

Bone Morphogenetic Protein 7 in the Development and Treatment of Bone Metastases from Breast Cancer

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Abstract

Bone morphogenetic protein 7 (BMP7) counteracts the physiological epithelial-to-mesenchymal transition (EMT), a process that is indicative of epithelial plasticity. Because EMT is involved in cancer, we investigated whether BMP7 plays a role in breast cancer growth and metastasis. In this study, we show that decreased BMP7 expression in primary breast cancer is significantly associated with the formation of clinically overt bone metastases in patients with ≥ 10 years of follow-up. In line with these clinical observations, BMP7 expression is inversely related to tumorigenicity and invasive behavior of human breast cancer cell lines. Moreover, BMP7 decreased the expression of vimentin, a mesenchymal marker associated with invasiveness and poor prognosis, in human MDA-MB-231 (MDA-231)-B/Luc⁺ breast cancer cells under basal and transforming growth factor- β (TGF- β)-stimulated conditions. In addition, exogenous addition of BMP7 to TGF- β -stimulated MDA-231 cells inhibited Smad-mediated TGF- β signaling. Furthermore, in a well-established bone metastasis model using whole-body bioluminescent reporter imaging, stable over-expression of BMP7 in MDA-231 cells inhibited *de novo* formation and progression of osteolytic bone metastases and, hence, their metastatic capability. In line with these observations, daily i.v. administration of BMP7 (100 μ g/kg/d) significantly inhibited orthotopic and intrabone growth of MDA-231-B/Luc⁺ cells in nude mice. Our data suggest that decreased BMP7 expression during carcinogenesis in the human breast contributes to the acquisition of a bone metastatic phenotype. Because exogenous BMP7 can still counteract the breast cancer growth at the primary site and in bone, BMP7 may represent a novel therapeutic molecule for repression of local and bone metastatic growth of breast cancer. [Cancer Res 2007;67(18):8742–51]

Introduction

Approximately 90% of cancer deaths result from the local invasion and distant metastasis of tumor cells (1). Better understanding of

the process of metastasis is, therefore, urgently needed. Increased motility and invasiveness of breast cancer cells is reminiscent of the epithelial-to-mesenchymal transition (EMT) that occurs during embryonic development (2, 3). This transition to a more mesenchymal, motile cellular phenotype is the result of a complex physiologic process that includes dissolution of adherens junctions, loss of cell polarity, a change to spindle-like cell morphology, cytoskeletal reorganization, increased cell motility, loss of epithelial markers, and induction of mesenchymal marker (2, 4). Increased vimentin expression and perturbation of E-cadherin-mediated cell adhesion appear as hallmarks of this process (4–8). However, in organogenesis, ‘mesenchymal’ cells may possess remarkable plasticity and can eventually regain a fully differentiated epithelial phenotype via a mesenchymal-to-epithelial transition (MET; refs. 9–11).

Members of the transforming growth factor- β (TGF- β) superfamily, which include bone morphogenetic proteins (BMP), are involved in the control of many different biological processes, including cell proliferation, differentiation, apoptosis, and regulation of invasiveness (12–14). In normal and nonmalignant epithelial cells, TGF- β is a potent growth inhibitor (15, 16). However, different types of carcinomas often escape this tumor-suppressing activity and become refractile to growth inhibition (15, 16). Even more, TGF- β can also potentiate tumorigenesis and contribute to the progression and invasiveness of various carcinomas (8, 17, 18). Accordingly, it has been shown that a blockade of TGF- β (signaling) inhibits tumor cell viability, migration, and metastasis (19), including the formation of bone metastases (20, 21).

The homodimeric protein BMP7 induces MET in normal and nontransformed cells (14, 22). For instance, during kidney development, BMP7 is essential for the condensation and epithelialization of the metanephric mesenchyme in the kidney, resulting in the formation of the tubular epithelium (9–11, 14). Furthermore, BMP7 seems to be involved in the preservation of the epithelial phenotype (23, 24), decreases fibrogenesis (23–25), and causes repression of inflammation (26, 27).

In this study, we present a cross-talk between BMP7 and TGF- β signaling in the regulation of EMT in breast cancer and identify BMP7 as a potential therapy for metastatic bone disease.

Materials and Methods

Cell lines and culture conditions. The human breast cancer cell lines MDA-MB-231 (MDA-231), T47D, and ZR-75-1 [American Type Culture

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Collection [ATCC] were cultured in DMEM (Invitrogen) containing 4.5 g glucose/L for MDA-231 or RPMI 1640 (ATCC) for T47D and ZR-75-1, supplemented with 10% FCS (Invitrogen), 100 units/mL penicillin, and 50 µg/mL streptomycin (Invitrogen). Culture medium for T47D was supplemented with 6.5 µg/mL insulin (Sigma Chemical Co.), and in culture medium for MDA-231-B/Luc⁺, a bone-seeking and luciferase-expressing subclone from MDA-231 (28), 800 µg/mL geneticin/G418 (Invitrogen) was added. MCF10A (29) and its fully malignant derivative MCF10CA1a (Barbara Ann Karmanos Cancer Institute, Detroit, MI; ref. 30) were cultured as described previously (30). All cell lines were grown in a humidified incubator at 37°C and 5% CO₂. *Mycoplasma* contamination was regularly tested by PCR, but never detected.

Putative BMP7 effects on luciferase expression by MDA-231-B/Luc⁺ cells have been tested and excluded (data not shown).

Generation of isogenic BMP7-overexpressing cell lines using targeted integration. MDA-231 cells that display a unique predilection for bone (MDA-231-BO2; ref. 31) were selected to generate a stable cell line that overexpresses BMP7. Stable cell lines were generated using the Flp-In system (Invitrogen) according to the manufacturer's protocol (32). In short, a MDA-231-BO2-Flp-in host cell line (MDA-231-BO2-Frt11) was generated by stable introduction of a single copy of a Flp recombinase target (FRT) site as an integral part of an antibiotic resistance gene in the genome of these cells. Subsequently, a luciferase-expressing subclone (MDA-231-BO2-Frt11/Luc⁺) was generated as described previously (28). This clone was used for the generation of isogenic stable cell lines by transient cotransfection of an FRT-targeting vector and a Flp recombinase expression vector. The FRT-targeting vector was either a pcDNA5/FRT vector (Invitrogen) expressing green fluorescent protein (GFP; control) or *BMP7* (target gene) under the control of the cytomegalovirus (CMV) promoter. Due to Flp-mediated recombination at the genomic FRT site, this targeting vector was incorporated in the genome. Simultaneously, a shift in antibiotic resistance was introduced allowing positive selection for integrants in the genomic FRT site only and negative selection for random integrants in one single step (32). This method allows the generation of isogenic stable cell lines, which only differ in sequence inserted in the genomic FRT site, thereby eliminating the need for clonal selection. It is important to note that both the MDA-231-BO2-Frt11 and MDA-231-BO2-Frt11/Luc⁺ cell line were validated for *in vivo* bone metastasis formation.

Patients. We retrospectively analyzed tumor tissue from 67 primary unilateral nonmetastatic ductal breast carcinomas excised from women treated at Centre René Huguenin (St. Cloud, France) from 1980 to 1994. The samples were examined histologically for the presence of tumor cells. A tumor sample was considered suitable if the proportion of tumor cells was >70%. Immediately after surgery, the tumor samples were stored in liquid nitrogen until RNA extraction.

