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Osteoprotegrin and the Bone Homing and Colonization Potential of Breast Cancer Cells

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Abstract Breast cancer cells preferentially metastasize to bone, leading to the formation of primarily osteolytic lesions. Osteoprotegerin (OPG) plays multifactorial roles in the development of osteolytic bone metastases. An increase in the ratio of receptor activator of nuclear factor κB ligand (RANKL) to OPG increases osteoclastogenesis within the bone microenvironment. OPG also acts as a survival factor for cancer cells by protecting them from tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis. This study compares OPG production in vitro in a number of breast cancer cell lines exhibiting both differences in metastatic capacity and in preferential metastasis to bone. Our studies demonstrated that OPG expression by MDA-231, MDA-MET, and MDA-231/K cancer cells was directly correlated with bone specific homing and colonization potential but not with metastasis of cancer cells to other organs; both in IL-1β stimulated and control cells. We also demonstrated expression of other bone-related markers including type I collagen, osteocalcin, osteopontin, and Runx2 in these cells. However, the generally lower expression of these markers in the bone selective cell line MDA-MET suggested that increased OPG expression in the bone specific variant was not merely a consequence of enhanced osteomimicry by these cells but that it has a significant role in the metastatic process. Co-culture of breast cancer cells with osteoblastic cells (hFOB 1.19) led to an overall downregulation in OPG production, which was not affected by the bone homing and colonization potential of the cell lines, suggesting that OPG alone is not indicative of osteolytic bone activity by breast cancer cells. J. Cell. Biochem. 103: 30–41, 2008. © 2007 Wiley-Liss, Inc.

Key words: BRMS1; osteoclastogenesis; OPG; RANK; RANKL; TRAIL

Breast cancer cells preferentially metastasize to bone where they commonly cause osteolytic metastases, a process that is at least partly dependent upon osteoclast-mediated bone resorption [Roodman, 2004]. Osteoclast activation occurs when receptor activator of nuclear factor κB ligand (RANKL), expressed by osteoblasts and stromal cells [Anderson et al., 1997; Wong et al., 1997; Yasuda et al., 1998] binds to its cognate signaling receptor activator of nuclear factor κB (RANK), which is expressed on the surface of osteoclast precursor cells and leads to their differentiation into active, fully mature osteoclasts capable of resorbing bone [Hsu et al., 1999; Li et al., 2000]. RANK–RANKL interactions are disrupted by osteoprotegerin (OPG), a decoy receptor secreted by

Abbreviations used: BRMS1, breast metastasis suppressor gene; °C, degree centigrade; FBS, fetal bovine serum; OPG, osteoprotegerin; PBS, phosphate buffered saline; RANK, receptor activator of nuclear factor κB; RANKL, receptor activator of nuclear factor κB ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

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osteoblasts, which acts to prevent excessive osteoclastogenesis [Simonet et al., 1997; Tsuda et al., 1997; Kostenuik and Shalhoub, 2001]. Under normal conditions, these molecules are tightly regulated so as to prevent excess osteoblast or osteoclast activities. Bone metastatic cancers alter this delicate balance, leading to increased osteoclastogenesis. The mechanism by which these cancer cells lead to a net increase in osteoclast activation at least partially involves alterations in osteoclastic and osteoblastic regulation of OPG/RANKL ratios [Thomas et al., 1999; Chikatsu et al., 2000; Guise, 2000].

In addition to their effects on osteoblast OPG and RANKL levels, some breast cancer cells also produce OPG in culture [Thomas et al., 1999]. Similarly, prostate cancer cells have been known to produce OPG in culture [Holen et al., 2002] and it has been demonstrated that OPG production by these cells protects them against tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis [Holen et al., 2002].

In this study, we examined the hypothesis that OPG production by breast cancer cells correlates with their bone homing and colonization potential to bone. Basal OPG levels in breast cancer cell lines with varying bone homing and colonization potential were examined to determine whether OPG production of a particular cell line correlated with its bone homing and colonization potential. A previous report found modest (or no) changes in the average gene expression of RANKL and OPG in different stage breast tumor tissues [Reinholz et al., 2002]. However, these studies were performed on primary tumors from different patients and therefore were genetically unrelated. In the study described here, OPG production was compared in genotypically related variants of the MDA-MB-435 (435) and MDA-MB-231 (231) cell lines to minimize background heterogeneity. OPG production in metastatic 435 and 231 cells was compared to expression in BRMS1 transfected, metastasis-suppressed counterparts. Basal OPG levels in a bone selective variant of the 231 cell line, MDA-MET [Bendre et al., 2002], were also quantified. OPG production by MDA-MET cells was compared to parental weakly metastatic 231/K cells [Bendre et al., 2002]. Since it has been demonstrated previously that breast cancer cells also inhibit OPG production in breast cancer cell-osteoblast/stromal cell co-cultures [Thomas et al., 1999; Chikatsu et al., 2000; Guise, 2000], we also examined the effect of the breast cancer cells used in this study on total OPG release in co-cultures of breast cancer cells and a fetal osteoblastic cell line, hFOB1.19 (hFOB), to determine if bone homing and colonization potential or site specific metastasis was related to differential inhibition of total OPG in co-cultures.

MATERIALS AND METHODS

Cell Lines, Cell Culture, and Reagents

Cell lines used in this study were as follows: the telomerase-immortalized, normal breast epithelial cell line HTERT-HME1 (HTERT) [Bodnar et al., 1998] was purchased from Clontech and was maintained in a specially formulated media (Clontech, Lexington, KY). Metastatic 435 and 231 cell lines obtained from ATCC and Dr. Janet Price at the University of Texas M.D. Anderson Cancer Center, respectively, were originally derived from the pleural effusion of a female patient with an infiltrating ductal carcinoma [Price et al., 1990]. These cell lines were maintained in DMEM/F-12 media (GIBCO, Life Technologies, Inc., Rockville, MD) supplemented with 5% FBS. 435/BRMS1 cells and 231/BRMS1 cells are 435 and 231 cells, respectively, expressing the metastasis suppressor gene BRMS1 [Seraj et al., 2000]. We examined two BRMS1 transfected clones each for 435/BRMS1 and the 231/BRMS1 cell lines. Clones 3 and 6 of the 435/BRMS1 cell line and clones 11 and 13 of the 231/BRMS1 cell line were used in our studies. 435/BRMS1 and 231/BRMS1 cell lines were maintained in 435/231 cell medium supplemented with geneticin (500 μg/ml). 231/K cells are 231 cells that were originally obtained from ATCC. The 231 cells from Dr. Jane Price and the 231/K cells from ATCC used in these studies have been passaged for extended periods of times in vitro and in different laboratories and therefore have diverged and very likely display instability for multiple phenotypes. Therefore, we gave them different designations to reflect this. 231/K cells are the cells from which the MDA-MET were derived by an in vivo selection process for bone metastasis. [Bendre et al., 2002] Thus, 231 and 231/BRMS1 are genetically more similar to one another than are MDA-MET and 231/K. Likewise, 231/K and MDA-MET are more similar to

