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Original Article

Pre-osteoblastic MC3T3-E1 cells promote breast cancer growth in bone in a murine xenograft model

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Abstract

The bones are the most common sites of breast cancer metastasis. Upon arrival within the bone microenvironment, breast cancer cells coordinate the activities of stromal cells, resulting in an increase in osteoclast activity and bone matrix degradation. In late stages of bone metastasis, breast cancer cells induce apoptosis in osteoblasts, which further exacerbates bone loss. However, in early stages, breast cancer cells induce osteoblasts to secrete inflammatory cytokines purported to drive tumor progression. To more thoroughly evaluate the role of osteoblasts in early stages of breast cancer metastasis to the bones, we used green fluorescent protein-labeled human breast cancer cell lines MDA-MB-231 and MDA-MB-435, which both induce osteolysis after intra-femoral injection in athymic mice, and the murine pre-osteoblastic cell line MC3T3-E1 to modulate osteoblast populations at the sites of breast cancer metastasis. Breast cancer cells were injected directly into the femur with or without equal numbers of MC3T3-E1 cells. Tumors grew significantly larger when co-injected with breast cancer cells and MC3T3-E1 cells than injected with breast cancer cells alone. Osteolysis was induced in both groups, indicating that MC3T3-E1 cells did not block the ability of breast cancer cells to cause bone destruction. MC3T3-E1 cells promoted tumor growth out of the bone into the extraosseous stroma. These data suggest that breast cancer cells and osteoblasts communicate during early stages of bone metastasis and promote tumor growth.

Key words MC3T3-E1, osteoblast, breast cancer, bone, metastasis

Breast cancer cells most frequently metastasize to the trabaecular bone, resulting in severe complications including contractile pain, fractures, and hypercalcemia[1-3]. These effects exacerbate bone metastasis and hinder therapeutic interventions. Breast cancer cells are known to secrete numerous factors within the bone environment, such as parathyroid hormone-related protein (PTHrP)[4], that up-regulate the expression of receptor activator of nuclear factor-κB (RANK) ligand (RANKL)[5] in osteoblasts. This up-regulation, in addition to other events, leads to an increased activity of osteoclasts, causing high rates of bone osteolysis and release of growth factors embedded in the bone matrix, such as transforming growth factor-β (TGF-β)[6,7]. These factors promote survival of the cancer cells, resulting in tumor growth and weakening of the bones.

Successful treatments have been developed to combat the interactions between breast cancer cells and osteoclasts. Bisphosphonates and RANK/RANKL inhibitors, which inhibit osteoclast activity, have provided a degree of success by slowing osteolysis; however, many patients with breast cancer are incapable of repairing the bone matrices that have been resorbed[8,9].

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This observation suggests an alteration or loss-of-function in remaining osteoblasts. These results are not entirely surprising because bone turnover is an intricate balance of formation and osteolysis mediated in tight cooperation by osteoclasts and osteoblasts[10-13]. We previously reported that breast cancer cells, in addition to increasing osteoclast activity, can induce apoptosis and inhibit differentiation of osteoblasts both *in vitro* and *in vivo*[14-16]. Our results suggest that a reduction in the number of osteoblasts in late stages of disease contributes to failure to repair bone defects. Breast cancer cells have been shown to inhibit osteoblast differentiation and induce cells of the osteoblast lineage to secrete inflammatory cytokines that alter progression of osteolytic disease[17,18]. Collectively, these data suggest that osteoblasts have multiple roles in osteolytic progression and interact differently with breast cancer cells depending on the stage of disease.

To elucidate the mechanisms underlying these clinical observations, we evaluated the effects of modulating osteoblast populations during early stages of breast cancer bone metastasis. Our laboratory previously established *in vivo* murine models of osteolytic breast cancer using enhanced green fluorescent protein (GFP) -labeled breast cancer cell lines MDA-MB-231 and MDA-MB-435[16]. Following intra-cardiac or intra-femoral injections, both breast cancer cell lines readily seeded the bones and eventually induced osteolytic lesions in the long bones (including femurs), vertebrae, and mandibles of athymic mice[16,19]. These models have some caveats, including altered immune system and failure to form osseous lesions from orthotopic tumors; however, both models provide high levels of consistency and effectively model tumor-induced osteolytic processes.

