Osteoactivin Promotes Breast Cancer Metastasis to Bone

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Abstract
The skeleton is a preferred site of metastasis in patients with disseminated breast cancer. We have used 4T1 mouse mammary carcinoma cells, which metastasize to bone from the mammary fat pads of immunocompetent mice, to identify novel genes involved in this process. In vivo selection of parental cells resulted in the isolation of independent, aggressively bone metastatic breast cancer populations with reduced metastasis to the lung. Gene expression profiling identified osteoactivin as a candidate that is highly and selectively expressed in aggressively bone metastatic breast cancer cells. These cells displayed enhanced migratory and invasive characteristics in vitro, the latter requiring sustained osteoactivin expression. Osteoactivin depletion in these cells, by small interfering RNA, also lead to a loss of matrix metalloproteinase-3 expression, whereas forced osteoactivin expression in parental 4T1 cells was sufficient to elevate matrix metalloproteinase-3 levels, suggesting that this matrix metalloproteinase may be an important mediator of osteoactivin function. Overexpression of osteoactivin in an independent, weakly bone metastatic breast cancer cell model significantly enhanced the formation of osteolytic bone metastases in vivo. Finally, high levels of osteoactivin expression in primary human breast cancers correlate with estrogen receptor-negative status and increasing tumor grade. Thus, we have identified osteoactivin as a protein that is expressed in aggressive human breast cancers and is capable of promoting breast cancer metastasis to bone. (Mol Cancer Res 2007; 5(10):1001–14)

Introduction
It is estimated that 65% to 75% of breast cancer patients with advanced disease develop skeletal metastases, making the bone a preferred site for metastatic dissemination of breast cancer (1). Numerous complications are associated with the development of osteolytic bone metastases, including pain, hypercalcemia, fracture, and spinal cord compression, resulting in a significant reduction in the patient’s quality of life (2). Although a better understanding of the processes controlling breast cancer metastasis to bone is emerging (3, 4), the identification of novel molecular mediators that can potentially be exploited as therapeutic targets for treating osteolytic bone metastases is needed.

Primary breast tumors are heterogeneous in nature, and cancer cells with vastly distinct metastatic capacities exist within a single tumor (5). Isolation of tumor cells from the metastatic site allows selection of subpopulations that are predisposed to metastasize to a particular secondary organ (6-8). This method has allowed the identification of genes that promote cancer metastasis to lung (7, 9), brain (10), and bone (6, 10). Indeed, breast cancer cells that preferentially metastasize to bone or lung express distinct and largely non-overlapping gene expression signatures (6, 7), providing insights into the mechanisms controlling organ-specific metastasis (8, 11, 12). Although these xenograft models have been very useful, they incompletely approximate the metastatic cascade, recapitulating only the final stages of metastasis— including dissemination of tumor cells through the circulation, extravasation into the secondary organ, and growth of the nascent lesion. In addition, human-derived cancer cells require the use of immunocompromised mice, which precludes study of cancer cell/immune cell interactions that facilitate cancer spread, including the formation of osteolytic bone metastases (13).

4T1 mouse mammary carcinoma cells possess the ability to form tumors that spontaneously metastasize from the mammary fat pad to distinct sites such as the bone, brain, liver, lung, and lymph node, making them an excellent model of aggressive stage IV breast cancer (14, 15). Importantly, the 4T1 model has proven useful in the identification and characterization of metastatic mediators, such as Twist, that are relevant to human breast cancer (16). We have used this cell model to enrich for breast cancer cells that aggressively metastasize to bone, by in vivo passage, using both spontaneous and experimental metastasis approaches. Using Agilent genomic profiling, we have identified osteoactivin, a cell surface glycoprotein, which is overexpressed in all in vivo selected bone metastatic populations.

Osteoactivin has previously been shown to be overexpressed in patients with glioblastoma multiforme, which correlated with poor outcome (17). Moreover, forced expression of osteoactivin
in transformed human astrocytes enhanced their motility and invasion in vitro and promoted local invasion after intracranial injection (18). However, the importance of osteoactivin in promoting breast cancer metastasis is unknown. We show that high levels of osteoactivin expression in the in vivo selected breast cancer cells are necessary for their enhanced invasiveness. Furthermore, forced osteoactivin overexpression in weakly bone metastatic cell lines is sufficient to increase their migratory and invasive characteristics in vitro and also significantly increases the formation of osteolytic bone metastases in vivo.

Results
In vivo Selection of Bone Metastatic 4T1 Breast Cancer Cells

To better approximate the entire metastatic cascade in an immunocompetent host, we have used the 4T1 murine mammary carcinoma cell line, which was isolated from a spontaneously arising mammary tumor in a BALB/c mouse (14). These cells form mammary tumors when injected into the mammary fat pads of BALB/c mice and spontaneously metastasize to the lung, liver, brain, and bone, matching the most common metastatic sites in breast cancer patients (14, 15, 19).