one another than 231 and MDA-MET. The bone selective MDA-MET breast cancer cell line and the parental cell line, 231/K were maintained in DMEM media (GIBCO, Life Technologies, Inc., Rockville, MD) supplemented with 10% FBS.

MDA-MB-231 cells have been reported to be relatively less metastatic than MDA-435 cells. MDA-MB-435 cells preferentially metastasize to lymph nodes and lungs and to a lesser degree to brain, heart, adrenal glands, muscle [Price et al., 1990], and bone [Phadke et al., 2006]. MDA-MB-231, and the variant 231/k, metastasizes to adrenal gland, ovary, brain, and bone [Yoneda et al., 2001; Bendre et al., 2002] while 231-MET metastasize exclusively to bone [Bendre et al., 2002].

hFOB 1.19 are human fetal osteoblastic cells conditionally immortalized with a gene encoding for a temperature sensitive mutant to A58 of the SV40 large T antigen[Harris et al., 1995]. At permissive temperatures (37°C) the temperature sensitive gene is expressed and this cell line proliferates rapidly while at restrictive temperatures (39.5°C) the gene is not expressed and the cells proliferate less rapidly and express increased alkaline phosphatase activity and osteocalcin levels relative to cells cultured at the permissive temperature. hFOB 1.19 cells were maintained in DMEM/F-12 media supplemented with 10% FBS and 1% penicillin–streptomycin as previously described [Harris et al., 1995; Donahue et al., 2000]. hFOB cells were cultured at 37°C not in differentiation media for all experiments and therefore are considered premature osteoblastic cells. Metastasizing breast cancer cells would come in contact with bone lining cells. Bone lining cells are quiescent or premature osteoblasts and therefore immature hFOB 1.19 cells are particularly good model for bone cells with which breast cancer cells would come in contact. All cell lines were used after one subculture from routine culture in standard T-75 flasks. Table I depicts the metastatic capacity of these cell lines.

Monocultures

Cells were plated on Day 0 at a density of 10^5 cells/ml (1 ml/well) in 24-well plates in DMEM/F-12 media containing 2% FBS and incubated at 37°C in a 5% CO_2 incubator. Media was collected on Day 3 and stored at −20°C until assayed for OPG. To allow normalization of data, cells were lysed and total protein was quantified using the Bradford method of protein estimation [Bradford, 1976]. Data were expressed in pmol/mg protein. The proliferation rates of all breast cancer cell lines used in these studies have been published and are similar [Samant et al., 2000; Seraj et al., 2000; Bendre et al., 2002]. Results were expressed as mean ± SD of six assays with five samples per assay.

Treatment of Monocultures With IL-1β

Cells were plated on Day 0 at a density of 10^5 cells/ml (1 ml/well) in 24-well plates in DMEM/F-12 media containing 2% FBS and incubated at 37°C in a 5% CO_2 incubator. The medium was changed on Day 2 to medium containing 1% FBS and IL-1β was added at 20 ng/ml. Parallel controls were run without IL-1β. Media was collected on Day 4 and stored at −20°C until assayed for OPG. The cells were lysed and total protein determined as before. Data were expressed in pmol/mg protein. Results were expressed as mean ± SD of two assays with five samples per assay.

Collection of Breast Cancer Cell Conditioned Media

Breast cancer cells were plated at a density of 10^5 cells/ml (1 ml/well) in 24-well plates on Day

TABLE I. Properties of Breast Epithelial Cells Used in This Study

Cell line	Tumorigenic	Metastatic	Sites of metastasis	References
HTERT	−	−	Non-metastatic	[Bodnar et al., 1998]
435	+	+++	Multiple organs	[Price et al., 1990]
435/BRMS1	+	+	Multiple organs	[Price et al., 1990], [Seraj et al., 2000]
231	+	+++	Multiple organs	[Price et al., 1990]
231/BRMS1	+	+	Multiple organs	[Price et al., 1990], [Bendre et al., 2002; Seraj et al., 2000]
MDA-MET	+	+++	Bone	[Bendre et al., 2002]
231/K	+	+	Few organs	[Bendre et al., 2002]

−, Negative; +, Positive.
435/BRMS1 cells are derived from 435 cells, 231/BRMS1 cells are derived from 231 cells and MDA-MET cells are derived from 231/K cells.

0 in DMEM/F-12 media containing 2% FBS and incubated at 37°C in a CO_2 incubator. On Day 3, the conditioned media was collected, filtered through 0.22 μm filters to remove any cells and used at a 50% concentration for the co-culture studies. The final value was multiplied by a factor of two to account for this dilution.

Co-culture of Osteoblasts With Breast Cancer Cells or Breast Cancer Conditioned Media

hFOB cells were plated on Day 0 at a density of 10^5 cells/ml (1 ml/well) in 24-well plates in DMEM/F-12 media containing 5% FBS and incubated at 37°C in a 5% CO_2 incubator. On Day 3, at which time cell monolayers were confluent, the old media was aspirated off and either fresh media (DMEM/F-12 containing 2% FBS) or breast cancer cells in fresh media were added at a density of 10^5 cells/well in a total volume of 1 ml/well. In other studies, 3-day-old breast cancer cell conditioned media was added onto hFOB cells. Cells were incubated at 37°C for three more days. Media was collected on Day 6 and stored at −20°C until assayed for OPG. Media could be stored for 2 months at −80°C without any loss of activity. Breast cancer monocultures were plated in parallel as controls on Day 3 and their media harvested on Day 6 for the assay. In hFOB monoculture controls, cells were plated on Day 0, their media was changed on Day 3 and they were incubated until Day 6, when their media was harvested. Since the total number of cells in the co-culture system was twice that of monocultures, OPG values in co-cultures were compared to predicted OPG values, that is, total combined OPG of both monocultures (OPG value of 3-day-old breast cancer cell monoculture plated on Day 3 and collected on Day 6 plus OPG value of 6-day-old hFOB cell monoculture). Data were expressed in pmol/L rather than pmol/mg protein in co-culture experiments becuase we were unable to account for the protein contributed by the individual cell types in co-cultures. We believe it is more accurate and better reflective our results to compare OPG values in terms of pmol/L. Results were expressed as mean ± SD of six assays with five samples per assay.