To assess the role of osteoblast involvement in early stages of bone metastasis, we co-injected the pre-osteoblastic cell line MC3T3-E1 and tumor cells into mouse femurs. MC3T3-E1 cells, which were isolated from the calvaria of C57BL/6 mice, differentiate into functional osteoblasts *in vitro* with the addition of ascorbic acid and inorganic phosphate and differentiate *in vivo* when injected into immunodeficient mice[20-22]. Therefore, the MC3T3-E1 cell line was chosen to modulate osteoblast populations within the femur at sites of breast cancer-induced osteolysis.

Materials and Methods

Cell lines

MDA-MB-231 and MDA-MB-435 metastatic breast cancer cell lines were kindly provided by Dr. Janet Price (University of Texas M. D. Anderson Cancer Center) and were subsequently transduced with GFP (MDA-MB-231GFP, MDA-MB-435GFP) using a human immunodeficiency virus (HIV) type 1-based lentiviral vector system as previously described[16,23]. Both cell lines are triple-negative basal type derived from pleural effusions[24,25] and can spontaneously metastasize to the lymph nodes and lungs after orthotopic implantation, although the MDA-MB-435 cell line is far more efficient at spontaneous metastasis than MDA-MB-231 in our lab. Neither cell line makes osseous metastases after orthotopic injection with experimentally useful efficiency, but both cell lines are highly efficient at osseous metastasis if delivered via intracardiac injection[16,23]. The origin of the MDA-MB-435 cell line has been questioned[26,27], but recent literature confirms its origin as breast cancer and legitimizes its use for these studies[28-30].

MDA-MB-231^{GFP} and MDA-MB-435^{GFP} cells were grown in Dulbecco's-modified Eagle's medium mixed 1: 1 (*V*:*V*) with Ham's F-12 medium (DMEM/F12; Invitrogen, Carlsbad, CA #11330) and supplemented with 2 mmol/L L-glutamine, 0.2 mmol/L non-essential amino acids, and 5% fetal bovine serum (FBS). MC3T3-E1 cells were grown in α-Minimum Essential Media Eagle (α-MEM) (Sigma #M0894) supplemented with 10% FBS. MC3T3-E1 cells in culture readily differentiated into mature osteoblasts when ascorbic acid and β-glycerolphosphate were added over 3 weeks as demonstrated by positive alkaline phosphatase and von Kossa staining (data not shown). All cell lines were tested and found to be free of *Mycoplasma an spp*. contamination using a PCR-based kit (Aligent Technologies, Santa Clara, CA #302108).

Animals

Athymic mice (Harlan Sprague Dawley, Indianapolis, IN) were maintained under the guidelines of the NIH and UAB. All protocols were approved by the UAB and U.S. Army Medical Research and Materiel Command Institutional Animal Care and Use Committees. Food and water were provided *ad libitum*.

Intra-femoral injections

Standard methods[31] were used with minor modifications. Briefly, mice were anesthetized with ketamine-HCl (129 mg/kg) and xylazine (4 mg/kg) and were subsequently tested for lack of response to toe pinch. Buprenorphine (analgesic) was administered at 1 mg/kg by subcutaneous injection prior to intra-femoral injection. The knee joint area was wiped with 70% ethanol and flexed to ~75°. A 27-gauge needle tip was placed between the condyles and access to the marrow was gained by applying gentle pressure with twisting. A

total of 5×10^3 MDA-MB-231^{GFP} or MDA-MB-435^{GFP} cells were injected into the left femur of each 6-week old female athymic mouse, with 7 mice in each group. For co-injections, 5×10^3 MDA-MB-231^{GFP} or MDA-MB-435^{GFP} cells were mixed with 5×10^3 MC3T3-E1 cells and injected together (1:1 ratio). Additionally, 5×10^3 MC3T3-E1 cells were injected alone and served as a control. Uninjected, contralateral femurs also served as controls. Mice were monitored for signs of post-injection pain or discomfort. All protocols were approved and monitored by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Fluorescence microscopy

