

## Tumorigenesis and Neoplastic Progression

# BMP7, a Putative Regulator of Epithelial Homeostasis in the Human Prostate, Is a Potent Inhibitor of Prostate Cancer Bone Metastasis *in Vivo*

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**Bone morphogenic protein 7 (BMP7) counteracts physiological epithelial-to-mesenchymal transition, a process that is indicative of epithelial plasticity. Because epithelial-to-mesenchymal transition is involved in cancer, we investigated whether BMP7 plays a role in prostate cancer growth and metastasis. BMP7 expression in laser-microdissected primary human prostate cancer tissue was strongly down-regulated compared with normal prostate luminal epithelium. Furthermore, BMP7 expression in prostate cancer cell lines was inversely related to tumorigenic and metastatic potential *in vivo* and significantly correlated to E-cadherin/vimentin ratios. Exogenous addition of BMP7 to human prostate cancer cells dose-dependently inhibited transforming growth factor  $\beta$ -induced activation of nuclear Smad3/4 complexes via ALK5 and induced E-cadherin expression. Moreover, BMP7-induced activation of nuclear Smad1/4/5 signaling transduced via BMP type I receptors was synergistically stimulated in the presence of transforming growth factor  $\beta$ , a growth factor that is enriched in the bone microenvironment. Daily BMP7 administration to nude mice inhibited the growth of cancer cells in bone. In contrast, no significant growth inhibitory effect of BMP7 was observed in intraprostatic xenografts. Collectively, our observations suggest that BMP7 controls and preserves the epithelial phenotype**

**in the human prostate and underscore a decisive role of the tumor microenvironment in mediating the therapeutic response of BMP7. Thus, BMP7 can still counteract the epithelial-to-mesenchymal transition process in the metastatic tumor, positioning BMP7 as a novel therapeutic molecule for treatment of metastatic bone disease. (Am J Pathol 2007, 171:1047-1057; DOI: 10.2353/ajpath.2007.070168)**

The phenotypic changes of increased motility and invasiveness of carcinoma cells are reminiscent of the epithelial-to-mesenchymal transition (EMT) that occurs during embryonic development and morphogenesis.<sup>1-3</sup> During EMT, epithelial cell layers lose polarity and cell-cell contact, undergo a dramatic remodeling of the cytoskeleton, express mesenchymal components, and manifest a migratory phenotype by resolving cell-matrix contacts and digesting adjacent basal lamina.<sup>4,5</sup> Whereas in the developing embryo EMT is a prerequisite for the formation of various tissues and organs, EMT in postnatal life is required for repair and remodeling of numerous tissues or organs.<sup>1,6</sup>

Plasticity resulting from cells shifting between epithelial and mesenchymal phenotypes is discernible either by EMT or the reverse process of mesenchymal-to-epithelial transition (MET).<sup>2</sup> Both processes have emerged as a fundamental principle for reprogramming of gene transcription and as a major determinant

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of stem cell fate in development and in adult tissue homeostasis. The process of EMT is currently considered a potential mechanism of the disease progression in malignant and fibrotic disorders.<sup>1</sup> An example of the latter is the persistence of a pathogenic insult of the kidney that causes disruption of the tubular basement membrane and eventually may lead to renal fibrosis due to transition of tubular epithelial cells into a migratory mesenchymal phenotype (EMT-derived fibroblasts in the interstitium).<sup>6</sup>

In epithelial cells transforming growth factor  $\beta$  (TGF $\beta$ ) has been identified as one of the main inducers of EMT during development and in fibrotic disorders,<sup>5–10</sup> although another member of the TGF $\beta$  superfamily, bone morphogenetic protein 7 (BMP7) or osteogenic protein-1, is involved in the maintenance of the epithelial phenotype by induction of MET.<sup>11–14</sup> Zeisberg et al<sup>13,14</sup> demonstrated that BMP7 was capable of counteracting TGF $\beta$ -induced EMT and could reverse chronic renal injury.

Prostate cancer is the second most frequently diagnosed cancer and the second leading cause of cancer death in the Western male population. Evidence is mounting that during carcinogenesis, developing prostate cancer cells acquire mesenchymal (and even osteoblastic) characteristics and migratory features concomitant with a loss of epithelial characteristics like E-cadherin expression.<sup>15,16</sup> In cancer progression, “oncogenic” EMT refers to clusters of malignant cells that lose epithelial characteristics and acquire self-sustained migratory and highly invasive phenotypes.<sup>5</sup> We hypothesized that the Gleason histological grading of prostate cancer parallels EMT starting from “well-differentiated” cells with well-defined boundaries that still resemble healthy prostate epithelium (Gleason patterns 1 and 2), via infiltrative cancer cells with less-defined boundaries with extensions into adjacent non-neoplastic prostate tissue (Gleason pattern 3), toward “poorly differentiated” highly migratory prostate cancer cells that have lost many epithelial characteristics and have acquired an invasive metastatic and mesenchymal phenotype (Gleason patterns 4 and 5).

Several defects in the canonical TGF $\beta$  signaling pathway have been reported in prostate cancer<sup>17,18</sup> and may contribute to the development and progression of prostate cancer as alterations in TGF $\beta$  and TGF $\beta$ -receptor 2 expression are associated with poor clinical outcome.<sup>19</sup> A growing number of *in vivo* studies demonstrate that inhibitors of TGF $\beta$  or TGF $\beta$  receptors may reduce the metastatic and/or invasive properties of a variety of experimental cancers by preventing EMT pathways,<sup>5,20,21</sup> thus underscoring the importance of TGF $\beta$  in oncogenic EMT associated with cancer progression.

This study was designed to investigate whether tumorigenicity and invasive behavior are associated with modulated BMP7 expression in clinical prostate cancer specimens and in human prostate cancer cell lines with different tumorigenic potential. Furthermore, we studied if systemic administration of BMP7 affects the growth of human prostate cancer cells in orthotopic tumor and bone metastasis models by whole-body bioluminescent imaging (BLI). We present evidence for cross talk between BMP7 and TGF $\beta$

signaling in the regulation of EMT in prostate cancer, and we identified BMP7 as a potential therapy for metastatic bone disease.

## Materials and Methods

### Tissue Sampling

Radical prostatectomy specimens were obtained at the Department of Urology of the University of Bern, Switzerland. Written informed consent was obtained from all patients, and tissue sampling was approved by the local ethical committee. Within 15 minutes of surgical excision, samples of prostate cancer tissue or noncancerous tissue of the prostate were taken and either snap-frozen or immersed in RNAlater (Qiagen, Basel, Switzerland).<sup>22</sup> Tissue adjacent to the respective samples was processed for paraffin embedding and served as histological control. Histological diagnosis and grading were performed by a pathologist (R.M.).

