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To determine if some malignant cells serve an accessory function and promote osteoclast differentiation, we cultured T47D human breast cancer cells with murine osteoclast precursors. We find bone resorbing osteoclasts develop with 7-10 days and do so in a cancer cell-dependent manner. Establishing osteoclast recruitment is not a universal property of all breast cancers. We have isolated T47D subclones incapable of osteoclastogenesis. Thus, formation of osteoclasts in the presence of breast cancer cells requires contact between the two cell types.

We also find that MDA-MD-231 human breast cancer cells selected for preferential adherence to bone marrow stroma, when injected intracardiac into nude mice, produce 6 times as many osteolytic metastases as their stroma non-adherent counterparts. Despite their preferential metastases to skeleton, bone resorbing tumor cells derived from the stroma-adherent in comparison to the non-adherent parental cells express lower levels of $\alpha_v\beta_3$ integrin. While lower $\alpha_v\beta_3$ expressing MDA-231 cells preferentially home to bone, the high integrin containing cells selectively metastasize to lungs. Thus, $\alpha_v\beta_3$ expression represents a marker of site-selective metastasis by a human breast cancer cell line.

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INTRODUCTION

Breast cancer often metastasizes to the skeleton causing osteolysis, a product not of the breast cancer cells, per se, but of the osteoclasts (OCs) accompanying the bone residing tumor. The proximity of OCs and metastatic breast cancer in osteolytic lesions suggests the malignant cells serve an accessory function and promote OC differentiation. Furthermore, the predominance of skeletal metastasis in breast cancer patients often portends a prognosis which differs from those with visceral tumors, suggesting that molecules on the surface of biologically distinct forms of breast cancer dictate their capacity to home to the skeleton. Thus, our goals were to determine:

1. IF HUMAN BREAST CANCER CELLS PROMOTE OC DIFFERENTIATION.

2. IF MOLECULES ON THE SURFACE OF BONE AS COMPARED TO VISCERA SEEKING BREAST CANCER CELLS DICTATE ORGAN SPECIFICITY.

STUDIES AND RESULTS

1. HUMAN BREAST CANCER CELLS PROMOTE OC DIFFERENTIATION

Breast cancer-induced osteolysis may be viewed as two sequential steps, namely, selective marrow-targeting of primary tumor cells and subsequent recruitment of OCs by the marrow-residing malignant cells. The evidence that OCs, the primary resorptive cells of bone, are pivotal to osteolysis induced by breast cancer metastases include the facts that: 1) In contrast to their ability to degrade soft tissues, cancer cells appear to have limited, if any, capacity to resorb bone (1). This may reflect the cancer cells' inability to bind to mineralized bone, a prerequisite to resorption (2). 2) OCs are abundant in the foci of metastatic breast cancer (3,4). 3) Breast cancer-induced osteolysis is diminished by bisphosphonates which inhibit osteoclastic activity (4,5). 4) Breast cancer cells are known to secrete potential osteoclastogenic agents such as eicosanoids (6,7), CSF-1 (7), and parathyroid hormone related protein (8-10). However, the capacity of breast cancer cells to directly promote OC differentiation has not been established.

OCs belong to the monocyte/macrophage family, and in mammals, differentiation of myeloid precursors into OCs requires accessory cells which include stromal cells or osteoblasts (11-14). We herein show that some, but not all, breast cancer cells may assume the role of accessory cells in osteoclastogenesis, in vitro. Similar to normal OC differentiation promoted by marrow stromal cells (14), breast cancer cell-induced osteoclastogenesis is dependent on the presence of 1,25-dihydroxyvitamin D_3 ($D_{3)}$ and contact between cancer cells and OC precursors. Breast cancer cell induced polykaryons exhibit the OC phenotype in that they express tartrate resistant acid phosphatase (TRAP), and are capable of resorbing bone.

Human breast cancer cells promote differentiation of OCs from marrow precursors. OC differentiation requires the interaction of myeloid precursors with accessory cells which include osteoblasts or marrow stromal cells (7,14,15). Because OCs are abundant in foci of metastatic breast cancer (3,4), we sought to determine if the cancer cells could serve as accessory cells and promote differentiation of OC precursors into mature, bone resorbing polykaryons. To test this hypothesis we used a modification of an established in vitro OC differentiation assay, in which non-adherent murine bone marrow macrophages (BMMs) are cocultured with the ST2 murine marrow stromal line in the presence of dexamethasone and D_3 (13,14). In the BMM/ST2 co-culture system, OCs normally appear as TRAP-positive multinucleated cells after 7-10 days. To determine if human

breast cancer cells promote osteoclastogenesis, we substituted the T47D human breast cancer cells for the ST2 cells in our co-culture system. When non-adherent murine bone marrow progenitors were cultured with T47D cells multinucleated OCs developed within 8-10 days (Fig. 1 A & B).