The mice were euthanized following anesthesia and cervical dislocation from 1 week to 6 weeks post-injection. Tumor size (mean tumor diameter in mm) was calculated by taking the square root of the product of the largest tumor diameter and perpendicular diameter as previously described[32]. Gross analysis of tumors and dissected femurs (soft tissue removed) was performed using a Leica MZFLIII dissecting microscope with bright field and GFP fluorescence filters [$\lambda_{excitation}$ = (480 ± 20) nm; $\lambda_{emission}$ = 510 nm barrier; Leica, Deerfield, IL]. Photomicrographs were collected using a MagnaFire digital camera (Optronics, Goleta, CA) and ImagePro Plus 5.1 software (Media Cybernetics, Silver Spring, MD).

Histomorphometry

Histomorphometry was performed at the University of Alabama at Birmingham Center for Metabolic Bone Disease Histomorphometry and Molecular Analysis Core Facility by the method of Parfitt *et al.*[33,34] using Bioquant image analysis software (R&M Biometrics, Nashville, TN). Intact, dissected femurs were placed in 25 mL glass scintillation vials and fixed in freshly prepared 4% paraformaldehyde in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS) and stored at 4℃[35] to maintain fluorescence. The bones destined for histological examination were subsequently removed and decalcified in 500 mmol/L EDTA and DPBS. The bones were dehydrated in increasing concentrations of ethanol and embedded in a mixture of 80:20 methyl methacrylate and dibutyl phthalate. Hematoxylin and eosin (HE) staining was performed on 5-μm serial sections. Tartrate resistant alkaline phosphatase (TRAP) staining was used to assess activity of osteoclasts.

X-ray analysis

Dissected femurs were exposed at 26 kV for 10 s on a Faxitron MX-20 Radiography system (Faxitron X-ray Corp., Buffalo, IL, USA). Bone osteolysis was observed qualitatively by the presence of dark areas (pits) in the exposed femurs.

Results

MC3T3-E1 cells increased breast cancer tumor size *in vivo*

MDA-MB-231^{GFP} or MDA-MB-435^{GFP} cells were injected into the left distal femur head with or without co-injection of MC3T3-E1 cells. The contralateral femurs injected with MC3T3-E1 cells alone served as controls. Mice were monitored and displayed no injection-related adverse events. At 5 weeks post-injection, 5 mice inoculated with MDA-MB-435^{GFP} and MC3T3-E1 cells exhibited large, palpable tumors [(13.1 ± 3.0) mm^2] in the left leg, whereas only 2 mice from the MDA-MB-435^{GFP}-injected group displayed palpable tumors, which were smaller [(5.5 ± 2.1) mm^2] (Figure 1A). The bulk of the palpable tumors exhibited high GFP expression and were noticeably large (Figure 1B).

Additionally, at 6 weeks post-injection, 5 mice inoculated with MDA-MB-231^{GFP} and MC3T3-E1 cells exhibited large tumors [(13.1 ± 6.7) mm^2], whereas only 1 mouse from the MDA-MB-231^{GFP}-injected group exhibited a palpable tumor (9.5 mm^2). The other 6 mice had no palpable tumors (Figure 1C). Dissected femurs were analyzed via fluorescence microscopy and revealed that MDA-MB-435^{GFP} cells and MDA-MB-231^{GFP} cells grew within the femur regardless of whether MC3T3-E1 cells were co-injected (Figure 2). Data for Figures 1 and 2 are summarized in Table 1.