Standard prognostic factors are presented in Supplementary Table S1. All patients were treated by hormone therapy only after surgery. The patients had physical examinations and routine chest radiography every 3 months for the first 2 years, followed by annual examination (mammograms). The median follow-up was 11.2 years (range, 1.5–20.3 years). Thirty-five patients were without relapse (≥10 years of follow-up), 17 patients developed exclusively visceral metastases (liver and/or lung), and 15 patients developed exclusively bone metastases. Immunohistochemical validation was done on fixed, paraffin-embedded tissue sections from primary breast tumor specimens (8 of 67).

ELISA for human BMP7. Levels of BMP7 in conditioned medium were measured with a commercially available specific ELISA kit using sandwich enzyme immunoassay technique (R&D Systems). Cells were routinely cultured for 4 days.

Real-time reverse transcription-PCR. Total RNA was extracted with the RNeasy Midi kit (Qiagen) from *in vitro* cultured cells at 70% to 80% confluence. Reverse transcription was done with random primers in the presence of RNase inhibitor (Roche Diagnostics). Quantitative real-time reverse transcription-PCR (qPCR) was done using commercially obtained exon-specific primers for BMP7, E-cadherin, vimentin, and β-actin (Applied Biosystems) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems). All experiments were done in duplo on four different samples.

For BMP7 mRNA detection in patients, qPCR (including thermal cycling conditions) was done as described previously (33). Each sample was normalized based on its TATA box-binding protein (TBP) content. Primers used were as follows: *BMP7* ATGGCCAACGTGGCAGAGAA (forward) and CAGCCAGGTCTCGGAAGCT (reverse) and *TBP* TGCACAGGAGCCAA-GAGTGAA (forward) and CACATCACAGCTCCCCACCA (reverse). All qPCR values were normalized using the comparative method of Livak and Schmittgen (34).

Western blot. Cells were seeded in six-wells plates at a density of 20,000/cm² in DMEM with 0.1% Fetal Clone II (FCII; HyClone, Perbio Science Nederland B.V.) for MDA-231-B/Luc⁺. After 18 h, cells were stimulated with recombinant human mature BMP7 [rhBMP7; 0.5 mg batches; Creative Biomolecules; rhBMP7 was freshly dissolved to a stock solution [1 mg/mL in 20 mmol/L acetate buffer with 5% mannitol (pH 4.5)] and was obtained from Dr. Vukicevic, Department of Anatomy, School of Medicine, Zagreb, Croatia; ref. 35] and/or porcine TGF-β2 (R&D Systems). After an additional 48 h, cells were lysed and collected in 250 µL lysis buffer [20 mmol/L Tris (pH 7.5), 20% glycerol, 400 mmol/L KCl, 1 nmol/L DTT, aprotinin (1:1,000), Roche protease inhibitor mix] followed by one or two rounds of freeze/thawing and used for Western blot analysis as described earlier (36). Rabbit polyclonal antibodies α-vimentin (diluted 1:500; ab7783, Abcam) and α-E-cadherin (diluted 1:500; Santa Cruz Biotechnologies, Tebu-bio), and a mouse monoclonal antibody α-β-actin (clone AC-15, Sigma-Aldrich) were used as primary antibodies.

Cells grown on chamber slides. MDA-231-B/Luc⁺ cells were seeded at a density of 20,000/cm² in DMEM with 0.1% FCII in eight chamber slides (Falcon, Becton Dickinson), and growth factors were added after 18 h. After 30 h, cells were dried for 5 min and fixed with 3.7% paraformaldehyde (pH 6.8; Merck, VWR) in PBS for 10 min and stained for vimentin as described for tissues, with the exception of the antigen retrieval step (see section 'histomorphometry, histochemistry, and immunohistochemistry'). Images were acquired using a color charge-coupled device (CCD) camera mounted on a Nikon Eclipse 610 microscope at a 20-fold magnification. Subsequently, cells were scored double blind for positive vimentin staining from four randomly chosen fields by two investigators (J.T.B. and P.G.M.v.O.).

Transient transfections and transcription reporter assays. MDA-231 cells were seeded at a density of 7,500 cells/cm² in DMEM with 10% FCII in 24-well plates. On the subsequent day, cells were transiently transfected with 1 µg of the indicated constructs using Fugene 6 (Roche) transfection reagent following manufacturer's protocol. To correct for transfection efficacy, 100 ng *Renilla* luciferase (pRL-CMV or pRL-CAGGS, both from Promega) was cotransfected. On day 3, cells were serum starved for 24 h before stimulation with TGF-β and/or BMP7 for a duration of 30 h. On day 5, luciferase activities were quantified using Dual-Luciferase Assay (Promega; ref. 37). Firefly luciferase activity was corrected for *Renilla* luciferase activity. The experiments were done in 4-fold and repeated at least twice. Values are expressed as mean ± SE.

Luciferase reporter gene constructs. For intracellular signaling of TGF-β, 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-β stimulation to a heterologous promoter reporter construct, whose activity depends on binding of activated Smad3/Smad4 transcription factor complexes (37).

The *BRE-luciferase* construct, which is based on the mouse Id1 promoter, was used to study the presence and functionality of BMP receptors (38).

Animals. Female nude mice (BALB/c *nu/nu*) were purchased from Charles River. Animals were housed in individual ventilated cages under sterile condition, and sterile food and water were provided *ad libitum*. Animal experiments were approved by the local committee for animal health, ethics and research of Leiden University and carried out in accordance with European Communities Council Directive 86/609/EEC. For surgical and analytical procedures, mice were anesthetized by i.p. injection of a 50 µL 1:1:1 mixture; ketamine HCl (stock solution of 100 mg/mL Nimatek, Vetimex Animal Health B.V.) + xylazine (2% Rompun, Bayer AG) + PBS (pH 6.8). At the end of the experimental period, animals were sacrificed by cervical dislocation.

Animal models. All cells were harvested at 70% to 80% confluence after changing to geneticin-free medium 24 h before inoculation.

For tumor growth in the bone marrow, single-cell suspensions of 2.5×10^5 MDA-231-B/Luc⁺ cells/10 μ L PBS were injected into the right tibiae of 6-week-old mice as described previously (28, 39).

Three days after intraosseous inoculation of MDA-231-B/Luc⁺ cells, the animals were equally distributed into three experimental groups based on a comparable tumor burden/mouse, as detected by whole-body bioluminescent reporter imaging (BLI). From this time point (day 0) and during a subsequent period of 24 days, all animals received vehicle or rhBMP7 (10 or 100 μ g/kg/d) treatment by tail vein injection. The progression of intraosseous growth was monitored by BLI at days 3, 10, 17, and 24 and by radiography at days 17 and 24 (28, 39).

Six-week-old mice were also intraosseous inoculated (1.0×10^5 cells/10 μ L PBS) with either MDA-231-BO2-Frt11(GFP)/Luc⁺ cells ($n = 8$) or MDA-231-BO2-Frt11(BMP7)/Luc⁺ cells ($n = 5$). The progression was monitored by BLI weekly and by radiography at the end of the experiment (day 28; refs. 28, 39).

For orthotopic tumor growth, single-cell suspensions of 1.0×10^6 MDA-231-B/Luc⁺ cells/10 μ L PBS were inoculated via a 0.5 mL U-100 insulin needle (29G 1/2, BD Micro-Fine, Becton Dickinson) into the mammary fat pads of 7-week-old mice. Animals were equally distributed into two experimental groups based on a comparable tumor burden/mouse, as detected by BLI. From this time point (day 0), all animals were given vehicle or BMP7 (100 μ g/kg/d) treatment by tail vein injection. Subsequently, the progression of orthotopically growing tumors was monitored weekly by BLI (28, 39).