The parental 4T1 cell population was subjected to two rounds of in vivo selection after mammary fat pad (Fig. 1A, top) or left cardiac ventricle (Fig. 1A, bottom) injection. After resection of the primary tumor, 47% of mice injected with parental 4T1 cells developed osteolytic bone metastases, as determined by X-ray imaging (Fig. 1B, left). In contrast, after two rounds of in vivo selection, three cell populations were identified that produced osteolytic metastases in 71% (590 BM2), 68% (592 BM2), and 80% (593 BM2) of mice (Fig. 1B, left). In comparison, 55% of mice injected intracardially with parental 4T1 cells developed bone metastases, which increased to 75% after two rounds of selection (606 BM2; Fig. 1A, bottom; data not shown). Interestingly, injection of the cardiac-derived 606 BM2 cells into the mammary fat pad also increased the frequency of bone metastasis relative to parental cells (Fig. 1B, left). Notably, another mammary fat pad–derived population (511 BM2) was carried through two rounds of in vivo selection but did not display the more aggressive bone metastatic phenotype exhibited by the 590, 592, 593, or 606 BM2 populations (Fig. 1B, left). The higher percentage of mice developing bone metastases was accompanied by significantly increased numbers of osteolytic lesions in animals injected with the 590, 592, 593, and 606 BM2 cell populations. The 511 BM2 population behaved like parental 4T1 cells with respect to the severity of the bone metastatic phenotype (Fig. 1B, right, and C). The animals in each cohort were sacrificed within the same time frame postinjection (44-49 days), which precludes the possibility that the enhanced bone metastatic phenotype displayed by the in vivo selected populations resulted from their prolonged presence in the animal.

Bone Metastatic 4T1 Subpopulations Are Highly Motile and Invasive in vitro

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Bone Metastatic 4T1 Subpopulations Do Not Display More Aggressive Tumor Growth or Soft Tissue Metastasis In vivo

To ensure that the enhanced bone metastatic phenotype did not result from elevated growth rates of the in vivo selected populations, we examined their primary mammary tumor outgrowth after mammary fat pad injection. These analyses did not reveal any significant differences in the outgrowth between any of the parental or in vivo selected cell populations (Fig. 2A). Moreover, the bone metastatic populations displayed a decreased propensity for local spread to the axillary lymph nodes and comparable levels of metastasis to the liver relative to 4T1p or 511 BM2 cells (Fig. 2B). Although all cell populations are lung metastatic, we observed a substantial decrease in the percentage of mice with lung metastases in the cardiac-selected population (606 BM2; Fig. 2B). Moreover, the overall tumor burden was decreased in the lungs of the bone metastatic populations (590, 592, 593, or 606 BM2) compared with mice injected with 4T1p or 511 BM2 cells (Fig. 2C and D). This reduction in the burden of lung metastases reached significance in the 592 and 606 BM2 populations ($P < 0.0109$ and $P < 0.0053$, respectively; Fig. 2C). Thus, the enhanced bone metastatic ability displayed by the in vivo selected 4T1 cells does not reflect a general increase in their overall aggressiveness with respect to tumor outgrowth or soft tissue metastasis.

Gene Expression Profiling Reveals a Small Subset of Genes Common to Mammary Fat Pad and Cardiac-Selected Bone Metastatic 4T1 Cell Populations

To identify mediators that are responsible for the increased bone metastatic potential of the in vivo selected populations, we did gene expression profiling experiments using Agilent whole mouse microarrays. Two independent isolates of the parental 4T1 population (4T1_A and 4T1_B) and 511 BM2 cells represented the weakly bone metastatic 4T1 populations. The mammary fat pad–derived (590, 592, and 593 BM2) and cardiac-selected populations (44 BM1 and 606 BM2) represented the aggressively bone metastatic populations. When these populations were clustered using all 43,790 features on the Agilent microarrays, the parental 4T1 replicates segregated...
into a distinct subgroup separate from both the mammary fat pad– and cardiac-selected populations (Fig. 4A). Three two-way comparisons were done between distinct 4T1 populations and differentially expressed genes were characterized by a greater than 2-fold change and a Holm-adjusted $P < 0.05$ (Fig. 4B). Comparison of the mammary fat pad–selected bone metastatic cells (590, 592, 593 BM2) with parental cells (4T1_A and 4T1_B) revealed 180 differentially expressed genes (123 genes with elevated expression and 57 genes with reduced expression). To control for changes in gene expression associated with mammary tumor outgrowth, the bone metastatic cells originating from the mammary fat pad–derived population that did not display an aggressive bone metastatic phenotype (511 BM2) were compared with the mammary fat pad–derived population (Fig. 4C). Interestingly, only 12 genes were found in this intersection, with 8 displaying elevated expression and 4 expressed at lower levels in aggressive versus weakly bone metastatic 4T1 cells (Fig. 4C).

