Pathogenesis and treatment of skeletal metastasis : studies in animal models

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Chapter 7

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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Abstract

Bone Morphogenic Protein 7 (BMP7) counteracts physiologic epithelial-to-mesenchymal transition (EMT), a process that is indicative of epithelial plasticity. Since EMT is involved in cancer we investigated whether BMP7 plays a role in prostate cancer growth and metastasis.

BMP7 expression in laser micro-dissected primary human prostate cancer tissue was strongly down-regulated compared to normal prostate luminal epithelium. Furthermore, BMP7 expression in prostate cancer cell lines was inversely related to their 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 TGF-ß induced activation of nuclear Smad-2/3 via ALK5 and induced E-cadherin expression. Moreover, BMP7-induced activation of nuclear Smad1/4/8 signaling transduced via BMPR-1A receptors was synergistally stimulated in the presence of TGF-ß, 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 intra-prostatic xenografts. Collectively, our observations suggest that BMP7 controls and preserves the epithelial phenotype in the human prostate, underscoring a decisive role of the tumor microenvironment in mediating the therapeutic response of BMP7. BMP7 can still counteract the EMT process in the metastatic tumor, positioning BMP7 as a novel therapeutic molecule for treatment of metastatic bone disease.

Introduction

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 [1-3].

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 [4,5]. 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 [1,6].

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) [2]. Both processes have emerged as a fundamental principle for reprogramming of gene transcription and as a major determinant 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 [1].

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) [6].

In epithelial cells TGF-ß has been identified as one of the main inducers of EMT during development and in fibrotic disorders [5-10], while another member of the TGF-ß superfamily, bone morphogenetic protein 7 (BMP7) alias osteogenic protein-1 (OP-1), is involved in the maintenance of the epithelial phenotype by induction of MET [11-14]. Zeisberg and co workers demonstrated that BMP7 was capable of counteracting TGF-ß-induced EMT and could reverse chronic renal injury [13,14].

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 [15,16]. In cancer progression, "oncogenic" EMT refers to clusters of malignant cells that loose epithelial characteristics and acquire self-sustained migratory and highly invasive phenotypes [5]. 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), towards "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-ß signaling pathway have been reported in prostate cancer [17,18] and may contribute to the development and progression of prostate cancer as alterations in TGF-ß and TGF-ß-receptor II expression are associated with poor clinical outcome [19]. A growing number of *in vivo* studies demonstrate that inhibitors of TGF-ß or TGF-ß-receptors may reduce the metastatic and/or invasive properties of a variety of experimental cancers by preventing EMT pathways [5,20,21], thus underscoring the importance of TGF-ß in oncogenic EMT associated with cancer progression.

This study was designed to investigate whether tumorigenicity and invasive behavior is 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-ß signalling 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 from surgical excision, samples of prostate cancer tissue or non-cancerous tissue of the prostate were taken and either snap-frozen or immersed in RNAlater (Qiagen, Basel, Switzerland) [22]. 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 µm cryo-sections were treated according to the HistoGene LCM Frozen Section staining Kit (Arcturus, Bucher Biotech, Basel, Switzerland) [22,23]. The P.A.L.M. Micro-Beam system (P.A.L.M. Microlaser Technologies, Bernried, Germany) was used to excise approximately thousand pure epithelial cells of non-cancerous prostate (NP) and prostate cancer (PC) tissue in each prostate. Total RNA was extracted with the Pico Pure RNA Isolation Kit (Arcturus).

Real-Time PCR Analysis

Total RNA (see above) was reverse transcribed using random primers in presence of RNase inhibitor (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCR was performed with

exon specific primers for human BMP7 (Hs_002333477_m1), GAPDH (Hs_99999905_m1) E-cadherin (Hs 00170423 m1), Vimentin (Hs_00185584_m1), and β-actin (Hs_99999903_m1) (primer catalogue Applied Biosystems, Rotkreuz, Switzerland) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). The resulting values were normalized to GAPDH or β-actin. Semi-quantitative PCR was performed on samples of experimentally-induced bone metastases from PC-3M-Pro4 after intracardiac inoculation into nude mice using the following exon-specific and human-specific BMP primers [22,24].

Cell lines and culture conditions

The human prostate cancer cell lines PC-3 (ATCC Number CRL-1435, ATCC-LGC Promochem, Molsheim Cedex, France) and PC-3M-Pro4 were grown in DMEM+ 10% FCS (Bio-Wittaker, 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 re-injecting cells from xenograft tumors back into the mouse prostate [25,26]. The human prostate cancer cell line LNCaP and LNCaP-derived cell lines C4-2 and C4-2B4 [27,28] were grown in T-Medium.

Cells at 70%-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 CMV-promoter driven mammalian expression vector for luciferase, CMV-luc, and one clone with the highest expression of luciferase expression (PC-3M-Pro4/Luc+) was successfully used for *in vivo* whole body bioluminescent reporter imaging (BLI) [29,30,32]. PC-3M-Pro4/Luc+ were cultured in DMEM+10% FCS and 800 µg/ml geneticyne/G418 (Gibco-BRL, Breda, the Netherlands). Cells were regularly certified free of mycoplasma contamination.

For intraosseous or orthotopic inoculation cell suspensions of PC-3M-Pro4/Luc+ (1 x 10^5 cells /10 µl PBS) were prepared [29-32].