Nude mice (4.5 weeks old) were inoculated (5.0×10^5 cells/100 μ L PBS) in the tail artery with either MDA-231-BO2-Frt11(GFP)/Luc⁺ cells ($n = 13$) or MDA-231-BO2-Frt11(BMP7)/Luc⁺ cells ($n = 8$), as described earlier (31). The progression was monitored by BLI weekly and by radiography at the end of the experiment (day 28; refs. 28, 39).

After the experimental periods, mice were sacrificed by cervical dislocation.

Mammary fat pads and tibiae with tumors were dissected and processed for further histomorphometrical and immunohistochemical analysis (see below).

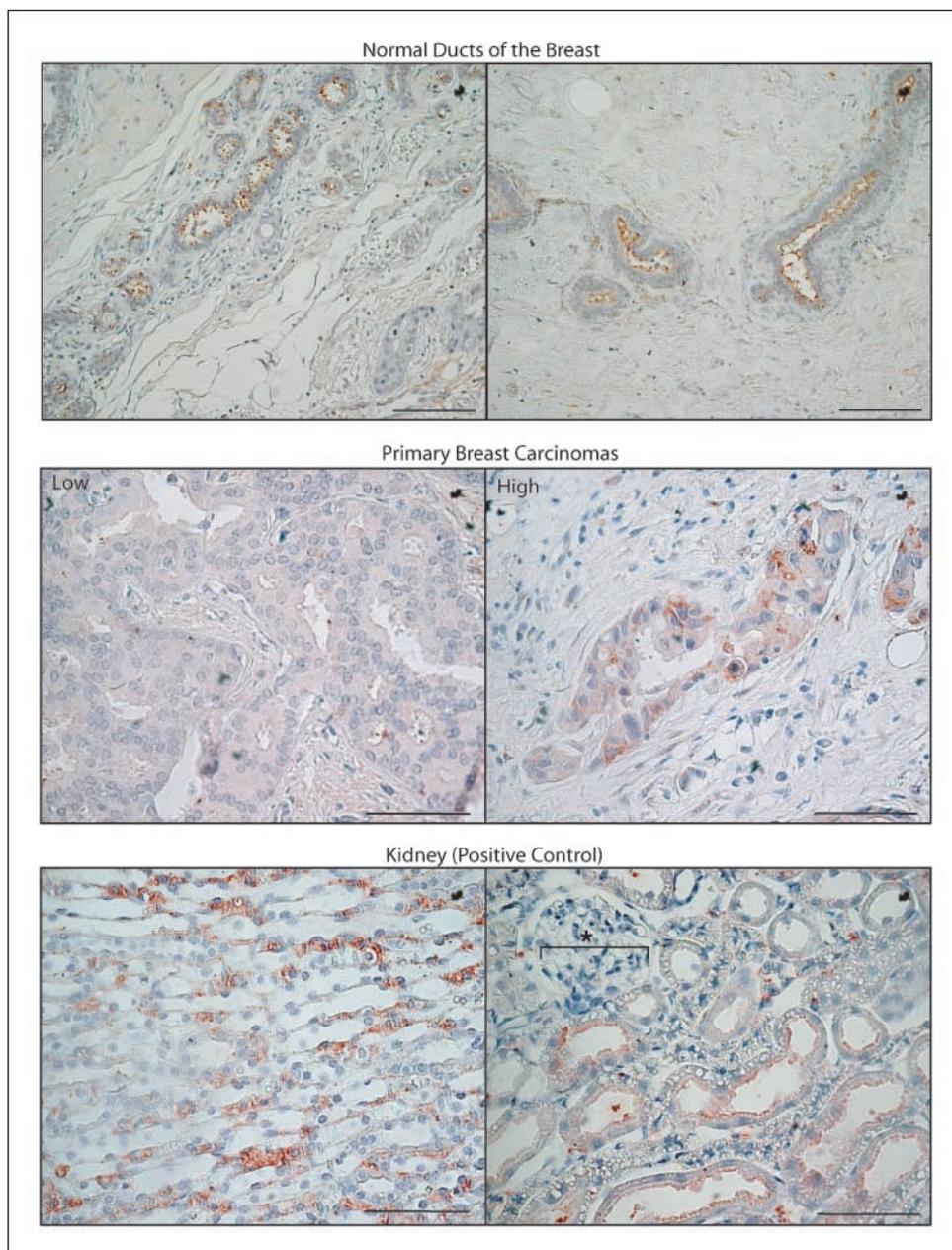


Figure 1. BMP7 mRNA expression levels and BMP7 immunolocalization in human primary breast cancer. BMP7 immunolocalization. *Top*, normal ducts of the breast [pronase (0.1% for 10 min at 37°C; Roche) was used as antigen retrieval step]. Immunolocalization of BMP7 in primary breast tumors with low (*middle left*) and high (*middle right*) levels of BMP7 mRNA (0.09 and 16.11, respectively). Rat kidney: medullary rays were used as a positive (*bottom left*) and glomeruli (as indicated by *) as negative control (*bottom right*). Adjacent to the negative glomerulus, convoluted tubuli stained positive for BMP7. Bars, 80 μ m (*top*) and 50 μ m (*middle and bottom*).

