B). Treating C4-2 cells with EB1089 (10^{-6} or 10^{-9} mol/L) for 72 hours significantly decreased PTHrP mRNA and secreted protein levels (Fig. 1C and D). We previously reported similar effects of 1,25(OH)_{2}D_{3} on PTHrP mRNA levels (13). Under the same conditions, the effects of EB1089 and 1,25(OH)_{2}D_{3} on PTHrP mRNA and secreted protein levels in PTHrP-overexpressing cells were significantly lower (Fig. 1C and D; data not shown). Treatment with EB1089 (10^{-6} or 10^{-9} mol/L) for 48 or 72 hours caused a significant decrease in the migration (∼45% decrease) and invasion (∼35% decrease) of control C4-2 cells (Fig. 1A and B; data not shown). At the same concentrations, EB1089 had a significantly smaller effect (<20%) on the migration and invasion of PTHrP-overexpressing C4-2 cells (Fig. 1A and B). Similar effects were obtained with 1,25(OH)_{2}D_{3} (data not shown). PTHrP had no effect on C4-2 apoptosis under serum-replete or serum-depleted conditions (data not shown). Treatment with 1,25(OH)_{2}D_{3} or EB1089 (10^{-9} to 10^{-7} mol/L) for 48 or 72 hours also had no significant effect on the apoptosis of parental, empty vector-transfected, or PTHrP-overexpressing C4-2 cells (data not shown).

Cell migration was also assessed using the monolayer scratch assay. Repair of the cell monolayer was significantly faster at 48 and 72 hours after wounding for cells overexpressing PTHrP compared with control cells, and was complete 72 hours after wounding (Fig. 1E; data not shown). There was no significant difference in cell migration and invasion, or in cell monolayer repair between parental cells and control cells (data not shown).

Both EB1089 and 1,25(OH)_{2}D_{3} inhibited monolayer repair of the control cells at 48 and 72 hours after wounding (Fig. 1E; data not shown). In agreement with the cell migration data (Fig. 1A), EB1089 and 1,25(OH)_{2}D_{3} exerted a negligible effect on the migration of PTHrP-overexpressing cells (Fig. 1E; data not shown). Because the proliferation rate of PTHrP-overexpressing cells is higher than that of the controls, and 1,25(OH)_{2}D_{3} compounds exert a significantly greater effect on the proliferation of control cells than on that of PTHrP-overexpressing cells (13), the differences in monolayer repair shown in Fig. 1D may be attributed to differences in cell migration as well as in cell proliferation.

**EB1089 Inhibits the PTHrP-Mediated Increase in Anchorage-Independent Cell Growth**

PTHrP facilitated soft agar clone formation; overexpressing PTHrP increased the size and number of colonies in soft agar after 15 days in culture (Fig. 2A–C). Increasing the incubation time from 15 to 21 days did not further increase the number of colonies formed (data not shown). Treating control cells with EB1089 or 1,25(OH)_{2}D_{3} significantly decreased both the size and number of colonies formed (Fig. 2; data not shown). EB1089 and 1,25(OH)_{2}D_{3} also caused a significant decrease in the size and number of colonies formed by PTHrP-overexpressing cells. The effect on colony number was more pronounced than that on colony size (Fig. 2; data not shown).

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EB1089 Reverses the Growth-Stimulatory Effects of PTHrP in a Xenograft Model

After s.c. injection, both control and PTHrP-overexpressing C4-2 cells produced tumors; the same incidence of tumor formation was observed at the later time points after injection (21 days). However, PTHrP increased both the rate of xenograft growth and the size of the tumors (Fig. 3A–C). At the time of sacrifice, tumors derived from PTHrP-overexpressing cells were, on average, ∼3.5-fold heavier than those from control cells (Fig. 3A and B). In addition, microvessels and mitotic cells were detected to a significantly greater extent in tumors from PTHrP-overexpressing cells than in those from control cells (Fig. 3D).

There were no significant differences in any of the variables measured between tumors from parental cells and empty vector transfectants (data not shown). No tumors were observed when cells were injected in the absence of Matrigel (data not shown), indicating that C4-2 cells require additional factors or stromal cells to induce tumor growth (29, 31).