OPG ELISA

The concentration of OPG present in media was determined using a sandwich ELISA kit (Cat # Bl-20402) from Alpco Diagnostics (Windham, NH 03087) as per manufacturer's instructions. Briefly, media was added to a 96-well plate pre-coated with a monoclonal anti-human OPG antibody. A biotinylated polyclonal goat-anti-human OPG antibody was used as the secondary antibody followed by detection with streptavidin-horseradish peroxidase and a 3′3′-5′5′-tetramethylbenzidine substrate. The reaction was stopped after 20 min by addition of 2 M sulfuric acid. Optical density was measured at 450 nm using an ELISA plate reader. An OPG standard curve was generated using 1.1–30 pmol/L recombinant human OPG. The concentration of OPG in the supernatant was determined by interpolation. The sensitivity of this assay was 0.14 pmol/L. All samples were assayed in duplicate.

Quantitative Real-Time RT-PCR Analysis for Osteoblastic Markers

Total RNA was isolated from 3-day-old confluent cells using the RNeasy kit from Qiagen according to the kit's instructions and was subjected to real-time RT-PCR using a Perkin-Elmer ABI Prism 7700 sequence detection system as described previously [Donahue et al., 2000]. The sequences for primers and probes used are described in Table II. We chose to examine type 1 collagen, osteopontin, osteocalcin, and Runx2 because their expression increases as osteoblastic cells differentiate in vitro and they are considered markers of

TABLE II. Sequences of the Primers and Probes Used for the Real-Time RT-PCR Reaction

Human genes	Forward primer	Reverse primer	Probe	Gene bank No.
Type I collagen	CGC ACG GCC AAG AGG A	ACG CAG GTG ATT GGT GGG	CAA GTC GAG GGC CAA GAC GAA GAC A	NM000088
Osteopontin	TTG CAG CCT TCT CAG CCA A	CAA AAG CAA ATC ACT GCA ATT CTC	CGC GGA CCA AGG AAA ACT CAC TAC CA	NM000582
Osteocalcin	AGT TGG CTG ACC ACA TCG G	ACC CTA GAC CGG GCC GT	TTT CAG GAG GCC TAT CGG CGC TTC	NM000711
Runx2	TGC TTC ATT CGC CTC ACA AA	TGC TGT CCT CCT GGA GAA AGT T	AAC CAC AGA ACC ACA AGT GCG GTG C	NM009820

differentiated osteoblastic cells [Aubin, 1998; Aubin and Triffitt, 2002].

These primers and probes were designed using sequence data from Gene Bank and real-time RT-PCR probe/primer design software Primer Express (version 1.0, Perkin-Elmer), which optimized the sequences for use in RT-PCR. These sequences were synthesized and PCR conditions were optimized with respect to concentrations of Mg^{2+}, probe and both primers to maximize the signal. For real-time RT-PCR analysis, 2.5 μl of RNA (20 ng/ml) was added to a reverse transcription reaction mix consisting of 0.5 μl RNAse inhibitor (40 U/μl), 2.0 μl 10× Taq-Man universal master mix buffer, 3.6 μl $MgCl_2$ (25 mM), 2.0 μl reverse primers for the genes (10 μM), 1.0 μl 18S reverse primer, 1.0 μl each of ATP, CTP, GTP, and UTP (10 mM), 0.44 μl murine leukemia virus RT (50 U/μl) and 5.5 μl DEPC treated deionized water. This mixture was placed in a thermocycler and the reaction carried out under the following parameters: 1 h at 42°C, 5 min at 72°C and 2 min at 25°C. Eight microliters of this mixture was then added to a PCR reaction mix consisting of 5 μl 10× Taq-Man universal master mix buffer, 4.0 μl $MgCl_2$ (25 mM), 2.0 μl each forward and reverse primers for the genes (10 μM), 1.0 μl forward and reverse 18S primers (0.25 μM), 1.0 μl 18S probe (1.0 μM), 1.0 μl each of ATP, CTP, GTP, and UTP (10 mM), 0.25 μl TAQ Gold (5 U/μl) and 18.35 μl deionized water and subjected to 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C in the Perkin-Elmer ABI Prism 7700. The fluorescent time history was recorded and the number of amplification steps required to reach an arbitrary intensity threshold (Ct) was counted. For example, for OPN, the ratio of OPN mRNA and 18S rRNA was calculated from the formula:

$$A_{OPN}/A_{18S} = 2Ct_{18S}/2Ct_{OPN} = 2(Ct_{18S} - Ct_{OPN})$$

where, A_{OPN} and A_{18S} are the initial concentrations of OPN mRNA and 18S rRNA, respectively, and Ct_{OPN} and Ct_{18S} are the number of cycles necessary to reach threshold intensity for OPN mRNA and 18S rRNA, respectively. Results were expressed as mean ± SD of three assays with triplicate runs per assay.

Statistical Analysis

A one-way ANOVA test was used to detect statistically significant differences between data sets. This was followed by the Student–Newman–Keuls test to calculate the level of significance between two different values. Statistical analysis was performed using the Graph Pad Instat program assuming a significance level of $P < 0.05$.