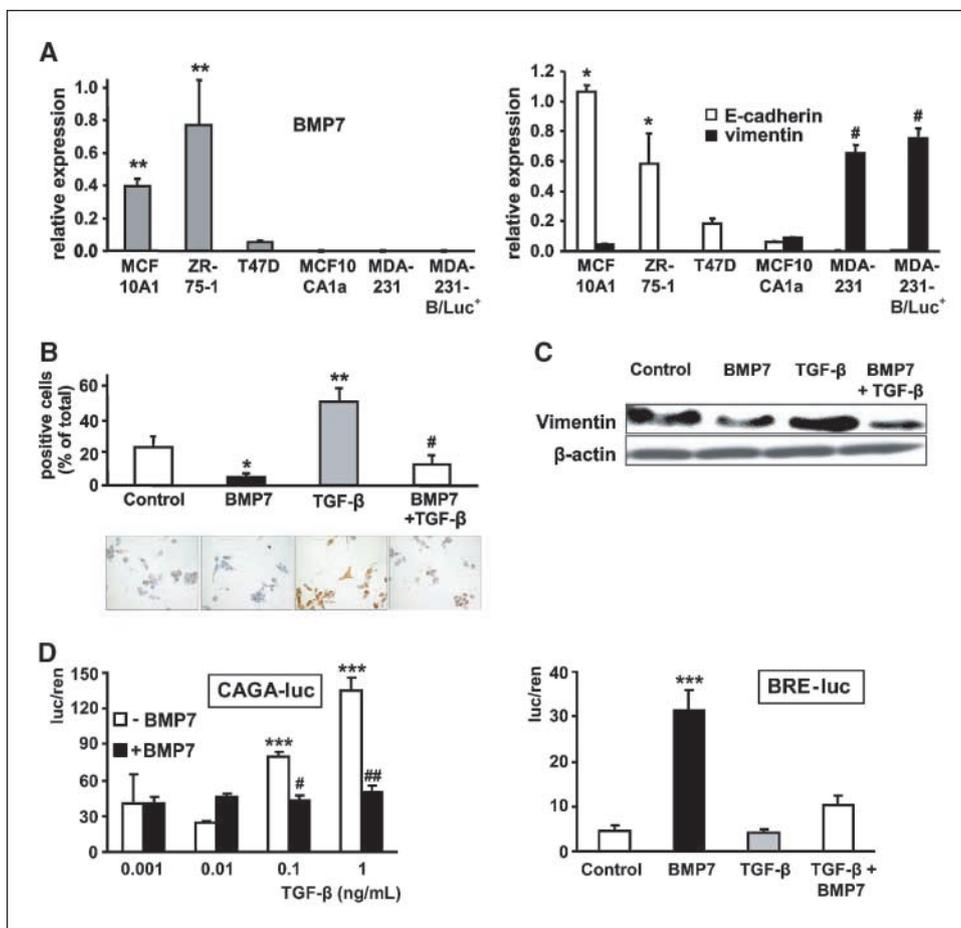


Figure 2. E-cadherin and vimentin expression in breast cancer cell lines. **A**, BMP7, E-cadherin, and vimentin mRNA expression in human breast cancer cell lines with increasing tumorigenic and metastatic potential (left to right) as detected by qPCR. E-cadherin expression in MDA-231 and MDA-231-B/Luc⁺ cells, vimentin expression in T47D and ZR-75-1 cells, and BMP7 expression in MCF10CA1a, MDA-231, and MDA-231-B/Luc⁺ cells were just above detection level. **, $P < 0.01$ versus BMP7 expression in T47D, MCF10CA1a, MDA-231, and MDA-231-B/Luc⁺ cell lines; *, $P < 0.05$ versus E-cadherin expression in all other cell lines; #, $P < 0.01$ MDA-231 cell lines versus the other cell lines. Expression values are normalized to β -actin (BMP7/10³ β -actin). **B**, vimentin immunolocalization of MDA-231-B/Luc⁺ cells after incubation with 1 μ g/mL BMP7 in the presence or absence of 10 ng/mL TGF- β . Representative examples. *, $P < 0.05$; **, $P < 0.01$ versus control; #, $P < 0.001$ versus TGF- β . **C**, expression of vimentin by MDA-231-B/Luc⁺ cells after incubation with 1 μ g/mL BMP7 in the presence or absence of 10 ng/mL TGF- β as detected by Western blotting. **D**, MDA-231 cells were transiently transfected with the CAGA-luciferase (CAGA-luc) reporter (left) and the BRE-luciferase (BRE-luc) reporter (right) constructs. The presence of functionally active TGF- β receptor complexes was shown with stimulation of the CAGA-luciferase reporter by TGF- β . ***, $P < 0.001$ versus all other conditions. Addition of BMP7 (500 ng/mL) to TGF- β -stimulated MDA-231 cells significantly inhibited TGF- β -driven CAGA-luciferase activity. #, $P < 0.01$; ##, $P < 0.001$. Values are normalized to *Renilla* (CAGA-luciferase/10⁵ pRL-CAGGS). Value for vehicle treated is 16.1 ± 1.1 . BMP7 (200 ng/mL), but not TGF- β (5 ng/mL), induces BRE-luciferase activity, indicating the presence and functionality of BMP receptors in MDA-231 cells. ***, $P < 0.001$ versus all other conditions. Values are normalized to *Renilla* (BRE4-luc/10⁻¹ pRL-CMV).

Whole-body BLI of isogenic BMP7- and GFP-overexpressing cell lines. In animals that were inoculated with either MDA-231-BO2-Frt11(GFP)/Luc⁺ or MDA-231-BO2-Frt11(BMP7)/Luc⁺, the luciferase activity was visualized through imaging of whole bodies with an intensified CCD video camera of the *in vivo* Imaging System (IVIS 100, Xenogen). The animals were anesthetized using the isoflurane anesthesia system (XGI-8, Xenogen) and injected i.p. with 2 mg D-luciferin sodium salt (Synchem OHG) dissolved in PBS. Animals were kept anesthetized, and measurements were done 5 min after the injection of D-luciferin. Bioluminescence imaging was acquired with a 15-cm FOV, a medium binning factor, and exposure times of 10 to 60 s. Imaging data were analyzed by using the program living image (Xenogen). Values are expressed as relative light units (RLU) in photons per seconds.

Radiographical analysis. Radiographic analyses of osteolytic lesions were assessed by radiography (Kodak X-OMAT TL film, Eastman Kodak Co.) using a Hewlett Packard X-ray system Faxitron 43805 and quantified using NIH Image 1.62b7 software as described earlier (39).

Histomorphometry, histochemistry, and immunohistochemistry. After orthotopic tumors and bone metastasis were fixed in 3.7% paraformaldehyde (pH 6.8) in PBS and processed, they were submitted to

Goldner staining, staining for tartrate-resistant acid phosphatase (TRAcP), H&E staining, or immunohistochemical staining as described previously (39, 40). Histomorphometric measurements of tumor burden were done on central sections through the tumor (largest tumor area). Tumor growth in bone could be readily identified by pancytokeratin staining alone or in combination with H&E staining. Total tumor areas, as an estimate of total tumor burden, was measured by image analysis using NIH Image 1.62b7 image analyses software as described previously (39). Subsequently, a distinction was made between the total tumor burden and the intraosseous and extraosseous tumor burden as described previously (39).

The following rabbit polyclonal antibodies were used at a concentration of 10 μ g/mL: α -human pancytokeratin (DAKO), α -human vimentin (ab7783), α -human BMP7 (2854ab, directed against prodomain of BMP7, obtained from Dr. Vukicevic; refs. 35, 41), α -phosphorylated Smad1 (PS1; ref. 42), and normal rabbit IgG (Jackson ImmunoResearch) antibodies as negative control. Goat α -rabbit IgG (DAKO) was used as secondary antibody. For antigen retrieval, slides were treated for 10 min at 37°C with 5 μ g/mL proteinase K (Invitrogen). To quantify PS1 and TRAcP staining, three histological sections per mouse ($n = 8$) were acquired using a color CCD camera mounted on a Nikon Eclipse 610 microscope at a 20-fold