**Osteoactivin Overexpression Correlates with a Bone Metastatic Phenotype and Is Required for the Invasive Phenotype of Bone Metastatic 4T1 Cells**

One of the genes overexpressed in both the mammary fat pad– and cardiac-selected 4T1 populations that aggressively metastasize to bone is *osteoactivin* (*Gpnmb, DC-HIL, HGFIN*). This gene was of immediate interest to us because it was recently shown to promote the motility and invasion of glioma cells (18). To verify that *osteoactivin* message is indeed elevated in our in vivo selected bone metastatic 4T1 populations in the manner indicated by the Agilent expression data (Fig. 5A), we did Northern blot analysis on several non–bone metastatic (67NR and 66cl4), weakly (4T1p and 511 BM2), and aggressively (590, 592, 593, and 606 BM2) bone metastatic populations. These results confirmed that *osteoactivin* transcripts are significantly overexpressed in those populations possessing a strong bone metastatic phenotype (Fig. 5B). Osteoactivin protein levels are also elevated in all bone metastatic populations but not in weakly or non–bone metastatic cells (Fig. 5C). Finally, we did immunoblot analysis on explants derived from primary mammary tumors originating from parental 4T1 cells to assess whether selection for high levels of osteoactivin expression can occur at the primary site. A range of osteoactivin expression was observed in these 4T1 tumor cell explants when compared with the levels of osteoactivin observed in parental 4T1 cells. Of the six tumor explants examined, only one (149 BT) displayed osteoactivin levels that were similar to those observed in bone metastatic 592 BM2.
cells (Fig. 5D). In the remaining samples, one possessed a strong increase (148 BT), three displayed modest increases (151 BT, 152 BT, and 154 BT), and one revealed no discernible change in osteoactivin expression (156 BT) when compared with parental 4T1 cells (Fig. 5D). Thus, mammary tumor outgrowth of 4T1 cells is not sufficient to induce high levels of osteoactivin expression. Instead, elevated osteoactivin levels in a small fraction of these cells correlate with an increased propensity to form osteolytic bone lesions. This conclusion is further supported by the fact that 4T1 cells isolated from bone metastases that developed after cardiac injection, in which no primary tumors were formed, also display high levels of osteoactivin expression (Fig. 5C).

To determine if osteoactivin is required for the enhanced migratory and invasive phenotypes displayed by the in vivo selected bone metastatic 4T1 cells, we did transient knockdown experiments using small interfering RNA (siRNA) approaches. Osteoactivin protein levels were ablated 72 h posttransfection in osteoactivin siRNA–transfected 592 and 593 BM2 cells relative to control siRNA-transfected cells (Fig. 6A and data not shown). Whereas 592 and 593 BM2 cells transfected with control or osteoactivin siRNAs did not exhibit any changes in motility (Fig. 6B and C, top panels), transient knockdown of osteoactivin resulted in a clear and statistically significant reduction in invasion compared with control siRNA–transfected cells (Fig. 6B and C, bottom panels). Previous studies have suggested that osteoactivin expression is capable of inducing the expression of matrix metalloproteinases, including matrix metalloproteinase (MMP)-3 and MMP-9 (18). Using quantitative real-time PCR, we showed that MMP-3 transcripts were indeed 3-fold higher in the in vivo selected bone metastatic populations compared with the parental 4T1 cells (Fig. 6D). The elevated MMP-3 expression was not reflected in the Agilent microarray data given that the increased MMP-3

FIGURE 2. Bone metastatic 4T1 subpopulations do not display a generalized increase in primary tumor growth or metastasis to other organs. A. Primary mammary tumor outgrowth after mammary fat pad injection of the indicated populations. Sample sizes, 4T1p (n = 37), 511 BM2 (n = 19), 593 BM2 (n = 21), and 606 BM2 (n = 23). B. The percentage of mice with soft tissue metastases at the time of necropsy for the indicated cell populations. Sample sizes, 4T1p (n = 36), 511 BM2 (n = 10), 590 BM2 (n = 12), 592 BM2 (n = 13), 593 BM2 (n = 12), and 606 BM2 (n = 13). C. Lung wet weights were determined from cohorts of mice that were uninjected (Uninject.) or injected with the indicated populations into the mammary fat pad. The primary tumor was removed once it reached a volume of 125 to 150 mm³. Sample sizes, uninjected (n = 12), 4T1p (n = 31), 511 BM2 (n = 12), 590 BM2 (n = 10), 592 BM2 (n = 11), 593 BM2 (n = 10), 606 BM2 (n = 13). *, P < 0.0109; **, P < 0.0053. D. Representative images of lungs at necropsy illustrating surface lesions (top) and corresponding H&E sections (bottom). Bar, 200 μm.
levels were not greater than 2-fold in all of the aggressively bone metastatic populations, the criteria used for our comparisons (Fig. 4). However, we confirmed that MMP-3 protein levels are low in parental 4T1 cells, increased in control-transfected 592 BM2 in vivo selected bone metastatic cells, and clearly diminished in cells with a siRNA-induced loss of osteoactivin expression (Fig. 6A, middle). Thus, our results indicate that osteoactivin expression is necessary for sustained MMP-3 expression and enhanced invasiveness of in vivo selected, bone metastatic 4T1 cells.

**Overexpression of Osteoactivin Is Sufficient to Induce Enhanced Migration of Parental 4T1 Cells**

To determine whether osteoactivin is sufficient to confer enhanced migratory and invasive phenotypes to parental 4T1 cells, we established pooled populations and clonal cell lines expressing osteoactivin to levels observed in 592 BM2 cells (Fig. 7A, top). Exogenous osteoactivin expression is sufficient to increase MMP-3 expression to levels at or above those observed in the bone metastatic 592 BM2 population. Moreover, progressively higher osteoactivin levels in clonal 4T1 stable cell lines resulted in correspondingly elevated MMP-3 expression levels (Fig. 7A, middle). Together with the results obtained from the osteoactivin knockdown experiments (Fig. 6), we show that osteoactivin expression is both necessary and sufficient for MMP-3 expression in breast cancer cells.