### Laser Capture Microdissection (LCM)

Six- $\mu$ m cryosections were treated according to the HistoGene LCM frozen section staining kit (Arcturus, Bucher Biotech, Basel, Switzerland).<sup>22,23</sup> The P.A.L.M. MicroBeam system (P.A.L.M. Microlaser Technologies, Bernried, Germany) was used to excise approximately 1000 pure epithelial cells of noncancerous prostate (NP) and prostate cancer tissue in each prostate. Total RNA was extracted with the Pico Pure RNA Isolation Kit (Arcturus).

### Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA (see above) was reverse transcribed using random primers in the presence of RNase inhibitor (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCR was performed with exon-specific primers for human BMP7 (Hs\_002333477\_m1), glyceraldehyde-3-phosphate dehydrogenase (Hs\_99999905\_m1), E-cadherin (Hs\_00170423\_m1), vimentin (Hs\_00185584\_m1), and  $\beta$ -actin (Hs\_99999903\_m1) (primer catalog, Applied Biosystems, Rotkreuz, Switzerland) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The resulting values were normalized to glyceraldehyde-3-phosphate dehydrogenase or  $\beta$ -actin. Semiquantitative PCR was performed on samples of experimentally induced bone metastases from PC-3M-Pro4 after intracardiac inoculation into nude mice using exon-specific and human-specific BMP primers.<sup>22,24</sup>

### Cell Lines and Culture Conditions

The human prostate cancer cell lines PC-3 (American Type Culture Collection number CRL-1435; ATCC-LGC Promochem, Molsheim Cedex, France) and PC-3M-Pro4 were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (BioWhittaker, Verviers, Belgium). The PC-3M-Pro4 cells were generated from PC-3M cells

by injecting PC-3M cells into athymic mouse prostates and selecting for variants with increasing metastatic potential by several rounds of reinjecting cells from xenograft tumors back into the mouse prostate.<sup>25,26</sup> The human prostate cancer cell line LNCaP and LNCaP-derived cell lines C4-2 and C4-2 B4<sup>27,28</sup> were grown in T-Medium. Cells at 70 to 80% confluence were used for RNA extraction using an RNeasy Midi RNA extraction kit (Qiagen). Quantitative real-time PCR analysis was performed as described above.

Human PC-3M-Pro4 prostate cancer cells were stably transfected with a cytomegalovirus promoter-driven mammalian expression vector for luciferase, CMV-luc, and one clone with the highest expression of luciferase expression (PC-3M-Pro4/luc<sup>+</sup>) was successfully used for *in vivo* whole-body BLI.<sup>29-31</sup> PC-3M-Pro4/luc<sup>+</sup> were cultured in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and 800 µg/ml geneticin/G-418 (Invitrogen, Breda, The Netherlands). Cells were regularly certified free of *Mycoplasma* contamination. For intraosseous or orthotopic inoculation, cell suspensions of PC-3M-Pro4/luc<sup>+</sup> ( $1 \times 10^5$  cells/10 µl of phosphate-buffered saline) were prepared.<sup>29-31</sup>

### Animals

Male nude (BALB/c nu/nu) mice were purchased from Charles River (L'Arbresle, France). Mice were housed in individual ventilated cages under sterile conditions according to the Dutch guidelines for the care and use of laboratory animals (DEC 4077). Surgical and analytical procedures were performed while mice were anesthetized.<sup>29,31,32</sup>

### Intraosseous Inoculation of PC-3M-Pro4/luc<sup>+</sup> Cells

A single cell suspension of PC-3M-Pro4/luc<sup>+</sup> cells was injected into the right tibiae.<sup>29-31</sup> The progression of cancer cell growth was monitored weekly by BLI. After the experimental period, the animals were sacrificed, and the tibiae were dissected and processed for further histomorphometrical and immunohistochemical analysis (see below).

### Inoculation of PC-3M-Pro4/luc<sup>+</sup> into the Mouse Prostate (Orthotopic Implantation)

A single-cell suspension of  $1 \times 10^5$  PC-3M-Pro4/luc<sup>+</sup> cells/10 µl of phosphate-buffered saline was surgically inoculated into the prostates of anesthetized 6-week-old male nude mice. The cutaneous wound was sutured. The progression of cancer cell growth was monitored weekly by BLI. The animals were subsequently treated daily with 100 µg/kg BMP7 or vehicle solution. After the experimental period, the animals were sacrificed. Directly after removal of the mouse prostate, BLI images of the animals were taken to establish the number of locoregional lymph node metastases. Tissues were dissected and processed for further histomorphometrical and immunohistochemical analysis (see below).

### Induction of Systemic Metastases by Intracardiac Injection of PC-3M-Pro4/luc<sup>+</sup> Cells

A single-cell suspension of  $1 \times 10^5$  PC-3M-Pro4/luc<sup>+</sup> cells/100 µl of phosphate-buffered saline was injected into the left cardiac ventricle, and cancer cell growth was monitored weekly by BLI and radiography.<sup>29-31</sup> Daily BMP7 treatment started 2 days before inoculation of the cancer cells until the end of the experiment.

### BMP7 Treatment and Prostate Cancer Growth in Vivo

Human BMP7 was obtained from Dr. S. Vukicevic (Laboratory of Mineralized Tissues, School of Medicine, Zagreb, Croatia). BMP7 was expressed, purified, and lyophilized.<sup>33</sup> When used, BMP7 was freshly dissolved to a stock solution containing 1 mg/ml in 20 mmol/L acetate buffer with 5% mannitol, pH 4.5. BMP7 (20 µl) or vehicle solution (20 µl, 20 mmol/L acetate buffer with 5% mannitol) was administered daily into the tail veins of mice for 21 to 23 days (100 µg/kg human BMP7).

### Whole-Body BLI and Quantification of the Bioluminescent Signal

BLI of tumors induced by the luciferase-expressing human prostate cancer cell lines was performed.<sup>29,31</sup> Analyses for each metastatic site were performed after definition of the region of interest and then quantified.<sup>31</sup> Values are expressed as relative light units.