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 condition according to the Dutch guidelines for the care and use of laboratory animals (DEC 4077). For surgical and analytical procedures were performed mice were anaesthetized [29,31,32].

Intraosseous inoculation of PC-3M-Pro4/Luc+ cells

A single cell suspension of PC-3M-Pro4/Luc+ cells was injected into the right tibiae [29-32]. The progression of cancer cell growth was monitored weekly by BLI. After the experimental period the animals were sacrificed, the tibia dissected and processed for further histomorphometrical and immunohistochemical analysis (see below).

Inoculation of PC-3M-Pro4/Luc+ into the mouse prostate (orthotopic implantation)

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

Induction of systemic metastases by intracardiac injection of PC-3M-Pro4/Luc+ cells

A single cell suspension of 1 x 10^5 PC-3M-Pro4/Luc+ cells /100 µl PBS was injected into the left cardiac ventricle and cancer cell growth was monitored weekly by BLI and radiography [29-32]. Daily BMP7 treatment started 2 days prior to 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. Vukicevic. BMP7 was expressed, purified and lyophilized [33]. When used, BMP7 was freshly dissolved to a stock solution containing 1 mg/ml in 20 mM acetate buffer with 5 % mannitol, pH 4.5. BMP7 (20 µl) or vehicle solution (20 µl, 20 mM acetate buffer with 5 % mannitol) was administered daily into the tail veins of mice for 21-23 days (100 µg/kg/d human BMP7).

Whole body bioluminescent reporter imaging (BLI) and quantification of the bioluminescent signal

BLI of tumors induced by the luciferase-expressing human prostate cancer cell lines was performed [29,32]. Analyses for each metastatic site were performed after definition of the region of interest and quantified [32]. Values are expressed as relative light units (RLU).

Histomorphometry, Histochemistry and Immunohistochemistry (IHC)

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) were used as the detection system. 3-Amino-9-ethyl-carbazole (AEC, Sigma, Buchs, Switzerland) served as chromogen (Sigma, Buchs, Switzerland).

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 [31] with PS1 antibodies directed against phosphorylated-Smad1 [34,35].

Immuno-blotting

Crude cell lysates (10 µg/lane) loaded were separated on SDS-polyacrylamide gels and blotted on Hybond-P membranes (Amersham Biosciences). E-Cadherin mAb (5µg/ml) and a monoclonal mouse anti-actin antibody (1:5000) 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) using the VersaDoc imaging system and QuantityOne imaging software (Bio-Rad).

Human BMP7 ELISA

PC-3M-Pro4 cells, and a clone stably transfected to overexpress rhBMP7, were seeded in a 6-wells plate (120.000 cells/ml) and conditioned medium was harvested and analysed for BMP7 protein synthesis by ELISA after 4 days of culture according to the manufacturer's protocol (R&D systems).

Transient transfections and transcription reporter assays

Transient transfections and transcription reporter assays were performed and values expressed as luciferase intensities (relative light units or RLU) [36]. The experiments were performed in four fold and repeated at least twice. Values are expressed as means ± s.e.m. Incubation time with TGF-ß or BMP7 was performed for 30 hours.

Luciferase Reporter Gene Constructs

For intracellular signaling of TGF-ß 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-ß, but not BMP-stimulation to a heterologous promoter reporter construct [37].

The BRE4-luciferase construct, that is based on the mouse Id1 promoter, was used to study the presence and functionality of BMP-receptors [37]. Signaling of BMP to stimulate the expression of BRE4-luciferase is transduced by BMPR-IA and mediated by Smad1, Smad4 and Smad5, which form a complex with this reporter construct [38].

The SBE4 construct consists of 4 Smad Binding Elements (SBEs) in the promoter of the JunB gene, an immediate early gene that is potently induced by TGF-ß, activin, and bone morphogenetic proteins [39].

The E-cadherin promoter-luciferase construct is based on the upstream fragment (positions -178 to +92). The pGL3-E-cadherin promoter plasmid [40] was a kind gift from Dr. Antonio Garcia de Herreros.

Statistical Analysis

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

using GraphPad Prism Version 3 (GraphPad Software Inc., USA). Data are presented as mean ± s.e.m. ANOVA was performed for statistical evaluation of whole body BLI data and reporter-luciferase assays. A p-value equal or superior to 0.05 was considered as non-significant.

Results

BMP7 mRNA Expression in Radical Prostatectomy Specimens

Samples of prostate cancer tissue (PC) and internal controls from the same patient (non-cancerous tissues of the prostate, 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 non-cancerous prostate (11/16, mean decrease 26 %), a non significant difference (Fig. 1*A,C*; P=0.1). However, the multi-focal 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 non-cancerous prostate epithelial cells by laser capture microdissection (LCM) from 14 patients. In 93% of those cancer cell samples BMP7 mRNA was strongly under-expressed when compared to patient-matched non-cancerous epithelial cells (13/14, mean decrease 70%, P=0.0019; Fig. 1*B,D*). Comparison of the BMP7 mRNA expression in non-cancerous prostate either derived from bulk tissue or from laser captured epithelial cells revealed a 9.4 x higher expression in the epithelial compartment alone than in bulk tissue. BMP7 expression in laser captured prostate cancer was also (2.8 x) higher when compared to 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 non-cancerous 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 non-cancerous prostate tissue, while hardly any BMP7 could be detected in basal cells (Fig. 1*E,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 (Fig. 1*G,H*).