In groups without MC3T3-E1 cell co-injection, most tumor cells remained confined to the bone, whereas tumors from groups co-injected with MC3T3-E1 cells extended beyond the bones into the extra-osseous environment. No fluorescence was observed in the non-injected leg, indicating that metastasis from injected femurs to contralateral femurs did not occur in this model during the experiment. Additionally, the lungs were, somewhat surprisingly, clear of overt or microscopic metastases in all groups because both tumor cell lines readily colonize lungs. No tumors were detected in mice injected with MC3T3-E1 cells alone.

MC3T3-E1 cells did not block the ability of breast cancer cells to induce osteolysis

Although a significant ($P < 0.05$) increase in tumor size was observed in the presence of MC3T3-E1 cells, we tested whether MC3T3-E1 cells affected the ability of the MDA-MB-435^{GFP} and MDA-MB-231^{GFP} cells to induce osteolysis. X-ray analysis revealed areas of bone

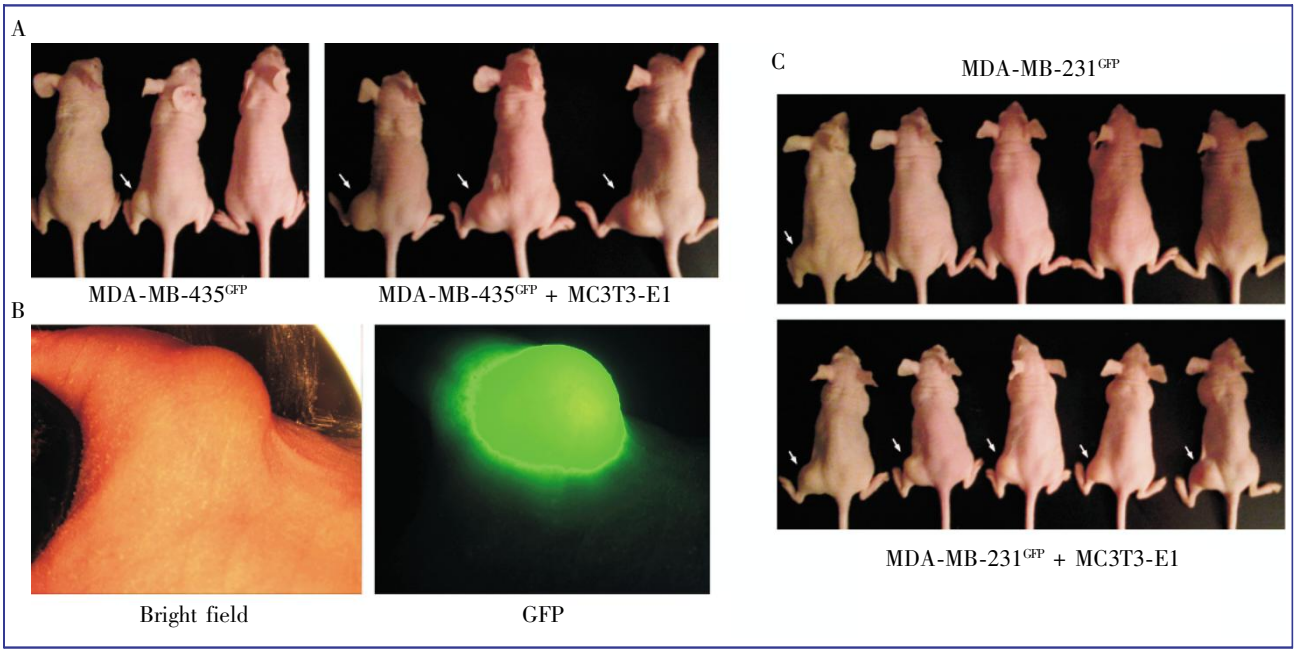

Figure 1. MC3T3-E1 cells promote tumor growth of breast cancer cells in athymic murine bone. A, MDA-MB-435^{GFP} cells co-injected with MC3T3-E1 cells form dramatically large tumors. A total of 5 × 10^3 MDA-MB-435^{GFP} cells were injected directly into the left femur of athymic mice with or without 5 × 10^3 MC3T3-E1 cells. At 5 weeks post-injection, the mice inoculated with MDA-MB-435^{GFP} and MC3T3-E1 cells displayed dramatically larger tumors than those injected with MDA-MB-435^{GFP} cells alone. B, representative image shows intense GFP expression within the tumor of a mouse injected with MDA-MB-435^{GFP} and MC3T3-E1 cells, demonstrating that MDA-MB-435^{GFP} cells accounted for the majority of tumor burden. C, intra-femoral injections of 5 × 10^3 MDA-MB-231^{GFP} cells and 5 × 10^3 MC3T3-E1 cells displayed more frequent and larger tumors than injection of MDA-MB-231^{GFP} cells alone, providing similar results to those obtained with co-injection of MDA-MB-435^{GFP} and MC3T3-E1 cells.