Small but statistically significant increases in cell migration were observed in pooled cell populations, as well as three individual clones expressing osteoactivin, when compared with 4T1 empty vector control cells (Fig. 7B and C). The observation that loss of osteoactivin expression does not affect the baseline motility of in vivo selected 4T1 cells that are metastatic to bone (Fig. 6) but is sufficient to enhance motility in parental 4T1 cells suggests that redundant mechanisms capable of promoting cell migration have been selected for in the explanted populations that are absent in parental 4T1 cells. In contrast, osteoactivin expression alone was not sufficient to further promote invasion of 4T1 cells (Fig. 7B and C). The fact that osteoactivin expression is necessary (Fig. 6) but not sufficient to induce 4T1 breast cancer cell invasion argues that it must function in conjunction with other mediators present in the in vivo selected bone metastatic 4T1 populations, to exert these effects. Indeed, the invasiveness of breast cancer cells requires additional capabilities that extend beyond their migratory characteristics. Thus, these results clearly indicate

**FIGURE 3.** Bone metastatic 4T1 populations are more migratory and invasive compared with nonmetastatic or weakly metastatic breast cancer cells. Motility (A and B) and invasion (C and D) of weakly and in vivo selected bone metastatic 4T1 populations. For each cell line, four digital images per well (triplicate wells per experiment) were quantified using Image J software. The data for both motility and invasion assays represent results from at least three independent experiments for each cell population. B. Representative images for each cell population are shown for both motility (B) and invasion (D) assays. A. *, 592 BM2 versus 4T1p, P < 1.2527E-05; **, 606 BM2 versus 4T1p, P < 0.0048. C. *, 592 BM2 versus 4T1p, P < 0.0013; **, 606 BM2 versus 4T1p, P < 0.0070.
that the ability of osteoactivin to modulate cell motility and invasion is influenced by additional changes that have occurred during the in vivo selection process, a cellular context that is distinct from the parental 4T1 cells.

Osteoactivin Expression Promotes Breast Cancer Metastasis to Bone

To determine whether osteoactivin can promote the ability of breast cancer cells to metastasize to bone, we derived pooled stables overexpressing osteoactivin in the 66cl4 breast cancer cell line, along with empty vector controls (Fig. 8A). These cells were chosen because they do not express endogenous osteoactivin (Figs. 5B and C and 8A) and have not previously been shown to metastasize to bone (14, 19), providing a rigorous test for the ability of osteoactivin to promote bone metastasis. Interestingly, osteoactivin expression was sufficient to significantly induce both the motility (Fig. 8B, top) and invasion (Fig. 8B, bottom) of 66cl4 cells compared with empty vector controls. To determine if osteoactivin could promote bone metastasis in vivo, we injected both the vector control and osteoactivin-expressing 66cl4 pooled cell populations into the left cardiac ventricle of BALB/c mice. Examination of blinded X-rays revealed that 81% (n = 13) of mice injected with osteoactivin-expressing 66cl4 cells developed osteolytic bone metastases compared with only 27% (n = 15) of mice injected with vector control cells (Fig. 8B, bottom). Moreover, mice injected with osteoactivin-expressing 66cl4 cells developed, on average, 2.5 times more osteolytic lesions compared with animals injected with the vector control cells (Fig. 8C, bottom left). Breast cancer cells flushed from osteolytic lesions derived from osteoactivin-expressing 66cl4 cells displayed significantly increased levels of both osteoactivin and MMP-3 transcripts, as determined by quantitative real-time PCR (Fig. 8D). These results confirm that osteoactivin is capable of enhancing the bone metastatic ability of weakly bone metastatic breast cancer cells, which may be mediated, in part, through the up-regulation of MMP-3 expression.

To determine its potential relevance to human breast cancer, we examined osteoactivin expression in several publicly available gene expression data sets. Interrogating the recently published data set from Neve et al. (20), we found that osteoactivin is expressed in many human-derived breast cancer cells at levels much higher than observed in MCF10A cells, an immortalized but nontransformed human breast epithelial cell line (Fig. 9A). Osteoactivin was expressed in breast cancer cell lines that are characterized as belonging to both luminal and basal phenotypes. We selected a subset of these cell lines and confirmed that osteoactivin was indeed expressed using quantitative real-time PCR and that these results were in good agreement with the microarray data (Fig. 9B). Furthermore, osteoactivin was also found to be expressed at higher levels in primary breast tumor samples compared with normal breast tissue (ref. 21; Fig. 9C). Intriguingly, high osteoactivin expression is also frequently associated with estrogen receptor α–negative breast tumors (ref. 22; Fig. 9D), a statistically significant correlation that is present in several independent microarray data sets (23-26). Moreover, increased osteoactivin expression also correlates with increasing breast tumor grade (ref. 27; Fig. 9E), which is reinforced by independent microarray studies (28, 29).