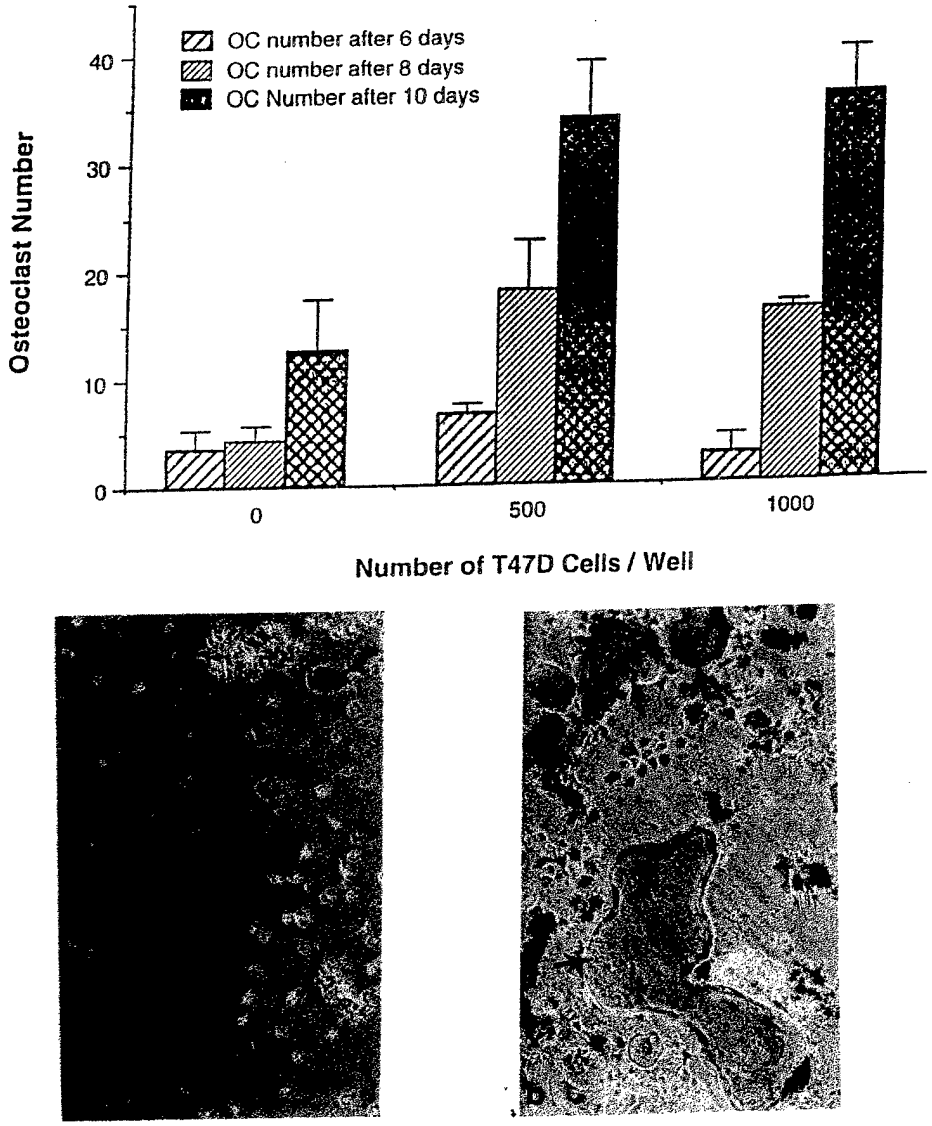

Fig. 1 The T47D human breast cancer cells promote OC differentiation in vitro. A) The indicated number of T47D cells were co-cultured with 10^4 OC progenitors, after 6, 8, or 10 days of culture the cells were fixed, stained for TRAP activity to identify OCs and the number of TRAP-positive cells counted. Significant number of OCs develop after day six. The results shown are average of triplicates and the error bars represent SEM. B) A representative micrograph of TRAP positive cells (arrows) induced in the absence (left) and presence of T47D breast cancer cells.

Increasing the number of T47D cells from 500 to 1000 induced a parallel enhancement of OC number. Based on this observation, we counted OCs after 10 days with a wider range of input T47D breast cancer cells. As seen in Fig. 2 the number of OCs generated parallels the abundance

of cancer cells until the number of tumor cells surpasses 2000 per well.

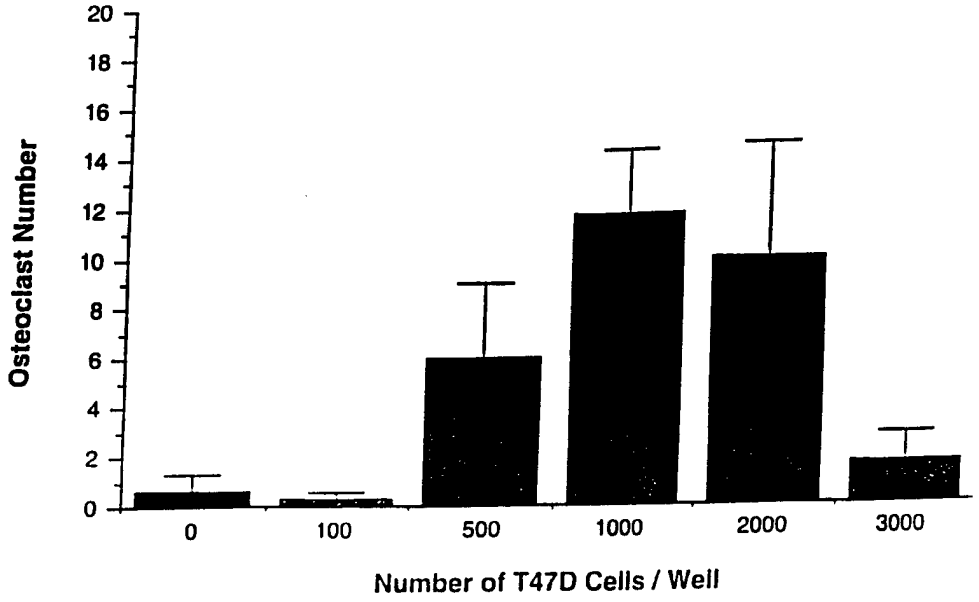

Fig. 2 OC differentiation promoted by human breast cancer cells is dependent on the initial number of tumor cells. Varying number of T47D cells, as indicated, were co-cultured with 10^4 OC progenitor for 10 days. The cells were fixed, stained for TRAP activity, and the number of TRAP-positive polykaryons counted. The results are the average of triplicates and the bars represent SEM.

Osteoclastogenesis declines with greater than 2000 cancer cells possibly due to competition for anchorage to the dish and/or nutrients between the tumor cells and the OCs. To assess the function of OCs generated from co-culture with human breast cancer cells, the experiments were repeated by plating the cells on bone slices which after 10 days were sonicated to remove the cells. The resorption lacunae were visualized by staining with toluidine blue (16). A representative bone slice is shown in Fig. 3, demonstrating that the cancer cell-induced polykaryons are, in fact, capable of bone resorption, and thus authentic OCs.

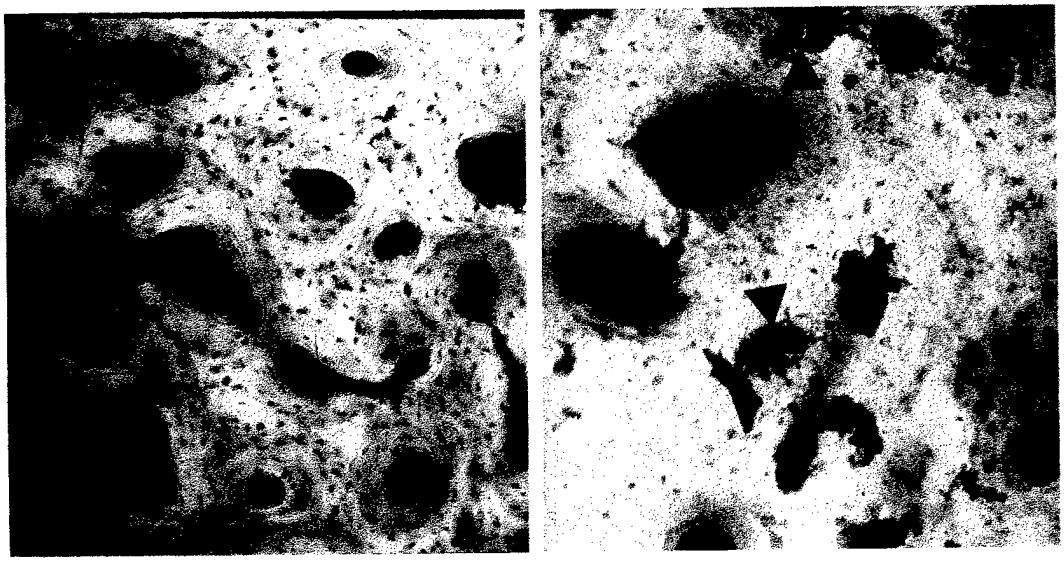

Fig. 3 OCs induced by breast cancer cells resorb bone. Bone marrow cells were cultured alone (left panel) or with T47D cells (right panel). After 10 days the bone was sonicated and stained with toluidine blue to demonstrate the resorption lacunae. These lacunae (arrows) are present only when the bone marrow cells are co-cultured with the T47D cells. The large black areas are Haversian canals and the small, concentric dots, are osteolytic lacunae (arrows).

