Journal of Cellular Physiology

Targeted Overexpression of BSP in Osteoclasts Promotes Bone Metastasis of Breast Cancer Cells

QISHENG TU,[1]* JIN ZHANG,[1,2] AMANDA FIX,[1] ERIKA BREWER,[1] YI-PING LI,[3] ZHI-YUAN ZHANG,[4] AND JAKE CHEN[1]**

[1]*Division of Oral Biology, Tufts University School of Dental Medicine, Boston, Massachusetts*

[2]*School of Dentistry, Shandong University, Jinan, Shandong Province, China*

[3]*Department of Cytokine Biology, The Forsyth Institute and Department of Developmental Biology, Harvard School of Dental Medicine, Boston, Massachusetts*

[4]*College of Stomatology, Shanghai Jiao Tong University, Shanghai, China*

Bone is one of the most common sites of breast cancer metastasis while bone sialoprotein (BSP) is thought to play an important role in bone metastasis of malignant tumors. The objective of this study is to determine the role of BSP overexpression in osteolytic metastasis using two homozygous transgenic mouse lines in which BSP expression is elevated either in all the tissues (CMV-BSP mice) or only in the osteoclasts (CtpsK-BSP mice). The results showed that skeletal as well as systemic metastases of 4T1 murine breast cancer cells were dramatically increased in CMV-BSP mice. In CtpsK-BSP mice, it was found that targeted BSP overexpression in osteoclasts promoted in vitro osteoclastogenesis and activated osteoclastic differentiation markers such as Cathepsin K, TRAP and NFAT2. MicroCT scan demonstrated that CtpsK/BSP mice had reduced trabecular bone volume and bone mineral density (BMD). The real-time IVIS Imaging System showed that targeted BSP overexpression in osteoclasts promoted bone metastasis of breast cancer cells. The osteolytic lesion area was significantly larger in CtpsK/BSP mice than in the controls as demonstrated by both radiographic and histomorphometric analyses. TRAP staining demonstrated a twofold increase in the number of osteoclasts in the bone lesion area from CtpsK/BSP mice compared with that from wild type mice. We conclude that host tissue-derived BSP also plays important roles in breast cancer metastasis through inducing tumor cell seeding into the remote host tissues. Furthermore, osteoclast-derived BSP promotes osteoclast differentiation in an autocrine manner and consequently promotes osteolytic bone metastasis of breast cancer.

J. Cell. Physiol. 218: 135–145, 2009. © 2008 Wiley-Liss, Inc.

Bone is one of the most common sites of breast cancer metastasis (Yoneda, 1998) and 64% of patients who die with breast cancer also have bone metastases. Most breast cancer metastatic lesions are osteolytic (bone destructive) and cause severe pain, pathological fractures, neural compression syndrome, and eventually death of the breast cancer patients (Mohla et al., 2003; Reddi et al., 2003). It is thus one of the major causes of morbidity of breast cancer patients. Unfortunately, relatively little is known about the specific molecular mechanisms that are responsible for colonization and preferential growth and invasion of breast carcinoma cells in skeletal tissue.

BSP is a major non-collagenous protein in mineralized connective tissues and is a member of the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family (Fisher et al., 2004). This glycoprotein is essentially specific for the tissues of the skeleton and is known to have a high binding affinity for calcium and hydroxyapatite (Fisher et al., 1990; Paz et al., 2005). It is a phosphorylated and sulfated glycoprotein with an Arg-Gly-Asp (RGD) sequence, which is a common recognition sequence for several integrins (Ruoslahti, 1996) such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$. It also has three polyglutamic acid domains to confer hydroxyapatite-binding abilities (Ganss et al., 1999). Using immunohistochemistry and in situ hybridization methods, it was shown that osteoclasts also express BSP gene at both protein and mRNA levels (Bianco et al., 1991). Raynal and colleagues found that BSP significantly stimulates bone resorption in a dose-dependent manner at concentrations as low as 50 nM (Raynal et al., 1996). They suggested that this stimulation could be partially due to an increase in osteoclast adhesion to bone via its RGD sequence or its acidic sequences. Using a cell culture system, another group found that BSP significantly increased the attachment of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts and suggested that post-translational modification of BSP phosphorylation is necessary for in vitro osteoclastic bone resorption (Razzouk et al., 2002). We recently have reported that BSP acts synergistically with the receptor activator of nuclear factor kB ligand (RANKL)/RANK signaling to induce osteoclastogenesis and bone resorption in RAW264.7 cells. Furthermore, BSP was shown to decrease RANKL-induced apoptosis (Valverde et al., 2005).

In addition to its association with de novo bone formation (Paz et al., 2005), the presence of BSP in human breast cancer has also been well documented (Bellahcene et al., 1994; Barnes et al., 2003, 2004). An analysis of 454 breast cancer patients reported that BSP expression is positively correlated with morbidity, tumor size, axillary lymph-node status, and the first

Qisheng Tu and Jin Zhang contributed equally to this work.

Contract grant sponsor: NIH;
Contract grant numbers: DE14537, DE16710.

*Correspondence to: Qisheng Tu, Division of Oral Biology, Tufts University School of Dental Medicine, One Kneeland Street, Boston, MA 02111. E-mail: qisheng.tu@tufts.edu

**Correspondence to: Jake Chen, Department of General Dentistry, Division of Oral Biology, Tufts University School of Dental Medicine, One Kneeland Street, Boston, MA 02111. E-mail: jk.chen@tufts.edu

Received 22 May 2008; Accepted 28 July 2008

Published online in Wiley InterScience (www.interscience.wiley.com.), 28 August 2008.
DOI: 10.1002/jcp.21576

site of distant metastasis (Bellahcene et al., 1996b). In patients with primary breast cancer, elevated serum bone sialoprotein (BSP) was reported as a prognostic marker of subsequent bone metastasis (Diel et al., 1999) and was highly associated with poor survival (Bellahcene et al., 1996a). Moreover, immunohistochemical staining demonstrated that BSP was higher in bone metastases than in visceral metastases in breast and prostate cancer patients (Waltregny et al., 2000). We also reported that BSP overexpression in MDA-231 human breast cancer cells promotes tumor cell migration through endothelial cells in vitro as well as through the chicken embryonic vasculature (Chen et al., 2003). In another tumor metastasis experiment, we used a mouse model to transplant breast cancer cells through left ventricle injection. We found that human breast cancer cells transfected with BSP caused extensive osteolytic lesions in metastases (Zhang et al., 2004) while repressed expression of BSP or antibodies against BSP inhibited these effects (Zhang et al., 2003). However, most of the BSP-associated metastasis studies focused on the roles of tumor-expressed BSP, and additional experimental work is required to understand whether bone tissue-derived BSP and BSP-associated osteoclastogenesis are also involved in the tumor metastasis. Therefore in this study two homozygous transgenic mouse lines with elevated BSP expression levels either in all the tissues or only in the osteoclasts were used to further elucidate the functions of BSP in tumor metastasis.

Materials and Methods

Generation of CMV-BSP transgenic mice

Full-length murine BSP cDNA was subcloned into pRc/CMV vector (Invitrogen, Valencia, CA). The 2,310 bp transgene fragment was then cleaved out from the resulted CMV-BSP plasmid and microinjected into CB6F1 mouse embryos. Slot blot analysis was performed to identify homozygous transgenics mice named as CMV-BSP transgenic mice. Polymerase chain reaction (PCR) was also performed to confirm the presence of the transgene (Valverde et al., 2008).

Construction of the osteoclast specific Cathepsin K (CtpsK) promoter and mouse BSP gene

The CtpsK-BSP transgene was constructed in a pBluescript (SK)-cathepsin K/promoter vector, which contains a 4.0 kb mouse cathepsin K promoter (Li and Chen, 1999). A 1.9-kb fragment of 3x Flag-SV40polyA cassette was released from pIRES-hrGFP-1a (Stratagene, La Jolla, CA) and subcloned into the blunted *Kpn*I site and *Sal*I site of pBluescript (SK)-cathepsin K/promoter vector and the resulted construct was named as pSK-CtpsK-3xFlag. A 1.0 kb fragment of mouse BSP cDNA including coding sequence (CDS) was amplified by PCR with a sense oligonucleotide *Eco*RV (AATCCGTGCCACTCACTCGA) and antisense oligonucleotide *Sal*I (CGCTGATGGTAGTAATAATTCT) using pRc/CMVmBSP (created in our lab) as a template, then digested with *Eco*RV and *Sal*I and subcloned downstream of the cathepsin K promoter into the same sites of pSK-ctpsK-3xFlag. The construct was designated as pCtpsK/BSP.