We also examined the effect of EB1089 (0.5 μg/kg) on tumor formation after injecting PTHrP-overexpressing and control cells. This dose of EB1089 has no effect on blood calcium (29, 32). We observed that EB1089-treated animals did not lose more weight than control animals, thereby supporting the absence of hypercalcemia. EB1089 treatment decreased the size of the tumors produced by both the PTHrP-overexpressing and control cells (Fig. 3A–C). Thus, starting at day 24, tumors from mice treated with EB1089 were significantly smaller than those from vehicle control-treated mice after injection of PTHrP-overexpressing cells. Similar effects were observed after injection of control cells, although significant changes were only observed at later time points (Fig. 3C). EB1089 also decreased microvessel formation in tumors from PTHrP-overexpressing cells (Fig. 3D).

The Effects of PTHrP on Tumor Size are Mediated via Increased Proliferation and Decreased Apoptosis whereas the Effects of EB1089 are Mediated via Decreased Proliferation

All immunohistochemical data presented are representative of a minimum of five sections each from xenografts obtained from three independent PTHrP-overexpressing clones and two empty vector–transfected clones. PTHrP and control cells.
expression was higher in xenografts from mice injected with PTHrP-overexpressing cells than in corresponding xenografts from control cells (Fig. 4A), confirming that cells within these tumors retained high PTHrP expression. PTHrP staining was evident both in the nucleus and cytoplasm (Fig. 4A). Xenografts from mice treated with EB1089 had lower nuclear and cytoplasmic PTHrP immunoreactivity (Fig. 4A). In all immunohistochemical data presented, no staining was observed when control IgG was used as the primary antibody (Fig. 4A; data not shown). There were no differences in staining between sections obtained from tumors derived from parental cells versus empty vector transfecants (data not shown).

We also compared cell apoptosis and proliferation in xenografts from PTHrP-overexpressing and control cells, and the effects of EB1089 on these processes. Using the TUNEL assay, we showed that PTHrP increases tumor cell viability (Fig. 4B). These effects were accompanied by increased staining for the antiapoptotic proteins Bcl-2 and BclXL and decreased staining for the proapoptotic protein Bax (Fig. 4B). In agreement with the in vitro data, treatment of animals with EB1089 did not alter the level of apoptosis or the levels of Bcl-2, BclXL, and Bax (data not shown). We also show that PTHrP increased the proliferative index of the tumor cells, an effect which was reversed by EB1089 (Fig. 4C). In agreement with the in vitro data (13), EB1089 exerted a significantly greater effect in xenografts from control cells than in those from PTHrP-overexpressing cells (Fig. 4C).

**PTHRP Expression Correlates with Gleason Score in Human Prostate Adenocarcinoma**

Normal prostate sections and prostate cancer sections from well-differentiated, moderately differentiated, and poorly differentiated specimens with Gleason scores ranging from 5 to 9 were analyzed for PTHrP expression. In normal prostate, low PTHrP expression was limited to the cells of the basal layer; staining in epithelial cells was largely negative (Fig. 4D). There was an increase in PTHrP expression with increasing Gleason score. Specifically, staining was

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**Figure 2.** Effect of EB1089 on anchorage-independent cell growth of PTHrP-overexpressing and control cells. Assays to determine colony formation in soft agar were done in 60 mm dishes containing a bottom layer consisting of 1.5 mL culture medium containing 0.4% (w/v) agar. After treating with EB1089 (10^{-8} or 10^{-9} mol/L) for 48 h, cells (1 × 10^4) were plated in a top layer of 0.3% agar. As control, cells were treated with ethanol (EtOH, vehicle control). After 2 weeks in culture in the presence of EB1089, the plates were photographed at ×40 magnification, and clone size was measured using ImageJ software (NIH). Photographs were also taken at ×10 magnification to measure clone frequency. All clones in focus >50 μm in size were measured. A, representative colonies. B, colony size. Columns, mean of 20 colonies for each of three independent clones overexpressing PTHrP (PTHrP) and two independent empty vector-transfected clones (V); bars, SE. C, average number of colonies. Columns, mean of three fields per plate for each of the clones described in B, bars, SE. *P < 0.001, significantly different from the corresponding vehicle-treated value; #, P < 0.001, significantly different from the empty vector-transfected vehicle control value (B and C).
more intense in samples from poorly differentiated cancers than in those from well-differentiated cancers (Fig. 4D). The well-differentiated specimens show well-formed glandular structures. Here, PTHrP immunoreactivity was predominantly confined to the basal layer, and was mainly cytoplasmic. As the cancer progressed to poorly differentiated, the glandular structures were lost; with more fused glands and solid tumor cells. This was accompanied by intense cytoplasmic and nuclear PTHrP immunoreactivity (Fig. 4D). This level of PTHrP staining was comparable to that of the xenografts from PTHrP-overexpressing cells (Fig. 4A). Moderately differentiated prostate cancer showed staining in the basal layer as well as in the areas in which the glandular structure was lost (Fig. 4D).