Isolation of T47D breast cancer subclones which fail to induce OC differentiation. To determine if osteoclastogenesis is a universal property of human breast cancer cells, we assessed the capacity of T47D subclones, generated by limiting dilution, to promote differentiation of BMMs into OCs. While the majority of the T47D clonal lines tested promoted OC differentiation, three clonal lines lacked this property (Fig. 4).

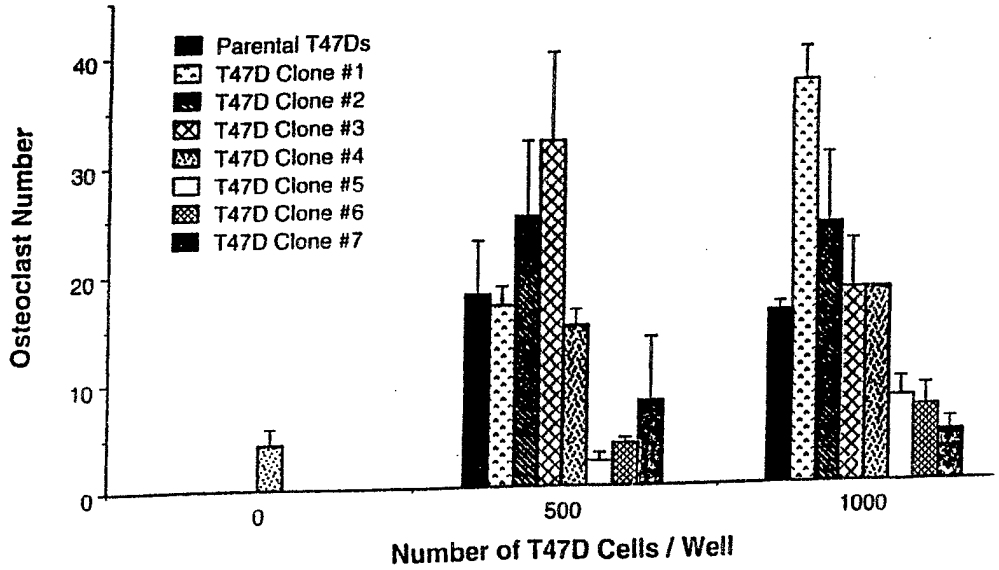

Fig. 4 Not all breast cancer cells support OC differentiation. Clonal lines of T47D cells were derived by limiting dilution. The osteoclastogenic potential of seven subclones is illustrated. While T47D clones 1-4 are as osteoclastogenic as the parental cells, clones 5-7 do not promote OC differentiation above background

OC differentiation is dependent on the presence of 1,25-dihydroxyvitamin D_3 (D_3). OC differentiation in vitro by coculture of BMMs and ST2 cells requires D_3 (13-15). To determine whether breast cancer induced OC differentiation is also dependent on the steroid, bone marrow precursors were co-cultured with T47D breast cancer cells with or without the steroid hormone. After 8 or 10 days the cells were fixed and stained for the OC marker, TRAP. As indicated in Fig. 5, OCs developed in the presence of D_3, but not in its absence.

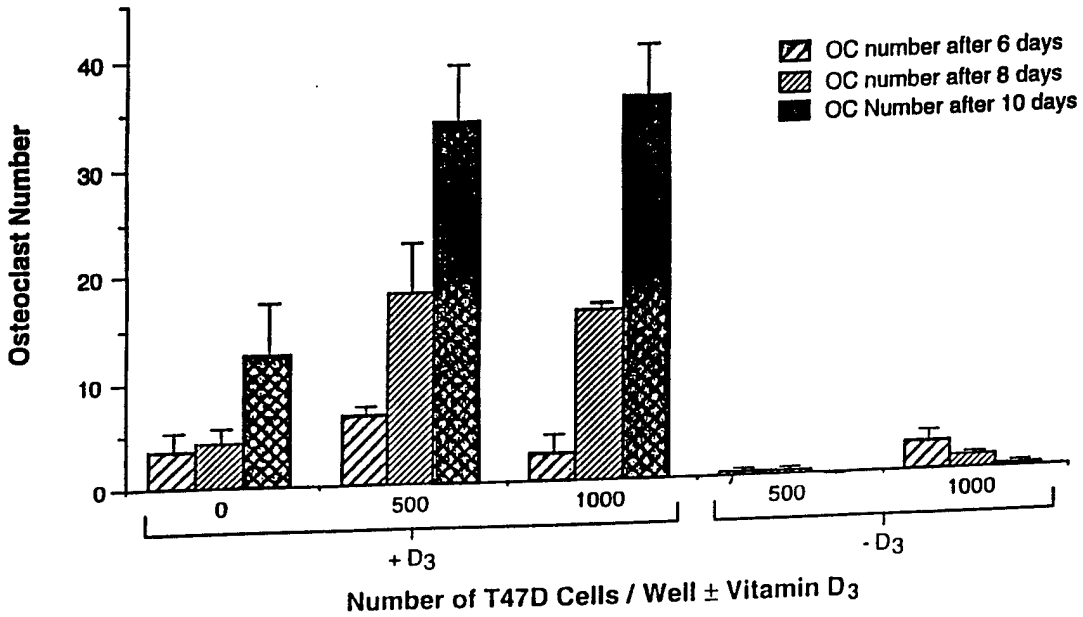

Fig. 5 Breast cancer induced osteoclastogenesis is dependent on D_3. The indicated number of T47D cells were co-cultured with 10^4 OC progenitors with or without 10^{-8} M D_3. After 6, 8, or 10 days of co-culture the cells were fixed, stained for TRAP and the number of TRAP-positive cells counted. Data are shown as average of triplicates with SEM.

Human breast cancer induced OC differentiation requires cell contact. Normal osteoclastogenesis requires contact between OC precursors and accessory cells (13-15), and we wished to determine if the same is true regarding breast cancer mediated recruitment of resorptive polykaryons. To this end, the cancer cells were cocultured, as in previous experiments, in contact with OC progenitors or separated from the BMCs by a filter in transwell dishes. After 10 days, the cells were fixed, and the number of TRAP-positive, multinucleated cells quantitated. As seen in Fig. 6 OCs do not develop when the tumor cells are separated from the BMCs, indicating that similar to normal osteoclastogenesis, that induced by breast cancer cells requires physical intimacy between accessory and progenitor cells. Similar results were obtained in experiments in which the cancer cells were separated from the OC progenitors by soft agar. Supporting the contention that cell

contact is prerequisite to tumor induced OC differentiation, media conditioned by T47D cells does not promote formation of TRAP-positive polykaryons.