Generation and identification of CtpsK-BSP transgenic mice

CtpsK/BSP DNA for microinjection was released from the vector backbone using BssH II, separated by agarose gel, and then gel-purified using QiAex II gel extraction (Qiagen, Valencia, CA) according to the manufacturer's instructions. The 6.9 kb CtpsK/BSP DNA construct was further purified using the EndoFree Kit to remove endotoxins. Transgene microinjection was performed at the Tufts Transgenic Facility into $B_6D_2F_1$ fertilized mouse eggs with DNA at a concentration of 2–3 ng/μl according to standard techniques (Chen et al., 1996; Paz et al., 2005). Founder mice and offspring were identified by Southern blot analysis and PCR using mouse tail genomic DNA. For Southern blots we used 10 μg of DNA from each mouse digested with *Hind*III separated on 0.8% agarose gel and hybridized by ^{32}P-radiolabeled cDNA probe as described in our protocols (Chen et al., 1996; Paz et al., 2005). Through interbreeding homozygotes for the transgene were established via slot blot screening (Li et al., 2005) and subsequent analyses were performed using the homozygote mice.

All the mice were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals prepared by the Institute on Laboratory Animal Resources, National Research Council (DHHS Publ. NIH 86-23, 1985), and by guidelines established by the Institutional Animal Care and Use Committee of the Tufts-New England Medical Center in Boston, MA.

In vitro osteoclast formation assay

Bone marrow monocyte/macrophages (BMM) were collected from the mouse femurs and tibias of sex-matched 6–8 week-old wild-type ($B_6D_2F_1$) and CtpsK-BSP transgenic mice as described by others (Sugatani and Hruska, 2005). Briefly, femurs and tibias were flushed with PBS using a 25-gauge needle and cultured in α-MEM supplemented with 20 ng/ml M-CSF overnight. Non-adherent cells were collected the following day and layered on Histopaque gradient (Sigma, St. Louis, MO). Cells at the gradient interface were transferred to 96 well plates and cultured in α-MEM with 10% heat-inactivated FBS, 1% glutamine, penicillin/streptomycin in the presence of 10 or 10 ng/ml M-CSF and 50 ng/ml RANKL for 5 days with change of medium every 3 days as recently described (Valverde et al., 2005). Cells were then subjected to semiquantitative RT-PCR analysis to determine BSP expression level. Specific primers (Li et al., 2005) for mouse BSP (GenBank Accession #L20232) were 5′-aacaatccgtgccactca-3′ (18 bases); 5′-ggaggggcttcactgat-3′ (18 bases) to amplify a 1,048 bp fragment. Cells were also cultured on 96 well plates in the presence of 10 ng/ml M-CSF and 50 ng/ml RANKL for 9 days (9D) and were then fixed and stained for TRAP activity using the K-ASSAY TRAP staining kit from Kamiya Biomedical Company (Seattle, WA) as described (Valverde et al., 2005). Digital images of the TRAP stainings were taken under bright field microscopy with a Nikon Eclipse E600 microscope and Spot Advanced software (Diagnostic Instruments, Sterling Hights, MI). Results were expressed as TRAP positive multinucleated cells ($TRAP^+$ MNC) per well. The numbers of positive cells with 5 or less and 10 or more nuclei were counted.

RNA isolation and RT-PCR analysis

Total RNA were extracted from unstimulated non-adherent cells (day 0) or from the adherent cell populations on days 1, 4, 5, 7, and 9 with an RNeasy Kit (Qiagen) following the manufacturer's instructions. One microgram of the RNA were used to perform the RT-PCR using SuperScript™ one-step RT-PCR with platinum Taq (Invitrogen). Specific primers (Li et al., 2005) for Ctps K were 5′-ACGGAGGCATCGACTCTGAA-3′ and 5′-GATGCCAAGCTTGCGTCGAT-3′ (20 bases); TRAP and NFAT2 (see reference 18 for primer sequences); and GAPDH, 5′-ACGACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ (20 bases). PCR products were electrophoresed on 1.5% agarose gel and the gel photographed and quantitated using UVP Image software. GAPDH amplification was performed for normalization purposes.

MicroCT measurement

The femurs from 6-week-old female CtpsK-BSP and wild-type mice were scanned by micro computed tomography (microCT) at 27 μm voxel resolution on a GE eXplore Locus Micro CT scanner. For image acquisition, the femurs were placed in a holder and the image consists of 200 slices with a voxel size of 9 mm in all three

axes. A measurement of trabecular bone structure was done in a region that starts at 0.1 mm from the lowest point of the growth plate and moving towards distally. Cancellous bone was separated from the cortical shaft. These parameters were measured: bone volume, bone surface, and surface-to-volume ratio. At a 3D level the following calculations were made as previously published (Lane et al., 2005): relative bone volume over total bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp). The diameter of spheres filling the structure was taken as Tb.Th, the thickness of the marrow spaces as Tb.Sp, and the inverse of the mean distances of the skeletal structure was calculated as Tb.N.

Intracardiac and intratibial inoculation of 4T1 mouse breast cancer cells in the recipient mice

For intracardiac inoculation 1×10^5 4T1 cells were injected into the left cardiac ventricle in 8-week-old CMV-BSP or CtpsK-BSP and their corresponding wild-type mice as previously described (Zhang et al., 2003). For intratibial inoculation of 4T1 cells a superficial incision was made in 8-week-old CtpsK-BSP and wild-type mice and the right proximal tibia was exposed. A 30-gauge needle was inserted into the medullary canal of tibia to create an initial core pathway, followed by insertion of a 29-gauge needle to make the final pathway into the bone. Then, 10 μl of culture medium containing 1×10^5 4T1 mouse breast cancer cells labeled with a luciferase reporter (Hiraga et al., 2004, 2005) were injected slowly using a 25 μl Hamilton syringe with a 29-gauge needle to prevent leakage of cells outside the bone. Cancer cells were inoculated into the right tibia. Resulting holes of 29-gauge Hamilton syringe were sealed with filling dental resin. Wound was closed using 6-0 nylon thread (Zhao et al., 2002).

Luciferase expression analysis by real-time IVIS imaging system and radiographic analysis

Three weeks after inoculation, the Luciferase expression was measured 12–15 min after luciferase substrate luciferin injection (1 mg luciferin/5 g body weight) by IVIS Imaging System 100 Series (Xenogen Corporation, Alameda, CA). Luciferase expression was detected as relative light units following a similar procedure described previously in our transgenic research (Paz et al., 2005). Radiographs were also taken using a radiographic inspection unit (Faxitron X-ray Corp., Wheeling, IL). Scanned radiographs were then analyzed with Scion Image Beta 4.02 software (Scion Corp., Frederick, MD) and the ratio of osteolytic area to total bone area in bones with osteolytic lesions was calculated.

Histological analysis and tartrate-resistant acid phosphatase histochemistry

After euthanasia, bone tissues with osteolytic lesions as demonstrated by real-time IVIS Imaging System and radiographic analysis in groups with intracardiac inoculation and the right tibial bones in groups with intratibial inoculation were collected and fixed in 10% formalin for 24 h and decalcified in 10% EDTA (pH 7.4) for 14 days at 4°C. Collected bone samples were sectioned from the mid-metaphysis for at least 30 sections (6 μm thick) and were mounted on glass slides. Hematoxylin and eosin staining was performed and histomorphometric analysis of osteolytic lesion areas and tumor sizes per total bone area was carried out using SPOT Advanced software (Diagnostic Instruments). TRAP staining was also performed as previously described (Park et al., 2007) to quantify the number of TRAP-stained osteoclasts in vivo. Briefly, the sections were stained for TRAP activities using a leukocyte acid phosphatase staining kit according to the manufacturer's instruction (Sigma). After incubation for 1 h at 37°C, the sections were counterstained with methyl green. The number of TRAP-positive osteoclasts existing in the tumor-bone interface was counted as previously described (Ida-Yonemochi et al., 2004) using a Nikon Eclipse E600 microscope and Spot Advanced software (Diagnostic Instruments). The number of TRAP-positive osteoclasts in 10 consecutive fields at 40× magnification of one section per specimen was counted.

Statistical analysis

All results are expressed as means ± SEM of 3 or more independent experiments. Chi-squared test and one-way ANOVA were used to test significance using the software package Origin 6.1 (OriginLab, Northampton, MA). Values of P lower than 0.05 were considered statistically significant.