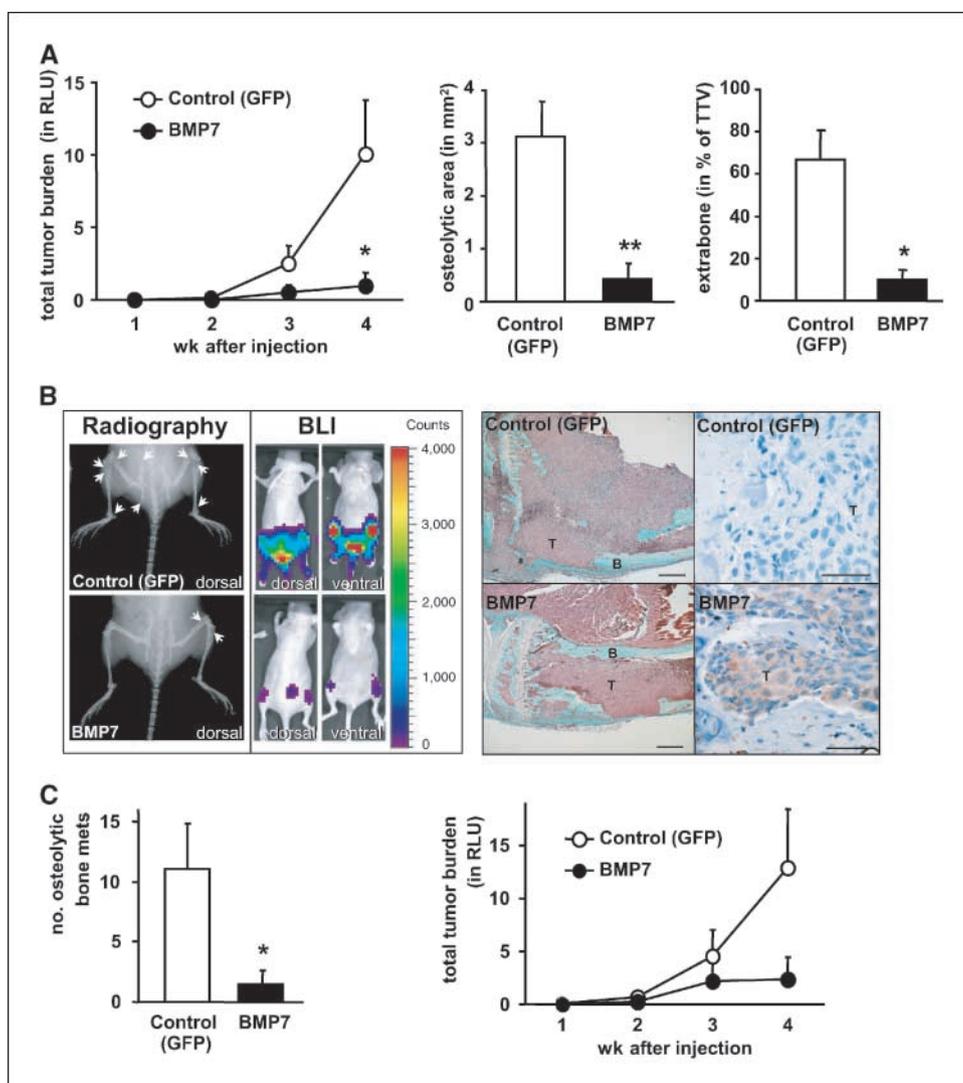


Figure 3. Bone metastatic and intrabone growth of MDA-231 cells expressing BMP7. *A*, in an experimental model of intraosseous implantation of breast cancer cells, stable BMP7 overexpression inhibited osteolysis as detected by radiographical analysis, total tumor burden as measured by BLI (RLU, 10^6 photons/s), and extrabone tumor volume determined as the percentage of total tumor volume (TTV) that has started to grow extramedullary. *B*, experimental model of bone metastasis formation after tail inoculation of breast cancer cells, from which representative examples of radiography, BLI, Goldner staining (bar, 200 μ m), and BMP7 immunolocalization (bar, 50 μ m; from left to right) are shown. Arrows, osteolytic lesions. T, tumor; B, bone. *C*, in the experimental bone metastasis model, stable BMP7 overexpression inhibited *de novo* formation of osteolytic bone metastases (mets; per mouse) as detected by radiographical analysis and total tumor burden (per mouse) as measured by BLI (RLU, 10^6 photons/s). *, $P < 0.05$; **, $P < 0.01$ versus control (GFP).

magnification. Subsequently, the number of cells that stained positive was scored single blind by two investigators (G.v.d.P. and P.G.M.v.O.).

BMP7 staining on patient material was done similarly, except that 5% normal goat serum (Jackson ImmunoResearch)/0.5% Boehringer Milk Powder (Boehringer Mannheim)/TTBS was used instead of 0.5% Boehringer Milk Powder/TTBS for incubation and first antibody dilution, and 0.01 mol/L citrate buffer (pH 6.0; 7 min at 98°C) was used as antigen retrieval step, unless stated otherwise.

Statistical analysis. Survival rates were determined using the log-rank test. Because BMP7 mRNA levels in patients did not follow a Gaussian distribution, (a) the mRNA levels in each subgroup of samples were characterized by their median values and ranges, rather than their mean values and coefficients of variation and (b) relationships between the molecular markers and clinical and histological variables were tested using the nonparametric Mann-Whitney *U* test. Other data are represented as mean \pm SE. *In vivo* tumor growth was analyzed by general linear model with repeated measurements using a least significant difference (LSD) post hoc test when applicable. Other statistical evaluations were carried out by ANOVA using a LSD post hoc test when applicable.

Results

BMP7 expression in human primary breast cancer and formation of distant metastasis. BMP7 mRNA expression levels

in primary breast tumors developing bone or visceral metastases were not significantly different from breast cancer patients without relapse (Supplementary Table S2, column 2 versus 1), and there was no significant difference observed that patients with primary breast tumors developing bone metastases had lower BMP7 mRNA expression levels ($P = 0.13$; Supplementary Table S2, column 3 versus 1) than patients without relapse. However, BMP7 mRNA expression levels in primary breast tumors developing bone metastases were significantly lower when compared with primary breast tumors developing visceral (lung and/or liver) metastases ($P = 0.027$; Supplementary Table S2, column 3 versus 4).

In patient-matched normal ducts of the breast, apical BMP7 staining could be observed (Fig. 1, top). Staining intensity of BMP7 protein in primary breast cancer specimens was in accordance with BMP7 mRNA levels (Fig. 1, middle). Adult kidneys were used as positive (Fig. 1, bottom left) and negative (Fig. 1, bottom right) control.

Tumorigenicity and BMP7 expression *in vitro*. We investigated whether BMP7 expression in human breast cancer cell lines is associated with tumorigenic and metastatic potential. The six cell lines examined in this study have progressively greater tumorigenic and metastatic potential when arranged in the

following order: MCF10A (normal breast epithelial cells), ZR-75-1, T47D, MCF10CA1a, MDA-231, and MDA-231-B/Luc⁺, where MCF10CA1a and MDA-231 and its derivate MDA-231-B/Luc⁺ are highly tumorigenic and metastatic. This arrangement of the cell lines inversely correlates with the expression of BMP7 (Fig. 2A). E-cadherin/vimentin expression ratios, indicative of epithelial phenotype, are also related to BMP7 expression (Fig. 2A), suggesting that acquisition of an invasive phenotype coincides with decreased BMP7 expression.