Together, these data argue that osteoactivin, which we have identified through a metastasis screen in mice, represents a target of interest in the progression of human breast cancer.
Discussion

We have used the 4T1 breast cancer cell line, which is capable of spreading to multiple organs and tissues from the primary site, in a fully immunocompetent host (14, 15, 19), to identify genes associated with the bone metastatic phenotype. By using both mammary fat pad and cardiac selection protocols, we have identified a common set of 12 genes that were differentially expressed (eight overexpressed and four underexpressed) in the aggressive versus weakly bone metastatic 4T1 cells. We have shown that one of these candidates, osteoactivin, is selectively expressed in aggressively bone metastatic breast cancer cells, promotes breast cancer cell motility and invasion, and significantly enhances bone metastasis of breast cancer cells that normally do not form osteolytic lesions in this site.

Our in vivo selection strategy is the first to isolate aggressively bone metastatic breast cancer cell populations from the orthotopic site in an immunocompetent host. Two previous studies have compared 4T1 breast cancer cells to sister populations that were isolated from the same primary tumor but display weaker metastatic abilities relative to 4T1 cells (16, 19). Although these experiments were designed to identify genes that are associated with the overall metastatic behavior of 4T1 cells, we have identified a novel set of genes that are associated with a bone metastatic phenotype.

Bone metastatic MDA-MB-231 human breast cancer cells have previously been isolated using an in vivo selection protocol in athymic mice; however, these cells cannot metastasize to bone from the orthotopic site and require the use of an immunocompromised host (6). Therefore, it may not be surprising, considering our stringent in vivo selection criteria, that we have identified novel candidate genes not observed in the in vivo selected MDA-MB-231 breast cancer cells. This raises the possibility that the novel genes identified in our study may contribute to tumor/host interactions that govern breast cancer cell metastasis to bone.

A recently published gene expression data set has been generated by comparing primary breast tumors taken from patients with known relapse to bone with breast cancer patients lacking bone involvement (30). Although no overlap exists between our 12 genes and those identified in the human samples, it is interesting that only one gene was common between bone metastatic MDA-MB-231 human breast cancer cells and primary breast tumors obtained from patients with known bone metastases (30). Two important differences between studies using breast cancer cell lines versus primary breast cancers are likely to account for the lack of overlapping candidate genes. First, gene expression profiles identified using cell-based models were derived from breast cancer cells flushed directly from bone metastases compared with those generated from primary breast tumors, in which only a small fraction of the cells represents the bone metastatic population. Second, the use of cell models permits the profiling of pure breast cancer cell populations in the absence of contaminating host cell types, which is not the case with primary breast tumor material. Therefore, the novel candidates that we have identified warrant further investigation into their role in promoting breast cancer metastasis to bone.

The identification of osteoactivin as a gene whose expression is associated with a bone metastatic phenotype was of immediate interest to us. Osteoactivin was identified as a
gene normally expressed in differentiating osteoblasts and has subsequently been implicated in osteoblast function (31-33). Osteomimicry is a term used to describe the observation that certain types of cancer that preferentially metastasize to bone acquire the expression of genes normally associated with osteoblasts. These osteomimetic characteristics were first ascribed to prostate cancer (34), but this phenomenon has also been observed with breast cancer cells (35). Examples of osteoblast genes expressed by breast cancer cells, which have been functionally implicated in breast cancer metastasis to bone, include bone sialoprotein, osteopontin, and Cbfa1 (6, 36, 37). Thus, osteoactivin may confer osteomimetic properties to breast cancer cells, which promote their metastatic outgrowth in the bone microenvironment.

Osteoactivin may function to promote metastasis of cancer cells through various mechanisms. For instance, osteoactivin expression in mouse dendritic cells enhances endothelial adhesion and transendothelial migration, two important steps for tumor cell extravasation (38). More recent studies have shown that forced osteoactivin expression in transformed human astrocytes or rat hepatoma cells results in enhanced invasiveness, both in vitro and in vivo (18, 39). Moreover, osteoactivin expression has been linked to the up-regulation of MMPs, such as MMP-3 and MMP-9, in transformed astrocytes and fibroblasts (18, 40). Our data illustrate that osteoactivin expression contributes to the migratory and invasive properties of 4T1 breast cancer cell populations and is both necessary and sufficient for MMP-3 expression. Moreover, osteoactivin-expressing 66cl4 cells flushed directly from osteolytic bone lesions retain high levels of osteoactivin and MMP-3 transcripts. Interestingly, studies have linked increased MMP-3 expression in osteoblasts under conditions associated with enhanced bone resorption, such as cytokine stimulation (41), estrogen withdrawal (42), or mechanical loading (43). The ability of osteoactivin to induce MMP-3 expression in bone metastatic breast cancer cells may be particularly relevant with respect to their ability to metastasize to bone. Recently, purified MMP-3 has been shown to cleave and solubilize receptor activator of nuclear factor-κB ligand (44), a key mediator of osteoclastogenesis. Therefore, osteoactivin-mediated MMP-3 expression in bone metastatic breast cancer cells may contribute to receptor activator of nuclear factor-κB ligand–induced osteoclastogenesis.