Results

Constitutive BSP overexpression in CMV-BSP transgenic mice dramatically enhances skeletal and systemic metastases of breast cancer cells

CMV-BSP transgenic mice were created to determine the effects of high BSP expression levels on bone metabolism in vivo (Valverde et al., 2008). In this homozygous transgenic mouse line, the constitutively overexpressed mouse BSP was driven by the CMV promoter. Semiquantitative RT-PCR and Western blot analyses confirmed the higher BSP expression levels in mineralized tissues and serum samples isolated from CMV-BSP mice in addition to the detection of BSP expression in all soft tissues isolated from CMV-BSP mice. In mineralized tissues, BSP expression in CMV-BSP mice was measured in calvaria, mandible and tibia to be, respectively 2.72, 2.68 and 2.55-fold higher than wild-type mice. In characterizing the phenotypical changes of this transgenic mouse line, it was found that osteoclastogenesis and bone resorption were significantly enhanced in CMV-BSP mice (Valverde et al., 2008). To investigate the effects of high serum BSP level and constitutive BSP overexpression in all the body tissues on breast cancer metastasis, we performed intracardiac inoculation of 4T1 murine breast cancer cells into 8-week-old CMV-BSP transgenic mice and the corresponding wild type mice. The real-time IVIS Imaging System demonstrated that among the mice receiving intracardiac inoculation, all the 10 CMV-BSP mice developed metastases 1-week after injection while only four out of seven wild type mice showed metastatic lesions. Twelve days after 4T1 cell inoculation, five out of ten CMV-BSP mice died of multiple metastatic lesions while only two of seven wild type mice failed to survive. To detect whether these mice developed osteolytic lesions, radiographic analysis was performed 12 days after tumor cell inoculation or immediately after death of the animals. Two of the ten CMV-BSP mice developed detectable osteolytic lesions while none of the wild type mice showed bone metastatic lesions. The results demonstrated that constitutive BSP overexpression and a high serum BSP level dramatically increase skeletal as well as systemic metastases of 4T1 murine breast cancer cells which originally show a primary characteristic of bone-seeking metastases. Autopsy, gross examination and histological analyses demonstrated that some of the CMV-BSP mice receiving 4T1 cell inoculation died soon due to multiple metastatic lesions involving vital organs such as liver, lung, and kidney before more osteolytic lesions were developed and could be detected (Fig. 1).

Generation and characterization of the CtpsK/BSP transgenic mice

The findings that constitutive BSP overexpression in CMV-BSP mice dramatically enhanced the bone metastasis of breast cancer cells strongly support the hypothesis that in addition to tumor-derived BSP, host tissue-derived BSP also functions in this pathological process. Considering that osteoclasts play important roles in the development of osteolytic lesions during bone metastasis, we generated another transgenic mouse line

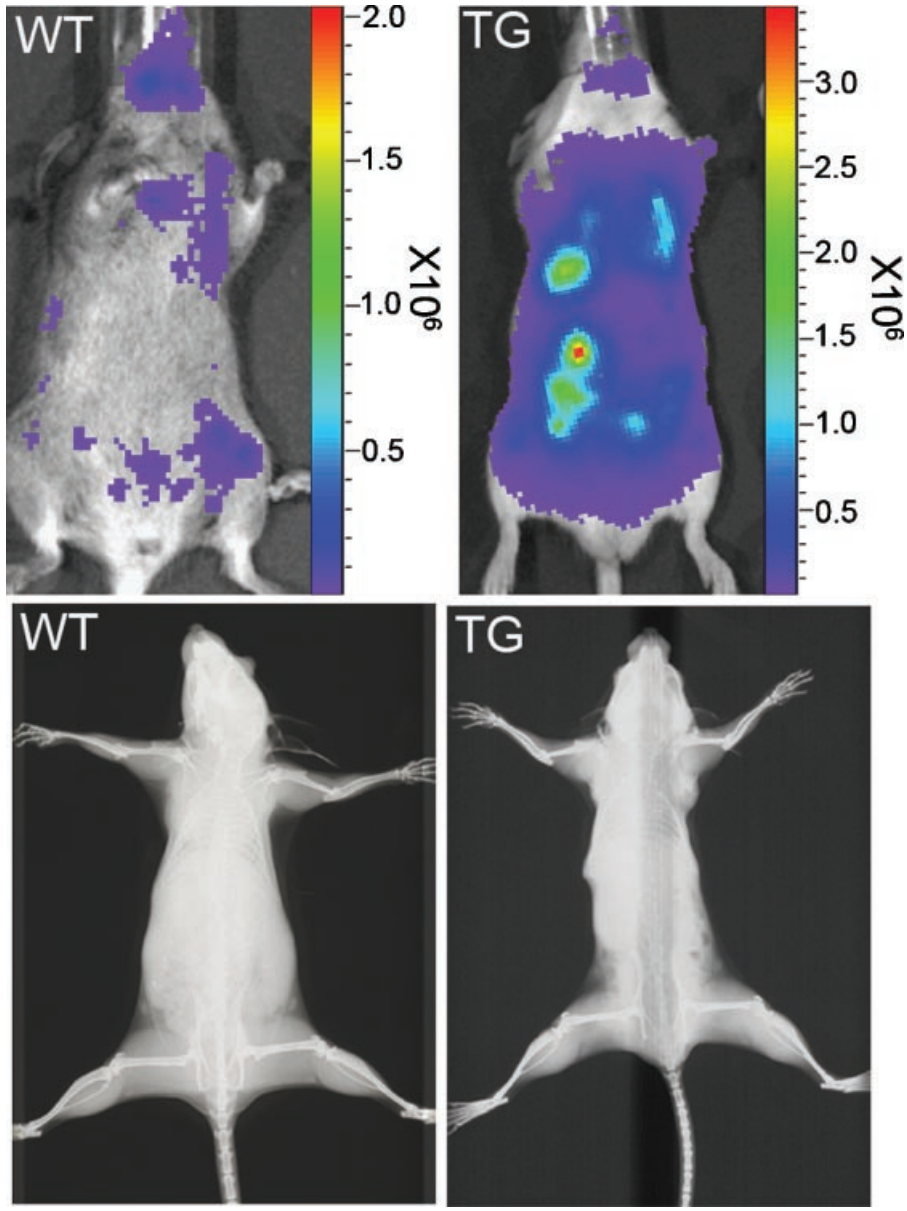

Fig. 1. BSP Overexpression in CMV-BSP transgenic mice dramatically enhanced systemic metastases of breast cancer cells. 1×10^5 4T1 murine breast cancer cells (labeled by luciferase reporter) were injected into the left cardiac ventricle in 8-week-old CMV-BSP and wild-type mice. The real-time IVIS Imaging System demonstrated that 9 of 10 CMV-BSP mice (upper right) developed multiple metastatic lesions 1-week after injection, while only four of seven wild type mice (upper left) developed less extensive metastatic lesions. Cancerous cachexia can be seen in the CMV-BSP mice that developed multiple metastatic lesions (lower right) while these conditions were not seen in wild type mice (lower left).

to focus on the role of osteoclasts-expressing BSP in breast cancer cells metastasis. This transgenic mouse line was named as CtpsK-BSP transgenic mice in which targeted BSP overexpression in osteoclasts was achieved by overexpressing mouse BSP cDNA driven by an osteoclast specific cathepsin K (CtpsK) promoter (Fig. 2A). Southern blot analysis confirmed the integration of CtpsK/BSP chimeric gene into the mouse genome (Fig. 2B). In RT-PCR analysis, bone tissues isolated from 3-day-old CtpsK/BSP transgenic mice demonstrated higher expression levels compared with those of wild-type mice (1.83-fold higher in calvaria, 1.80-fold higher in the mandible and 1.69-fold higher in tibia), whereas soft tissues isolated from both CtpsK/BSP and wild-type mice, such as heart, kidney, liver and muscle, showed negligible expression of BSP (Fig. 2C).

Overexpression of BSP in osteoclasts promoted in vitro osteoclastogenesis and activated osteoclastic differentiation markers

To investigate whether osteoclastogenesis in CtpsK/BSP transgenic mice is increased, BMM cells isolated from CtpsK/BSP and wild-type mice were cultured in the presence of M-CSF or M-CSF and RANKL to induce their proliferation or differentiation into osteoclasts, respectively. RT-PCR analysis was performed to confirm the increased BSP expression level in these osteoclast precursor cells. The results demonstrated that BSP expression in BMM cultures isolated from CtpsK-BSP mice was about twofold higher than in those from wild-type mice 5 days after the induction by 10 or 10 ng/ml M-CSF and 50 ng/ml

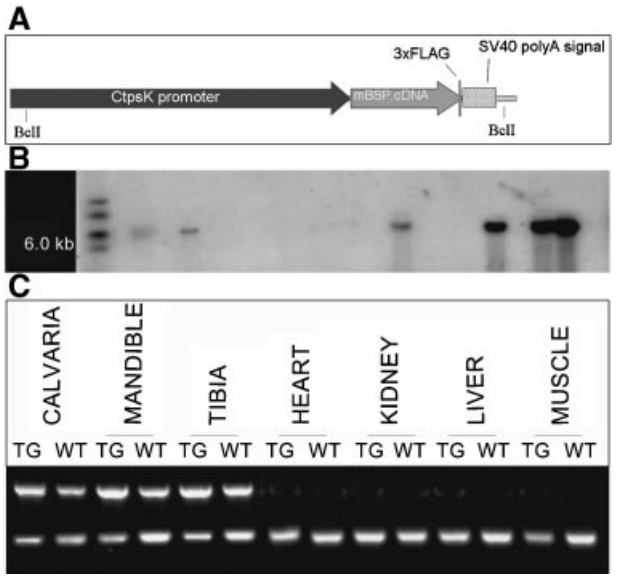

Fig. 2. Generation and Characterization of the CtpsK-BSP Transgenic Mice. A: Schematic drawing of the linearized construct, pCtpsK/BSP, used in microinjection for generation of CtpsK-BSP transgenic mice. CtpsK, cathepsin K; BSP, bone sialoprotein. B: Southern blot analysis of founder mice. Lane 1 showed molecular weight marker. Six positive founders (CtpsK-BSP) were shown in the picture. C: Generation of a homozygous CtpsK-BSP transgenic mouse line that exhibits constitutive overexpression of BSP. RT-PCR analysis of BSP (upper bands) and GAPDH (lower bands) expression levels in soft and mineralized tissues of 3-day old wild-type (WT) and transgenic (TG) mice.