RESULTS

Breast Cancer Cell Lines Produce Different Amounts of OPG

ELISA analyses demonstrated that 'normal' HTERT breast epithelial cells do not produce detectable levels of OPG in culture (Fig. 1). With one notable exception, 435 cells, the breast cancer cell lines examined produced significantly more OPG than did HTERT cells. Compared to one another, the highly metastatic breast cancer cell lines used in these studies expressed dramatically different levels of OPG. Four hundred and thirty-five breast cancer cells did not secrete detectable levels of OPG, whereas the 231 breast cancer cell line produced significant amounts of OPG in culture. OPG production was compared next in genotypically related phenotypic variants of the 231 cell line to eliminate cell line related differences (Table I). No statistical differences in the levels of OPG released by metastasis-competent 231 cells and their metastasis-suppressed 231/BRMS1 variants (Fig. 1) were observed. However, the bone selective MDA-MET cells demonstrated a significant (1.5-fold) upregulation in OPG production as compared to parental, multiorgan metastasizing 231/K breast cancer cells (Fig. 1).

The Bone Resorbing Cytokine IL-1β Increases OPG Expression in Breast Cancer Cell Lines

IL-1β is a pleiotropic cytokine that is normally secreted by activated macrophages and blood monocytes [Treves et al., 1983; Schwamberger et al., 1991]. Since 231 cells do not produce IL-1β [Liu and Gudas, 2002] and IL-1β is a metastasis-inducing cytokine [Hoosein, 1998] that has effects on OPG in other cell systems [Penno et al., 2002], on growth of cancer cells [Hoosein, 1998] and on metastasis to bone marrow [Hoosein, 1998], we examined the effects of IL-1β on OPG production by these breast cancer cells and differences, if any, in OPG induction by IL-1β in metastasis capable and suppressed cells.

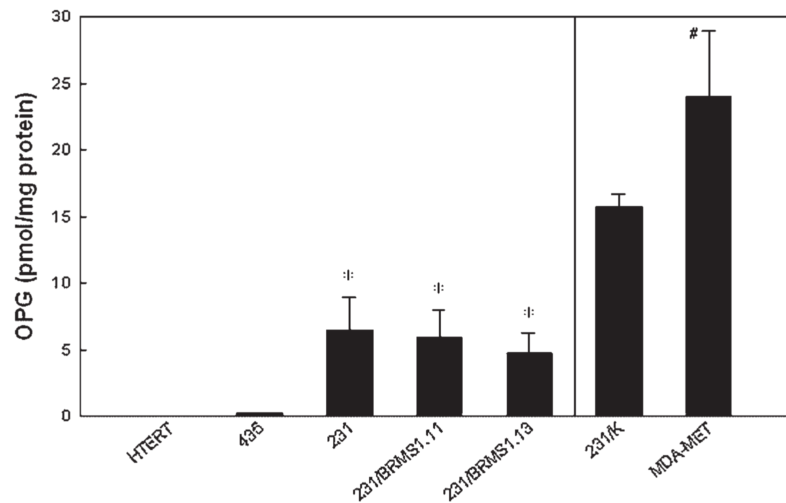

Fig. 1. OPG release by cells of differing metastatic potential as quantified by ELISA. Results are mean ± SD values of five experiments done in quadruplicate. *Significantly different from HTERT or 435 cells ($P < 0.001$), #significantly different from 231/K cells, $P < 0.001$.

ELISA demonstrated that treatment of breast cancer cells with 20 ng/ml IL-1β for 48 h, led to a significant increase in OPG production by all cell lines capable of producing measurable levels of OPG. This increase, however, reached the greatest level of significance in the bone selective, osteolytic MDA-MET cell line (Fig. 2).

Osteoblastic Mimicry by Both MDA-MET and 231 Cells

We quantified expression of osteoblastic markers in 'normal' mammary epithelial HTERT cells, bone selective, osteolytic MDA-MET cells, its parental cell line 231, and the 231 cell line's variant 231/K. Real-time RT-PCR analysis demonstrated that all these cell lines expressed type I collagen, osteocalcin, osteopontin, and Runx2 to a greater degree than did HTERT cells (Fig. 3). However, the expression of these bone-related markers, with the exception of osteopontin, did not show a direct correlation with bone-related homing and colonization potential. In fact, 231/K cells expressed greater mRNA levels of these bone-related markers, with the exception of osteopontin, than did MDA-MET cells (Fig. 3).

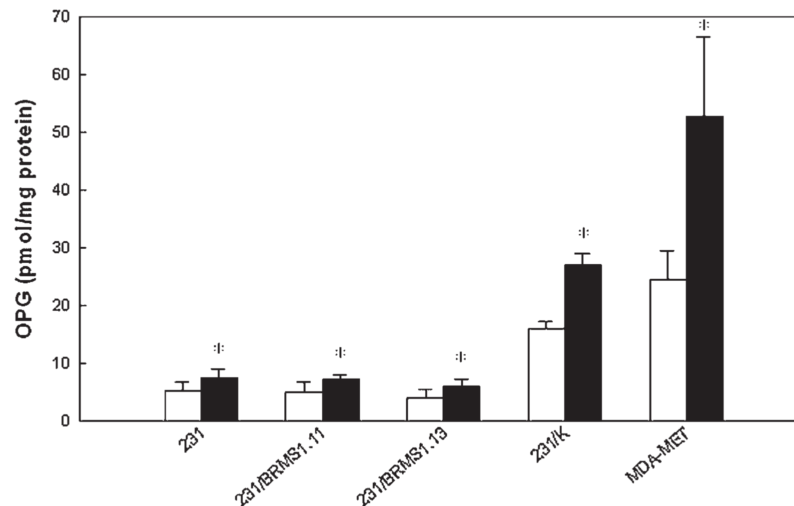

Fig. 2. Effect of IL-1β (20 ng/ml) on OPG release by breast cancer cells of differing metastatic potential as quantified by ELISA. □, Untreated cells; ■, IL-1β treated cells. Results are mean ± SD values of two experiments done in quintriplicate. *Significantly different from untreated controls, $P < 0.05$.