**PTHRP Exerts Proangiogenic Effects which are Reversed by EB1089**

Tumor angiogenesis was examined by immunohistochemical staining for platelet/endothelial cell adhesion molecule-1 and vascular endothelial growth factor. The intratumoral microvessel density was significantly higher in tumors derived from PTHrP-overexpressing cells than in those from control cells (Fig. 5A and B). EB1089 significantly decreased intratumoral microvessel density in xenografts from both the PTHrP-overexpressing and control groups (Fig. 5A and B). Vascular endothelial growth factor expression was also higher in tumors from PTHrP-overexpressing cells than in those from empty vector transfected animals (Fig. 5C). EB1089 reversed these effects (Fig. 5C).

**PTHRP Enhances Metastasis of C4-2 Cells to the Bone and Increases the Degree of Osteolytic versus Osteoblastic Lesions whereas EB1089 Exerts a Protective Effect**

Four weeks after intracardiac injection, GFP fluorescence was observed in the facial region of 70% of the mice receiving PTHrP-overexpressing cells, but not in any of the mice receiving control cells. The earliest time point at which fluorescence was observed in mice injected with the control cells was 6 weeks postinjection (Fig. 6A). At this time point, there...
was significantly more fluorescence in mice injected with PTHrP-overexpressing cells (Fig. 6A). The mice were sacrificed at this time point because 8 of 10 of the mice receiving PTHrP-overexpressing cells had lost ∼30% of body weight and had marked cachexia. Whole-body imaging revealed little or no fluorescence in other areas of the body in the animals injected with PTHrP-overexpressing or control cells.

EB1089 significantly decreased metastasis, and no fluorescence was detected 6 weeks after injection of control cells. In contrast, fluorescence was observed in the facial area of mice injected with control cells and treated with sesame oil (vehicle controls; Fig. 6A). Treating mice injected with PTHrP-overexpressing cells with EB1089 also caused a significant decrease in fluorescence, compared with vehicle control–treated mice, although fluorescence could still be detected (Fig. 6A).

To confirm the presence of tumor cells in the areas that showed fluorescence, the bones were excised for immunohistochemical analysis. In control mice, C4-2 cells caused predominantly osteoblastic lesions (Fig. 6B). Conversely, in

![Figure 4](https://example.com/figure4.jpg)