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

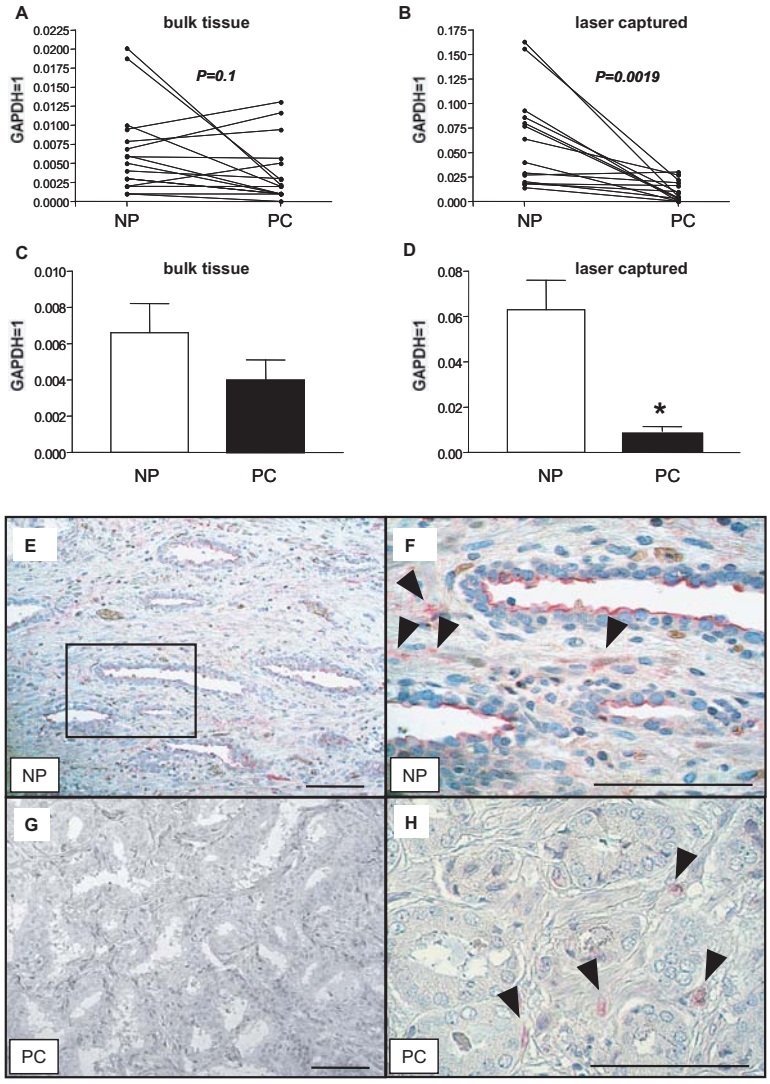

Figure 1 **BMP7 mRNA expression in non-cancerous prostate epithelium (NP) and prostate cancer cells (PC) of radical prostatectomy specimens.** *A*, BMP7 mRNA expression in patient matched specimens consisting predominantly of NP and PC tissue (n=16, P=0.1, paired t-test). *B*, BMP7 mRNA expression in NP and neoplastic epithelial cells isolated by LCM (PC) (n=14, P=0.0019, paired t-test). *C*, mean BMP7 mRNA expression values ± s.e.m. in bulk tissue in NP and PC both normalized to GAPDH (P=0.1, paired t-test). *D*, mean BMP7 mRNA expression values ± s.e.m. in microdissected non cancerous epithelium (NP) and neoplastic epithelium (PC) both normalized to GAPDH (P=0.0019, paired t-test). BMP7 mAb immuno-reactivity and in clinical specimens of NP (E,F) and PC (G,H). *E*, boxed area is magnified in *F,* BMP7 immuno-reactivity 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, H*, prostate cancer cells lack BMP7 immuno-reactivity, whereas BMP7 was still detectable in parts of the stroma (arrowhead). Bars = 100 µm.

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, 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 (Fig. 2*A*). Vimentin is a characteristic marker for the mesenchymal phenotype of cancer cells and enhanced vimentin expression was previously found in motile prostate cell lines and in poorly differentiated and metastatic prostate carcinoma [41]. 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 (Fig. 2*A*).

BMP7 mRNA levels in prostate cancer cell lines strongly correlated to E-cadherin/vimentin ratio (Fig. 2*A,B*) whereas no association was observed for other BMPs (results not shown) [24]. 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 (Fig. 2*B*) and protein synthesis *in vitro* (human specific BMP7 ELISA; 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 (Fig. 2*D*), suggesting that the bone microenvironment is unable to induce BMP7 expression in the 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 (Fig. 2*B*). 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, Fig. 2*C*).

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 if systemic administration of rhBMP7 affected the growth of human prostate cancer cells in orthotopic tumor and bone metastasis animal models by whole body bioluminescent reporter imaging (BLI) of human PC-3M-Pro4/Luc+ cells.