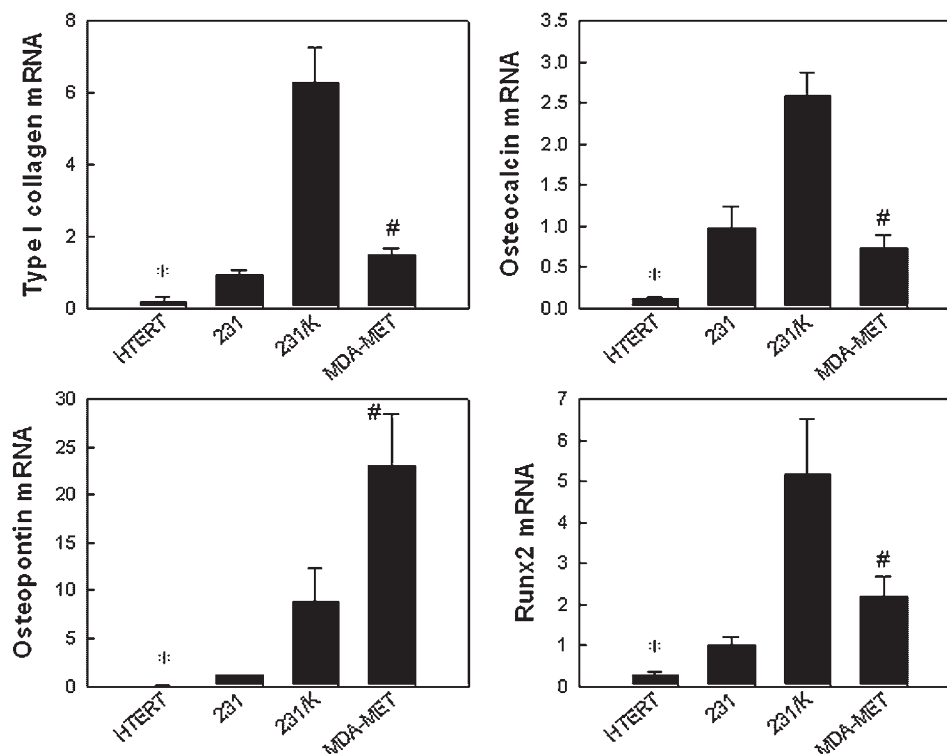

Fig. 3. Quantitative real-time RT-PCR analysis for steady state levels of type 1 collagen, osteocalcin, osteopontin, and Runx2 mRNA. Two hundred and thirty-one is the positive control and the ratio of type I collagen, osteocalcin, osteopontin, and Runx2 mRNA to 18S mRNA in 231 cells has been set to 1. Bars are means ± SE. Results are representative of three assays performed in triplicate. *Significantly different from mRNA levels in breast cancer cell lines, #significantly different from mRNA levels in 231/K, $P < 0.05$.

Breast Cancer Cells Inhibit Total OPG Production in Breast Cancer—hFOB Human Fetal Osteoblastic Cell Co-Cultures

Breast cancer-hFOB co-culture resulted, both in the case of the 435 cells and in the case of the 231 cells, in a net inhibition in OPG levels over predicted values (Fig. 4). We were unable to distinguish between hFOB secreted OPG and breast cancer cell secreted OPG in our co-culture system. This raised the question as to whether it was hFOB secreted OPG or breast cancer secreted OPG or both, which was inhibited in co-cultures. To address this issue, we examined the results of Figure 4 further.

Figure 4 depicts OPG in the 435-hFOB co-culture system. OPG in co-cultures could be secreted from hFOB cells, 435 cells or both, though the contribution of 435 OPG to the total secreted OPG would probably be quite low since 435 cells released barely detectable amounts of OPG in monoculture. OPG secreted into medium of co-cultures, most of which was probably contributed by hFOB cells, were about 50% less than the predicted OPG values. Since a 50% reduction, relative to predicted values, is much more than would occur from inhibition of release of OPG from 435 cells alone, OPG release from hFOB cells must have been inhibited. This suggests that hFOB cells co-cultured with 435 cells released less OPG than hFOB cells in monoculture. Similar results were observed with 435/BRMS1 cells. Total OPG in the 231-hFOB co-culture system was also decreased relative to predicted values. Similar results were observed with 231/BRMS1 cells.

Interestingly, neither MDA-MET, a 231 clone that selectively colonizes bone, nor its parental cell line 231/K, when in co-cultured with hFOB, had significantly different OPG levels than breast cancer cell monocultures (Fig. 5). However, as seen with other breast cancer cell-hFOB co-culture studies, the OPG value in these co-cultures was still significantly lower than the predicted OPG value (Fig. 5). Since MDA-MET and 231/K secrete levels of OPG similar to hFOB cells we could not determine whether the

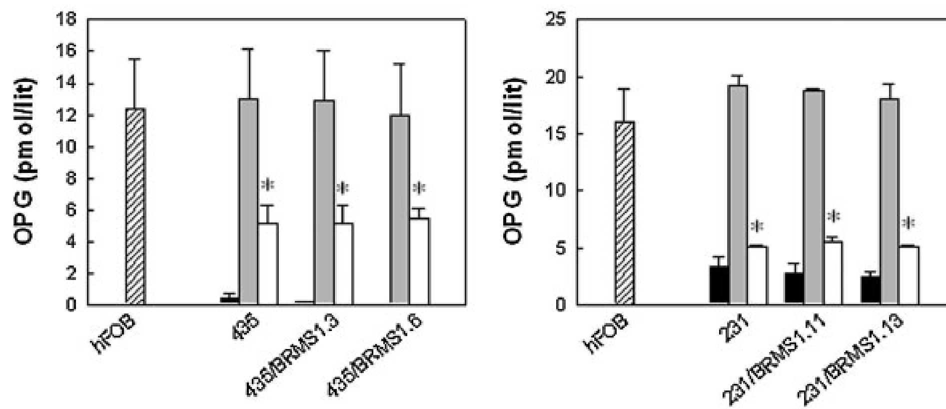

Fig. 4. Effect of co-culture with breast cancer cells on OPG release by osteoblastic hFOB cells. ▒, hFOB monocultures; ■, breast cancer cell monocultures; ▨, predicted values; □, hFOB-breast cancer cell co-cultures. Bars represent mean ± SD of five experiments done in quadruplicate. *Significantly different from respective predicted values or respective monocultures, $P < 0.01$.

decrease OPG in co-culture is the result of decreased release of OPG from hFOB or the breast cancer cell lines.

Conditioned media from 435 cells reduced co-culture OPG to levels similar to those induced by 435 cells themselves suggesting that the inhibition of total OPG production in co-cultures was mediated by a factor released into the media (data not shown).