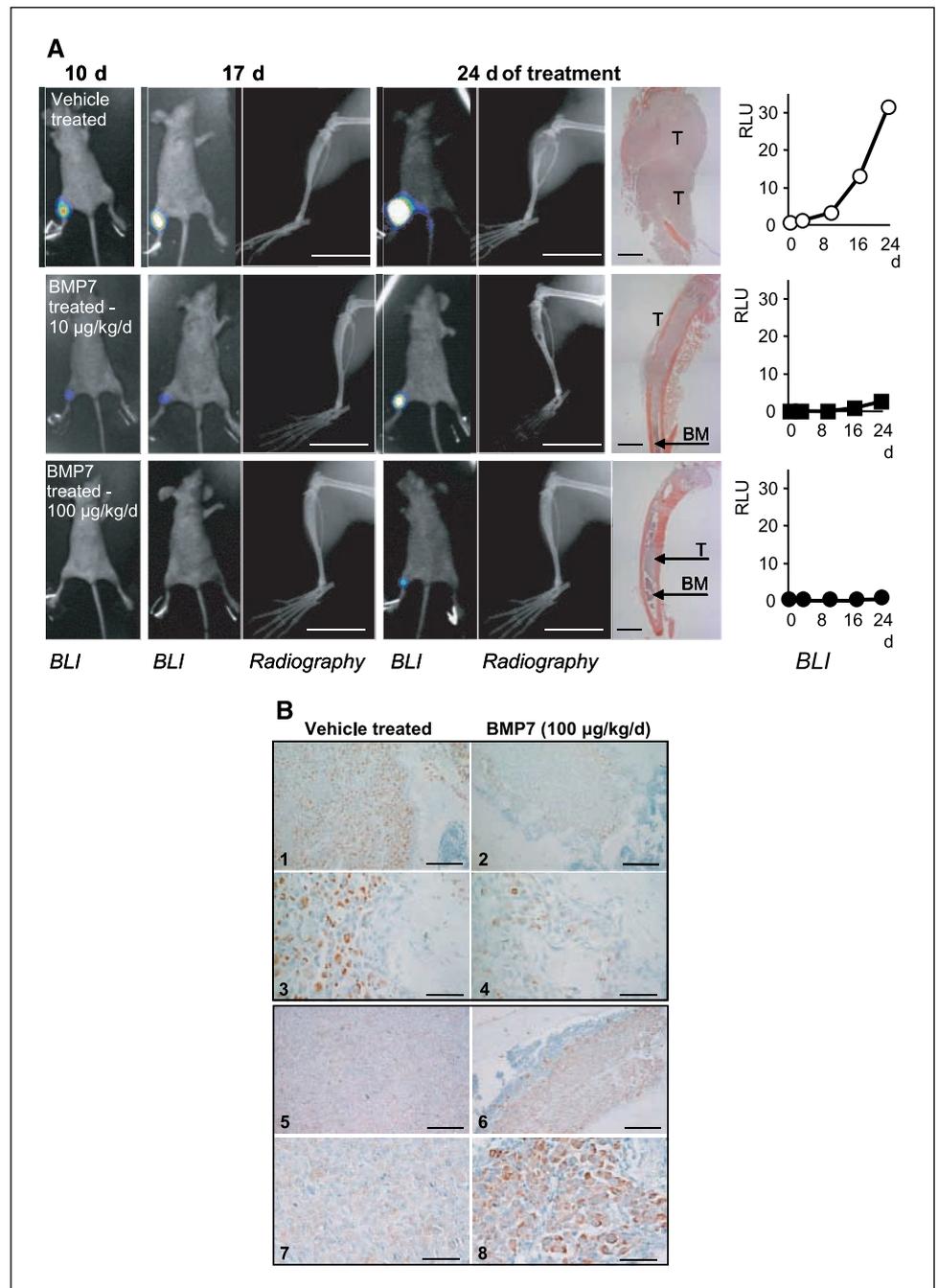
Immunohistochemical staining of MDA-231-B/Luc⁺ cells for vimentin showed a heterogeneous population with 22% vimentin-positive cells (Fig. 2B). Stimulation with BMP7 significantly decreased the percentage of vimentin-positive cells (5%; *P* =

0.038 versus control), whereas stimulation with TGF- β significantly increased the percentage of vimentin-positive cells (49%; *P* = 0.002 versus control). This increase in vimentin expression by TGF- β could be blocked completely by coincubation of BMP7 (12% vimentin-positive cells; *P* < 0.001 versus TGF- β -treated cells). These results were further confirmed by Western blotting (Fig. 2C).

Next, we tested whether BMP7 acts on MDA-231 cells to inhibit the acquisition of an invasive, mesenchymal phenotype by antagonizing Smad-dependent TGF- β signaling.

The presence of functionally active TGF- β receptor complexes, particularly ALK5, in MDA-231 cells was shown by the dose-dependent activation of the CAGA-luciferase reporter, whose activity depends on activated Smad3/Smad4 transcription factor

Figure 4. Representative examples of BMP7 treatment and intrabone growth of MDA-231-B/Luc⁺ cells. *A*, Left intrabone growth of tumor cells and tumor-induced osteolysis (BLI, radiography, and histology). Bars, 10 mm (X-rays) and 1 mm (histological sections). *T*, tumor; *BM*, bone marrow. Right, BLI is quantified in RLU (10⁵ photons/s). *B*, expression of vimentin (1–4) and pancytokeratin (5–8) in MDA-231-B/Luc⁺ cells growing in bone marrow after vehicle (1, 2, 5, and 6) and 100 μ g/kg/d BMP7 (3, 4, 7, and 8) treatment. Bars, 150 μ m (1, 2, 5, and 6) and 40 μ m (3, 4, 7, and 8).



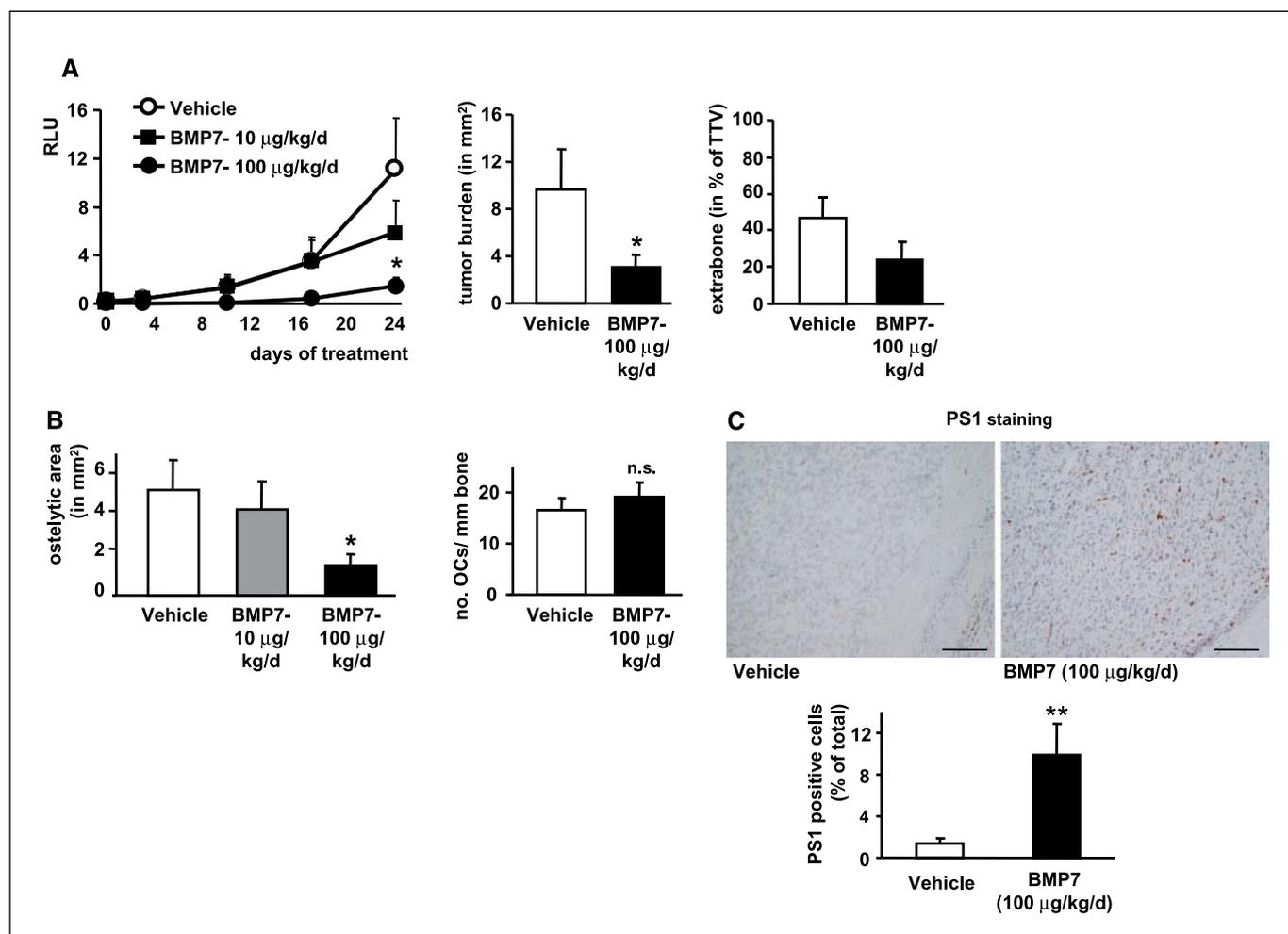


Figure 5. Effects of BMP7 treatment on intrabone tumor growth, osteolysis, and BMP7 signaling of MDA-231-B/Luc⁺ cells. *A*, tumor burden as measured by BLI (RLU, 10⁵ photons/s) and histomorphometric analysis, and the extraosseous tumor volume determined as the percentage of total tumor volume that has started to grow extramedullary. *B*, radiographical analysis of tumor-induced osteolysis in tibia at the end of the experimental treatment (24 d; *left*) and number of osteoclasts (OCs) per millimeter bone cortex (*right*). *C*, PS1-positive cells in the tumor at the end of the experimental treatment. The animals were either vehicle or BMP7 treated 6 h before explantation and fixation of the tibiae. N.S., not significant; *, $P < 0.05$; **, $P < 0.01$ versus vehicle. Bar, 80 μm .

complexes (Fig. 2D). Addition of BMP7 to TGF- β -stimulated MDA-231 cells significantly inhibited the TGF- β -driven CAGA-luciferase activity ($P < 0.001$).