Three days after intra-bone inoculation of PC-3M-Pro4/Luc+ cells into the tibiae of BALB/c *nu/nu* mice, the animals were treated daily via tail i.v. 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 (Fig. 3*A-C*). Systemic administration of BMP7 directly acts on bone-residing PC-3M-Pro4/Luc+ tumor cells as visualized by nuclear staining for Phospho-Smad1 while this is significantly lower in vehicle–treated animals (Fig. 3*D*). Inoculation of PC-3M-Pro4/

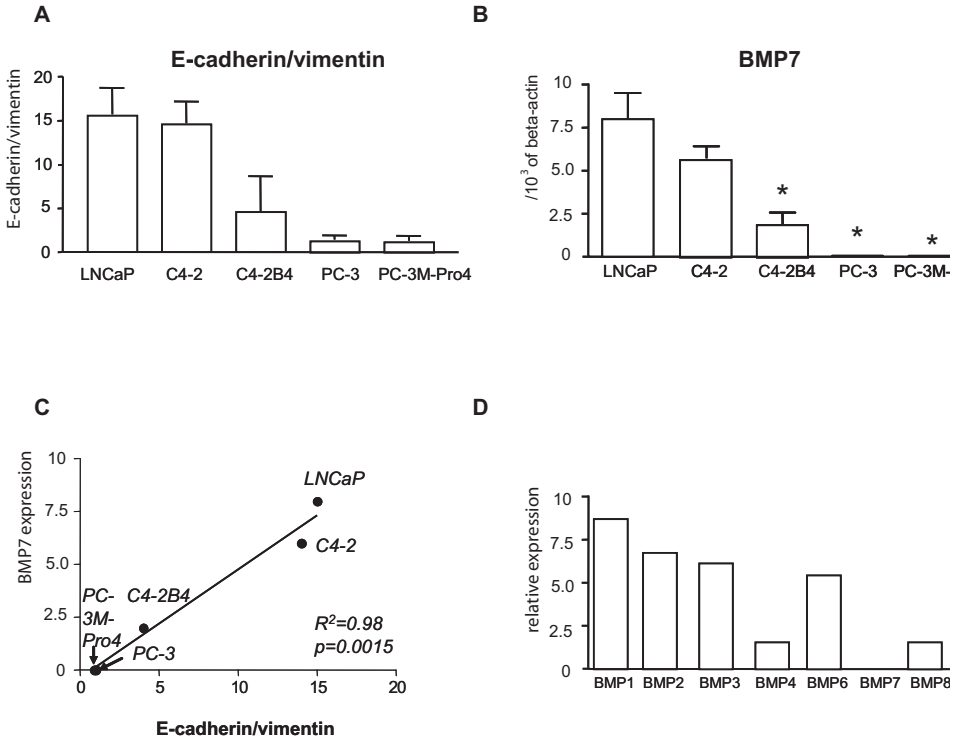

Figure 2 **mRNA expression levels of E-cadherin, vimentin and BMPs in prostate cancer cell lines with different tumorigenic potential** *in vitro* **and in experimentally-induced bone metastases.** *A*, E-cadherin/vimentin ratios in prostate cancer cell lines. *B*, BMP7 mRNA expression in prostate cancer 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 BMP mRNA expression levels in an experimentally-induced bone metastasis from PC-3M-Pro4 cells in nude mice (β2-μglobulin was used for normalization as described previously [31]). All panels: mRNA levels (± SEM) were quantified by real-time RT-PCR.

Luc+ cells into the left cardiac ventricle of nude mice resulted in the formation of multiple bone metastases [29-32]. Continuous daily treatment with BMP7 did not significantly inhibited the number of bone metastases, despite a tendency toward a decrease. However, the average tumor burden per bone lesion was significantly decreased upon BMP7 treatment (Fig. 3*E*).

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+ cells did not alter tumor growth (Fig. 4*A*). After removal of the prostate containing the orthotopically growing tumor, multiple loco-regional lymph nodes harbored bioluminescent PC-3M-Pro4/Luc+ cells (Fig.