**FIGURE 6.** Osteoactivin expression is required for the invasive phenotype of in vivo selected bone metastatic 4T1 breast cancer cells. A. Seventy-two hours posttransfection, immunoblot analyses were done on control and osteoactivin (OA) siRNA–transfected 592 and 593 BM2 cells, using antibodies against osteoactivin (top), MMP-3 (middle), and α-tubulin (bottom; data not shown for 593 BM2 cells). B. Motility (top) and invasion (bottom) assays were done as described in Fig. 3. Significant differences in invasion were observed between 592 and 593 BM2 cells treated with osteoactivin siRNAs compared with 592 and 593 BM2 cells treated with control siRNAs (*, P < 0.01; **, P < 0.03). Results are derived from at least three independent experiments. C. Representative images are shown for both motility (top) and invasion (bottom). D. Quantitative real-time PCR analysis was done to examine MMP-3 expression in the in vivo selected bone metastatic populations compared with parental 4T1 cells. MMP-3 expression was first normalized to GAPDH levels and expressed as the fold change over 4T1 parental cells.
differentiation and bone destruction. The importance of MMP-3 in mediating breast cancer metastasis to bone in vivo remains an open question. Finally, osteoactivin/DC-HIL has recently been characterized as a negative regulator of T-cell activation (45). Osteoactivin/DC-HIL expression by breast cancer cells may facilitate the suppression of antitumor immunity and facilitate metastasis. This may explain why osteoactivin was identified in our screen using immunocompetent BALB/c mice and not when similar experiments were done using human breast cancer–derived cell populations in athymic animals (6).

Although we have identified osteoactivin in a screen for molecules that promote breast cancer metastasis to bone, it is possible that it may play a more general role in metastasis to multiple organs. Indeed, high osteoactivin expression has been associated with malignant glioblastoma multiforme and poor patient prognosis (17). Although originally associated with weakly metastatic melanoma cells (46), a recent survey of metastatic melanoma cell lines and clinical specimens has revealed that osteoactivin is expressed in the majority of these samples and that an osteoactivin-specific antibody linked to a cytotoxic agent resulted in the regression of s.c. melanoma formation in xenograft models (47). Interestingly, a recent report examining mutations in breast and colon cancers identified osteoactivin (GPNMB) missense mutations specifically in breast tumor samples at a higher frequency than expected from the background mutation rate (48).

The importance of osteoactivin in human breast cancer is also supported by numerous gene expression microarray data sets generated from primary breast tumors. Osteoactivin is expressed in many breast cancer–derived cell lines at levels higher than observed in normal mammary epithelial cells (MCF10A; ref. 20) and is more highly expressed in breast tumor samples compared with normal breast epithelium (21). Interestingly, statistically significant correlations exist between high osteoactivin expression and estrogen receptor α–negative status (22-26), increasing grade (27-29), and p53 mutational status (27, 49). Thus, osteoactivin expression correlates with several features that are associated with an aggressive breast cancer phenotype. Together with these observations, our discovery that osteoactivin is selectively overexpressed in aggressively bone metastatic breast cancer cells and that osteoactivin expression is sufficient to confer a bone metastatic phenotype to weakly bone metastatic cells suggests an important role for this molecule in the progression to metastatic breast cancer.

Materials and Methods

Cell Culture and Transfections

The 4T1 murine mammary carcinoma cell line was obtained from the American Type Culture Collection. Nonmetastatic

![FIGURE 7.](image_url)
67NR and lung-metastatic 66cl4 murine mammary carcinoma cell lines were kindly provided by Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI). All cell lines were grown in DMEM supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 1.5 g/L sodium bicarbonate, penicillin/streptomycin, and fungizone. The pEF1/osteoactivin vector was constructed by ligating the full-length murine osteoactivin cDNA (Open Biosystems; clone ID: 4164706) into a pEF1/V5-His expression vector (Invitrogen) using 5¢ EcoRI and 3¢ NotI restriction enzyme sites. 4T1 and 66cl4 cell lines were engineered to express osteoactivin by LipofectAMINE 2000 (Invitrogen)–mediated transfection of the pEF1/osteactivin vector. Stable cell lines were maintained under 1 mg/mL G418 antibiotic selection.

Experimental and Spontaneous Metastasis Assays
Female BALB/c mice (4-6 weeks) were purchased from Charles River Laboratories. The mice were housed in facilities managed by the McGill University Animal Resources Centre, and all animal experiments were conducted under a McGill University–approved Animal Use Protocol in accordance with guidelines established by the Canadian Council on Animal Care. Experimental metastasis assays were performed by injecting the 4T1 mammary carcinoma cells (10⁵ cells) into the left cardiac ventricle of 4- to 5-week-old BALB/c mice as previously described (6). For the spontaneous metastasis studies, 4T1 mammary carcinoma cells were harvested from subconfluent plates, washed once with PBS, and resuspended (10⁶ cells) in 50 μL of a 50:50 solution of Matrigel (BD Biosciences) and PBS. This cell suspension was injected into the right abdominal mammary fat pad of BALB/c mice and measurements were taken beginning on day 7 postinjection for the time periods indicated. Tumor volumes were calculated using the following formula: \(V = \frac{1}{2}LW^2\), where \(L\) is the length and \(W\) is the width of the tumor. Tumors were surgically removed, using a cautery unit, once they reached a volume between 100 and 125 mm³.
Radiographic Analysis of Bone Metastases

Immediately before sacrifice, mice were anesthetized and digital X-rays were obtained with a Faxitron Specimen Radiography System (model MX-20 digital). At the termination of these experiments, all digital X-rays were blinded and scored by two independent researchers. Each X-ray was examined for the presence of osteolytic lesions in the following six sites: proximal humerus, distal femur, and proximal tibia (left and right side). Each X-ray was given a score between 0 and 6 depending on the number of affected sites. The number of mice possessing at least one osteolytic lesion was divided by the total number of animals in each cohort to determine the percentage of mice developing bone metastases. The number of metastatic lesions produced by the injection of each 4T1 in vivo selected population is the average of the six-point scoring system for all animals in the cohort. At the time of necropsy, lungs and hind limbs were removed and fixed in 4% paraformaldehyde. Fixed tissues were paraffin embedded, sectioned, stained with H&E, and examined by light microscopy. Routine histologic services were provided by the Centre for Bone and Periodontal Research (McGill University) histology platform.