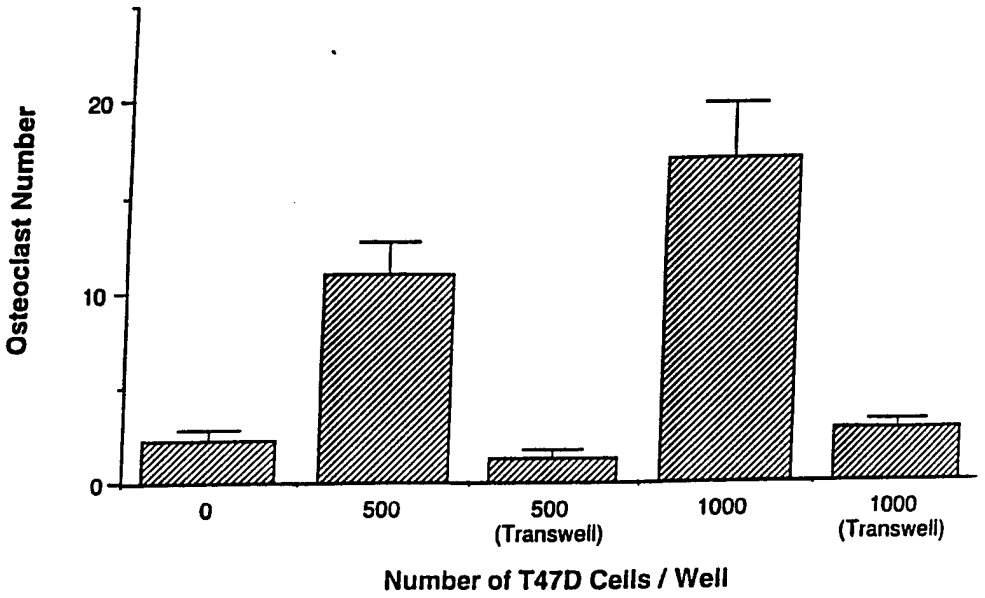

Fig. 6 OC induction by T47D cells requires cell contact. The indicated number of T47D cells and OC progenitors were either co-culture or separated into two compartments of transwells to determine if OC induction by the tumor cells is mediated through soluble of cell contact mediated factor(s). After 10 days, the TRAP-positive cells were counted. Data are shown as average of triplicates with SEM.

2. THE $\alpha_v\beta_3$ INTEGRIN DICTATES ORGAN SPECIFIC METASTASIS OF BREAST CANCER

We hypothesized that selective metastasis of breast cancer cells to bone likely involves adhesion of circulating malignant cells to specific ligands on stromal cells or their extracellular matrix. Using a bone metastasis model in which tumor cells are injected intracardiac into nude mice (17), we demonstrated that the MDA-231 human breast cancer cells selected, in vitro, for preferential attachment to primary human marrow stromal cells metastasize to bone more frequently than marrow stroma-nonadherent cells. Intriguingly, the stroma-adherent tumor cells express substantially less $\alpha_v\beta_3$ integrin than their nonadherent counterparts. Supporting the contention that low $\alpha_v\beta_3$ expression is a potential marker of skeletal, as opposed to visceral, targeting breast cancer, $\alpha_v\beta_3$-non-expressing MDA-231 cells, selected by cell sorting, metastasize to bone approximately seven times more frequently than tumor cells expressing a high level of the integrin. In contrast, mice administered high $\alpha_v\beta_3$ expressing MDA-231 cells develop more pulmonary metastases than animals receiving low $\alpha_v\beta_3$ expressing cells.

Characterization of integrin mediating attachment of human breast cancer cells to bone marrow

stroma. We reasoned that selective metastasis of breast cancer to bone likely involves attachment to marrow stromal cells and/or their extracellular matrix. To characterize the interaction between cancer and stromal cells, adherent cells from the mononuclear fraction of human rib marrow were isolated, and grown to confluence (18). These human bone marrow stromal cells (HBMCS) were fixed, and used as substrate for attachment of the MDA-231 human breast cancer cell line. We first showed that the MDA-231 cells adhere to the HBMSC in a saturable manner (data not shown). Furthermore, attachment of MDA-231 cells to HBMSC is inhibited dose dependently by the peptide, GRGDSP, but not GRGESP, indicating that RGD-dependent integrins play an important role in breast cancer-marrow stroma recognition (Fig. 7).

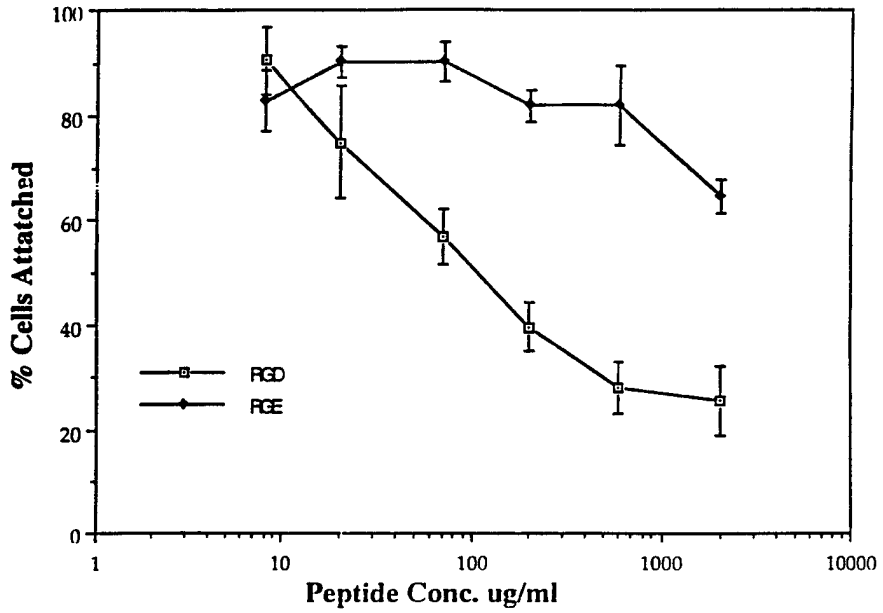

Fig. 7 RGD-dependent integrins mediate attachment of human breast cancer cells to bone marrow stroma. 5 x 10^5 MDA-231 cells were incubated with fixed stromal cells in the presence of indicated concentrations of the peptides GRGDSP, or GRGESP for 30 minutes at 37°C. After removing the unbound cells, the adherent cells were stained with crystal violet, and the bound dye quantitated using a plate reader. Each data point represents the average of triplicates and the error bars represent the standard deviation.

Selective adherence of human breast cancer cells to bone marrow stroma, in vitro, leads to increased frequency of bone metastasis, in vivo. To test the hypothesis that tumor cell interactions with marrow stroma are critical to development of bone metastases, we isolated, by cell panning, stroma-adherent and nonadherent subpopulations of the MDA-231 cells. The bone metastatic potential of each population was determined using a mouse model in which the tumor cells were injected into the left ventricle of nude mice (17,19). In this experimental metastasis model, the animals develop radiographically apparent, osteolytic lesions primarily in bones containing red marrow, which are similar to the skeletal metastases experienced by breast cancer patients (Fig. 8) (20). Twice as many mice injected with the stroma-adherent cells developed bone metastases as animals injected with the stroma-nonadherent cells (Table 1). In addition, the stroma-adherent cells produce, on average, three times more osteolytic lesions than their nonadherent counterparts

(Table 1). Taken together, these data indicate that stroma-adherent cells are six times as likely as stroma-nonadherent cells to produce osteolytic metastases. Thus, panning for HBMSC adherent cells likely resulted in selection of specific cell adhesion molecules responsible for the increased metastasis to bone.