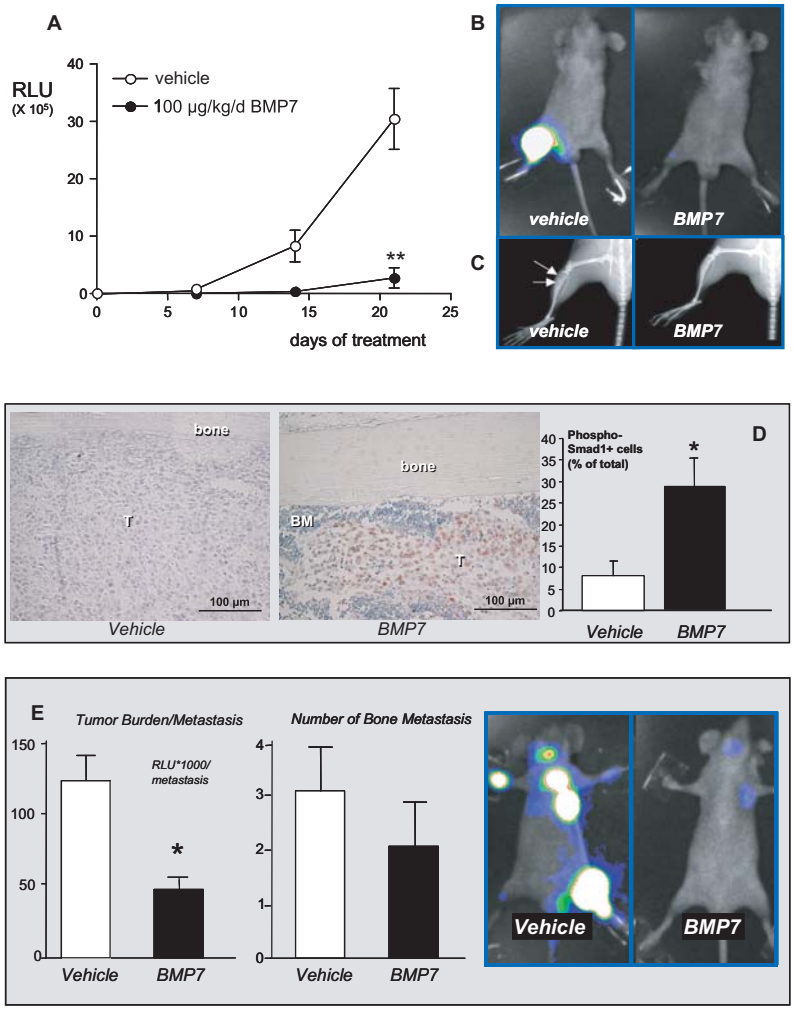

Figure 3 **Effects of systemic administration of 100 µg/kg/d rhBMP7 on the growth of human prostate cancer cells in bone marrow and bone metastasis** *in vivo* **after intraosseous transplantation or intracardiac inoculation of human PC-3M-Pro4/Luc+ cells using whole body bioluminescent reporter imaging.** *A*, BMP7 treatment of PC-3M-Pro4/Luc+ 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 intraosseous transplantion of PC-3M-Pro4/Luc+ cells growing in bone marrow (arrows indicate bone lesions in tibiae of nude mice). *D*, immunohistochemical analyses of phosho-Smad1localization at day 21 after intraosseous transplantation of PC-3M-Pro4/Luc+ cells growing in bone marrow. The animals were either vehicle treated (upper left panel) or treated with rhBMP7 (upper right panel) 6 hours prior to explantation and fixation of the tibiae. The number of phosphoSmad1 positive cells in the BMP7 treated group are increased significantly. *E*, effect of BMP7 treatment on the formation and growth of bone metastases by PC-3M-Pro4/Luc+ in nude mice as monitored by BLI (tumor burden, number of bone metastases, representative examples of BLI at day 19). BLI was performed once weekly starting at day 0 after intraosseous implantation or intracardiac inoculation of the prostate cancer cells and quantified in relative light units (RLU) ± s.e.m. n=8 for each experimental group for intraosseous transplantation experiment (A-D); n=10 for intracardiac inoculation (E). Values are expressed as means ± s.e.m. *p<0.05, **p<0.001 (ANOVA)