RANKL (Fig. 3A). Using semiquantitative RT-PCR analysis, we measured the induction of Cathepsin K, TRACP and NFAT2 mRNA during the differentiation process of BMM towards osteoclasts. We found elevated expression levels of all these osteoclastic differentiation markers in BMM cultures derived from CtpsK/BSP mice compared with those from wild-type mice (Fig. 3B). TRAP staining was also performed to determine the number of differentiated osteoclasts induced by M-CSF and RANKL. Considering that large osteoclasts with 10 or more nuclei have been reported to resorb bone more efficiently in vitro than small osteoclasts with 5 or less nuclei (Lees et al., 2001), we counted the $TRAP^+$ MNC containing 5 or less and 10 or more nuclei separately. The results showed that 9 days after the induction by M-CSF and RANKL, the numbers of $TRAP^+$ MNC with 5 or less and 10 or more nuclei were both higher in cultures obtained from CtpsK/BSP mice than in those from wild-type mice (Fig. 3C,D).

CtpsK-BSP transgenic mice demonstrated reduced bone mineral density (BMD) and trabecular bone volume

To determine whether the targeted overexpression of BSP in osteoclasts increases bone resorption, femurs isolated from CtpsK/BSP and wild-type mice underwent quantitative microCT scan. Compared with the wild-type mice, CtpsK/BSP mice demonstrated decreased BMD. The total trabecular volume (BV/TV) in CtpsK/BSP mice decreased by 14.7%, and the Tb.N decreased by 53.4% when compared with the wild-type mice. Tb.Th was also 31.4% lower in CtpsK/BSP mice than in wild-type mice, whereas Tb.Sp was increased by 33.4% in CtpsK/BSP mice compared with wild-type mice (All $P < 0.05$, Table 1).

Targeted BSP overexpression in osteoclasts promoted bone metastasis of breast cancer cells

Using the in vivo bone metastasis models and the real-time IVIS Imaging System, we found that when 4T1 murine breast cancer cells (1×10^5, labeled by luciferase reporter) were injected into the left cardiac ventricle 7 of 10 CtpsK/BSP mice developed large osteolytic bone lesions 4 weeks after injection. In the CtpsK-BSP group mice with metastatic bone lesions developed multiple osteolytic lesions in the clavicles, the front limbs and the hind limbs. In contrast, only 4 of 10 wild type mice developed metastatic bone lesions and osteolytic bone lesions were less than those observed in the CtpsK-BSP group (Fig. 4A,B). Radiographic and gross histological analysis demonstrated that the lesion area was significantly larger in CtpsK-BSP mice than in the wild-type mice (Fig. 4C,D). Histomorphometric analysis further confirmed that metastatic bone lesion area in CtpsK-BSP mice was larger than in the wild type mice (Fig. 4E). Using TRAP staining we observed a twofold increase in the number of TRAP-positive, multinuclear osteoclasts in the bone lesion area from CtpsK-BSP mice when compared with that from wild-type mice (Fig. 4F).

Targeted BSP overexpression in osteoclasts resulted in larger osteolytic bone lesions in bone metastasis of breast cancer cells

Tumor metastasis begins with tumor cells departing from the primary site, followed by their entering circulation, and then ended with their seeding and growing in a remote site. In order to focus on the effects of targeted BSP overexpression in osteoclasts on the final step of bone metastasis, we performed intratibial inoculation of 4T1 murine breast cancer cells (1×10^5, labeled by luciferase reporter). Among the mice receiving intratibial inoculation, 9 of 10 CtpsK-BSP mice developed osteolytic bone lesions 3 weeks after injection while only 4 of 9 wild-type mice showed bone lesions as demonstrated by the real-time IVIS Imaging System (Fig. 5A,B). Radiographic and gross histological analysis also demonstrated that the lesion area was significantly larger in CtpsK-BSP mice than in the wild-type mice (Fig. 5C,D). H&E staining sections showed that cortical bone, trabecular bone, growth plate, and bone marrow cavity in CtpsK-BSP mice with osteolytic bone lesions were almost completely destructed and replaced by tumor tissues. In contrast, bone destruction in wild type mice with bone lesions was less extensive (Fig. 5E). Consistent with the results of radiographic analysis, histomorphometric analysis also demonstrated that metastatic bone lesion area in CtpsK-BSP mice was twofold larger than in the wild type mice (Fig. 5E). TRAP staining also demonstrated a twofold increase in the number of TRAP-positive, multinuclear osteoclasts in the bone lesion area from CtpsK-BSP mice when compared with that from wild-type mice (Fig. 5F).

Discussion

Breast cancer is among the most common cancers that disseminate to bone (Yoneda, 1998) with bone metastases found in virtually all advanced breast cancer patients. Since 1994 when it was reported that human breast cancer cells express BSP, numerous studies have been focused on investigating the mechanisms underlying the tendency of breast cancer cells metastasizing to bone tissues. It has been reported that in non-skeletal sites (e.g., lungs) where the capillaries are of smaller diameters, mechanical arrest of cancer cells is more common than in the wide-channeled sinusoids of bone. Therefore, the high specificity of breast cancer metastasis to bone needs additional adhesive interactions between tumor cells and the endothelial cells of sinusoids. In the pathogenesis of breast cancer metastasis to bone, several bone- and