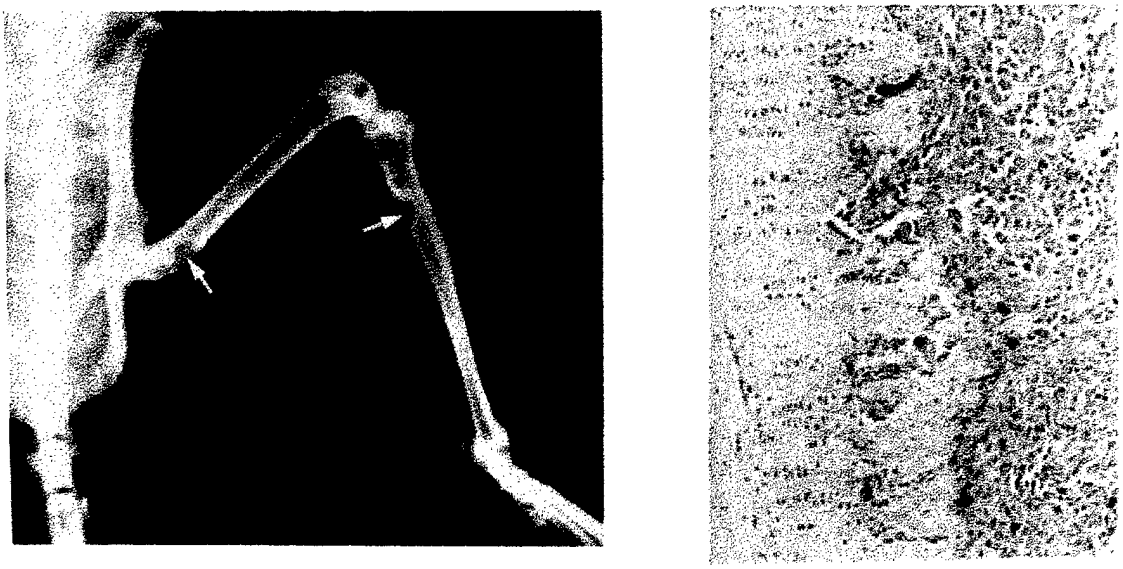

Fig. 8 Human breast cancer cells metastasize to bone and cause osteolytic lesions. X-ray of a mouse injected, intracardiac, with MDA-231 cells six weeks earlier (left panel). Note the presence of large osteolytic lesions in the proximal femur and tibia (arrows). Histological section of a metastatic bone tumor stained for tartrate-resistant acid phosphatase showing abundant OCs recruited by the malignant cells (right panel).

TABLE 1

MDA-231 subpopulation	Number of mice injected	Number of mice with osteolytic lesions	Average number of osteolytic lesions/mouse	Number of weeks to detection of bone metastases
Stroma-adherent	38	13	6.1	6-8
Stroma-nonadherent	43	7	2.3	6-8

Marrow stroma-adherent MDA-231 cells, which metastasize to bone more frequently than their nonadherent counterparts, express less $\alpha_v\beta_3$ integrin. The data shown in Figure 8 indicate that RGD-dependent integrins play a significant role in breast cancer-marrow recognition. Thus, we analyzed, by FACS, the abundance of nine integrins, α_2-α_6, α_v, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and β_1 on the surface of cells derived from bone metastases produced by stroma-adherent and nonadherent MDA-231 breast cancer cells, respectively. We found a difference only in $\alpha_v\beta_3$ expression between the two populations. Whereas the parental and non-adherent MDA-231 cells, i.e. those poorly metastasizing to bone, express moderate levels of $\alpha_v\beta_3$, the marrow stroma-adherent cancer cells, which metastasize to bone more frequently, express essentially undetectable levels of this integrin (Fig. 9).

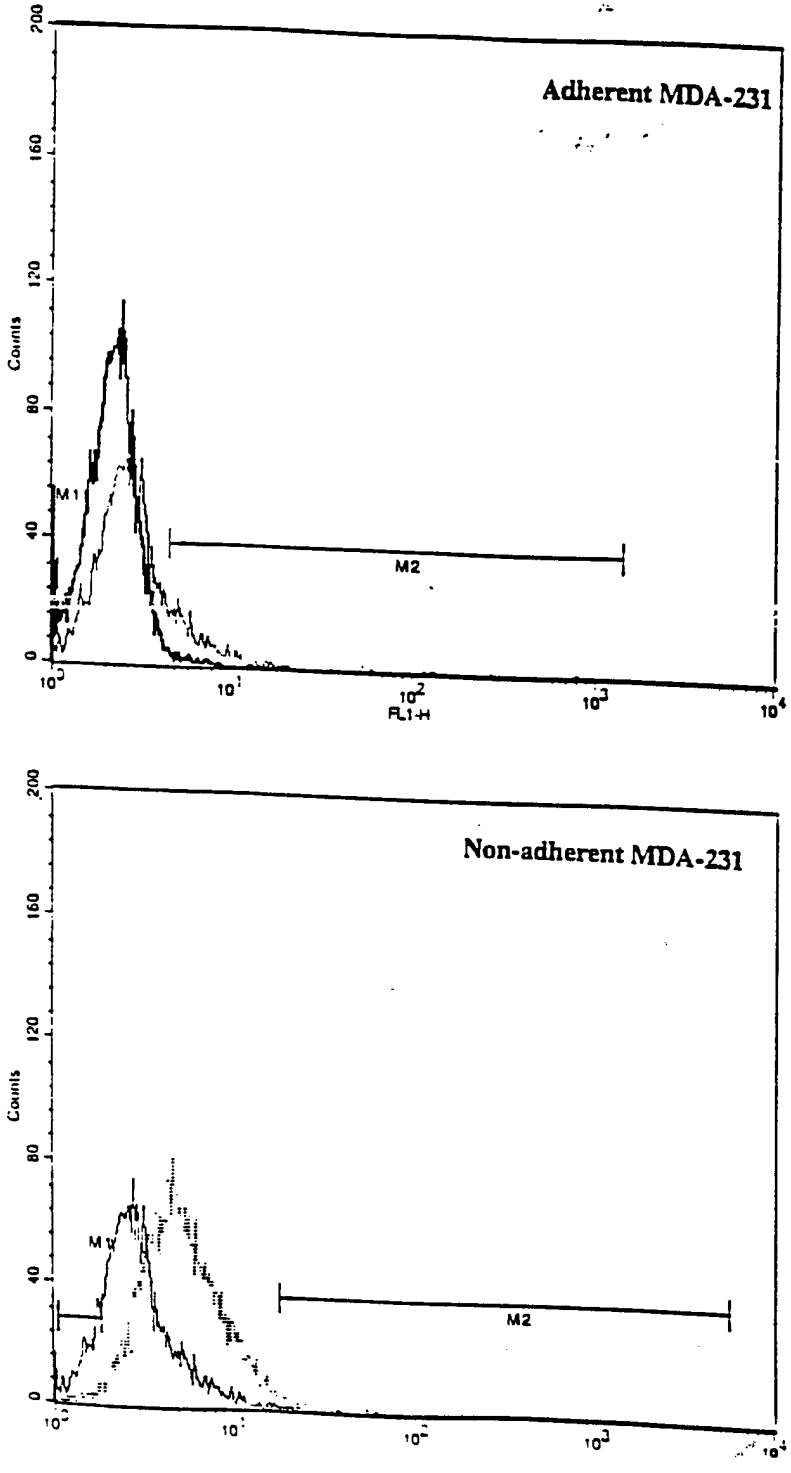

Fig. 9 Bone marrow stroma-adherent MDA-231 cells express lower levels of $\alpha_v\beta_3$ integrin than the stroma-nonadherent cancer cells. FACS analysis of bone tumor cells derived from the adherent (top panel)and nonadherent (bottom panel) parental MDA-231 cells stained with LM609 (dotted lines). In each panel, control cells (solid lines) were stained only with the second antibody.