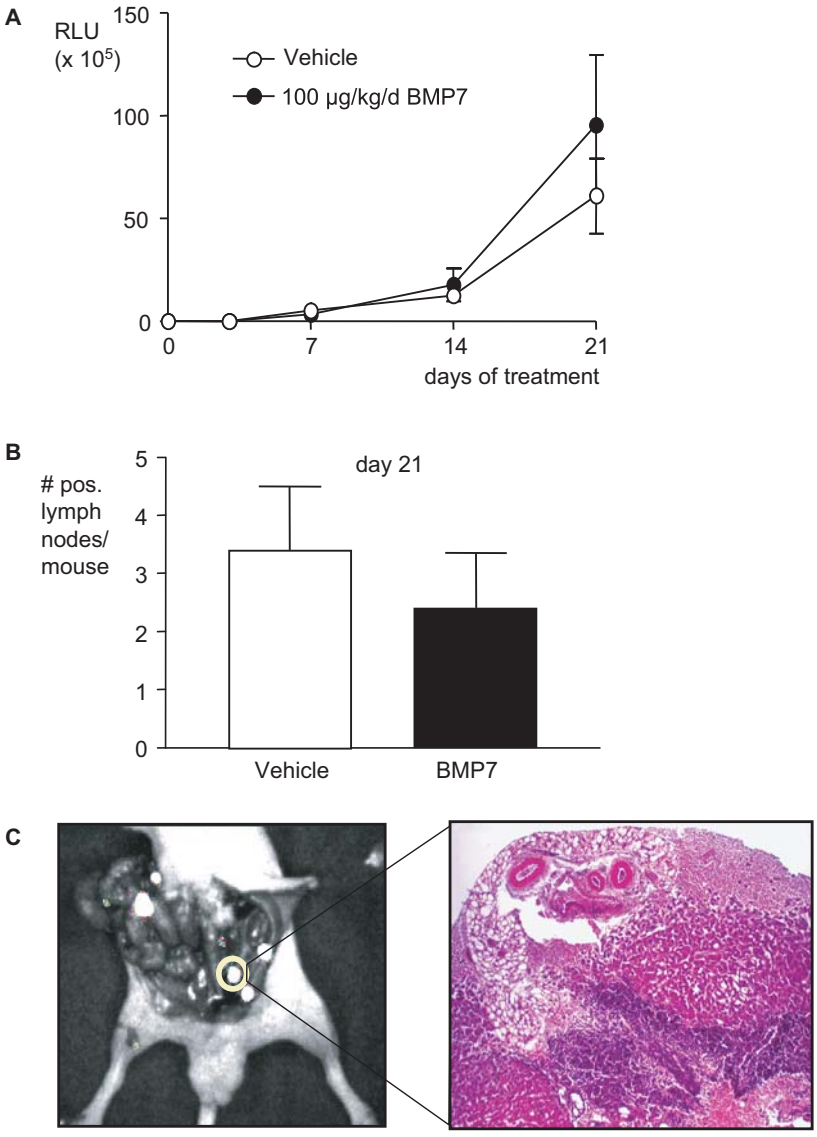

Figure 4 **Effects of systemic administration of 100 µg/kg/d rhBMP7 on intra-prostatic growth of human PC-3M-Pro4/Luc+ cells and formation of loco-regional lymph node metastases nude mice.** *A*, tumor take of PC-3M-Pro4/Luc+ cells growing in prostate upon treatment with vehicle solution or 100 µg/kg/d rhBMP7 as monitored by BLI. *B*, 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 intra-prostatic inoculation of PC-3M-Pro4/Luc+ cells. The mouse prostate was removed prior to bioluminescent reporter imaging to obtain sensitive measurements of affected lymph nodes (panel B). The presence of micrometastatic deposits in lymph nodes was confirmed by HE-staining. BLI was performed at least once weekly starting at day 0 after intra-prostatic implantation of the prostate cancer cells and quantified in Relative light units (RLU) ± s.e.m. n=8 for each experimental group. Values are expressed as means ± s.e.m. (ANOVA).

4*B*). Administration of BMP7, however, did not result in decreased formation of locoregional lymph node metastases (Fig. 4*C*).

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 acquistion of an invasive, mesenchymal phenotype by antagonizing Smad-dependent TGF-ß signaling. For this, we first studied the functionality of both TGF-ß and BMP receptors in PC-3M-Pro4 prostate cancer cells. Challenging PC-3M-Pro4/Luc+ cells by both BMP7 and TGF-ß, that were transiently transfected with SBE4-luciferase, resulted in a significant induction of reporter expression (Fig. 5*A*). This indicates that functional TGF-ß and BMP receptors are present in these cells, thus confirming our earlier observations [22,24]. Exogenous addition of BMP7, but not of TGF-ß, strongly stimulated BRE4-luciferase activity in PC-3M-Pro4 cells and indicated the presence of functioning, activated type I BMP-receptor complexes in these prostate cancer cells (Fig. 5*B*). Strikingly, when BMP7 and TGF-ß were given simultaneously, BRE4-luciferase activity was significantly stimulated in a synergistic manner (Fig. 5*B*).

The presence of functionally active TGF-ß receptor complexes, particularly ALK5 (TGF-ß type I receptor), in PC-3M-Pro4 cells was demonstrated by the CAGA-luciferase reporter. The CAGA boxes confer TGF-ß, but not BMP, stimulation to the promoter reporter constructs in PC-3M-Pro4 cells in a dose-dependent manner (Fig. 5 *C,D*). Addition of BMP7 to TGF-ß-stimulated PC-3M-Pro4 cells dose-dependently inhibited TGF-ß-driven CAGA-luciferase activity. This suggests that BMP7 counteracts TGF-ß-induced nuclear activation of Smad3/4 (Fig. 5*C,D*) in human prostate cancer cells, whereas TGF-ß can stimulate BRE4-luciferase only in the presence of BMP7 (Fig. 5*B*).

The observed anti-tumor effects of BMP7 *in vivo* may, therefore, be caused by inhibition of TGF-ß mediated growth stimulatory responses in bone metastasis. This may be particularly true for the bone microenvironment since bone is a storehouse for TGF-ß and it has been shown that activated TGF-ß is released from bone matrix by bone-resorbing osteoclasts [29,31,32,42,43].

The expression level of E-cadherin appears to be inversely related with prostate cancer grade and its loss considered a hall mark of EMT [23,44,45]. Recombinant human BMP7 dose-dependently induced E-cadherin promoter activity (> 150% at 1 µg/ml BMP7, $p<0.01$) while TGF-ß had no effect (Fig. 6*A*). Strikingly, E-cadherin promoter activity was stimulated in a synergistic, dose-dependent and significant manner by BMP7 in the presence of TGF-ß (Fig. 6*B*, 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 (Fig. 6*C*). These data, therefore, suggest that BMP7 can counteract the acquisition of an invasive, metastatic phenotype by re-expressing the key epithelial marker E-cadherin, that is an absolute requirement for epithelial integrity by decreasing cell motility, invasion and migration.