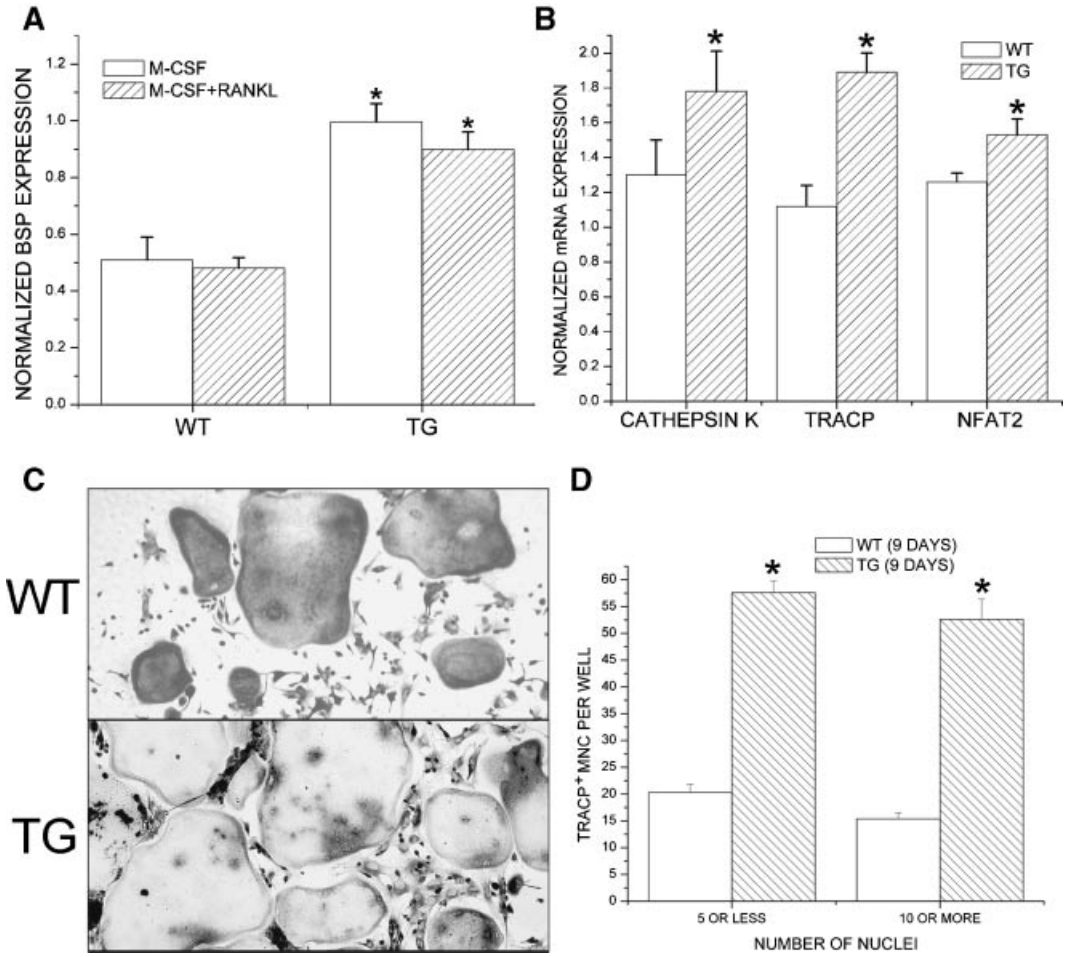

Fig. 3. Overexpression of BSP in Osteoclasts Promoted in vitro Osteoclastogenesis and Activated Osteoclastic Differentiation Markers. A: BMMs from 6 to 8-week-old wild type (WT) and transgenic mice (TG) were cultured in the presence of 10 ng/ml M-CSF or 10 ng/ml M-CSF and 50 ng/ml RANKL for 5 days and then subjected to semiquantitative RT-PCR analysis. Levels of BSP expression were normalized with those of GAPDH. BSP expression in BMM cultures isolated from CtpsK-BSP mice was about twofold higher than in those from wild-type mice 5 days after the induction. B: The induction of Cathepsin K, TRACP and NFAT2 mRNA during the differentiation process of BMM towards osteoclasts was also determined using semiquantitative RT-PCR analysis. Levels of mRNA expression were normalized with those of GAPDH. C: BMM cells were also cultured on 96 well plates in the presence of 10 ng/ml M-CSF and 50 ng/ml RANKL for 9 days (9D) to evaluate their differentiation by TRACP staining. D: The number of TRAP positive multinucleated cells were counted and data are presented as mean ± SE of four different cultures. (TG, CtpsK-BSP transgenic mice; WT, wild-type mice; *$P < 0.05$ TG vs. WT).

marrow-derived chemotactic factors such as BSP, osteonectin, and osteopontin play an important role (Mastro et al., 2003). It has also been reported that the binding of BSP to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ enhances migration, adhesion, and proliferation of the MDA-MB-231 human breast cancer cells (Sung et al., 1998). In a recently published article, Nam et al. (2006) reported that BSP is an important mediator of tumor cell-targeted prometastatic activity of TGF-β and knockdown of BSP in 4T1 cells significantly reduced TGF-β induced tumor invasion in vitro.

However, most of the available research results regarding BSP-associated bone metastasis focused on the roles of tumor-derived BSP. Considering that BSP stored in bone tissues is currently recognized to serve as a regulator of osteoblast and osteoclast function (Shankar et al., 1995), host tissue-derived BSP may also affect breast cancer cells and is involved in the pathophysiology of bone metastasis. Consistent with our previous findings (Chen et al., 2003), it can be postulated that a high serum BSP level might facilitate the tumor cells migration through the endothelial cells and promote vascular invasion. Furthermore, consistent with its roles in increasing tumor cell proliferation, adhesion, and migration (Sung et al., 1998; Byzova et al., 2000), BSP may further help seeding and growing of the tumor cells at a saturated level in the entire body tissues. Strongly supporting this hypothesis, our

TABLE 1. MicroCT analysis of femurs in 6-week-old female CtpsK-BSP and wild-type mice

Genotype	Wild-type	CtpsK-BSP
BMD (mg/cm^3)	26.239 ± 1.552	20.848 ± 1.3241*
BV/TV (%)	68.58 ± 3.256	58.52 ± 4.56*
Tb.Th (μm)	236 ± 12.6	161.9 ± 8.9*
Tb.N (mm^{-1})	3.268 ± 0.012	1.524 ± 0.006*
Tb.Sp (μm)	172.57 ± 6	230.14 ± 12*

Micro CT scan was performed to assess bone mineral density (BMD) and three-dimensional trabecular bone architecture of femur bones from 6–week-old female wild-type (WT) and CtpsK-BSP (TG) mice. The bone volume density (BV/TV), the trabecular thickness (Tb.Th), the trabecular Number (Th.N) and the trabecular separation (Tb.Sp) were measured. Data are mean ± SEM from five individual mice.
*$P < 0.05$, wild-type mice versus CtpsK-BSP mice.