### Histomorphometry, Histochemistry, and Immunohistochemistry

Five-micrometer paraffin sections of patient-matched normal prostate and prostate cancer tissue were rehydrated, and a rabbit polyclonal antibody against BMP7 pro-domain was applied at a dilution of 1:100. Rabbit IgG (Jackson ImmunoResearch, La Roche, Switzerland) served as negative control. A species-specific biotinylated anti-IgG antibody followed by streptavidin/alkaline phosphatase conjugate (Amersham Biosciences, Roosendaal, The Netherlands) was used as the detection system. 3-Amino-9-ethyl-carbazole (Sigma, Buchs, Switzerland) served as chromogen (Sigma). Dissected tissues from animal studies were fixed in 4% paraformaldehyde (pH 6.8), decalcified (only bones) as described previously, and processed for paraffin embedding, sectioning, and staining<sup>32</sup> with PS1 antibodies directed against phosphorylated Smad1.<sup>34,35</sup>

### Immunoblotting

Crude cell lysates (10 µg/lane) were separated on sodium dodecyl sulfate-polyacrylamide gels and blotted on Hybond-P membranes (Amersham Biosciences). E-cadherin monoclonal antibody (5 µg/ml; Zymed Laboratories, Invitrogen), vimentin polyclonal rabbit antibody (ab7783; Abcam, Cambridge, UK), and a monoclonal

mouse anti-actin antibody (1:5000; Transduction Laboratories, Lexington, KY) were used as primary antibodies. Binding was detected with a peroxidase-labeled anti-mouse secondary antibody (Amersham Biosciences) and the ECL Advanced chemiluminescence substrate (Amersham Biosciences) using the VersaDoc imaging system and QuantityOne imaging software (Bio-Rad, Venendaal, The Netherlands).

### Human BMP7 Enzyme-Linked Immunosorbent Assay

PC-3M-Pro4 cells and a clone stably transfected to overexpress rhBMP7 were seeded in a six-well plate (120,000 cells/ml), and conditioned medium was harvested and analyzed for BMP7 protein synthesis by enzyme-linked immunosorbent assay after 4 days of culture according to the manufacturer's protocol (R&D Systems, Abingdon, UK).

### Transient Transfections and Transcription Reporter Assays

Transient transfections and transcription reporter assays were performed and values expressed as luciferase intensity (relative light units).<sup>36</sup> The experiments were performed in quadruplicate and repeated at least twice. Values are expressed as means  $\pm$  SEM. Incubation time with TGF $\beta$  or BMP7 was 30 hours.

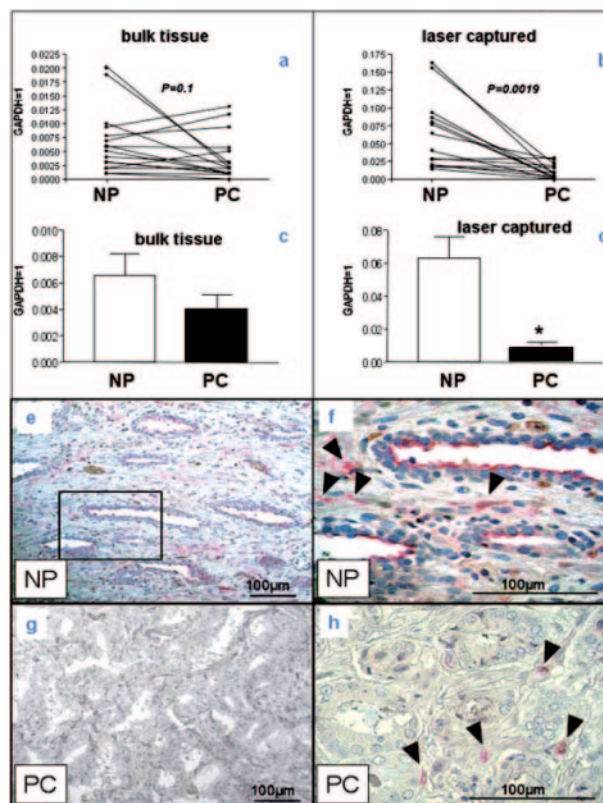
### Luciferase Reporter Gene Constructs

For intracellular signaling of TGF $\beta$ , the CAGA-luciferase construct, consisting of 12 Smad3/Smad4 binding sequences (CAGA boxes), and the luciferase-coding sequence was used. The CAGA boxes confer TGF $\beta$  but not bone morphogenetic protein (BMP) stimulation to a heterologous promoter reporter construct.<sup>37</sup>

The BRE<sub>4</sub>-luciferase construct, which is based on the mouse Id1 promoter, was used to study the presence and functionality of BMP receptors.<sup>37</sup> Signaling of BMP to stimulate the expression of BRE<sub>4</sub>-luciferase is transduced by BMP type I receptors and mediated by Smad1, Smad4, and Smad5, which form a complex with this reporter construct.<sup>38</sup> The SBE<sub>4</sub> construct consists of four Smad-binding elements (SBEs) in the promoter of the *JunB* gene, an immediate early gene that is potentially induced by TGF $\beta$ , activin, and bone morphogenetic proteins.<sup>39</sup> The *E-cadherin* promoter-luciferase construct is based on the upstream fragment (positions -178 to +92). The pGL3-E-cadherin promoter plasmid<sup>40</sup> was a kind gift from Dr. Antonio Garcia de Herreros (Institut Municipal d'Investigació Mèdica, Barcelona, Spain).

### Statistical Analysis

The paired *t*-test was performed for statistical evaluation of patient-matched mRNA expression, and the unpaired *t*-test was performed for comparison of mRNA expression in bulk and laser-captured tissue using GraphPad Prism



**Figure 1.** BMP7 mRNA expression in noncancerous prostate epithelium (NP) and prostate cancer cells (PC) of radical prostatectomy specimens. **a:** BMP7 mRNA expression in patient-matched specimens consisting predominantly of NP and PC tissue ( $n = 16$ ,  $P = 0.1$ , paired *t*-test). **b:** BMP7 mRNA expression in NP and neoplastic epithelial cells isolated by LCM (PC) ( $n = 14$ ,  $P = 0.0019$ , paired *t*-test). **c:** Mean BMP7 mRNA expression values  $\pm$  SEM in bulk tissue in NP and PC both normalized to glyceraldehyde-3-phosphate dehydrogenase ( $P = 0.1$ , paired *t*-test). **d:** Mean BMP7 mRNA expression values  $\pm$  SEM in microdissected NP and neoplastic epithelium (PC), both normalized to glyceraldehyde-3-phosphate dehydrogenase ( $*P = 0.0019$ , paired *t*-test). BMP7 monoclonal antibody immunoreactivity and in clinical specimens of NP (**e** and **f**) and PC (**g** and **h**). **e:** Boxed area is magnified in **f**. BMP7 immunoreactivity was detected in the apical cytoplasm of the luminal cell layer of the normal acinar epithelium and parts of stroma (arrowhead), but weak or no staining of the basal cells was found. **g** and **h:** Prostate cancer cells lack BMP7 immunoreactivity, whereas BMP7 was still detectable in parts of the stroma (arrowhead). Scale bars = 100  $\mu$ m.

Version 3 (GraphPad Software Inc.). Data are presented as means  $\pm$  SEM. Analysis of variance was performed for statistical evaluation of whole-body BLI data and reporter-luciferase assays. A *P* value equal or more than 0.05 was considered not significant.