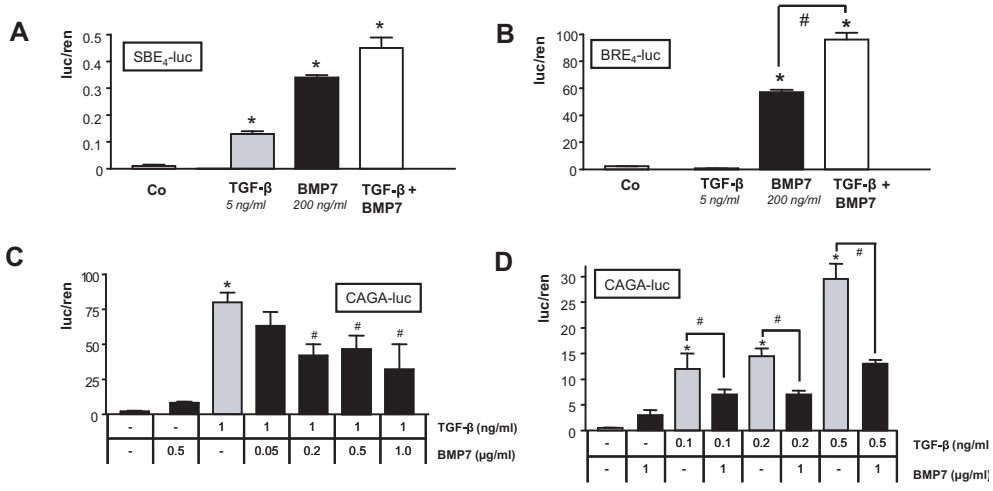

Figure 5 **The effects of BMP7 on gene promoter luciferase constructs in human PC-3M-Pro4 prostate cancer cells.** *A*, challenge of the SBE4 construct, demonstrates the presence of functional BMP7 and TGF-ß receptors (Smad-mediated signaling). *B*, BMP7, but not TGF-ß, induces BRE4-luciferase actvity, indicating the presence and functionality of BMP-receptors. Signaling of BMP to stimulate the expression of BRE4-luciferase is transduced by BMPR-IA and mediated by Smad1, Smad4 and Smad5, both of which form a complex with this reporter construct. Strikingly signaling of BMP7 to stimulate BRE4-luciferase is significantly enhanced when co-incubated in the presence of TGF-ß. *C,D,* the presence of functionally active TGF-ß receptor complexes, particularly ALK5 (TGF-ß type I 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 TGF-ß, but not BMP, stimulation to the promoter reporter constructs. Addition of BMP7 to TGF-ß-stimulated PC-3M-Pro4 cells dose-dependently inhibited TGF-ß-driven CAGA-luciferase activity. All panels: * p < 0,01 vs. Co; # p < 0,01 vs. TGF-ß

When TGF-ß was added to PC-3M-Pro4/Luc+ cells the intermediate filament and mesenchymal marker vimentin was redistributed in the prostate cancer cells (Fig. 6*D*). This TGF-ß-induced vimentin expression and cellular redistribution was completely abolished by BMP7 (Fig. 6*D*).

Discussion

In this report novel evidence is provided for the importance of BMP7 (and TGF-ß) in the development of an epithelial cancer and metastastic behavior with prostate cancer as a model. Our clinical findings suggest that decreased BMP7 expression is implicated in