Addition of BMP7, but not TGF- β , stimulated BRE4-luciferase activity, indicating the presence of functioning, activated type I BMP receptor complexes in MDA-231 cells (Fig. 2D). TGF- β antagonizes BMP7-induced BRE-luciferase activity ($P < 0.001$).

It is important to note that BMP7 did not affect proliferation of MDA-231-B/Luc⁺ cells *in vitro* using different cell culture conditions (1% FCS and 10% FCS; Supplementary Fig. S1).

BMP7 overexpression and experimental bone metastasis. As detected with ELISA, overexpression of BMP7 in MDA-231 cells [MDA-231-BO2-Frt11(BMP7)/Luc⁺] resulted in substantial secretion of BMP7 protein in the medium, 6.55 ng BMP7 protein/10⁶ cells/d. In contrast, BMP7 protein was not detectable in the control cell line [MDA-231-BO2-Frt11(GFP)/Luc⁺], <0.03 ng BMP7 protein/10⁶ cells/d.

Overexpression of BMP7 in MDA-231 cells [MDA-231-BO2-Frt11(BMP7)/Luc⁺] significantly inhibited both the intrabone growth ($P = 0.034$), osteolytic area ($P = 0.007$), and extraosseous

extension ($P = 0.042$) when compared with control [MDA-231-BO2-Frt11(GFP)/Luc⁺; Fig. 3A]. Moreover, in an experimental model of bone metastasis using tail inoculation of breast cancer cells, overexpression of BMP7 in MDA-231 cells [MDA-231-BO2-Frt11(BMP7)/Luc⁺] was shown to significantly inhibit the number of osteolytic lesions ($P = 0.025$; Fig. 3B and C). In line with the ELISA data described above, overexpression of BMP7 protein in tumor cells was also detected in histological sections in bone metastasis from MDA-231 cells overexpressing BMP7 [MDA-231-BO2-Frt11(BMP7)/Luc⁺], but not in the GFP control cell line (Fig. 3B). In addition, a trend was noticed for less overall total tumor burden as detected by BLI ($P = 0.10$; Fig. 3C). Furthermore, it is important to note that BMP7 overexpression did not affect the growth rate *in vitro* [doubling time MDA-231-BO2-Frt11(BMP7)/Luc⁺ cells: 21.0 h versus MDA-231-BO2-Frt11(GFP)/Luc⁺ cells: 20.9 h].

Experimental BMP7 treatment. Figure 4 depicts a representative example from each experimental group (vehicle treatment, 10 $\mu\text{g}/\text{kg}/\text{d}$ BMP7, or 100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7) showing different imaging methods (BLI, radiography, and histology) that have been used to detect MDA-231-B/Luc⁺ breast cancer cells growing in

bone. Breast cancer growth in bone and the formation of osteolytic lesions was strongly inhibited, particularly in the high-dose BMP7 group (100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7; Fig. 4A). Remarkably, mice receiving 100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7 had tumors that express higher levels of cytokeratins and lower levels of vimentin when compared with vehicle-treated mice (Fig. 4B). The number of strong cytokeratin-positive cells was increased by 25% on BMP7 treatment. This indicates that BMP7 treatment provokes a more epithelial-like phenotype.

In the vehicle-treated group, the tumor burden increased strongly over time (Fig. 5A). Tumor growth in mice receiving 10 $\mu\text{g}/\text{kg}/\text{d}$ BMP7 was not significantly different from vehicle-treated animals, whereas 100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7 strongly and significantly inhibited tumor progression ($P = 0.038$; Fig. 5A). This result was confirmed by histomorphometric analysis of total tumor burden ($P = 0.049$; Fig. 5A). In addition, no significant difference was observed in extraosseous tumor volume on BMP7 treatment (100 $\mu\text{g}/\text{kg}/\text{d}$; $P = 0.15$; Fig. 5A). Furthermore, radiographic analysis of the tibiae revealed that mice receiving 100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7 show significantly smaller bone lesions ($P = 0.046$), although number of osteoclasts per millimeter bone cortex seemed not to be different at the end of the experiment ($P = 0.528$; Fig. 5B). Systemic administration of BMP7 for the duration of the experiment can directly act on MDA-231-B/Luc⁺ bone metastatic cells as visualized by significantly enhanced nuclear staining for PS1 when compared with vehicle-treated animals ($P = 0.0097$; Fig. 5C).

Next, we studied the effects of BMP7 treatment on the growth of MDA-231-B/Luc⁺ cells that were orthotopically implanted into the mammary fat pads of nude mice (Fig. 6A–C). Figure 6B depicts a representative example from both experimental groups (vehicle and 100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7) as detected by BLI and histomorphometry. Tumor growth of the orthotopically implanted breast cancer cells in mice receiving 100 $\mu\text{g}/\text{kg}/\text{d}$ BMP7 was significantly inhibited versus vehicle-treated animals ($P = 0.049$; Fig. 6C).

Discussion

Although BMP7 has been proposed as a marker of differentiation in normal and breast cancer cells (43), the role of BMP7 in breast cancer progression has remained largely elusive (43–45). In this report, novel evidence is provided for a role of BMP7 in breast cancer development and bone metastases. We describe here that BMP7 expression in patients with primary breast tumors exclusively developing bone metastases is significantly lower than in primary breast tumors developing exclusively visceral (lung and/or liver) metastases. These clinical findings suggest that decreased BMP7 expression may confer a bone metastatic potential to human breast cancer cells. Normal ducts of the breast display strong apical BMP7 protein expression. BMP7 protein expression in patient-matched lymph node metastases was low or absent and, as a consequence, generally lower than in normal ducts and primary