### Results

#### BMP7 mRNA Expression in Radical Prostatectomy Specimens

Samples of prostate cancer (PC) tissue and internal controls from the same patient (NP) were obtained after radical prostatectomy, and BMP7 mRNA was measured by real-time PCR. In bulk tissue BMP7 mRNA expression was generally lower than in whole tissue specimens of noncancerous prostate (11/16, mean decrease 26%), a nonsignificant difference (Figure 1, a and c;  $P = 0.1$ ).

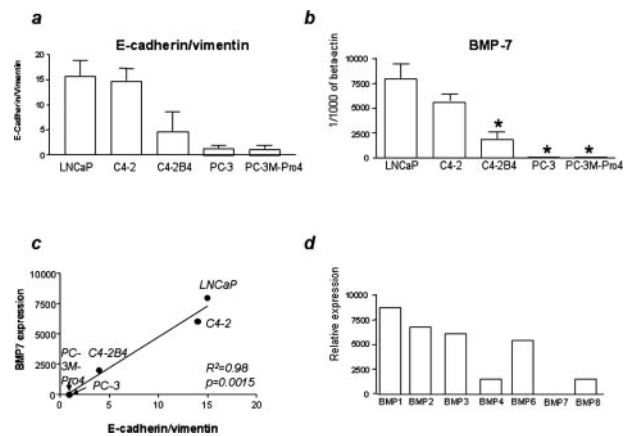
However, the multifocal nature of prostate cancer and the coexistence of epithelial and stromal tissues may cross-contaminate and dilute cell-specific gene expression analyzed in bulk tissue specimens. Therefore, we next collected pure populations of >1000 prostate cancer cells and patient-matched noncancerous prostate epithelial cells by LCM from 14 patients. In 93% of those cancer cell samples, BMP7 mRNA was strongly underexpressed when compared with patient-matched noncancerous epithelial cells (13/14, mean decrease 70%,  $P = 0.0019$ ; Figure 1, b and d). Comparison of the BMP7 mRNA expression in noncancerous prostate derived either from bulk tissue or from laser-captured epithelial cells revealed a 9.4 times higher expression in the epithelial compartment alone than in bulk tissue. BMP7 expression in laser-captured prostate cancer was also higher (2.8 times) when compared with bulk prostate cancer tissue, but these differences were less pronounced. These data strongly suggest that BMP7 is expressed mainly in the epithelial compartment of the prostate gland, particularly in the glandular epithelium.

Immunohistochemical analyses were performed for BMP7 on primary prostate cancer specimens and noncancerous tissue adjacent to the respective samples, which served as a histological control. Histological diagnosis and grading were performed by a certified pathologist (R.M.). BMP7 immunoreactivity was present in the cytoplasm of the luminal cell layer of the normal acinar epithelium in samples of noncancerous prostate tissue, although hardly any BMP7 could be detected in basal cells (Figure 1, e and f). In addition, BMP7 protein expression was also found in the prostate stromal compartment and blood vessels. In contrast, prostate cancer cells showed lack of BMP7 immunoreactivity (Figure 1, g and h).

Our LCM and immunohistochemistry data reveal that BMP7 expression was enriched in noncancerous prostate epithelium compared with prostate cancer cells in the same patients, suggesting that BMP7 down-regulation may facilitate prostate carcinogenesis, dissemination, and/or metastasis. Moreover, BMP7 expression (ratio prostate cancer/NP) appeared inversely correlated to Gleason patterns, but the distribution of patients for each pattern was too small to reach statistical significance (results not shown).

### BMP7 mRNA Expression in Prostate Cancer Cell Lines

We further investigated whether BMP7 expression in prostate cancer cell lines was associated with tumorigenicity, metastatic potential, and EMT degree. The prostate cell lines examined have progressively greater tumorigenic and metastatic potential in the following order: LNCaP, C4-2, C4-2B4, PC-3, and PC-3M-Pro4. This arrangement of cell lines also parallels the gradual loss of expression of epithelial markers (E-cadherin epithelial marker) and acquisition of mesenchymal characteristics (Figure 2a). Vimentin is a characteristic marker for the mesenchymal phenotype of cancer cells, and enhanced



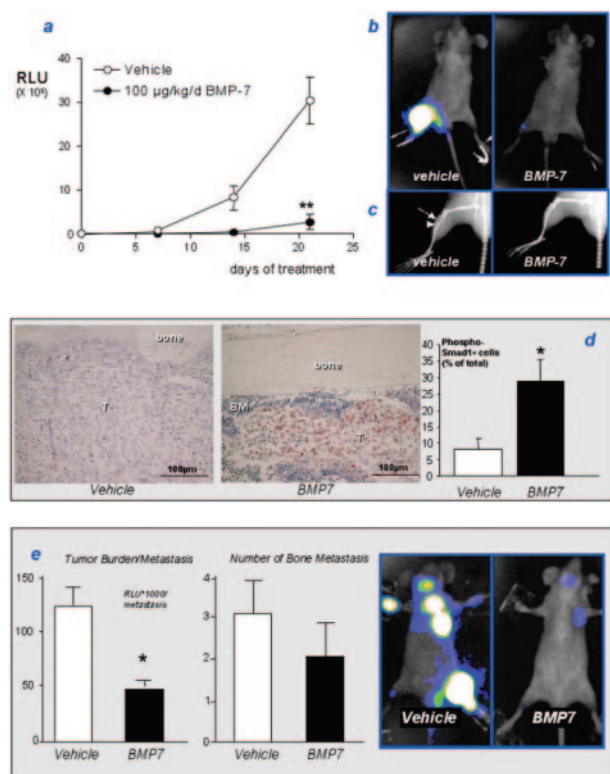
**Figure 2.** Real-time mRNA expression of E-cadherin, vimentin, and BMPs in prostate cancer cell lines with different tumorigenic potential *in vitro* and in experimentally induced bone metastases. **a:** E-cadherin/vimentin ratios in prostate cancer cell lines. **b:** BMP7 mRNA expression in prostate cancer cell lines. **c:** Correlation between E-cadherin/vimentin ratio and BMP7 mRNA expression in prostate cancer cell lines with different tumorigenic potential ( $R^2 = 0.98$ ,  $P = 0.0015$ ). **d:** Relative expression levels of different BMP mRNA levels in an experimentally induced bone metastasis from PC-3M-Pro4 cells in nude mice. The mRNA levels ( $\pm$ SEM) were quantified by real-time reverse transcriptase-PCR relative to  $\beta$ -actin endogenous control, except for **d**, where  $\beta$ 2-microglobulin was used for normalization as described previously.<sup>32</sup>

vimentin expression was previously found in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma.<sup>41</sup> In line with these observations, we found that vimentin expression increased with tumorigenicity and that E-cadherin/vimentin ratios decreased with augmented invasiveness and aggressiveness (Figure 2a).