osteolysis for both breast cancer cell lines injected alone, or with MC3T3-E1 cells (Figure 3A), although rates of osteolysis were not measured. No apparent osteolysis was detected from injection of MC3T3-E1 cells alone (Figure 3A). In support of this, TRAP staining of bone sections revealed higher levels of osteoclast activity from all groups injected with breast cancer cells compared to MC3T3-E1 alone (Figure 3B).

Discussion

Metastasis to the bones is one of the most common and severe complications of breast cancer. Within the bone microenvironment, breast cancer cells can coordinate tumor growth and bone destruction, specifically by up-regulating osteoclast activity. Interactions between osteoclasts and breast cancer cells are, in part, mediated by osteoblasts through expression of RANKL. Although data exist for direct cancer cell-osteoclast interactions[14,36-45], much less attention has been given to tumor cell interactions with osteoblasts at sites of bone metastasis. The focus on osteoclast activation is partly due to observations that osteoblast populations are reduced at sites of progressing osteolysis and may not be necessary for tumor growth

Table 1. **Effects of co-injection of MC3T3-E1 cells with breast cancer cells on tumor growth and size**

Group	Tumor growth in femur	Palpable tumors	Tumor size (mm^2)
MC3T3-E1	0	0	0
MDA-MB-231^{GFP}	7	1	9.5
MDA-MB-231^{GFP} + MC3T3-E1	7	5	13.1 ± 6.7
MDA-MB-435^{GFP}	7	2	5.5 ± 2.1
MDA-MB-435^{GFP} + MC3T3-E1	7	5	13.1 ± 3.0

Each group contained 7 mice. The data of tumor size are presented as mean ± standard deviation of tumors in 7 mice of each group.