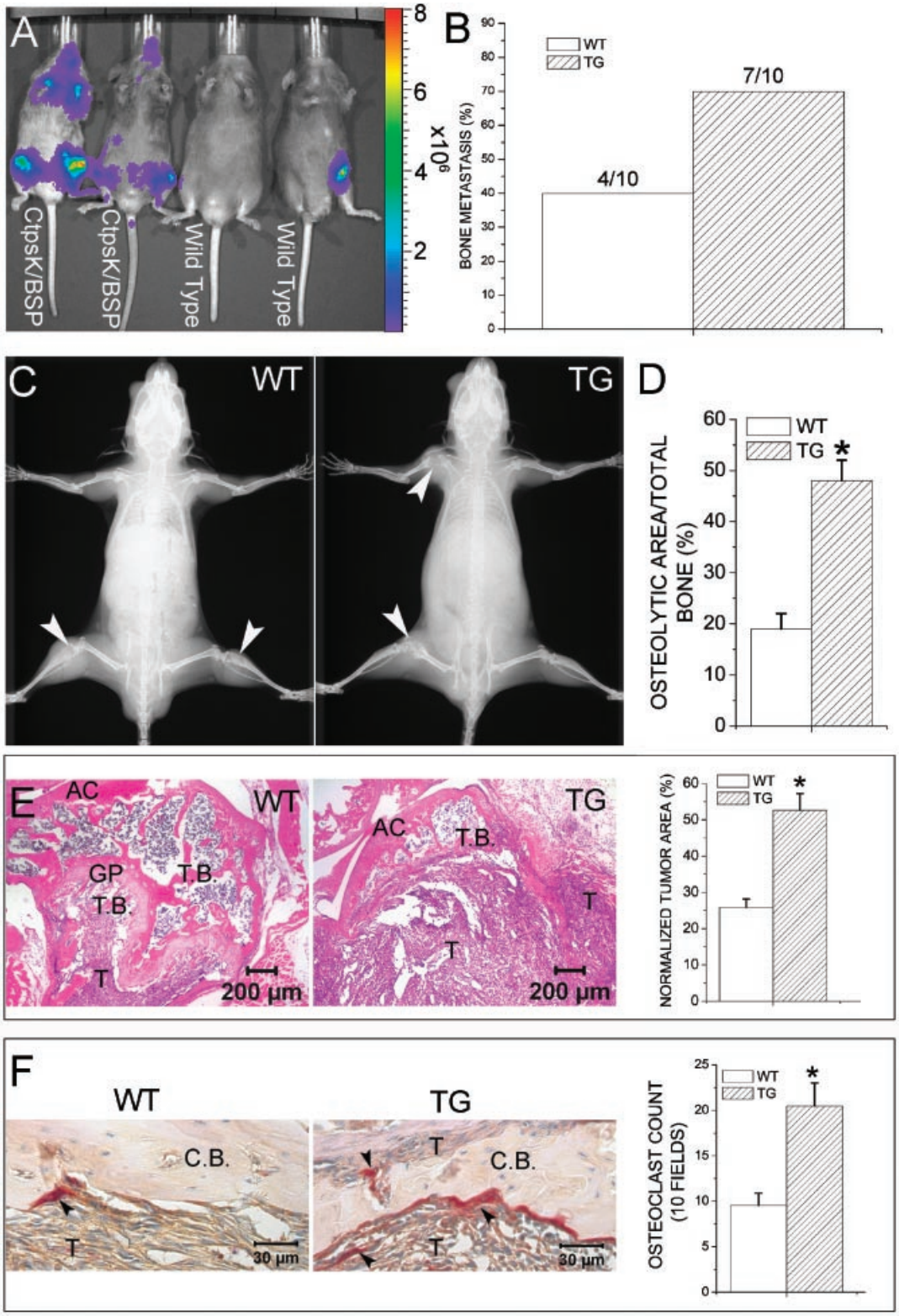

Fig. 4. Targeted BSP overexpression in osteoclasts promoted bone metastasis of breast cancer cells in bone metastasis mouse models. (A, B) 1×10^5 4T1 murine breast cancer cells (labeled by luciferase reporter) were injected into the left cardiac ventricle in 8-week-old CtpsK-BSP and wild-type mice. The real-time IVIS Imaging System demonstrated that 7 of 10 CtpsK-BSP mice developed large osteolytic bone lesions 4 weeks after injection. In contrast, only 4 of 10 wild type mice developed metastatic bone lesions and osteolytic bone lesions were less than those observed in the CtpsK-BSP group. C: Representative radiographs of the wild type mice (left) and CtpsK-BSP (right) 4 weeks after intracardiac inoculation. D: Computerized software analysis of the scanned radiographs showed that the osteolytic lesion area in CtpsK-BSP mice was significantly larger than in the wild type mice. E: H&E staining sections of osteolytic bone lesions from wild type mice (left) and CtpsK-BSP mice (center). Histomorphometric analysis demonstrated that metastatic bone lesion area in CtpsK-BSP mice was twofold larger than in the wild type mice (right). F: Tartrate-resistant acid phosphatase (TRAP) staining of osteolytic lesions from wild type mice (left) and CtpsK-BSP mice (center). The number of TRAP-positive, multinuclear osteoclasts in the bone lesion area from CtpsK-BSP mice was twofold higher than that of wild type mice (right; AC, articular cartilage; GP, growth plate; T.B., trabecular bone; T, tumor; C.B., cortical bone; WT, wild type mice; TG, CtpsK-BSP transgenic mice; white arrow head, osteolytic bone lesions; black arrow head, TRAP positive osteoclast; *$P < 0.05$ TG vs. WT).

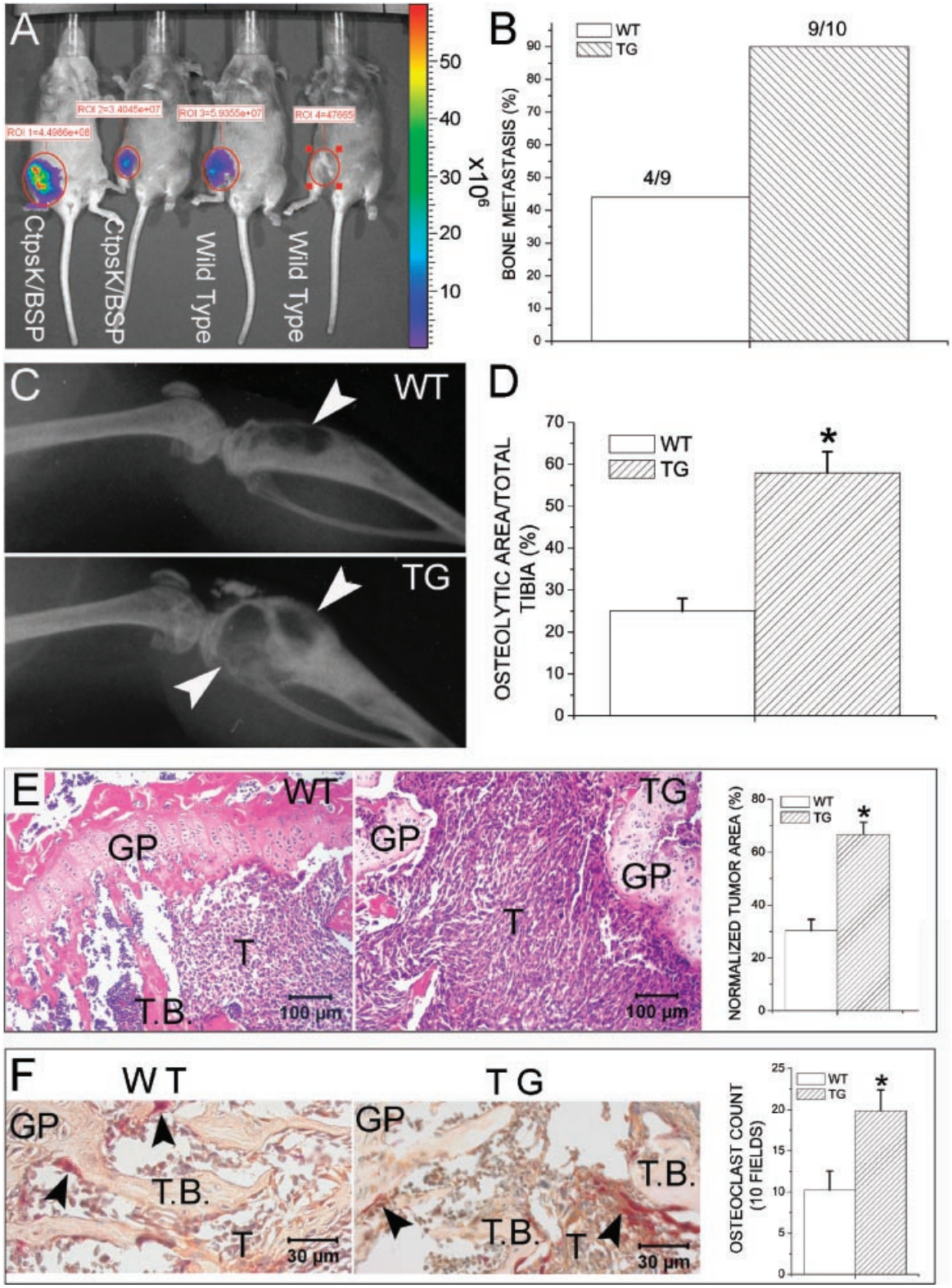

Fig. 5. Targeted BSP overexpression in osteoclasts resulted in larger osteolytic bone lesions in bone metastasis mouse model with intratibial inoculation of breast cancer cells. (A, B) For intratibial inoculation, 1×10^5 4T1 murine breast cancer cells (labeled by luciferase reporter) were injected into the right tibia of 8-week-old CtpsK-BSP and wild type mice. Among these mice, 9 of 10 CtpsK-BSP mice developed osteolytic bone lesions 3 weeks after injection while only 4 of 9 wild type mice showed bone lesions as demonstrated by the real-time IVIS Imaging System. C: Representative radiographs of the right hindlimbs from wild type (left upper) and CtpsK-BSP (left lower) mice 3 weeks after intratibial inoculation. D: Computerized software analysis of the scanned radiographs showed that the osteolytic lesion area in CtpsK-BSP mice was significantly larger than in the wild type mice (right). E: H&E staining sections of osteolytic bone lesions from wild type mice (left) and CtpsK-BSP mice (center). Histomorphometric analysis demonstrated that metastatic bone lesion area in CtpsK-BSP mice was twofold larger than in the wild type mice (right). F: Tartrate-resistant acid phosphatase (TRAP) staining of osteolytic lesions from wild type mice (left) and CtpsK-BSP mice (center). The number of TRAP-positive, multinuclear osteoclasts in the bone lesion area from CtpsK-BSP mice was twofold higher than that of wild type mice (right; GP, growth plate; T.B., trabecular bone; T, tumor; WT, wild type mice; TG, CtpsK-BSP transgenic mice; white arrow head, osteolytic bone lesions; black arrow head, TRAP positive osteoclast; $^*P < 0.05$ TG vs. WT).

results demonstrated that in CMV-BSP transgenic mice, constitutive BSP overexpression and a high serum BSP level enhanced multi-organ metastases of 4T1 breast cancer cells that have a preferable tendency of bone-seeking metastasis. We found that after intracardiac inoculation of 4T1 cells, all the 10 CMV-BSP mice developed metastases 1-week after injection while only four out of seven wild type mice showed metastatic lesions. In addition, five of ten CMV-BSP mice died only 12 days after tumor cell innoculation, while only two of seven wild type mice failed to survive. Increased serum BSP levels have shown to mediate endothelial cell attachment and migration, promoting angiogenesis allowing continuous tumor growth at the metastatic site (Bellahcene et al., 2000). Therefore, in the CMV-BSP mice, tumor migration was facilitated throughout the soft organs as well as the bone, causing extensive metastasis and rapid demise.

In breast cancer, bone metastases are predominately osteolytic and destructive. While the metastasizing cells invade the bone, osteoclasts in the area are stimulated to resorb bone, a pathologic process called neoplastic osteolysis. In addition to the previous findings that RANKL/RANK and the decoy receptor, osteoprotegerin (OPG), are the key regulators of bone remodeling (Lacey et al., 1998; Yasuda et al., 1998), we reported that BSP can act synergistically with RANKL/RANK signaling to induce osteoclastogenesis and bone resorption in RAW264.7 cells. Osteoclasts isolated from bone marrow after overexpression of BSP in CMV-BSP mice exhibited threefold increased resorptive activity on dentin and hydroxyapatite slices, compared to wild-type (Valverde et al., 2008). Furthermore, BSP was shown to decrease RANKL-induced apoptosis (Valverde et al., 2005). These previous findings of the involvement of BSP in osteoclastogenesis and the widely reported presence of BSP in human breast tumors, together with the CMV-BSP bone metastasis results, strongly indicated enormous importance of further investigating the major roles of BSP in osteoclastogenesis, osteoclast survival/apoptosis, and bone resorption during the process of bone metastasis.