RNA Amplification, Labeling, and Hybridization to Agilent Microarrays

4T1 parental and individual in vivo selected bone metastatic subpopulations were plated (10^6 cells) in 10-cm tissue culture dishes and RNA was extracted 48 h later using RNeasy Mini Kits and QIAshredder columns (Qiagen). One microgram of purified total RNA was subjected to T7-based amplification using the Amino Allyl MessageAmp II kit (Ambion), and the resulting aRNA was conjugated to Cy3 and Cy5 dyes (Amersham). RNA concentration and dye incorporation was measured using a UV-VIS spectrophotometer (Nanodrop ND-1000). RNA quality was assessed by electrophoresis through a 1% agarose gel (1:0 MOPS, 0.67% formaldehyde) followed by staining with ethidium bromide. The same labeling procedure was used for universal mouse reference RNA (Stratagene). Hybridization solutions were prepared with the In situ Hybridization Kit Plus (Agilent Technologies) and dye swaps (Cy3 and Cy5) were preformed for RNA extracted from each population. Labeled RNA was hybridized to 44K whole mouse genome microarray gene expression chips (Agilent Technologies) for 17 h at 60°C. Microarray chips were then

FIGURE 9. Osteoactivin is highly expressed in human breast cancer and correlates with an aggressive tumor phenotype. A. Osteoactivin expression in a series of human-derived breast cancer cell lines from a recently published microarray data set (20). The data are expressed as the fold change in osteoactivin relative to MCF10A cells, an immortalized but nontransformed human breast epithelial line. Only those breast cancer cells lines with a 5-fold or greater increase in osteoactivin expression are shown. B. Quantitative real-time PCR analysis done on selected human breast cancer cell lines confirmed osteoactivin expression. Analysis of publicly available gene expression data sets reveal that high levels of osteoactivin are found in primary breast tumors versus normal breast epithelium (C; P = 1.7 × 10^-5; ref. 21) and is significantly correlated with estrogen receptor (ERα)–negative tumors (D; P = 7.5 × 10^-5; ref. 22) and increasing grade (E; P = 0.001; ref. 27). In each box plot (D and E), the upper and lower limits of the box indicate the 75th and 25th percentile, respectively, whereas the lines (whiskers) emerging from above and below the box indicate the 90th and 10th percentiles. Black dots, maximum and minimum values within the data set. The sample sizes in each category are indicated in parentheses and statistical significance was calculated using a Student’s t test.

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washed, dried with gaseous N₂, and immediately scanned using a DNA Microarray Scanner (Model G2565BA, Agilent Technologies).

**Gene Expression Analysis**

Microarray data were feature extracted using Feature Extraction Software (v. 7.11) available from Agilent using the default variables. Outlier features on the arrays were flagged by the same software package. Data preprocessing and normalization was automated using the BIAS system (50). Raw feature intensities were background corrected using the RNA background correction algorithm (51, 52), and the resulting expression estimates were converted to log 2 ratios. Within-array normalization was done using spatial and intensity-dependent loess (53). Median absolute deviation scale normalization was used to normalize between arrays (54).

The hierarchical clustering was done using Ward’s minimum variance method with a correlation distance metric. The significance of the clusters was done using 1,000 permutations with the pvclust package in R (55). Heatmaps are generated by scaling each row (gene) by its mean and dividing by its SD. Above-average expression is in red, whereas below-average expression is in blue. The dendrograms are generated as defined for hierarchical clustering.

Differential expression was done using Linear Models for Microarray Analysis (refs. 56, 57; R Development Core Team, 2006). If a gene is represented by several probes, only the probe with the largest interquartile range is used. Probes that could not be mapped to any gene were ignored. A gene is considered differentially expressed if it displays fold change of ≥2 and a Holm-adjusted P value of 0.05 or below between the two categories (58).

**Northern Blotting**

RNA was extracted and purified as described above. Ten micrograms of purified RNA were separated on a 1% agarose gel and Northern blots were done as previously described (59) with the following modifications. The membranes were hybridized in Express Hyb (BD Biosciences) at 65°C with a 32P-labeled probe to osteoactivin (full-length mouse cDNA, Genbank accession no. NM_053110), stripped with boiling 0.5% SDS, and subsequently reprobed for GAPDH (full-length rat cDNA, Genbank accession no. X02231) as a loading control. Following exposure to X-ray films, the membranes were exposed to phosphorimagre screens, and signal intensity was quantified with a Storm imaging system (GE Healthcare) and ImageQuant software.