BMP7 mRNA levels in prostate cancer cell lines strongly correlated to E-cadherin/vimentin ratio (Figure 2, a and b), whereas no association was observed for other BMPs (results not shown).<sup>24</sup> The highly tumorigenic and metastatic human prostate cancer cell line PC-3 and its derivative PC-3M-Pro4 did not show detectable BMP7 mRNA expression (Figure 2b) and protein synthesis *in vitro* (human specific BMP7 enzyme-linked immunosorbent assay; all <10 pg/ml hBMP7,  $n = 4$ ). The lack of BMP7 expression by PC-3M-Pro4 cells *in vitro* was confirmed *in vivo* in bone metastases (Figure 2d), suggesting that the bone microenvironment is unable to induce BMP7 expression in metastatic cancer cells. In contrast, the LNCaP, C4-2, and C4-2B4 cell lines, characterized by a much lower tumorigenic and metastatic potential, showed substantial expression of BMP7 mRNA (Figure 2b). E-cadherin/vimentin ratio together with BMP7 expression therefore decreased with increasing tumorigenic potential, and a strong correlation exists between BMP7 expression and E-cadherin/vimentin ratio ( $R^2 = 0.98$ ,  $P = 0.0015$ ; Figure 2c).

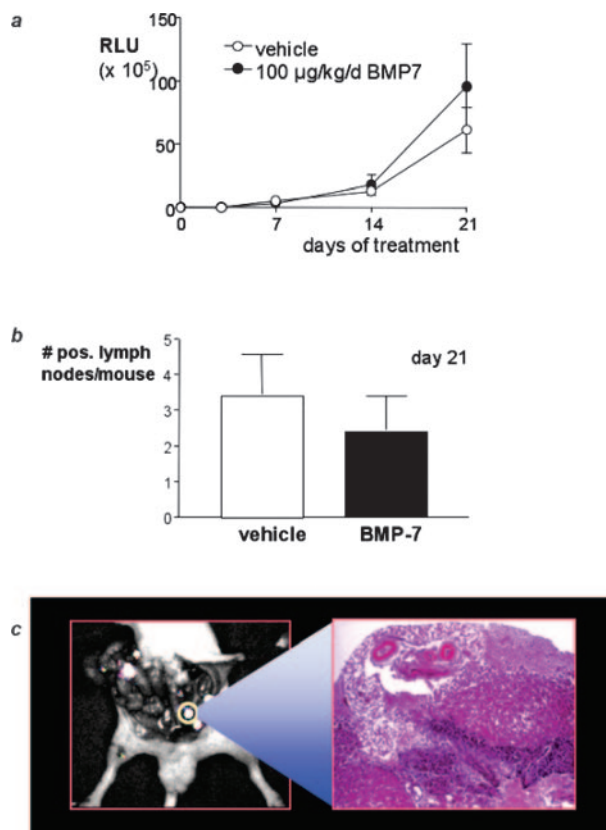
### BMP7 Treatment of Bone Metastasis and Orthotopic Prostate Cancer Growth *In Vivo*

To test whether the observed decrease of BMP7 expression during prostate cancer progression may contribute to the acquisition of an invasive metastatic phenotype, we investigated whether systemic administration of rhBMP7 affected the growth of human prostate cancer cells in



**Figure 3.** Effects of daily systemic administration of 100 µg/kg rhBMP7 on the growth of human prostate cancer cells in bone marrow (a–d) and bone metastasis (e and f) *in vivo* after intrasosseous transplantation or intracardiac inoculation of human PC-3M-Pro4/luc<sup>+</sup> cells using whole body bioluminescent reporter imaging. **a:** BMP7 treatment of PC-3M-Pro4/luc<sup>+</sup> cells growing in bone marrow of nude mice as monitored by BLI. **b:** Representative examples of bioluminescent photon emission in a vehicle- and BMP7-treated animal at day 21. **c:** Representative radiographs at day 21 after intrasosseous transplantation of PC-3M-Pro4/luc<sup>+</sup> cells growing in bone marrow (arrows indicate bone lesions in tibiae of nude mice). **d:** Immunohistochemical analyses of phospho-Smad1 localization at day 21 after intrasosseous transplantation of PC-3M-Pro4/luc<sup>+</sup> cells growing in bone marrow. The animals were either vehicle-treated (upper left panel) or treated with rhBMP7 (top right) 6 hours before explantation and fixation of the tibiae. The number of phospho-Smad1-positive cells in the BMP7-treated group is increased significantly. **e:** Effect of BMP7 treatment on the formation and growth of bone metastases by PC-3M-Pro4/luc<sup>+</sup> in nude mice as monitored by BLI (tumor burden, number of bone metastases, representative examples of BLI at day 19). Relative light units (±SEM) emitted was quantified at least once weekly starting at day 0 after intrasosseous implantation or intracardiac inoculation of the prostate cancer cells. *n* = 8 for each experimental group for intrasosseous transplantation experiment (a–d); *n* = 10 for intracardiac inoculation (e). Values are expressed as means ± SEM. \**P* < 0.05, \*\**P* < 0.001 (analysis of variance).

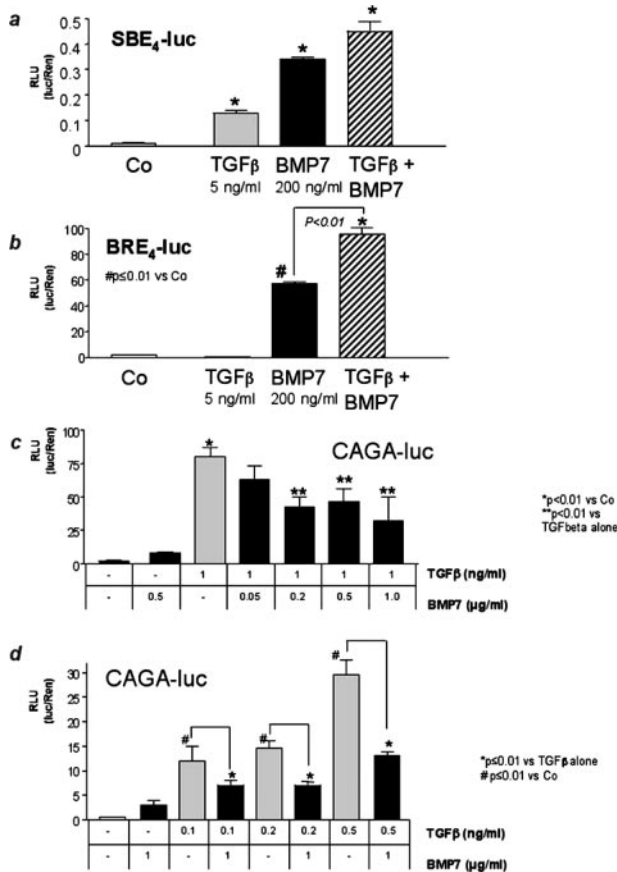
orthotopic tumor and bone metastasis animal models by whole-body BLI of human PC-3M-Pro4/luc<sup>+</sup> cells. Three days after intrabone inoculation of PC-3M-Pro4/luc<sup>+</sup> cells into the tibiae of BALB/c nu/nu mice, the animals were treated daily via tail intravenous injections of 100 µg/kg rhBMP7 or a vehicle solution, and growth of prostate cancer cells was monitored weekly by BLI and radiography. Administration of BMP7 for the duration of the experiment resulted in a significant and sustained inhibition of tumor cell growth and tumor-induced osteolysis in bone marrow (Figure 3, a–c). Systemic administration of BMP7 acted directly on bone-residing PC-3M-Pro4/luc<sup>+</sup> tumor cells as visualized by nuclear staining for phospho-Smad1, whereas this was significantly lower in vehicle-treated animals (Figure 3d). Inoculation of PC-3M-Pro4/luc<sup>+</sup> cells into the left cardiac ventricle of nude mice