We, therefore, developed another homozygous transgenic mouse line (CtpsK-BSP mice) that constitutively over-expressed mouse BSP cDNA driven by an osteoclast specific cathepsin K (CtpsK) promoter. CtpsK is a lysosomal cysteine protease which has a specialized role in osteoclast-mediated bone resorption. Mouse CtpsK mRNA was demonstrated to be selectively expressed in osteoclasts and a TRAP-CtpsK dual immunostaining technique also showed that CtpsK is initially expressed at the preosteoclast stage and lasts throughout the mature osteoclast stage (Li and Chen, 1999). The chimeric gene used for microinjection in this study consists of a CtpsK promoter and a 1 kb mouse BSP gene. Southern blot analysis and RT-PCR analysis confirmed that the transgenic mice derived from this construct showed targeted BSP over-expression in preosteoclasts and osteoclasts. This transgenic mouse line provided us a useful in vivo tool to quantitatively and functionally analyze the autocrine effects of osteoclast-expressed BSP in promoting osteoclastogenesis and bone resorption either in normal condition or in tumor metastatic condition. In vitro osteoclast formation assay using BMM collected from the CtpsK-BSP transgenic mice and wild-type mice showed that targeted overexpression of BSP in osteoclasts promoted in vitro osteoclastogenesis, which is indicated by more large $TRAP^+$ MNC with 10 or more nuclei in BMM cultures obtained from CtpsK-BSP mice than in those from wild-type mice 9 days after M-CSF and RANKL induction. Furthermore, expression levels of several osteoclastic differentiation markers such as Cathepsin K, TRAP and NFAT2 in BMM cultures derived from CtpsK-BSP mice were elevated compared with those from wild-type mice. MicroCT scan also demonstrated reduced BMD and trabecular bone volume in CtpsK-BSP transgenic mice, which indicated an increase in bone resorption. These results further confirmed our previous findings that BSP can act synergistically with RANKL/RANK signaling to induce osteoclastogenesis and bone resorption.

Consistent with our results achieved using CMV-BSP mice, left vetricle inoculation of 4T1 cells induced more and larger metastatic bone lesions in CtpsK-BSP transgenic mice when compared with that in wild-type mice, which indicated that targeted BSP overexpression in osteoclasts also enhanced bone metastasis of breast cancer cells. In addition to the previous findings that BSP may bind to breast cancer cells and promote their seeding into the bones, it has also been reported that osteoclasts bind to intact bone matrix via $\alpha_v\beta_3$, using osteopontin and BSP for attachment (Ross et al., 1993). Furthermore, activation of osteoclast $\alpha_v\beta_3$ by RGD-containing peptides induces the formation of a signaling complex that includes the calcium-dependent tyrosine kinase Pyk2, c-Src and c-Cbl (Sanjay et al., 2001). Together with these findings, our results suggested that osteoclast-derived BSP not only induces tumor cell seeding into the bones, but also promotes osteoclast attachment as well as osteoclast differentiation in metastatic osteolysis of breast cancer in an autocrine manner. In addition, although it is not appropriate to compare these two transgenic mouse lines directly due to the different genetic background, we could still make some conclusions based on the comparison of these two transgenic mouse lines with their corresponding wild type mice. In the CMV-BSP mice tumor migration was facilitated throughout the soft organs, causing more extensive metastasis and rapid demise, which is the result of BSP overexpression in all cell types and a high serum BSP level. In contrast, the tumor migration was limited to the bone in the CtpsK-BSP mice, but the osteolytic lesions were larger compared with those in the wild type mice, which is considered to be the result of restricted BSP overexpression in osteoclasts.

Bone metastasis is a complex process including tumor cells detaching from the original site, penetrating the vasculature (intravasation), seeding into the target tissues, and growing in the target tissues. To further investigate into the roles of targeted BSP overexpression in osteoclasts in the final steps of bone metastasis, we performed intratibial inoculation of breast cancer cells into the CtpsK-BSP mice. Through intratibial injection of luciferase-labeled 4T1 murine breast cancer cells, we found that CtpsK-BSP mice developed more and larger osteolytic bone lesions compared with wild-type mice as demonstrated by the real-time IVIS Imaging System, radiographic and histological analyses. TRAP staining further demonstrated that the number of TRAP-positive, multinuclear osteoclasts in the bone lesion area from CtpsK-BSP mice is increased when compared with that from wild-type mice, which indicated an increased osteoclastogenesis and osteolytic activities. In conclusion, our results served as further evidence to demonstrate that the complex interplay between tumor cells and host tissues may enable and nourish the establishment of a microenvironment that facilitates tumor progression. For the first time we reported here that osteoclast-derived BSP plays an important role in these osteolytic processes. Bone-derived BSP is involved in the pathophysiology of bone metastasis through at least two distinct mechanisms: to induce tumor cell seeding into the bones and to enhance tumor growth by increasing osteolytic activities in metastatic sites.

To summarize, our results provided further evidence that osteoclasts differentiate via the interaction with secreted matrix proteins and host tissue-derived BSP also plays an important role in breast cancer bone metastasis. We also demonstrated that in an autocrine manner, osteoclast-expressed BSP promotes osteoclast differentiation in metastatic osteolysis of breast cancer and consequently promotes bone metastasis of breast cancer. These studies provided the molecular basis of and treatment for bone destruction associated with osteolytic metastasis (Fig. 6).

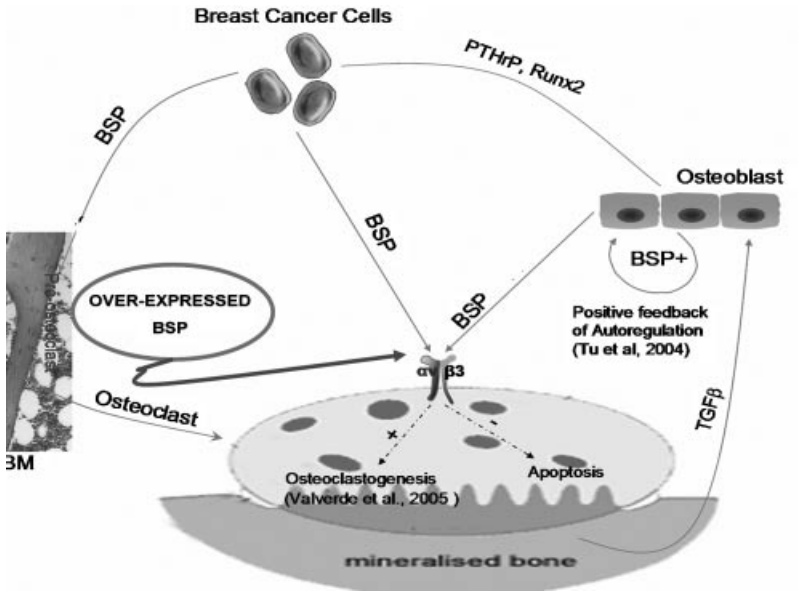

Fig. 6. The possible roles of bone sialoprotein (BSP) in the vicious cycle of osteolytic metastasis of breast cancer. Through BSP-mediated signal transduction pathway BSP increases resorption activity and survival of mature osteoclasts which leads to osteolysis. Destruction of bone will further lead to the release of BSP and other factors to worsen the vicious cycle.

Literature Cited

Barnes GL, Javed A, Waller SM, Kamal MH, Hebert KE, Hassan MQ, Bellahcene A, Van Wijnen AJ, Young MF, Lian JB, Stein GS, Gerstenfeld LC. 2003. Osteoblast-related transcription factors Runx2 (Cbfa1/AML3) and MSX2 mediate the expression of bone sialoprotein in human metastatic breast cancer cells. Cancer Res 63:2631–2637.

Barnes GL, Hebert KE, Kamal M, Javed A, Einhorn TA, Lian JB, Stein GS, Gerstenfeld LC. 2004. Fidelity of Runx2 activity in breast cancer cells is required for the generation of metastases-associated osteolytic disease. Cancer Res 64:4506–4513.

Bellahcene A, Merville MP, Castronovo V. 1994. Expression of bone sialoprotein, a bone matrix protein, in human breast cancer. Cancer Res 54:2823–2826.

Bellahcene A, Kroll M, Liebens F, Castronovo V. 1996a. Bone sialoprotein expression in primary human breast cancer is associated with bone metastases development. J Bone Miner Res 11:665–670.

Bellahcene A, Menard S, Bufalino R, Moreau L, Castronovo V. 1996b. Expression of bone sialoprotein in primary human breast cancer is associated with poor survival. Int J Cancer 69:350–353.