**Quantitative Real-time Reverse Transcription-PCR**

Total RNA was extracted from cell lines and purified as described above. One microgram of total RNA was converted to DNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Following the reverse transcription reaction, all samples were diluted 1:333.3 in double-distilled water and 1 µL (mouse cell lines) or 10 µL (human cell lines) were subjected to real-time PCR analysis with SYBR Green PCR Master Mix (Applied Biosystems). Primers were used at a concentration 200 fmol/µL in a total reaction volume of 25 µL. For mouse cell lines, the cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles each consisting of 95°C for 15 s and 58°C for 1 min. For human-derived cell lines, cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles each consisting of 95°C for 15 s and 60°C for 1 min. Incorporation of SYBR green dye into the PCR products was monitored with a 7500 real-time PCR system (Applied Biosystems). The integrity and specificity of the amplified PCR products were confirmed by dissociation curve analysis (SDS 2.0 software, Applied Biosystems) and by agarose gel electrophoresis. To normalize the degradation of total RNA used in cDNA synthesis, the threshold cycle (Ct) values were determined for target genes (mouse osteoactivin, mouse MMP-3, human osteoactivin) and corresponding housekeeping genes (mouse GAPDH, human β-actin) in each sample, and the target gene/housekeeping gene ratio was calculated from the following formula:

$$\frac{C_{\text{housekeeping gene}} - C_t \text{[target gene]}}{2}$$

Relative osteoactivin or MMP-3 mRNA levels were expressed in terms of fold induction rate over control cell lines (4T1p, 66cl4 vector control, or MCF10A). All measurements were done in triplicate and three independent experiments were done. Human primer sequences are as follows: osteoactivin (sense) 5'-CACCTCCTCAATTTGCTAC-3', osteoactivin (antisense) 5'-TAAAGAAGGGGTGTTTCTG-3', β-actin (sense) 5'-CC-AACCGGGAGAGTGACACC CGACATGT-3', β-actin (antisense) 5'-TTGAGGATCTATGAGGATCTGCA-3'. Mouse primer sequences are as follows: GAPDH (sense) 5'-CAAGTATGATGACATCAAGAAGGTGG-3', GAPDH (antisense) 5'-GGAAAAGTGTTGATTCTGTGTG-3', osteoactivin (sense) 5'-TTCCCTGGCAAAAGCCCAAT-3', osteoactivin (antisense) 5'-TTTGTACAGAAGATTGTAAC-CATG-3', MMP-3 (sense) 5'-CTTGTGAACCATTTGTTCTC-3', MMP-3 (antisense) 5'-AGCTATGCTTCTCAATATGCGG-3'.

**Immunoblotting**

Subconfluent cells were lysed for 20 min on ice in TNE lysis buffer. Protein concentrations were determined by Bradford assay (Bio-Rad) and 45 µg of total protein were subjected to immunoblot using the following antibodies: osteoactivin (1:10,000 dilution; R&D Systems), α-tubulin (1:10,000 dilution; Sigma), and MMP-3 (1:1,000 dilution; R&D Systems). The appropriate horseradish peroxidase–conjugated secondary antibodies (The Jackson Laboratory) were used at a dilution of 1:50,000, and membranes were visualized with Chemiluminescent HRP Substrate (Immobilon) on Bioflex scientific imaging film (Clonex Corp.).

**Motility and Invasion Assays**

Motility and invasion assays were done as previously described (60) with minor modifications. Cells (10⁶), resuspended

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in serum-free medium, were added to the top chamber of the transwell inserts (Falcon) and allowed to migrate through 8-μm pores toward complete medium over a 24 h period. For invasion assays, the transwell inserts were precoated with a 5% Matrigel solution. At the termination of each experiment, cells were fixed in formalin and stained with crystal violet (Sigma). Five images were taken for each insert and the cells were quantified using Scion Image software (Scion Corporation). Data for each insert are represented as the average pixel count from the five images. All experiments were done a minimum of three.

siRNA-Mediated Depletion of Osteoactivin

Transient knockdown of osteoactivin in 592 and 593 BM2 cells was accomplished by two sequential transfections (LipofectAMINE 2000) using 1 nmol/L of the following dicer substrate RNA interference duplex: 5′-GGCUAGGUG-UUGUUAACUAGC-3′ and 5′-GCUAUUACAACCACUCUCCUAAC-CCAC-3′ (Integrated DNA Technologies, Inc.) at t = 0 h and t = 24 h. An HPRT RNA interference duplex (5′-GCGAGACUUGGUUAAUUGATT-3′ and 5′-UUCCGGGUGAAACCAUCAACCUU-3′; 592 BM2 cells) or a scrambled RNA interference duplex (5′-CUUCCUCU-CUUUCUCUCCCCUGA-3′ and 5′-AGGAAGGAGAGAACGAAAC-3′; 593 BM2 cells) were used as a controls. Protein lysates were taken at the beginning (t = 48 h) and end (t = 72 h) of the migration/invasion assays to confirm efficient osteoactivin knockdown over the duration of the experiment.

Statistical Analysis

Statistical significance (P values) for bone metastasis severity and lung wet weight were assessed with Mann-Whitney rank sum test. Statistical significance values for motility and invasion assays were obtained by using a two-tailed, heteroscedastic Student’s t test.

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April A.N. Rose, François Pepin, Caterina Russo, et al.


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