In agreement with these in vivo results, attachment of MDA-231 cells to HBMSC was inhibited by only 20% by the anti-$\alpha_v\beta_3$ specific antibody LM609 (data not shown). Thus, the mechanisms by which MDA-231 cells adhere to marrow stromal cells do not involve $\alpha_v\beta_3$.

Low $\alpha_v\beta_3$ expressing variants of MDA-231 cells are highly metastatic to bone. To further test the possibility that low $\alpha_v\beta_3$ expression by human breast cancer cells is associated with increased bone metastasis, we isolated $\alpha_v\beta_3$ high and non-expressing sublines of the parental MDA-231 cell line. High $\alpha_v\beta_3$ expressing MDA-231 cells were isolated by repeated cell sorting using the $\alpha_v\beta_3$ heterodimer specific antibody LM609 (Fig. 10) (21,22).

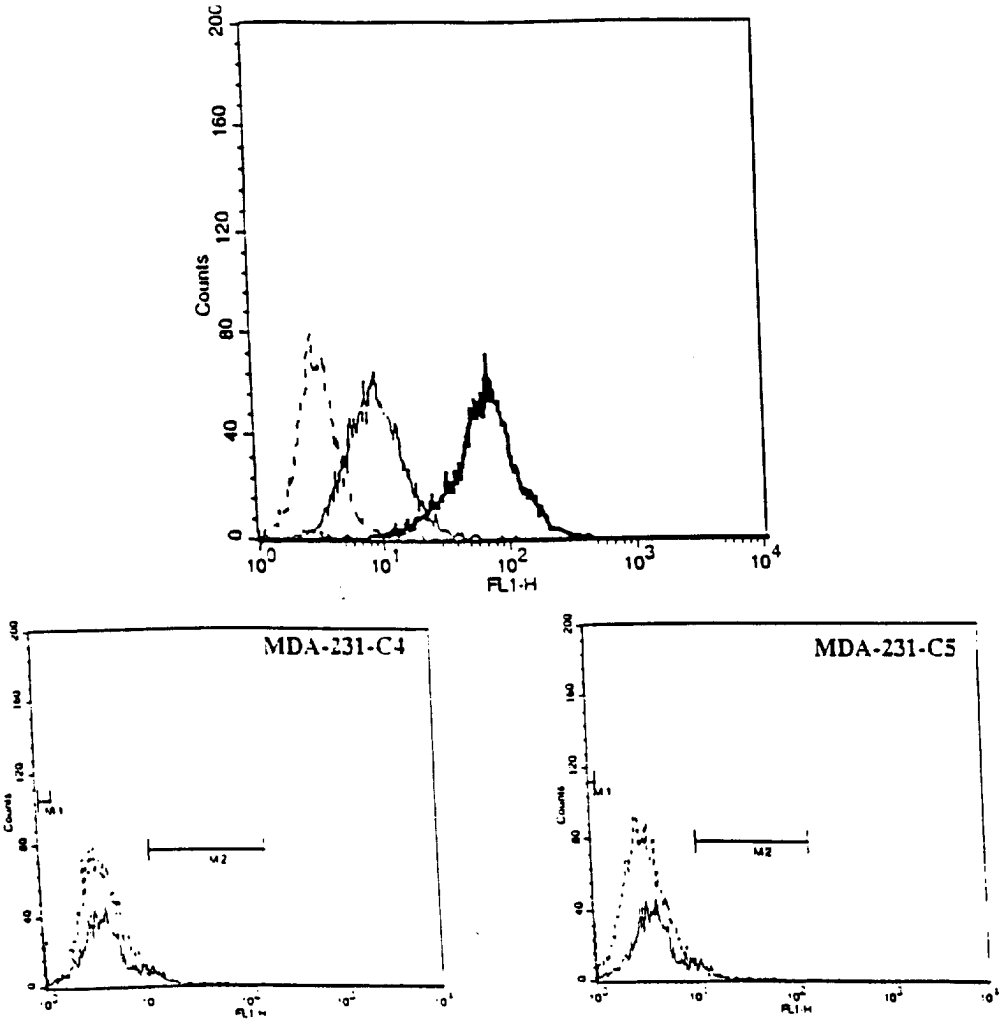

Fig. 10 Isolation of high $\alpha_v\beta_3$ expressing population, and two non-expressing clonal lines of MDA-231 cells. Top panel: The high expressors (heavy solid line) were selected by reiterative sorting for top 0.5% of cells stained with the $\alpha_v\beta_3$ specific antibody, LM609. For comparison, the parental MDA-231 cells stained with LM609 (solid line), and control cells stained with only the secondary antibody (dotted line) are also shown. Lower panels: $\alpha_v\beta_3$ non-expressing MDA-231 cells were isolated by cloning. The selected cells were stained with LM609 (solid lines) and controls (dotted lines) with the secondary antibody alone.

Because a similar strategy to generate a population of $\alpha_v\beta_3$ non-expressing cells failed, we isolated several clonal lines of MDA-231 lacking detectable expression of $\alpha_v\beta_3$ (Fig. 10). In vivo metastasis assays demonstrate that the high $\alpha_v\beta_3$ expressing MDA-231 cancer cells produce osteolytic lesions in 11% of the mice with an average of two lesions per animals (Table 2). In contrast, the two $\alpha_v\beta_3$ non-expressing clonal lines metastasized to bone with a frequency of 33% and 75%, respectively, and are detectable in a shorter time. In addition, the $\alpha_v\beta_3$ non-expressing MDA-231 cells produced, on average, 2-6 fold more osteolytic lesions per mouse than the high $\alpha_v\beta_3$-expressing cells (Table 2). Analysis of $\alpha_v\beta_3$ expression in bone metastatic tumor cells derived from the low $\alpha_v\beta_3$-expressing parental lines shows the same level of $\alpha_v\beta_3$ as in the parental line, indicating that growth of the cells in vivo does not induce expression of this integrin (Fig. 11).