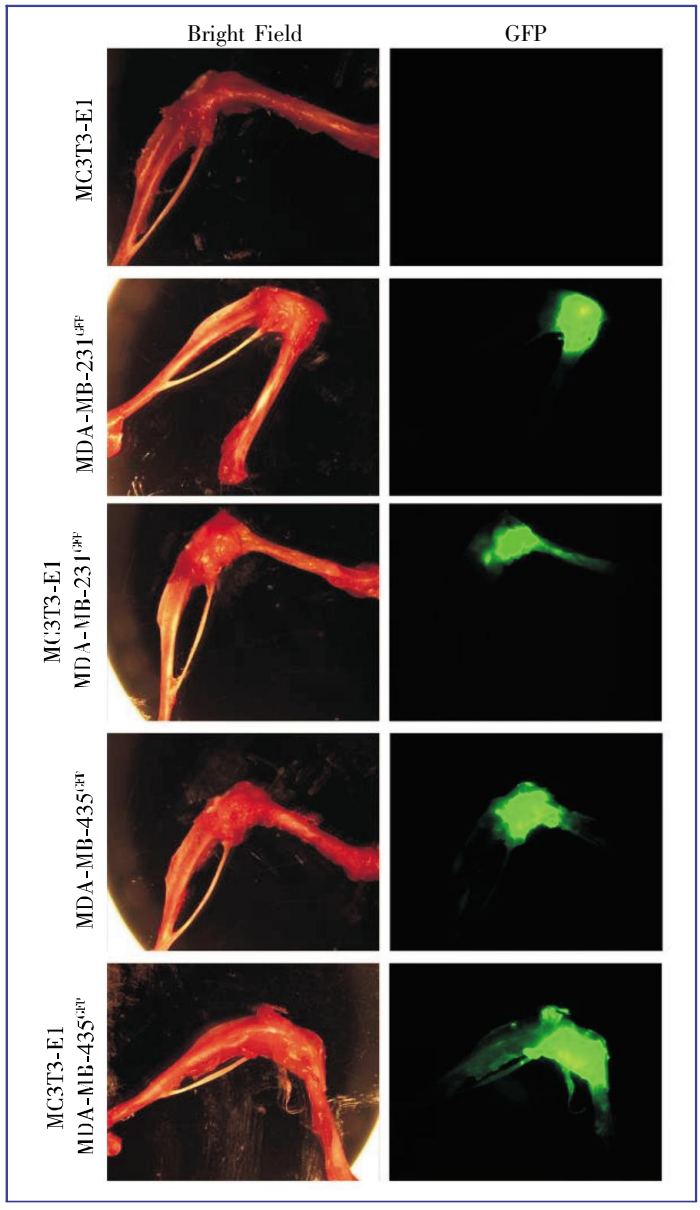

Figure 2. Representative fluorescence analysis of the femurs from experiments described in Figure 1. The femurs from the legs injected with MDA-MB-231^{GFP} and MDA-MB-435^{GFP} cells were dissected and analyzed by fluorescence microscopy after removal of soft tissues. Although large differences existed in the size of tumors, all groups injected with GFP-expressing breast cancer cells showed strong growth within the femurs. MC3T3-E1 cells injected alone served as a control and exhibited no GFP fluorescence.

mediated by the tumor-osteoclast axis in late stages of disease. We previously reported that osteoblasts are sensitive to apoptosis-inducing signals from breast cancer cells *in vivo* [15,16] and that breast cancer cells induce production of cytokines from osteoblasts[17,18].

To further explore connections between these data, we used established models of breast cancer-induced osteolysis in athymic mice and modulated the populations of osteoblasts at sites of osteolysis with murine pre-osteoblastic MC3T3-E1 cells, which differentiate into functional osteoblasts in immunodeficient murine models.

MDA-MB-231^{GFP} and MDA-MB-435^{GFP} cells were directly injected into the distal femur and induced bone osteolysis within 5 to 6 weeks. Co-injection with MC3T3-E1 cells was performed to increase the osteoblast-to-tumor cell ratio before osteolytic lesions developed. Unexpectedly, a dramatic increase in tumor size and expansion into the extra-osseous space was observed when MC3T3-E1 cells were also present. These findings illustrate the importance of this tumor-stromal interaction, as femurs injected with only MC3T3-E1 cells did not have tumors or detectable osteolysis.

To assess whether MC3T3-E1 cells affected breast cancer-induced osteolysis, X-rays were performed on dissected femurs. Bone osteolysis was observed in both the presence and absence of MC3T3-E1 cells by X-ray.

EBV VCA-IgA, Epstain-Barr virus viral capsid antigen-IgA antibody.