**Figure 4.** A, PTHrP immunostaining in xenografts from PTHrP-overexpressing and control C4-2 cells after EB1089 (EB) treatment. IgG, sections stained with an antirabbit IgG antibody (negative control). Representative nuclear staining (arrows). Magnification, ×100. B, level of apoptosis, measured using the TUNEL assay, and by immunohistochemical analysis for the antiapoptotic proteins Bcl-2 and BclXL, and the proapoptotic protein Bax. The apoptotic index was calculated by counting the brown cells in TUNEL sections. Data are expressed relative to the values obtained in sections from mice injected with empty vector–transfected cells and treated with sesame oil (set at 100%). *, *P* < 0.001, significantly different from the empty vector value. C, proliferative index, measured by staining for Ki67, which recognizes proliferating cells (brownish purple). Representative Ki67-positive cells (arrows). The proliferative index was calculated by counting the number of Ki67-positive cells per field. Data are expressed relative to the values obtained in sections from mice injected with empty vector–transfected cells and treated with saeose oil (set at 100%). *, *P* < 0.001, significantly different from the corresponding sesame oil–treated value; #, *P* < 0.001, significantly different from the empty vector–transfected values. Columns, mean of three fields for each of five sections from each of six tumors from three independent PTHrP-overexpressing clones and two empty vector–transfected clones, each treated with EB1089 or sesame oil; bars, SE histograms in B and C. A to C, each panel is representative of five sections for each of six tumors from mice injected with the clones described above. D, immunohistochemical staining of human prostate cancer specimens for PTHrP expression. Well, section from well-differentiated prostate cancer (Gleason score 5); moderate, section from moderately differentiated prostate cancer (Gleason score 7); poor, section from poorly differentiated prostate cancer (Gleason score 9).
PTHrP-overexpressing cells, the lesions were predominantly osteolytic, with tumor cells replacing the bone and bone marrow (Fig. 6B). EB1089 treatment of mice injected with control C4-2 cells largely reversed the osteoblastic lesions and decreased the tumor burden (Fig. 6B). In mice injected with PTHrP-overexpressing C4-2 cells, EB1089 also reduced the tumor burden and the extent of osteolysis (Fig. 6B). Thus, after EB1089 treatment, the ratio of tumor/bone areas was significantly decreased compared with sesame oil treatment (Fig. 6B). Treating naïve mice with EB1089 did not affect their bone morphology (Fig. 6B). Treatment with sesame oil also did not affect the bone morphology of naïve or treated mice (data not shown). At the time of sacrifice, a low degree of micrometastasis was detected in spinal column sections, and no tumor cells were detected in the long bones (data not shown).

Increased PTHrP immunoreactivity was observed in tumor cells within the bone lesions that developed after injection of cells overexpressing PTHrP versus those from control cells (Fig. 6B, inset). Treatment with EB1089 decreased PTHrP immunoreactivity (Fig. 6B, inset).

**Discussion**

The mechanisms by which tumor cells become invasive and eventually metastatic are a crucial issue in cancer biology and medicine (33). The metastatic process requires that cells acquire new capabilities, including an increased ability to migrate and invade surrounding tissues to reach the vasculature and lymphatics (33). This process is accompanied by neangiogenesis (34). PTHrP has been identified as a contributing factor for the pathogenesis and progression of prostate cancer (35). In this study, we show that PTHrP increases C4-2 prostate cancer cell migration and invasion, anchorage-independent growth in vitro, and xenograft growth in vivo. Both control and PTHrP-overexpressing C4-2 cells metastasized to the bone after intracardiac injection into nude mice. However, PTHrP altered the metastatic profile from predominantly osteoblastic to osteolytic.

Multiple features of PTHrP action account for its effects on cancer progression. Here, we show that PTHrP increases C4-2 cell migration and Matrigel invasion in vitro and cell growth in the absence of adhesion to the extracellular...
matrix. PTHrP also increases cell proliferation (13). In addition, whereas PTHrP has no significant effect on the basal level of apoptosis, it protects C4-2 cells from doxorubicin-induced apoptosis (30). PTHrP also decreases apoptosis, but has no effect on the proliferation of Mat-Ly-Lu prostate cancer cells (15), indicating that the PTHrP effects may be cell type-specific, and may also depend on the insult to which cells are exposed. PTHrP overexpression activates the phosphatidylinositol 3-kinase/Akt pathway and up-regulates proinvasive integrin α6β4 expression in multiple cell lines (27, 28, 30). Synergistic signaling between integrin α6β4 and growth factor receptors activates phosphatidylinositol 3-kinase, resulting in increased cancer progression (36). We recently established that integrin α6β4 provides the link between PTHrP and phosphatidylinositol 3-kinase/Akt activation (30).