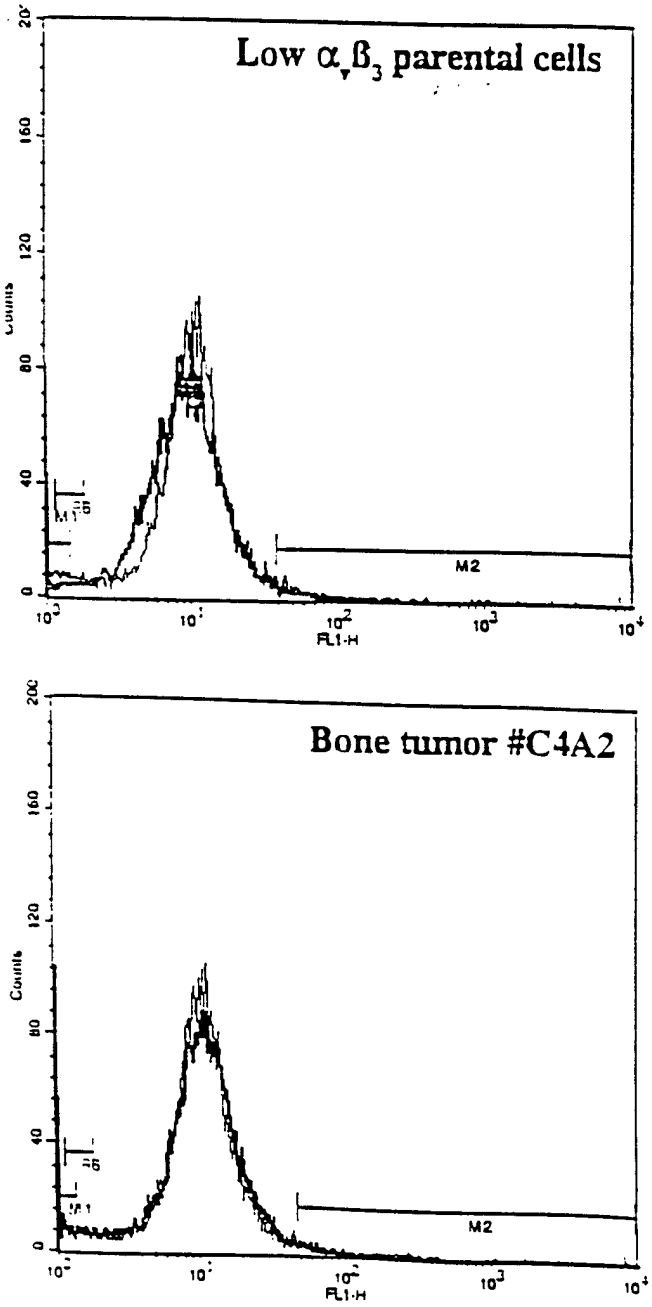

Fig. 11 Bone tumor cells derived from the low $\alpha_v\beta_3$ expressing MDA-231 cells maintain the same low level of $\alpha_v\beta_3$ expression as their parental line. Fluorescence intensity of low $\alpha_v\beta_3$-expressing parental (top panel) and bone-derived tumor cells (bottom panel) incubated with LM609 (heavy lines) and compared to that of control cells (thin lines) incubated with the secondary antibody alone.

TABLE 2

MDA-231 subpopulation	Number of mice injected	Number of mice with osteolytic lesions	Average number of osteolytic lesions/mouse	Number of weeks to detection of bone metastases
High $\alpha_v\beta_3$ MDA-231	26	3	2.0	6-8
Low $\alpha_v\beta_3$ MDA-231 clone C4	27	10	5.4	4-6
Low $\alpha_v\beta_3$ MDA-231 clone C5	40	30	12.2	4-6

These data suggest that absence of detectable $\alpha_v\beta_3$ may serve as a marker of bone-targeting breast cancer.

High $\alpha_v\beta_3$-expressing MDA-231 cells preferentially metastasize to the lungs. In the previous experiments, we observed that mice injected with high $\alpha_v\beta_3$ expressing MDA-231 cells developed more lung tumors than animals injected with low $\alpha_v\beta_3$ expressing variants of MDA-231 cells. Although these data suggested that $\alpha_v\beta_3$ expression correlates with increased lung metastasis, the results, we believe, were inconclusive because it is possible that, during intracardiac injection, tumor cells may have been directly seeded into the lungs. To determine if $\alpha_v\beta_3$ expressing cells in fact target preferentially to the lungs, high and low $\alpha_v\beta_3$ expressing MDA-231 variants were injected into the tail vein. After three months, 100% (n=5) of the mice injected with the high $\alpha_v\beta_3$ expressing and only 20% (n=5) of the mice injected with the $\alpha_v\beta_3$ non-expressing (clone C5) MDA-231 cells developed macroscopically visible lung tumors (Fig. 12).

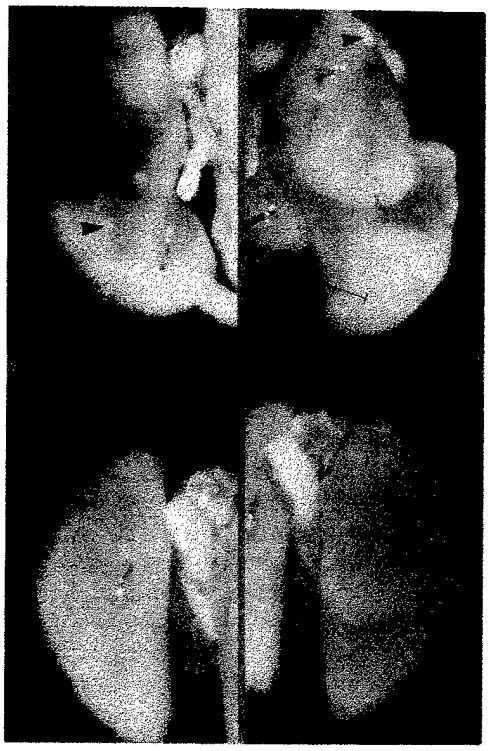

Fig. 12 High $\alpha_v\beta_3$ expressing breast tumor cells preferentially metastasize to lungs. Top panels represent lungs of a nude mouse injected, via the tail vein, with high $\alpha_v\beta_3$ expressing MDA-231 cells. The metastatic nodules are indicated by arrows. Bottom panels show the absence of metastatic nodules in the lungs of a mouse injected, via the tail vein, with the $\alpha_v\beta_3$ non-expressing MDA-231 C5 cells.

CONCLUSIONS

We have established that human breast cancer cells may serve an inductive role in OC generation. Such induction requires contact between the supportive breast cancer cells and OC precursors. Thus, we document for the first time that breast cancer cells, per se, are responsible for recruitment of OCs, thereby generating the format for osteolysis. Inhibiting the means by which breast cancer promotes osteoclastogenesis presents itself as a potential means of preventing tumor osteolysis and its consequences.

We have also documented that cell surface molecules may dictate organ targeting of breast cancer metastasis. In the case of the $\alpha_v\beta_3$ integrin, its expression homes the cells to viscera rather than bone. Thus, presently extant agents, capable of blocking the $\alpha_v\beta_3$ integrin, may potentially blunt visceral metastasis of breast cancer.

METHODS

Reagents. Sources of anti-integrin antibodies used in this study were: LM609 and anti-$\alpha_v\beta_3$ (21,22) generously provided by Dr. David Cheresh (Scripps Research Institute); P1E6 anti-α_2, P1B5 anti-α_3, P4C2 anti-α_4, P1D6 anti-α_5, and P1F6 anti-$\alpha_v\beta_3$ (all purchased from Gibco/BRL); GoH3 anti-α_6, MAR4 anti-β_1 (both purchased from Pharmingen); and L230 anti-α_v (American Type Culture Collection).