Bellahcene A, Bonjean K, Fohr B, Fedarko NS, Robey FA, Young MF, Fisher LW, Castronovo V. 2000. Bone sialoprotein mediates human endothelial cell attachment and migration and promotes angiogenesis. Circ Res 86:885–891.

Bianco P, Fisher LW, Young MF, Termine JD, Robey PG. 1991. Expression of bone sialoprotein (BSP) in developing human tissues. Calcif Tissue Int 49:421–426.

Byzova TV, Kim W, Midura RJ, Plow EF. 2000. Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. Exp Cell Res 254:299–308.

Chen J, Thomas HF, Jin H, Jiang H, Sodek J. 1996. Expression of rat bone sialoprotein promoter in transgenic mice. J Bone Miner Res 11:654–664.

Chen J, Rodriguez JA, Barnett B, Hashimoto N, Tang J, Yoneda T. 2003. Bone sialoprotein promotes tumor cell migration in both in vitro and in vivo models. Connect Tissue Res 44:279–284.

Diel IJ, Solomayer EF, Seibel MJ, Pfeilschifter J, Maisenbacher H, Gollan C, Pecherstorfer M, Conradi R, Kehr G, Boehm E, Armbruster FP, Bastert G. 1999. Serum bone sialoprotein in patients with primary breast cancer is a prognostic marker for subsequent bone metastasis. Clin Cancer Res 5:3914–3919.

Fisher LW, McBride OW, Termine JD, Young MF. 1990. Human bone sialoprotein. Deduced protein sequence and chromosomal localization. J Biol Chem 265:2347–2351.

Fisher LW, Jain A, Tayback M, Fedarko NS. 2004. Small integrin binding ligand N-linked glycoprotein gene family expression in different cancers. Clin Cancer Res 10:8501–8511.

Ganss B, Kim RH, Sodek J. 1999. Bone sialoprotein. Crit Rev Oral Biol Med 10:79–98.

Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. 2004. Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res 1 10:4559–4567.

Hiraga T, Hata K, Ikeda F, Kitagaki J, Fujimoto-Ouchi K, Tanaka Y, Yoneda T. 2005. Preferential inhibition of bone metastases by 5′-deoxy-5 fluorouridine and capecitabine in the 4T1/luc mouse breast cancer model. Oncol Rep 14:695–699.

Ida-Yonemochi H, Ishibashi O, Sakai H, Saku T. 2004. Recruitment of osteoclasts in the mandible of osteopetrotic (op/op) mice. Eur J Oral Sci 112:148–155.

Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 93:165–176.

Lane NE, Yao W, Nakamura MC, Humphrey MB, Kimmel D, Huang X, Sheppard D, Ross FP, Teitelbaum SL. 2005. Mice lacking the integrin beta5 subunit have accelerated osteoclast maturation and increased activity in the estrogen-deficient state. J Bone Miner Res 20:58–66.

Lees RL, Sabharwal VK, Heersche JN. 2001. Resorptive state and cell size influence intracellular pH regulation in rabbit osteoclasts cultured on collagen-hydroxyapatite films. Bone 28:187–194.

Li YP, Chen W. 1999. Characterization of mouse cathepsin K gene, the gene promoter, and the gene expression. J Bone Miner Res 14:487–499.

Li L, Zhu J, Tu Q, Yamauchi M, Sodek J, Karsenty G, Tang J, Chen J. 2005. An in vivo model to study osteogenic gene regulation: Targeting an avian retroviral receptor (TVA) to bone with the bone sialoprotein (BSP) promoter. J Bone Miner Res 20:1403–1413.

Mastro AM, Gay CV, Welch DR. 2003. The skeleton as a unique environment for breast cancer cells. Clin Exp Metastasis 20:275–284.

Mohla S, Weilbacher KN, Cher ML, Oyajobi BO, Poznak CV, Clohisy DR. 2003. Third North American Symposium on Skeletal Complications of Malignancy: Summary of the scientific sessions. Cancer 97:719–725.

Nam JS, Suchar AM, Kang MJ, Stuelten CH, Tang B, Michalowska AM, Fisher LW, Fedarko NS, Jain A, Pinkas J, Lonning S, Wakefield LM. 2006. Bone sialoprotein mediates the tumor cell-targeted prometastatic activity of transforming growth factor beta in a mouse model of breast cancer. Cancer Res 66:6327–6335.

Park BK, Zhang H, Zeng Q, Dai J, Keller ET, Giordano T, Gu K, Shah V, Pei L, Zarbo RJ, McCauley L, Shi S, Chen S, Wang CY. 2007. NF-kappaB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF. Nat Med 13:62–69.

Paz J, Wade K, Kiyoshima T, Sodek J, Tang J, Tu Q, Yamauchi M, Chen J. 2005. Tissue- and bone cell-specific expression of bone sialoprotein is directed by a 9.0 kb promoter in transgenic mice. Matrix Biol 24:341–352.

Raynal C, Delmas PD, Chenu C. 1996. Bone sialoprotein stimulates in vitro bone resorption. Endocrinology 137:2347–2354.

Razzouk S, Brunn JC, Qin C, Tye CE, Goldberg HA, Butler WT. 2002. Osteopontin posttranslational modifications, possibly phosphorylation, are required for in vitro bone resorption but not osteoclast adhesion. Bone 30:40–47.

Reddi AH, Roodman D, Freeman C, Mohla S. 2003. Mechanisms of tumor metastasis to the bone: Challenges and opportunities. J Bone Miner Res 18:190–194.

Ross FP, Chappel J, Alvarez JI, Sander D, Butler WT, Farach-Carson MC, Mintz KA, Robey PG, Teitelbaum SL, Cheresh DA. 1993. Interactions between the bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin alpha v beta 3 potentiate bone resorption. J Biol Chem 268:9901–9907.

Ruoslahti E. 1996. RGD and other recognition sequences for integrins. Ann Rev Cell Develop Biol 12:697–715.

Sanjay A, Houghton A, Neff L, DiDomenico E, Bardelay C, Antoine E, Levy J, Gailit J, Bowtell D, Horne WC, Baron R. 2001. Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrin-mediated signaling, cell adhesion, and osteoclast motility. J Cell Biol 152:181–195.

Shankar G, Gadek TR, Burdick DJ, Davison I, Mason WT, Horton MA. 1995. Structural determinants of calcium signaling by RGD peptides in rat osteoclasts: Integrin-dependent and -independent actions. Exp cell Res 219:364–371.

Sugatani T, Hruska KA. 2005. Akt1/Akt2 and mammalian target of rapamycin/Bim play critical roles in osteoclast differentiation and survival, respectively, whereas Akt is dispensable for cell survival in isolated osteoclast precursors. J Biol Chem 280:3583–3589.

Sung V, Stubbs JT, III, Fisher L, Aaron AD, Thompson EW. 1998. Bone sialoprotein supports breast cancer cell adhesion proliferation and migration through differential usage of the alpha(v)beta3 and alpha(v)beta5 integrins. J Cell Physiol 176:482–494.

Valverde P, Tu Q, Chen J. 2005. BSP and RANKL induce osteoclastogenesis and bone resorption synergistically. J Bone Miner Res 20:1669–1679.

Valverde P, Zhang J, Fix A, Zhu J, Ma L, Tu Q, Chen J. 2008. Overexpression of bone sialoprotein leads to an uncoupling of bone formation and bone resorption in mice. J Bone Miner Res [Epub ahead of print].

Waltregny D, Bellahcene A, de Leval X, Florkin B, Weidle U, Castronovo V. 2000. Increased expression of bone sialoprotein in bone metastases compared with visceral metastases in human breast and prostate cancers. J Bone Miner Res 15:834–843.

Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T. 1998. Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Nat Acad Sci 95:3597–3602.

Yoneda T. 1998. Cellular and molecular mechanisms of breast and prostate cancer metastasis to bone. Eur J Cancer 34:240–245.

Zhang JH, Tang J, Wang J, Ma W, Zheng W, Yoneda T, Chen J. 2003. Over-expression of bone sialoprotein enhances bone metastasis of human breast cancer cells in a mouse model. Int J Oncol 23:1043–1048.

Zhang JH, Wang J, Tang J, Barnett B, Dickson J, Hahsimoto N, Williams P, Ma W, Zheng W, Yoneda T, Pageau S, Chen J. 2004. Bone sialoprotein promotes bone metastasis of a non-bone-seeking clone of human breast cancer cells. Anticancer Res 24:1361–1368.

Zhao M, Harris SE, Horn D, Geng Z, Nishimura R, Mundy GR, Chen D. 2002. Bone morphogenetic protein receptor signaling is necessary for normal murine postnatal bone formation. J Cell Biol 157:1049–1060.