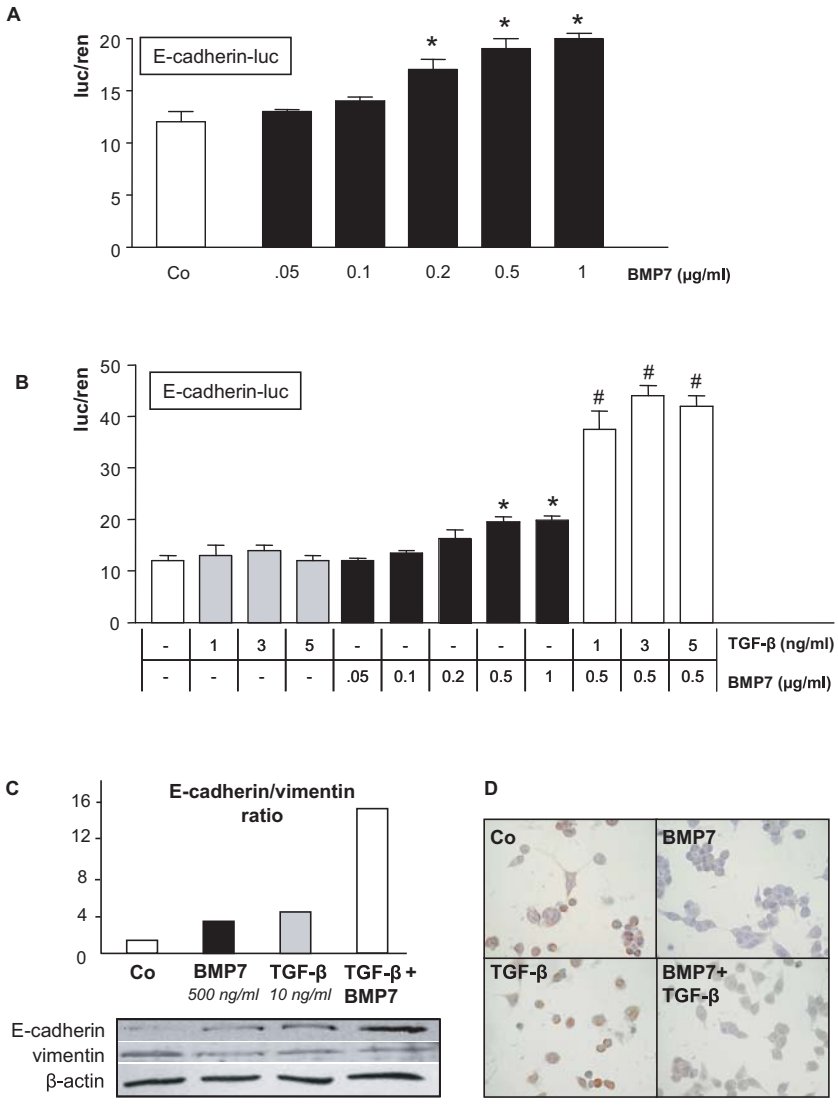

Figure 6 **The effects of BMP7 on E-cadherin promoter luciferase constructs,and protein expression of E-cadherin/vimentin in human PC-3M-Pro4 prostate cancer cells.** *A,B,* BMP7 induces a dose-dependent and significant increase in E-cadherin promoter-luciferase construct activity (* p<0.05 vs. Co; # p<0.01 vs. BMP7 aloneCo). E-cadherin promoter activity is significantly and synergistically enhanced when BMP7 and TGF-ß were coincubated (P<0.05 vs BMP7 alone). *C,* E-cadherin and vimentin protein expression in PC-3M-Pro4 cells in the presence or absence of rhBMP7 and/or TGF-ß for 48 hours as determined by immuno-blotting. Treatment of PC-3M-Pro4 cells with 0.5 µg/ml rhBMP7 in the presence of TGF-ß induces protein expression of E-cadherin. Values are expressed as E-cadherin/Vimentin ratio. *D,* vimentin mAb immuno-reactivity of cultured PC-3M-Pro4 cells. In the presence of BMP7 the TGF-ß–induced vimentin expression and cellular redistribution was completely abolished. Values are expressed as means ± s.d. (n=4, ANOVA).

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

Members of the TGF-ß superfamily play crucial roles in embryonic development, certain fibrotic diseases, inflammation and cancer. Although TGF-ß 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 [2,5,10,17,46-49,63]. 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 micro-dissection 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 non-cancerous epithelial cells from the same patient we observed significant and consistent BMP7 mRNA under-expression in invasive primary prostate carcinoma. In contrast, BMP7 mRNA expression in bulk tissue specimens of non-cancerous prostate and prostate cancer tissue showed only a tendency toward under-expression 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 [22]. Clearly, LCM is preferred to establish putative changes in gene expression repertories in both epithelial and stromal compartments.

Emerging evidence from a variety of tumors suggests that the effects of BMPs (and TGF-ß) are cell specific and could be either pro-tumorigenic or anti-tumorigenic [48]. For instance, BMP7 was shown to either counteract EMT by inducing MET, to induce EMT or to have no effect [2,4,10,49,50]. In the non-cancerous 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 analysis 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 appears, therefore, inversely related to their tumorigenic and metastatic potential *in vivo*.

Daily systemic administration of recombinant BMP7 did not affect the growth of orthopically implanted PC-3M-Pro4/Luc+ 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+ prostate cancer cells in bone marrow was inhibited significantly, whereas the process of bone marrow colonization per sé 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 TGF-ß-induced EMT in PC-3M-Pro4 prostate cancer cells. In these cells, BMP7 counteracts TGF-ß-induced activation of Smad3/4 signaling. 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 co-incubation with TGF-ß 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 intra-bone growth. In this context it is important to note that TGF-ß, which is concentrated in high inactive amounts in bone matrix, can be released and activated by osteoclastic resorption. TGF-ß may act as paracrine growth factors for neighboring cancer cells that may have colonized the bone marrow [31,32,42,43]. 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 [32,43,51]. 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, CSFs and cytokines like Wnts, that are critically important for the maintenance of the hematopoietic stem cells niche (and that regulate hematopoiesis), may also be essential for colonization, survival and growth of cancer cells. Interestingly, TGF-ß/BMPs and other pathways like wingless/Wnt, Notch, Hedgehog, appear not only to be involved in bone development/homeostasis and hematopoiesis but also in prostate cancer initiation and progression [2,8,52-55]. Our *in vitro* and *in vivo* data further support the notion BMP7 can antagonize TGF-ß signaling routes in human prostate cancer cells that are metastatic to the skeleton.

EMT provides mechanisms for prostate epithelial cells to overcome physical constraints imposed on them by intercellular junctions and 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-ß/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 [1,2]. EMT/MET can thus be viewed as a prime example of such cell plasticity. Considering the importance of TGF-ß superfamily in bone metastasis [42], 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 (TGF-ß-driven?) EMT. Clinical studies also seem to underline the cellular plasticity of prostate cancer during dissemination and bone metastasis [56,57]. The transition from a well-differentiated epithelial phenotype to an invasive mesenchymal phenotype may, of course, involve molecular mechanisms other than those described here (reviewed in [58]). Other studies have provided evidence that motility and invasiveness can be enhanced without inducing a complete conversion of cellular identity [58]. 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 [56-58]. The observed reversion to an epithelial morphology in prostate cancer seems possible even at bone metastatic sites [56-58] 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 (PC-3 cells), particularly the EMT/MET interconversion induced by different culture conditions (monolayer vs. 3D cultures) [59].

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 BLI of luciferase-expressing human prostate cancer cells revealed that growth of micrometastatic deposits in bone marrow are significantly inhibited by systemic administration of BMP7. Similar observations were made by us for other epithelial cancers, including breast cancer [62,63]. 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-like, tumor-initiating precursors [60]. Accumulating evidence suggests that cancer stem cells with tumor-initiating potential exist in human prostate cancer [61]. 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 metastastic) potential. Studies are currently on-going 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-ß 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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