Cell Culture. MDA-231 (American Type Culture Collection) cells were cultured in α-MEM containing 10% fetal bovine serum (α-10-MEMl Gibco/BRL). The cells were passaged by trypsinization twice weekly.

Human bone marrow stromal cells (HBMSC) were obtained from the ribs of cardiac surgery patients as described by Cheng, et al. (18). Briefly, the ribs were cleaned of adherent connective tissue, the marrow was flushed out with α-MEM, and the mononuclear cells pelleted on top of a cushion of 1.077 g/ml Ficoll-Hypaque (Pharmacia). The mononuclear cells were plated at a density of 4×10^5 cells/cm^2, and allowed to attach for 7 days. These adherent cells were fed with fresh α-MEM twice per week until confluence, at which time they were lifted by trypsin and subcultured at 10^4 cells/ml in 96 well microtiter plates. At confluence, the cells were washed with phosphate buffered saline (PBS) and fixed for 10 minutes with 3.7% formaldehyde in PBS. The fixative with washed out with PBS and the cells used as attachment substrate for the MDA-231 cancer cells.

Bone marrow-derived OC progenitors. Bone marrow cells were flushed from femora and tibiae of 4-5 week old female C3H/hen mice (Harlan Sprague Dawley, Indianapolis, IN). The cells were incubated for 24 hours in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum (both from Gibco/BRL, Gaithersburg, MD) and 1000 U/ml of CSF-1 (prepared from L929 conditioned media as previously described (23,24)). The non-adherent cells were layered on a 1.077 g/ml Ficoll-Hypaque (Pharmacia) cushion and centrifuged at 1500 rpm for 15 minutes. The cells at the interface, enriched for mononuclear cells, were collected and used as the source of OC precursors. We and others (13,14,25-27) have previously shown that a high proportion of these cells differentiate to mature OCs, in vitro, when co-cultured with appropriated bone marrow derived stromal cells, such as ST2, in the presence of D_3 (gift of Dr. Milan Uskokovic, Hoffman-LaRoche, Nutley, NJ).

Cancer cells. The human breast cancer cell line T47D was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in α-MEM supplemented with 10% fetal bovine serum. T47D subclones were obtained by limiting dilution of the cells in 96 well plates and only those wells that contained a single cell clone were expanded and used in the OC differentiation assays described below.

In vitro differentiation of OC precursors. All experiments were performed in 24 well tissue culture dishes. T47D or subclones of it were plated at zero, 500 or 1000 cells per well as indicated. OC precursors were added subsequently at 10^4 cells per well. The cells were maintained in α-MEM supplemented with 10% FBS, and containing final concentrations of 1000 U/ml of CSF-1, 10 nM D_3, and 10 nM dexamethasone. OCs were defined as TRAP expressing multinucleated cells. After 6, 8 or 10 days of co-culture the cells were fixed, stained for TRAP activity, an OC specific maker, and the number of TRAP-positive cells counted.
Enzyme histochemistry. The cultures were fixed and stained for tartrate-resistant acid

phosphatase producing cells using a commercial kit (Sigma, St. Louis, MO). The stained cells were manually counted by light microscopy.

In vitro bone resorption. Bone slices, 100 μM thick, were prepared from adult bovine femur according to Arnett and Dempster (16). The slices were sterilized with 70% ethanol, and placed in the culture wells together with the OC precursors and the T47D cells. After 10 days, the slices were fixed in formalin, sonicated to remove the cells, and the resorption lacunae stained with toluidine blue.

Cell attachment assays. MDA-231 cells were grown to approximately 80% confluence, at which time they were lifted non-enzymatically using cell dissociation medium (Sigma). The cells were washed, counted using a hemocytometer, and the concentration adjusted as indicated in the figure legends. Appropriate concentrations of cancer cells in 0.1 ml of α-MEM (without serum) was added to microtiter plates containing fixed HBMSC. Plates were incubated in a humidified incubator at 37°C for 30 minutes, washed three times with PBS, fixed with 3.7% formaldehyde and stained with 0.5% crystal violet. The number of adherent MDA-231 cells was quantitated using an ELISA reader after solubilizing the bound crystal violet with 1% sodium dodecyl sulfate (SDS).

Selection of HBMSC-adherent and non-adherent cancer cells. HBMSC-adherent MDA-231 cells were selected by repeating the attachment assay described above, except that the incubation time was limited to five minutes. After washing, the adherent cells were lifted by trypsin and plated in tissue culture dishes containing α-10-MEM. Non-adherent cells were selected by repeating the above assay for 60 minutes and culturing the unattached cells. For each cell type the panning procedure was repeated, sequentially, four times.

In vivo metastasis assays. For skeletal metastasis, subconfluent MDA-231 cells were harvested non-enzymatically with cell dissociation medium (Sigma), washed, and the concentration adjusted to 10^6 cells/ml in PBS. These cells were kept at 4°C until injected into mice. Athymic nude mice were anesthetized with 65 mg/kg body weight of pentobarbital administered intraperitoneal. Each animal was injected with 10^5 cells (0.1 ml) in the left ventricle, by insertion of a syringe needle between the second and third rib, and 2-3 mm to the left of the sternum (17,19). Entry of oxygenated (bright red) blood in the needle assured injection in the left ventricle (17). Progression of osteolytic metastases was monitored by x-ray of the animals using a CGR Sonograph 550T Dedicated Mammography Unit with Kodak MinR film at weekly intervals.

For lung metastasis, the MDA-231 cells were prepared as above, and 10^5 cells in 0.1 ml of PBS were injected into the lateral tail vein. The animals were sacrificed after three months, and the lungs examined macroscopically and histologically for the presence of metastatic tumors.

Isolation of high $\alpha_v\beta_3$-expressing population and $\alpha_v\beta_3$ non-expressing C4 and C5 clonal variants of MDA-231.

Subconfluent MDA-231 cells were lifted from culture plates with cell dissociation medium, washed into serum-free α-MEM, concentrated to 10^7 cells/ml, incubated on ice with 10 µg/ml of anti-$\alpha_v\beta_3$ heterodimer-specific antibody LM609 (21,22) for 60 minutes. The primary antibody was washed, and the cells incubated with a fluorescein labeled anti-mouse secondary (Jackson Immunochemicals). After washing excess secondary antibody, the cells were sorted using a

FACSCAN (Becton-Dickinson). The highest and the lowest 0.5% fluorescent-labeled cells were selected and grown. This procedure was repeated four times to isolate the high $\alpha_v\beta_3$ expressing subpopulations of MDA-231 cells. Isolation of a low $\alpha_v\beta_3$ expressing population was unsuccessful, as following each round of cell sorting a fraction of the cells re-expressed $\alpha_v\beta_3$ and these cells eventually took over the population. Since this approach failed to yield a low $\alpha_v\beta_3$ expressing population, the FACS was repeated except that the low $\alpha_v\beta_3$ expressing cells were cloned by limiting dilution to obtain clonal lines. The $\alpha_v\beta_3$ expression of each line was measured by FACS and only clones, MDA-231 C4 and C5, expressing undetectable expression of the integrin, were further propagated.

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