The in vitro effects of PTHrP are accompanied by enhanced xenograft growth in vivo; these effects may be mediated both via increased cell proliferation and decreased apoptosis. The antiapoptotic effects of PTHrP in the xenograft model contrast with its in vitro effects, indicating that additional factors absent in the in vitro culture system may mediate the antiapoptotic effect of PTHrP in vivo. Aggressive cancer progression also significantly correlates with the degree of tumor vascularity, and multiple studies have shown that angiogenesis is of fundamental importance for several physiologic and pathologic processes, including tumor growth and metastasis (37, 38). Notably, here we show that one of the pathways via which PTHrP supports tumor progression in vivo is through increased angiogenesis, as evidenced by increased vascular endothelial growth factor expression and endothelial components of xenograft vasculature. Utilizing the chorioallantoic membrane angiogenesis assay, Bakre et al. (39) reported that PTHrP inhibits angiogenesis in vivo via a protein kinase A-dependent pathway. In that study, chorioallantoic membranes were treated with

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**Figure 6.** Analysis of metastatic lesions to the bone from PTHrP-overexpressing and control C4-2 cells in mice treated with EB1089. C4-2 cells overexpressing PTHrP fused to GFP, and control GFP-expressing C4-2 cells were injected into the left ventricle of nude mice. A, fluorescence observed by whole-body imaging 6 weeks after injection. Areas of fluorescence (arrows). B, representative H&E staining of sections from areas of the bone that showed fluorescence in A. Bone sections after injection of PTHrP-overexpressing (PTHRP) and empty vector–transfected control (V) C4-2 cells in mice treated with EB1089 or sesame oil (vehicle control). Naive mice did not receive any cell injections. Each panel is representative of three sections from each of 10 mice injected with one of three independent PTHrP-overexpressing clones or two empty vector–transfected clones, then treated with EB1089 or sesame oil. B, bone; BM, bone marrow; T, tumor cells. Inset, PTHrP immunostaining in sections from the corresponding bone specimens.
NH2-terminal PTHrP fragments or transfected with a PTHrP-expressing construct (39). PTHrP activates protein kinase A via an autocrine/paracrine pathway involving an interaction with the parathyroid hormone/PTHrP 1 receptor (PTH1R; ref. 40). PTHrP also functions via an intracrine pathway after translocating to the nucleus (40), and its effects on cell proliferation, survival, migration, and invasion are mediated via this pathway in multiple cell lines (14, 41, 42). Moreover, opposing effects of PTHrP on cell proliferation have been reported, depending on whether it is acting via an autocrine/paracrine or intracrine pathway (41, 42). Thus, autocrine/paracrine PTHrP action may decrease angiogenesis (39), whereas intracrine PTHrP action may enhance angiogenesis.

Multiple studies have established a role for PTHrP in the osteolytic lesions accompanying breast cancer metastasis to the bone (reviewed in ref. 43). MCF-7 and MDA-MB-231 breast cancer cells overexpressing PTHrP (1–139) induce significantly more bone metastases than do parental cells (43). Unlike the case with breast cancer, a central role for PTHrP in the development of prostate cancer–induced bone metastases is not as strong. After intracardiac injection of Mat-Ly-Lu cells, both a lack of effect of PTHrP on the extent and nature of bone metastasis (44) and a PTHrP-mediated increase in osteoclast number (45) were observed. In contrast, PTHrP was reported to increase primary tumor growth after intratibial injection of PC-3, DU145, and Mat-Ly-Lu cells into nude mice (15, 46). These studies have led to the general conclusion that the role of PTHrP is not in the actual metastasis process, but in the bone response to prostate carcinoma within the bone microenvironment. However, the studies using the intracardiac model of prostate cancer metastasis used highly aggressive cells (15, 44–46). In this study, we show that after intracardiac injection of the less invasive C4-2 cells, PTHrP decreases the lag time for C4-2 cell metastasis to the bone, and alters the nature of the metastasis from predominantly osteoblastic to osteolytic. Moreover, we observed a correlation between PTHrP expression and the Gleason score in human prostate adenocarcinoma sections; the magnitude of the increase in PTHrP staining observed in poorly differentiated versus well-differentiated and normal prostate sections seems comparable to that observed in sections from xenografts from PTHrP-overexpressing versus control C4-2 cells (Fig. 4). The elevated expression of PTHrP in xenografts from PTHrP-overexpressing cells was accompanied by increased proliferation and angiogenesis and decreased apoptosis. These data indicate that PTHrP expression may play a central role in prostate cancer at the primary site, and this may in turn lead to increased metastasis.