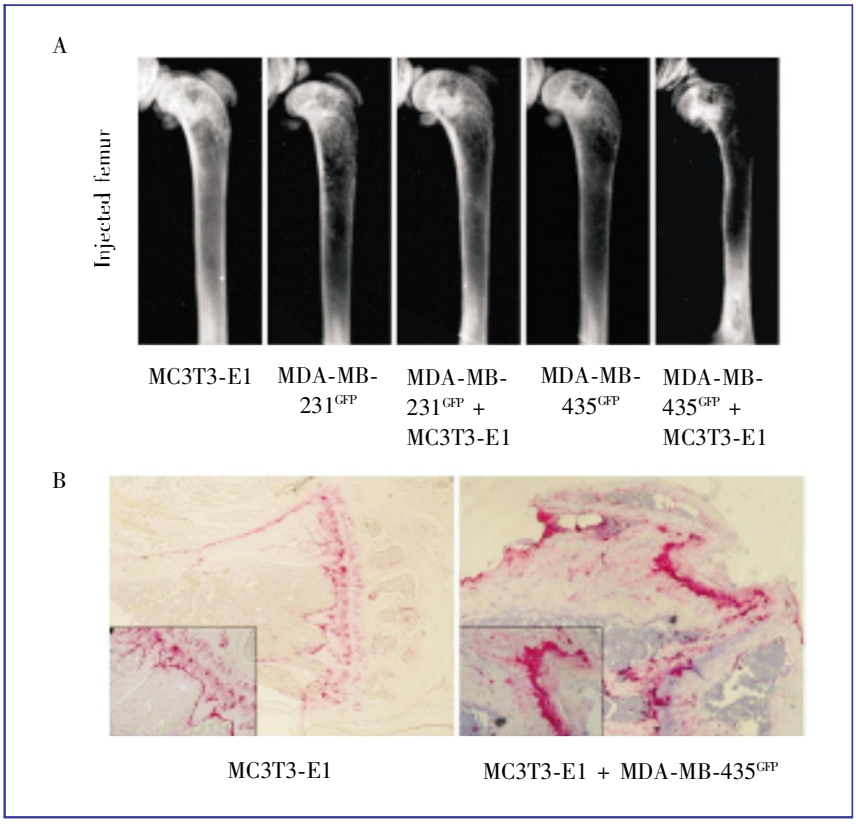

Figure 3. Representative X-ray and histomorphometric analysis of MC3T3-E1 and human breast carcinoma cells. A, representative X-ray analysis reveals bone osteolysis (dark areas) within the femurs of the mice injected with breast cancer cells. Co-injection of MC3T3-E1 cells with MDA-MB-231^{GFP} or MDA-MB-435^{GFP} cells did not inhibit the induction of osteolysis over 5 to 6 weeks. Injection of MC3T3-E1 cells alone did not induce osteolysis within the femur. B, comparison of TRAP staining in the bones after injection of MC3T3-E1 cells alone or with breast cancer cells. In the femurs containing only MC3T3-E1 cells, TRAP staining could be visualized within the growth plate, whereas the femurs containing either MDA-MB-231^{GFP} or MDA-MB-435^{GFP} cells exhibited TRAP staining throughout the femurs with disruption of bone architecture.

Although sensitivity of X-rays (*i.e.* >50% bone osteolysis required to detect a lesion) is low, the findings suggest that MC3T3-E1 cells affected tumor size but did not affect the ability of cancer cells to induce osteolysis. These findings were supported by histomorphometric data showing increased TRAP-positive staining within the lesions (Figure 3B). Therefore, a different signaling axis may exist between breast cancer cells and osteoblasts. Additionally, since the tumors were largely composed of GFP-expressing breast cancer cells, we reasoned it was unlikely that the MC3T3-E1 effect was solely via physical contact.

Our initial data cannot yet explain how the growth effect is sustained by MC3T3-E1 cells during the experiment, and efforts are currently underway to examine this. MC3T3-E1 cells may act to alter stromal cell function, recruit other cell types, or act in a direct manner on breast cancer cells. While much remains unclear about osteoblast signaling in regions of cancer-mediated bone osteolysis, the preliminary results presented here show that crosstalk between tumor cells and cells of osteoblast lineage may be independent of signaling axes involved in breast cancer cell-induced regulation of osteoclasts. With two independently established breast cancer cell lines, the stimulated growth results were dramatic and similar. The possibility that osteoblasts may initially play roles in tumor growth warrants further investigation into the communication between these two cell types. Our results further support important roles for a tumor-osteoblast axis during bone metastasis of breast cancer, which have been underappreciated in the context of well-established and well-studied tumor cell-osteoclast activation.

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