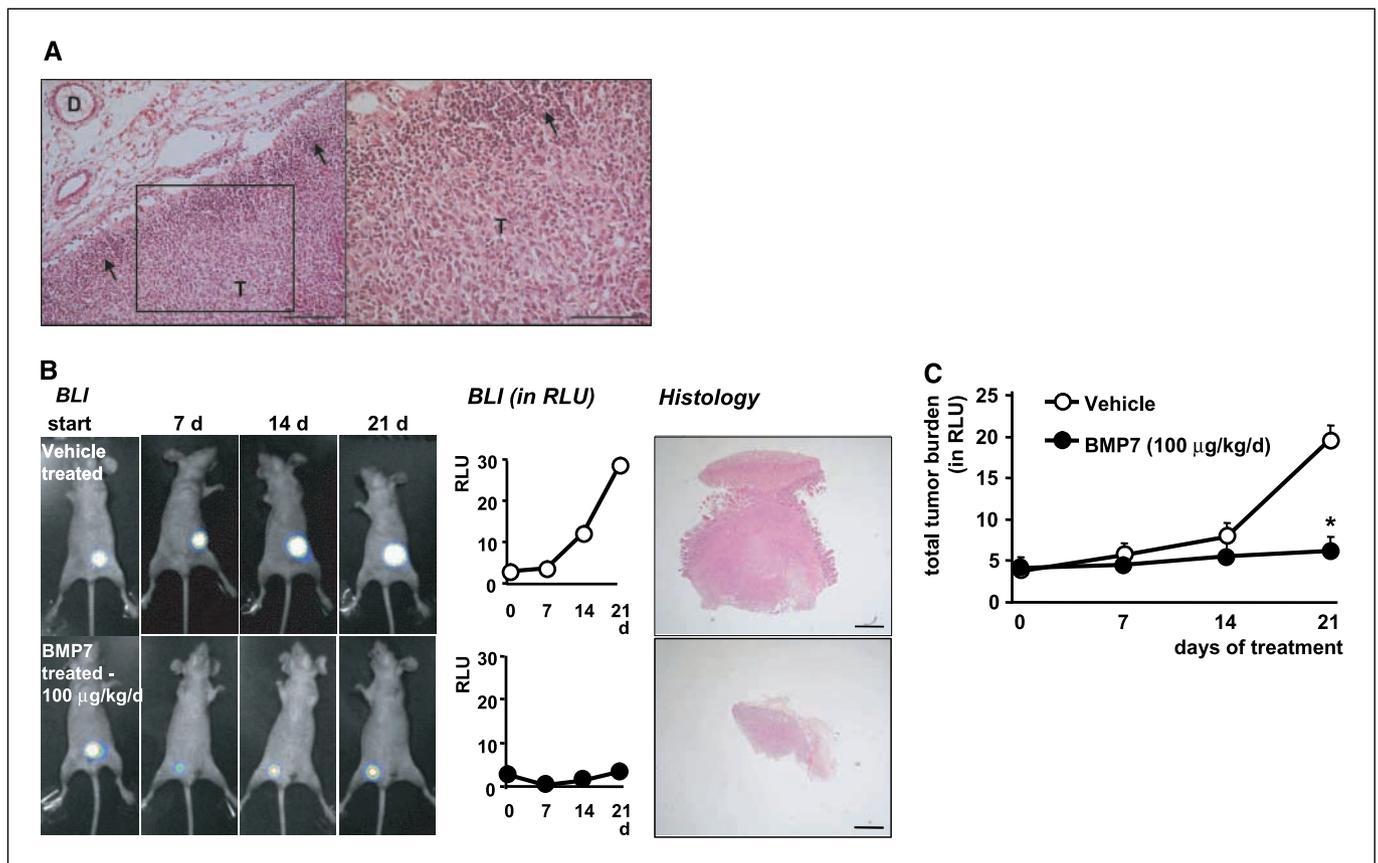


Figure 6. BMP7 treatment and growth of MDA-231-B/Luc⁺ cells in mammary fat pads. **A**, orthotopically implanted MDA-231-B/Luc⁺ cells have formed a tumor located in the mammary fat pad as shown by H&E staining. The figure on the right is a magnification from the box in the left figure. Lymphocytic infiltrates (arrows) in tumors could be observed. Bars, 80 μm (left) and 50 μm (right). **D**, mammary duct; **T**, tumor. **B**, representative examples for BLI and H&E staining. BLI is quantified in RLU (10^5 photons/s). Bar, 500 μm . **C**, tumor burden as measured by BLI (RLU, 10^5 photons/s). *, $P < 0.05$ versus vehicle.

breast carcinomas.⁹ Strikingly, BMP7 seems to be expressed predominantly in tumor cells in the solid tumor area rather than in migratory tumor cells outside this area (43), indicating that low BMP7 expression may support the acquisition of a more migratory invasive phenotype.

It has been well established that the process of EMT provides mechanisms for breast epithelial cells to overcome physical constraints imposed on them by intercellular junctions and adopt a motile phenotype (3, 8, 46). It is becoming increasingly clear that this local invasive process, representing initial stages of the metastatic cascade during carcinogenesis, is adapted from the remarkable epithelial plasticity (EMT) that occurs during embryonic development and postnatal tissue maintenance (2, 47). In many cancers, TGF- β is a protumorigenic factor that stimulates 'oncogenic' EMT (8, 17, 18). In contrast, BMP7 is a strong inducer of the reverse process (MET) during embryonic development (9–11, 14) and in renal fibroblasts (22). Moreover, BMP7 can inhibit TGF- β -induced fibrosis (25) and counteracts TGF- β -induced EMT in normal renal epithelial cells (24).

We show here that BMP7 mRNA expression is inversely related to tumorigenicity and invasive behavior of human breast cancer cell lines. Functional studies reveal that BMP7 overexpression by breast cancer cells inhibits *de novo* formation of osteolytic bone metastases and, hence, the metastatic capability of breast cancer cells in our *in vivo* bone metastasis model. Furthermore, BMP7 overexpression significantly inhibited tumor growth in bone. In keeping with these functional data, daily systemic administration of BMP7 strongly and significantly impairs both the orthotopic and intraosseous growth of human MDA-231-B/Luc⁺ breast cancer cells in nude mice.

Our *in vitro* studies reveal for the first time that BMP7 is a potent inhibitor of TGF- β -induced EMT in MDA-231 cancer cells. In these cells, BMP7 is able to counteract Smad-dependent TGF- β signaling. These actions of BMP7 may be of critical importance and could explain the observed beneficial effects of experimental BMP7 treatment on orthotopic growth and skeletal metastasis. In this context, it is important to note that inactive TGF- β is concentrated

and stored in high amounts in extracellular bone matrix and can be released and activated by osteoclastic resorption. Activated bone matrix-derived TGF- β may act as a paracrine growth factor for neighboring osteolytic cancer cells that may have colonized the bone marrow (21, 40, 48). Our *in vivo* data further support this notion because BMP7 antagonizes TGF- β signaling routes in human breast cancer cells that are metastatic to the skeleton. 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 (49).

Exogenous BMP7, however, could not restore E-cadherin expression in MDA-231-B/Luc⁺ cells. Recent observations support the notion that hypermethylation of the E-cadherin promoter in MDA-231 cells is involved in E-cadherin expression (6). Apparently, BMP7 cannot overcome this epigenetic effect.

It is important to note that comparable data have been obtained in clinical samples of prostate cancer (50) and uveal melanoma.¹⁰ Moreover, in human prostate cancer, we observed recently that BMP7 antagonizes TGF- β -induced EMT concomitant with an induction of E-cadherin expression (50).

Collectively, our data suggest that BMP7 regulates epithelial homeostasis in the human mammary gland by preserving the epithelial phenotype. Decreased BMP7 expression during breast cancer progression may, therefore, contribute to the acquisition of a bone metastatic phenotype. Furthermore, exogenous BMP7 can still inhibit breast cancer growth at the primary site and in bone marrow. Therefore, BMP7 may represent a novel therapeutic molecule for repression of local and bone metastatic growth of human breast cancer.

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¹⁰ I.C. Notting, et al. BMP7 inhibits tumor growth of uveal melanoma. *Invest Ophthalmol Vis Sci* 2007, in press.

⁹ J.T. Buijs, unpublished observation.

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Bone Morphogenetic Protein 7 in the Development and Treatment of Bone Metastases from Breast Cancer

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