In our studies, metastasis was observed predominantly in the oral and maxillofacial region. Previous studies using C4-2 cells injected intracardially did not report metastasis in this region; rather, 20% of the injected mice presented with a paraspinal tumor mass (47). However, metastasis to the oral and maxillofacial region has been observed after intracardiac injection of MDA-MB-231 cells (48). In our study, we only observed a low degree of micrometastasis in spinal column sections. The reason for the differences in the primary location of the metastases between the study by Wu et al. (47) and our study is at present unknown.

Given the observed role of PTHrP in prostate cancer growth and progression in vitro and in vivo, targeting PTHrP production in prostate cancer may prove therapeutically beneficial. Because the options for treating metastatic prostate cancer are currently limited, there is a need to develop well-tolerated alternative treatments to slow progression. The natural hormone 1,25(OH)2D3 and its analogues inhibit proliferation and induce apoptosis of numerous cancer cell types, including those derived from prostate cancer. 1,25(OH)2D3 also inhibits tumor cell migration, metastasis, and angiogenesis (49). Here, we show that EB1089 decreases C4-2 cell migration, invasion, and anchorage-independent cell growth in vitro, as well as xenograft growth and bone metastasis in vivo. 1,25(OH)2D3 and EB1089 also down-regulate endogenous PTHrP expression in prostate cancer cell lines (21–23), and we previously showed that regulation of PTHrP expression by 1,25(OH)2D3 plays a role in the anti-proliferative effects of 1,25(OH)2D3 in C4-2 cells (13). Here, we show that regulation of endogenous PTHrP expression by 1,25(OH)2D3 and EB1089 also plays a role in their anti-migratory and anti-invasive effects, in that the effects of these compounds on migration and Matrigel invasion were significantly attenuated in cells overexpressing PTHrP (~36 to +139), which lacks the negative vitamin D response element required for regulation by 1,25(OH)2D3. Thus, we conclude that regulation of endogenous PTHrP expression by 1,25(OH)2D3 is a major pathway via which 1,25(OH)2D3 exerts its protective effects in prostate cancer cells. However, pathways downstream of PTHrP may also be operational because EB1089 decreased the anchorage-independent growth of PTHrP-overexpressing cells, and the in vivo studies showed a decrease in PTHrP levels after EB1089 treatment of mice injected with PTHrP-overexpressing cells. In the in vivo studies, it is also possible that secreted PTHrP may diffuse away from the intercellular spaces to a significantly greater extent from the smaller tumors (after EB1089 treatment) than from the larger tumors (in vehicle-treated controls).

The direct effects of 1,25(OH)2D3 and its analogues on bone are complex. We show that treating mice with EB1089 decreased the incidence and severity of bone lesions after intracardiac injection of C4-2 cells. Similar results were observed in an intracardiac model of breast cancer metastasis (32). These effects were attributed at least in part to the effect of EB1089 on tumor cell growth within bone. As in C4-2 cells, 1,25(OH)2D3 and EB1089 decrease MDA-MB-231 cell proliferation but have no effect on apoptosis (32). Another low-hypercalcemic 1,25(OH)2D3 analogue, JK-1626-2, also decreased the incidence of bone lesions induced by MDA-PCa-2b cells injected into the tibia (50). However, in that study, treatment with the analogue only prevented the development of bone lesions; tumor cells were still present in the diaphysis of ~70% of treated mice (50). Differences in the protective effects of these analogues may be attributed to properties of the analogues themselves.
different cell lines used in the two studies, and/or the routes of administration used (intracardiac versus intratibial).

In conclusion, we show that PTHrP increases C4-2 cell migration, invasion, and anchorage-independent cell growth. PTHrP also increases xenograft growth in vivo. Furthermore, PTHrP enhances metastasis to the bone and shifts the metastatic lesion to a predominantly osteolytic profile. Regulation of PTHrP expression by 1,25(OH)2D3 and its analogues plays a role in the antimigratory and antiinvasive effects of 1,25(OH)2D3. EB1089 exerts a strong protective effect on PTHrP-mediated xenograft growth and metastasis in vivo. Using noncalcemic vitamin D analogues to target PTHrP expression in prostate cancer may prove therapeutically important in the prevention and treatment of prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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