DISCUSSION

Survival and proliferation at the secondary site are required steps in the metastatic cascade. Since OPG is a decoy receptor for TRAIL [Emery et al., 1998] and cancer cells are sensitive to TRAIL induced apoptosis [Kim et al., 2000], OPG production by breast cancer cells might correlate with cell survival and metastatic capability. Accordingly we quantified basal OPG production by breast cancer cells with different bone homing and colonization potential. OPG was not produced by 'normal' breast epithelial HTERT cells. Some breast cancer cells produced OPG in culture (231 cells) and some did not (435 cells), making it difficult to correlate this molecule with breast cancer metastasis. Therefore, OPG production was compared in genotypically related phenotypic variants of the 231 cell line of variable bone homing and colonization potential (i.e., 231 and 231/K) to minimize cell line related differences.

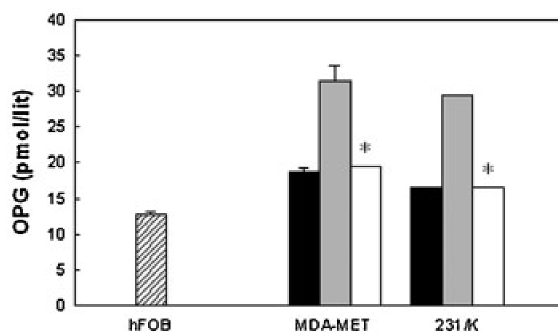

Fig. 5. Comparative effects of 231/K and its bone metastatic variant MDA-MET on OPG release by osteoblastic hFOB cells. ▒, hFOB monocultures; ■, breast cancer monocultures; ▨, predicted values, □, hFOB-breast cancer cell co-cultures. Results are mean ± SD values of two experiments done in quintriplicate. *Significantly different from respective predicted values or hFOB's ($P < 0.01$).

The 231 breast cancer cell line was used as a model system in the present study for three reasons. We had available genotypically related phenotypic variants of this cell type with variable bone homing and colonization potential, 231 cells produce OPG (Fig. 1) [Thomas et al., 1999] and, more importantly, previous studies have demonstrated that 231 cells are sensitive to TRAIL induced apoptosis [Keane et al., 1999]. Thus, these cells fulfill the criteria to study the correlation of OPG with bone homing and colonization potential. Since one of these cell lines preferentially colonizes bone and is osteolytic, this unique model system also affords us the ability to study the correlation of OPG with site-specific metastasis to bone and with osteolytic capability.

The lack of differences in OPG production by metastasis-competent 231 cells and their metastasis-suppressed 231/BRMS1 variants

suggested initially that there were no differences in OPG production between metastasis competent and suppressed cells. However, the highly significant (1.5-fold) ($P < 0.001$) upregulation of OPG production by the bone selective MDA-MET cell line [Bendre et al., 2002], compared to parental, multiorgan-metastasizing 231/K cells, suggests that OPG expression by breast cancer cells may correlate with bone homing and colonization but not overall metastatic potential. It is important to emphasize that the metastatic potential of MDA-MET and 231/K have been compared to one another (15) and the lung metastatic potential of 435, 435/BRMS1, 231 and 231/BRMS1 to one another. However, the metatstic potential of MDA-MET, 231 and 231/K have not been compared. Therefore, it is not appropriate to compare OPG levels across these groups but rather only within these groups. Within the 231 and 231/BRMS1 group the more multitissue metastatic 231 display a slight non-significant increase in OPG production. Within the MDA-MET and 231/k group the more bone metastatic MDA-MET produce significantly more OPG than the non-bone metastatic 231/K. Therefore, within a group of cancer cells strongly differing in their bone homing and colonization potential there was an increase in OPG. Since OPG is protective against TRAIL induced apoptotic effects in a number of cancer cells [Holen et al., 2002; Shipman and Croucher, 2003] this elevation of OPG production by bone selective MDA-MET cells may lend a survival advantage to them within the bone microenvironment.

Our studies demonstrated a modest but statistically significant increase in OPG production in all 231 and 231-BRMS1 cells treated with IL-1β. However, 231/K and MDA-MET displayed more significant increases in OPG following exposure to IL-1β. Importantly, IL-1β-stimulated OPG secretion was greater in MDA-MET cells suggesting a role for IL-1β-stimulated OPG in preferential metastasis of these breast cancer cells to bone.

Breast cancer cells are osteomimetic [Barnes et al., 2003] and partial mimicry of an osteoblastic phenotype is an important component of the preferential metastasis of cancer cells to skeletal sites [Cohen, 2001; Barnes et al., 2003]. Since OPG was elevated in bone selective MDA-MET cells in comparison to 231/K controls, we were interested in examining whether OPG increases were specifically correlated with bone metastatic potential or if elevation of OPG by the bone metastatic variant was merely a reflection of enhanced osteomimicry by these cells.

All of the 231 variants displayed increased mRNA levels of type 1 collagen, osteocalcin, osteopontin, and Runx2, relative to non-metastatic HTERT cells. This suggests that in general these breast cancer cells display osteomimicry. However, three of four of the bone markers were decreased in MDA-MET relative to 231/K and only one was increased. This suggests that the preferential metastasis of MDA-MET to bone, relative to 231/K, is not related to osteomimicry. However, our results do suggest a role of osteopontin expression in the process of bone metastasis, as has been suggested by previous studies [Weber, 2001; Hotte et al., 2002; Carlinfante et al., 2003].

OPG production was detected in hFOB monocultures, as has been demonstrated previously [Hofbauer et al., 1998]. Co-culture of 435 and 231 breast cancer cell lines with hFOB cells led to a significant inhibition of overall OPG levels. While we could not distinguish between breast cancer cell and hFOB cell OPG in our co-culture system, our studies demonstrated an inhibition of OPG release by hFOB cells in co-cultures. Park et al. [2003] previously demonstrated that co-culture of MDA-231 cells with osteoblastic MC3T3-E1 cells or ST2 bone stromal cells decreased OPG mRNA levels in the co-cultured cells. Presumably this reflected a decrease in OPG mRNA levels in the bone cell lines as minimal OPG mRNA was detected in the MDA-231 cells in monoculture. Our results are consistent with those of Park et al. and extend the findings to human cell lines and secreted OPG, which is more reflective of OPG activity. Our results are also consistent with those of Chikatsu et al. [2000] demonstrating that murine Balb/c-MC breast cancer cells decrease OPG secretion by murine bone marrow stromal cells.