**Figure 4.** Effects of daily systemic administration of 100 µg/kg rhBMP7 on intraprostatic growth of human PC-3M-Pro4/luc<sup>+</sup> cells and formation of loco-regional lymph node metastases in nude mice. **a:** Tumor take of PC-3M-Pro4/luc<sup>+</sup> cells growing in prostate on treatment with vehicle solution or daily 100 µg/kg rhBMP7 as monitored by BLI. **b:** The number of affected lymph nodes in nude mice after 21 days as detected by BLI. **c:** Representative example of bioluminescent photon emission of a mouse with multiple loco-regional lymph node metastases 21 days after intraprostatic inoculation of PC-3M-Pro4/luc<sup>+</sup> cells. The mouse prostate was removed before bioluminescent reporter imaging to obtain sensitive measurements of affected lymph nodes (b). The presence of micrometastatic deposits in lymph nodes was confirmed by H&E staining. Relative light units emitted was quantified at least once weekly starting at day 0 after intraprostatic implantation of the prostate cancer cells. *n* = 8 for each experimental group. Values are expressed as means ± SEM (analysis of variance).

resulted in the formation of multiple bone metastases.<sup>29–31</sup> Continuous daily treatment with BMP7 inhibited the growth of bone metastases (*P* < 0.05) despite a tendency toward a decrease. As a result, the average tumor burden per bone lesion was significantly decreased on BMP7 treatment (Figure 3e).

Our data therefore suggest that BMP7 inhibits the growth of bone metastases by human prostate cancer cells in nude mice. In contrast to its inhibitory effect on bone metastases, BMP7 treatment of orthotopically implanted PC-3M-Pro4/luc<sup>+</sup> cells did not alter tumor growth (Figure 4a). After removal of the prostate containing the orthotopically growing tumor, multiple loco-regional lymph nodes harbored bioluminescent PC-3M-Pro4/luc<sup>+</sup> cells (Figure 4b). Administration of BMP7, however, did not result in decreased formation of loco-regional lymph node metastases (Figure 4c).



**Figure 5.** The effects of BMP7 on gene promoter luciferase constructs in human PC-3M-Pro4 prostate cancer cells. **a:** Challenge of the SBE<sub>4</sub> construct, consisting of four SBEs, demonstrates the presence of functional BMP7 and TGFβ receptors (Smad-mediated signaling). \**P* ≤ 0.01 versus Co. **b:** BMP7, but not TGFβ, induces BRE<sub>4</sub>-luciferase activity, indicating the presence and functionality of BMP receptors. Signaling of BMP to stimulate the expression of BRE<sub>4</sub>-luciferase is transduced by BMP type I receptors and mediated by Smad1, Smad4, and Smad5, which form a complex with this reporter construct. Strikingly, signaling of BMP7 to stimulate BRE<sub>4</sub>-luciferase is significantly enhanced when coincubated in the presence of TGFβ. \**P* < 0.01 versus Co; \**P* < 0.01 versus BMP7. **c** and **d:** The presence of functionally active TGFβ receptor complexes, particularly ALK5 (TGFβ type 1 receptor), in PC-3M-Pro4 cells was demonstrated by the CAGA-luciferase reporter, whose activity depends on binding of activated Smad3/Smad4 transcription factor complexes. The CAGA boxes confer TGFβ but not BMP stimulation to the promoter reporter constructs. The addition of BMP7 to TGFβ-stimulated PC-3M-Pro4 cells dose-dependently inhibited TGFβ-driven CAGA-luciferase activity. \**P* < 0.01 versus Co, \*\**P* < 0.01 versus TGFβ alone (**c**); and \**P* < 0.01 versus TGFβ alone, #*P* < 0.01 versus Co (**d**).

### BMP7 and Epithelial-to-Mesenchymal Transition in Prostate Cancer Cells

Next, we tested whether BMP7 acts on human PC-3M-Pro4 prostate cancer cells to inhibit the acquisition of an invasive mesenchymal phenotype by antagonizing Smad-dependent TGFβ signaling. For this, we first studied the functionality of both TGFβ and BMP receptors in PC-3M-Pro4 prostate cancer cells. Challenge of PC-3M-Pro4/luc<sup>+</sup> cells with both BMP7 and TGFβ, which were transiently transfected with SBE<sub>4</sub>-luciferase, resulted in a significant induction of reporter expression (Figure 5a). This indicates that functional TGFβ and BMP receptors are present in these cells, thus confirming our earlier observations.<sup>22,24</sup> Exogenous addition of BMP7 but not of

TGFβ strongly stimulated BRE<sub>4</sub>-luciferase activity in PC-3M-Pro4 cells and indicated the presence of functioning, activated type 1 BMP-receptor complexes in these prostate cancer cells (Figure 5b). Strikingly, when BMP7 and TGFβ were given simultaneously, BRE<sub>4</sub>-luciferase activity was significantly stimulated in a synergistic manner (Figure 5b).

The presence of functionally active TGFβ receptor complexes, particularly ALK5 (TGFβ type 1 receptor), in PC-3M-Pro4 cells was demonstrated by the CAGA-luciferase reporter. The CAGA boxes confer TGFβ but not BMP stimulation to the promoter-reporter constructs in PC-3M-Pro4 cells in a dose-dependent manner (Figure 5, c and d). The addition of BMP7 to TGFβ-stimulated PC-3M-Pro4 cells dose-dependently inhibited TGFβ-driven CAGA-luciferase activity. This suggests that BMP7 counteracts TGFβ-induced nuclear activation of activated Smad3/4 complexes (Figure 5, c and d) in human prostate cancer cells, whereas TGFβ can stimulate BRE<sub>4</sub>-luciferase only in the presence of BMP7 (Figure 5b).