The lack of differences in overall OPG inhibition by parental or BRMS1 transfected cells in co-culture suggests that metastatic potential of a breast cancer cell line is not related to overall OPG inhibition. Furthermore, the lack of differences in overall OPG inhibition by MDA-MET and 231/K cells in co-culture also suggests that site specific metastasis of a breast cancer cell line does not affect inhibition of overall OPG in co-cultures. Thus, breast cancer cells are able

to cause an inhibition in osteoblast or overall OPG production but this inhibition of OPG does not correlate with either the metastatic capability (as in the case of 435/BRMS1 and 231/BRMS1) or with the site-specific metastasis (as in the case of MDA-MET and 231/K) of the cells.

Our studies do not explain the mechanism by which osteoblast or breast cancer OPG is inhibited in co-cultures. Previous studies suggest the involvement of breast cancer derived PTHrP [Thomas et al., 1999; Chikatsu et al., 2000; Guise, 2000] and more recently the role of osteoblastic cell apoptosis induced by breast cancer cells both in vitro and in vivo [Mastro et al., 2004; Phadke et al., 2006] in inhibition of osteoblast OPG. It is likely that these and a multitude of other factors may contribute to the inhibition of OPG within the bone milieu leading to a net osteoclastogenic effect.

Interestingly, while there is no difference in the inhibition of overall OPG in breast cancer cell-osteoblastic cell co-cultures by either MDA-MET or 231/K cells, the MDA-MET cell line has been previously demonstrated to be more osteoclastogenic than 231/K cells [Bendre et al., 2002, 2003] despite the fact that MDA-MET cells produce higher levels of OPG in vitro. This finding initially seems confusing since OPG producing tumor cells normally form osteosclerotic rather than osteolytic lesions at the bone interface [Brown et al., 2001; Penno et al., 2002]. Thus, one would expect the MDA-MET cells, which produce greater amounts of OPG than 231/K cells in vitro, to be less osteoclastogenic than 231/K cells. However, OPG alone is not an absolute indicator of the osteolytic potential of a cell line. RANKL to OPG ratios are important predictors of osteoclastogenic activity [Fazzalari et al., 2001]. Our previous studies [Bendre et al., 2003] have shown that MDA-MET cells produce high levels of the alpha chemokine interleukin 8 (IL-8), which has the capacity to both directly (independent of RANKL) and indirectly (upregulation of RANKL on osteoblasts) stimulate osteoclast formation and bone resorption [Bendre et al., 2002, 2003]. This suggests that while OPG is elevated in the bone selective MDA-MET cells, the level of IL-8 as well as the RANKL to OPG ratio in the bone milieu is high enough to support the dramatic elevation of osteolysis indicative of MDA-MET cells [Bendre et al., 2003]. Moreover, these results suggest that differences in the osteoclastogenic potential of breast cancer cells may rely on differences in the elaboration of osteoclastogenic factors, such as IL-8, as well as osteoblast RANKL upregulation, rather than merely the down-regulation of absolute OPG expression.

In summary, our data suggest a correlation between OPG secretion, either basal or IL-1β induced, with preferential metastasis of MDA-MET breast cancer cells to bone. However, inhibition of OPG levels in human breast cancer-human osteoblast co-cultures is not correlated with metastatic potential or site-specific metastasis of breast cancer cell lines.

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REFERENCES

Anderson DM, Maraskovsky E, Billingsley WL, Dougall WC, Tometsko ME, Roux ER, Teepe MC, DuBose RF, Cosman D, Galibert L. 1997. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 390:175–179.

Aubin JE. 1998. Advances in the osteoblast lineage. Biochem Cell Biol 76:899–910.

Aubin JE, Triffitt JT. 2002. Mesenchymal stem cells and osteoblast differentiation. San Diego, CA: Academic Press.

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.

Bendre M, Gaddy D, Nicholas RW, Suva LJ. 2003. Breast cancer metastasis to bone: It is not all about PTHrP. Clin Orthop Relat Res 415(suppl):S39–S45.

Bendre MS, Gaddy-Kurten D, Mon-Foote T, Akel NS, Skinner RA, Nicholas RW, Suva LJ. 2002. Expression of interleukin 8 and not parathyroid hormone-related protein by human breast cancer cells correlates with bone metastasis in vivo. Cancer Res 62:5571–5579.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349–352.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

Brown JM, Corey E, Lee ZD, True LD, Yun TJ, Tondravi M, Vessella RL. 2001. Osteoprotegerin and rank ligand expression in prostate cancer. Urology 57:611–616.

Carlinfante G, Vassiliou D, Svensson O, Wendel M, Heinegard D, Andersson G. 2003. Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma. Clin Exp Metastasis 20:437–444.

Chikatsu N, Takeuchi Y, Tamura Y, Fukumoto S, Yano K, Tsuda E, Ogata E, Fujita T. 2000. Interactions between cancer and bone marrow cells induce osteoclast differentiation factor expression and osteoclast-like cell formation in vitro. Biochem Biophys Res Commun 267:632–637.

Cohen MM, Jr. 2001. RUNX genes, neoplasia, and cleidocranial dysplasia. Am J Med Genet 104:185–188.

Donahue HJ, Li Z, Zhou Z, Yellowley CE. 2000. Differentiation of human fetal osteoblastic cells is partially dependent on gap junctional intercellular communication. Am J Physiol (Cell) 278:C315–322.

Emery JG, McDonnell P, Burke MB, Deen KC, Lyn S, Silverman C, Dul E, Appelbaum ER, Eichman C, DiPrinzio R, Dodds RA, James IE, Rosenberg M, Lee JC, Young PR. 1998. Osteoprotegerin is a receptor for the cytotoxic ligand TRAIL. J Biol Chem 273:14363–14367.

Fazzalari NL, Kuliwaba JS, Atkins GJ, Forwood MR, Findlay DM. 2001. The ratio of messenger RNA levels of receptor activator of nuclear factor kappaB ligand to osteoprotegerin correlates with bone remodeling indices in normal human cancellous bone but not in osteoarthritis. J Bone Miner Res 16:1015–1027.