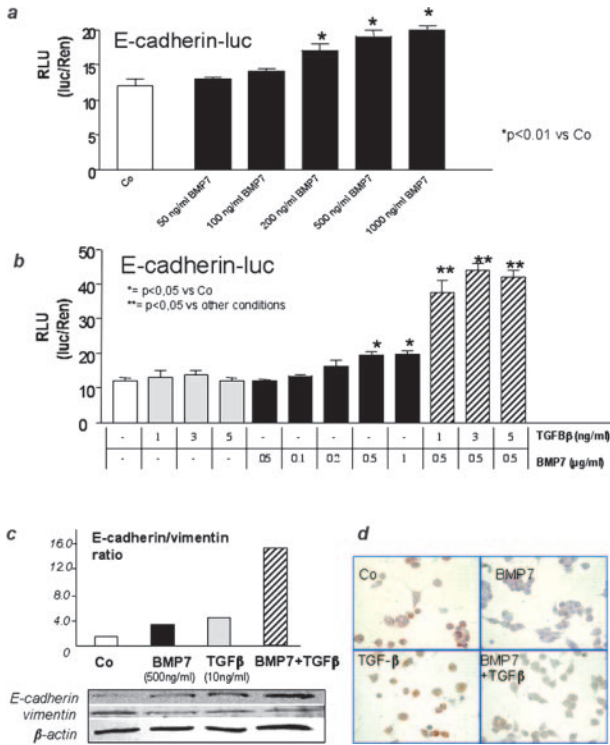
The observed antitumor effects of BMP7 *in vivo* may therefore be caused by inhibition of TGFβ-mediated growth-stimulatory responses in bone metastasis. This may be particularly true for the bone microenvironment, because bone is a storehouse for TGFβ, and it has been shown that activated TGFβ is released from bone matrix by bone-resorbing osteoclasts.<sup>29,31,32,42,43</sup>

The expression level of E-cadherin appears to be inversely related with prostate cancer grade, and its loss is considered a hallmark of EMT.<sup>23,44,45</sup> Recombinant human BMP7 dose-dependently induced E-cadherin promoter activity (>150% at 1 μg/ml BMP7, *P* < 0.01), whereas TGFβ had no effect (Figure 6a). Strikingly, E-cadherin promoter activity was stimulated in a synergistic, dose-dependent, and significant manner by BMP7 in the presence of TGFβ (Figure 6b, 350%). In line with this observation, Western blot analysis revealed that E-cadherin/vimentin ratio at the protein level was strongly up-regulated under these circumstances (Figure 6c). These data therefore suggest that BMP7 can counteract the acquisition of an invasive metastatic phenotype by re-expressing the key epithelial marker E-cadherin, which is an absolute requirement for epithelial integrity, by decreasing cell motility, invasion, and migration.

When TGFβ was added to PC-3M-Pro4/luc<sup>+</sup> cells the intermediate filament and mesenchymal marker vimentin was redistributed in the prostate cancer cells (Figure 6d). This TGFβ-induced vimentin expression and cellular redistribution were completely abolished by BMP7 (Figure 6d).

### Discussion

In this report, novel evidence is provided for the importance of BMP7 (and TGFβ) in the development of an epithelial cancer and metastatic behavior with prostate cancer as a model. Our clinical findings suggest that decreased BMP7 expression is implicated in human prostate cancer, and we postulate a role for BMP7 in controlling epithelial homeostasis of the prostate gland. In line with these clinical findings, experimental treatment



**Figure 6.** The effects of BMP7 on E-cadherin promoter luciferase constructs and protein expression of E-cadherin/vimentin in human PC-3M-Pro4 prostate cancer cells. **a** and **b**: BMP7 induces a dose-dependent and significant increase in E-cadherin promoter-luciferase construct activity ( $P < 0.01$  at  $>0.2 \mu\text{g/ml}$  BMP7). E-Cadherin promoter activity is significantly and synergistically enhanced when BMP7 and TGFβ were coincubated ( $P < 0.05$  versus BMP7 alone).  $*P \leq 0.01$  versus Co;  $*P < 0.05$  versus Co,  $**P < 0.05$  versus other conditions (**b**). **c**: E-cadherin and vimentin protein expression in PC-3M-Pro4 cells in the presence or absence of rhBMP7 and/or TGFβ for 48 hours as determined by immunoblotting. Treatment of PC-3M-Pro4 cells with  $0.5 \mu\text{g/ml}$  rhBMP7 in the presence of TGFβ induces protein expression of E-cadherin. Values are expressed as E-cadherin/Vimentin ratio. **d**: Vimentin monoclonal antibody immunoreactivity of cultured PC-3M-Pro4 cells. In the presence of BMP7, the TGFβ-induced vimentin expression and cellular redistribution was completely abolished. Values are expressed as means  $\pm$  SD ( $n = 4$ , analysis of variance).

of bone metastases from human prostate cancer *in vivo* positions BMP7 as a good candidate for the treatment of skeletal metastasis from prostate cancer.

Members of the TGFβ superfamily play crucial roles in embryonic development, certain fibrotic diseases, inflammation, and cancer. Although TGFβ inhibits the proliferation of normal prostate cells and functions as a tumor suppressor in early tumorigenesis, it acts as a tumor promoter in later stages of tumor progression and mediates oncogenic EMT.<sup>2,5,10,17,46–50</sup> Prostate cancers often grow in a variety of growth patterns that are classified by Gleason histological grading. Prostate cancer classification according to this “gold standard” is predictive of disease status and also seems to parallel EMT. Laser capture microdissection of prostate epithelium/cancer was used because the epithelial compartment in the normal prostate represents only a small percentage (~5%) of the entire organ. Furthermore, infiltrative prostate cancer patterns vary among individual cases. The latter can lead to misinterpretation of epithelium-specific gene expression among the various clinical specimens. By comparing pure populations of laser-captured cancer

cells and noncancerous epithelial cells from the same patient, we observed significant and consistent BMP7 mRNA underexpression in invasive primary prostate carcinoma. In contrast, BMP7 mRNA expression in bulk tissue specimens of noncancerous prostate and prostate cancer tissue showed only a tendency toward underexpression in prostate cancer. Our observations support concerns that high-throughput RNA expression analysis performed on bulk tissue samples, either on primary tumors or its metastases, may not detect differences in relevant gene expression restricted to the epithelial or mesenchymal compartment.<sup>22</sup> Clearly, LCM is preferred to establish putative changes in gene expression repertoires in both epithelial and stromal compartments.