Guise TA. 2000. Molecular mechanisms of osteolytic bone metastases. Cancer 88:2892–2898.

Harris SA, Enger RJ, Riggs BL, Spelsberg TC. 1995. Development and characterization of a conditionally immortalized human fetal osteoblastic cell line. J Bone Miner Res 10:178–186.

Hofbauer LC, Dunstan CR, Spelsberg TC, Riggs BL, Khosla S. 1998. Osteoprotegerin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphogenetic protein-2, and cytokines. Biochem Biophys Res Commun 250:776–781.

Holen I, Croucher PI, Hamdy FC, Eaton CL. 2002. Osteoprotegerin (OPG) is a survival factor for human prostate cancer cells. Cancer Res 62:1619–1623.

Hoosein NM. 1998. Neuroendocrine and immune mediators in prostate cancer progression. Front Biosci 3:D1274–1279.

Hotte SJ, Winquist EW, Stitt L, Wilson SM, Chambers AF. 2002. Plasma osteopontin: Associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. Cancer 95:506–512.

Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L, Xia XZ, Elliott R, Chiu L, Black T, Scully S, Capparelli C, Morony S, Shimamoto G, Bass MB, Boyle WJ. 1999. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proc Natl Acad Sci USA 96:3540–3545.

Keane MM, Ettenberg SA, Nau MM, Russell EK, Lipkowitz S. 1999. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Res 59:734–741.

Kim K, Fisher MJ, Xu SQ, el-Deiry WS. 2000. Molecular determinants of response to TRAIL in killing of normal and cancer cells. Clin Cancer Res 6:335–346.

Kostenuik PJ, Shalhoub V. 2001. Osteoprotegerin: A physiological and pharmacological inhibitor of bone resorption. Curr Pharm Des 7:613–635.

Li J, Sarosi I, Yan XQ, Morony S, Capparelli C, Tan HL, McCabe S, Elliott R, Scully S, Van G, Kaufman S, Juan SC, Sun Y, Tarpley J, Martin L, Christensen K, McCabe J, Kostenuik P, Hsu H, Fletcher F, Dunstan CR, Lacey DL, Boyle WJ. 2000. RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism. Proc Natl Acad Sci USA 97:1566–1571.

Liu L, Gudas LJ. 2002. Retinoic acid induces expression of the interleukin-1beta gene in cultured normal human mammary epithelial cells and in human breast carcinoma lines. J Cell Physiol 193:244–252.

Mastro AM, Gay CV, Welch DR, Donahue HJ, Jewell J, Mercer R, DiGirolamo D, Chislock EM, Guttridge K. 2004. Breast cancer cells induce osteoblast apoptosis: A possible contributor to bone degradation. J Cell Biochem 91:265–276.

Park HR, Min SK, Cho HD, Kim DH, Shin HS, Park YE. 2003. Expression of osteoprotegerin and RANK ligand in breast cancer bone metastasis. J Korean Med Sci 18:541–546.

Penno H, Silfversward CJ, Frost A, Brandstrom H, Nilsson O, Ljunggren O. 2002. Osteoprotegerin secretion from prostate cancer is stimulated by cytokines, in vitro. Biochem Biophys Res Commun 293:451–455.

Phadke PA, Mercer RR, Harms JF, Jia Y, Frost AR, Jewell JL, Bussard KM, Nelson S, Moore C, Kappes JC, Gay CV, Mastro AM, Welch DR. 2006. Kinetics of metastatic breast cancer cell trafficking in bone. Clin Cancer Res 12:1431–1440.

Price JE, Polyzos A, Zhang RD, Daniels LM. 1990. Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res 50:717–721.

Reinholz MM, Iturria SJ, Ingle JN, Roche PC. 2002. Differential gene expression of TGF-beta family members and osteopontin in breast tumor tissue: Analysis by real-time quantitative PCR. Breast Cancer Res Treat 74:255–269.

Roodman GD. 2004. Mechanisms of bone metastasis. N Engl J Med 350:1655–1664.

Samant RS, Seraj MJ, Saunders MM, Sakamaki TS, Shevde LA, Harms JF, Leonard TO, Goldberg SF, Budgeon L, Meehan WJ, Winter CR, Christensen ND, Verderame MF, Donahue HJ, Welch DR. 2000. Analysis of mechanisms underlying BRMS1 suppression of metastasis. Clin Exp Metastasis 18:683–693.

Schwamberger G, Flesch I, Ferber E. 1991. Tumoricidal effector molecules of murine macrophages. Pathobiology 59:248–253.

Seraj MJ, Samant RS, Verderame MF, Welch DR. 2000. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res Adv Brief 60:2764–2769.

Shipman CM, Croucher PI. 2003. Osteoprotegerin is a soluble decoy receptor for tumor necrosis factor-related apoptosis-inducing ligand/Apo2 ligand and can function as a paracrine survival factor for human myeloma cells. Cancer Res 63:912–916.

Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan HL,

Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L, Hughes TM, Hill D, Pattison W, Campbell P, Boyle WJ. 1997. Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89:309–319.

Thomas RJ, Guise TA, Yin JJ, Elliott J, Horwood NJ, Martin TJ, Gillespie MT. 1999. Breast cancer cells interact with osteoblasts to support osteoclast formation. Endocrinology 140:4451–4458.

Treves AJ, Barak V, Tal T, Fuks Z. 1983. Constitutive secretion of interleukin 1 by human monocytes. Eur J Immunol 13:647–651.

Tsuda E, Goto M, Mochizuki S, Yano K, Kobayashi F, Morinaga T, Higashio K. 1997. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun 234: 137–142.

Weber GF. 2001. The metastasis gene osteopontin: A candidate target for cancer therapy. Biochim Biophys Acta 1552:61–85.

Wong BR, Rho J, Arron J, Robinson E, Orlinick J, Chao M, Kalachikov S, Cayani E, Bartlett FSIIIrd, Frankel WN, Lee SY, Choi Y. 1997. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 272:25190–25194.

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 Natl Acad Sci USA 95:3597–3602.

Yoneda T, Williams PJ, Hiraga T, Niewolna M, Nishimura R. 2001. A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. J Bone Miner Res 16:1486–1495.