Emerging evidence from a variety of tumors suggests that the effects of BMPs (and TGFβ) are cell-specific and could be either protumorigenic or antitumorigenic.<sup>48</sup> For instance, BMP7 was shown to counteract EMT by inducing MET, to induce EMT, or to have no effect.<sup>2,4,10,49,51</sup> In the noncancerous human prostate, expression of BMP7 protein was restricted mainly to the glandular epithelium, whereas primary prostate cancers show low or undetectable BMP7 protein expression. It also appeared that BMP7 expression is negatively correlated to Gleason score (data not shown), but the number of patients per Gleason pattern were too small for statistical analysis. Clearly more LCM and real-time PCR analyses are warranted to address this issue. In line with these clinical observations, the level of BMP7 expression in tested prostate cancer cell lines correlated with an epithelial phenotype and seems, therefore, inversely related to their tumorigenic and metastatic potential *in vivo*.

Daily systemic administration of recombinant BMP7 did not affect the growth of orthotopically implanted PC-3M-Pro4/luc<sup>+</sup> prostate cancer cells and subsequent formation of lymph node metastasis in nude mice. However, the growth of micrometastatic deposits from human PC-3M-Pro4/luc<sup>+</sup> prostate cancer cells in bone marrow was inhibited significantly, whereas the process of bone marrow colonization per se appeared unaffected. Our findings suggest, therefore, that the tumor microenvironment is an important determinant of the therapeutic response to BMP7.

Our *in vitro* studies reveal for the first time that BMP7 is a potent inhibitor of TGFβ-induced EMT in PC-3M-Pro4 prostate cancer cells. In these cells, BMP7 counteracts TGFβ-induced activation of activated Smad3/4 complexes. BMP7 induced a dose-dependent expression of E-cadherin that led to an overall increase in E-cadherin/vimentin ratio as an established indicator of less malignant and more epithelial phenotype. Surprisingly, we observed that these BMP7-mediated effects were synergistically enhanced by coinubation with TGFβ at both transcriptional and protein levels. These actions of BMP7 may be of critical importance for explaining the successful experimental treatment of skeletal metastasis and intrabone growth. In this context, it is important to note that TGFβ, which is highly concentrated as inactive form in bone matrix, can be released and activated by osteoclastic resorption. TGFβ may act as paracrine growth factors for neighboring cancer cells that may have colonized the

bone marrow.<sup>31,32,42,43</sup> Compelling evidence suggests that the formation of micrometastatic deposits in bone marrow and subsequent development into clinically overt (macro)metastasis are critically dependent on the tumor-bone (marrow) interactions, in particular bone remodeling.<sup>31,43,52</sup> Although little is known regarding the impact of bone (marrow) stromal cells in prostate cancer bone metastasis, it can be argued that the production and release of specific growth factors, colony stimulating factors, and cytokines like Wnts, which are critically important for the maintenance of the hematopoietic stem cell niche (and which regulate hematopoiesis), may also be essential for colonization, survival, and growth of cancer cells. Interestingly, TGF $\beta$ /BMPs and other pathways like wingless/Wnt, Notch, and Hedgehog seem not only to be involved in bone development/homeostasis and hematopoiesis but also in prostate cancer initiation and progression.<sup>2,8,53-56</sup> Our *in vitro* and *in vivo* data further support the notion that BMP7 can antagonize TGF $\beta$  signaling routes in human prostate cancer cells that are metastatic to the skeleton.

Epithelial-to-mesenchymal transdifferentiation provides a mechanism for prostate epithelial cells to overcome physical constraints imposed on them by intercellular junctions and to adopt a motile phenotype. We hypothesize that the activation process of micrometastases in bone marrow may bear similarities to EMT that occurs at the primary site in various epithelial cancers and during ontogeny. TGF $\beta$ /BMPs and their signaling molecules have been implicated in the cellular plasticity that occurs during organogenesis, tissue repair/remodeling, and carcinogenesis, where cells can shift between epithelial and mesenchymal phenotypes.<sup>1,2</sup> EMT/MET can thus be viewed as a prime example of such cell plasticity. Considering the importance of the TGF $\beta$  superfamily in bone metastasis,<sup>42</sup> our findings suggest that the efficacy of BMP7 in the experimental treatment of bone metastases is interfering with recapitulation of such cell plasticity of bone marrow micrometastatic cancer cells, particularly the acquisition of an invasive phenotype by (perhaps TGF $\beta$ -driven) EMT. Clinical studies also seem to underline the cellular plasticity of prostate cancer during dissemination and bone metastasis.<sup>57,58</sup> The transition from a well-differentiated epithelial phenotype to an invasive mesenchymal phenotype may, of course, involve molecular mechanisms other than those described here.<sup>59</sup> Other studies have provided evidence that motility and invasiveness can be enhanced without inducing a complete conversion of cellular identity.<sup>59</sup> After migrating to new organ or tissue territories, metastatic prostate cancer cells can regain epithelial morphology by a phenomenon known as MET and re-establish E-cadherin expression and epithelial junctions.<sup>57-59</sup> The observed reversion to an epithelial morphology in prostate cancer seems possible even at bone metastatic sites<sup>57-59</sup> and is in full agreement with our *in vitro* and *in vivo* data presented here. Furthermore, previous studies demonstrated cellular plasticity of human prostate cancer cells (PC3 cells), particularly the EMT/MET interconversion induced by different culture conditions (monolayer versus three-dimensional cultures<sup>60</sup>).

In many prostate cancer patients, micrometastatic deposits may already exist in bone marrow after removal of the

primary tumor that cannot be identified at the time of diagnosis (minimal residual disease). Whole-body bioluminescent reporter imaging of luciferase-expressing human prostate cancer cells revealed that growth of micrometastatic deposits in bone marrow is significantly inhibited by systemic administration of BMP7. Similar observations were made by us for other epithelial cancers, including breast cancer.<sup>50,61</sup> Interestingly, recent data suggest that BMPs can inhibit the tumorigenic potential of human brain tumor-initiating cells, mediated via a significant reduction in the stem cell-like, tumor-initiating precursors.<sup>62</sup> Accumulating evidence suggests that cancer stem cells with tumor-initiating potential exist in human prostate cancer.<sup>63</sup> Although speculative at present, the observed therapeutic effects of BMP7 described here may be mediated, at least in part, by prostate cancer cells with tumor-initiating (and metastatic) potential. Studies are currently ongoing to address these important issues.

In conclusion, our data suggest that BMP7 controls the epithelial homeostasis in the human prostate gland by preserving the epithelial phenotype. Loss of BMP7 expression during prostate cancer progression could stimulate the TGF $\beta$ -stimulated EMT and thus contribute to the acquisition of an invasive phenotype. However, exogenous BMP7 can still counteract the EMT process in the metastatic tumor, thus underscoring tumor plasticity, but its therapeutic response may be determined by the microenvironment. Therefore, BMP7 may represent a novel therapeutic molecule for repression of systemic prostate cancer progression.

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