A Mouse Model of Human Breast Cancer Metastasis to Human Bone

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Abstract

Currently, an in vivo model of human breast cancer metastasizing from the orthotopic site to bone does not exist, making it difficult to study the many steps of skeletal metastasis. Moreover, models used to identify the mechanisms by which breast cancer metastasizes to bone are limited to intracardiac injection, which seeds the cancer cells directly into the circulation, thus bypassing the early steps in the metastatic process. Such models do not reflect the full process of metastasis occurring in patients. We have developed an animal model of breast cancer metastasis in which the breast cancer cells and the bone target of osteotropic metastasis are both of human origin. The engrafted human bone is functional, based on finding human IgG in the mouse bloodstream, human B cells in the mouse spleen, and normal bone histology. Furthermore, orthotopic injection of a specific human breast cancer cell line, SUM1315 (derived from a metastatic nodule in a patient), later resulted in both bone and lung metastases. In the case of bone, metastasis was to the human implant and not the mouse skeleton, indicating a species-specific osteotropism. This model replicates the events observed in patients with breast cancer skeletal metastases and serves as a useful and relevant model for studying the disease. (Cancer Res 2005; 65(14): 6130-38)

Introduction

Breast cancer is the second leading cause of malignancy-related death in American women with ~216,000 new cases diagnosed annually (1). Despite successful treatment of the primary malignancy, relapse and subsequent metastatic spread can still occur at distant sites, including bone, lung, liver, and brain (2–4). Development of skeletal metastasis occurs in over 80% to 90% of breast cancer cases that metastasize (2–4) and signals the entry of the disease into an incurable phase.

Many of the serious complications associated with breast cancer are due to these secondary tumors within bone, which can cause pathologic fractures, disability, pain, nerve compression (including spinal cord compression), anemia, and hypercalcemia (4, 5). Whereas important advances have been made in understanding the pathogenesis and treatment of the primary malignancy, little progress has been made in treating skeletal metastasis. Although there has been some recent success with the use of bisphosphonates in the prevention of bony metastases before clinical diagnosis, significant long-term success for patients who are treated with bisphosphonates after clinical diagnosis is not as promising (4). Hence, there is a compelling need to understand at the molecular level the mechanisms by which breast cancer metastasizes to the skeleton to develop agents to treat and prevent skeletal metastases.

The majority of bone lesions associated with breast cancer metastases are osteolytic (4–7). It is thought that breast cancer cells secrete factors that act in a paracrine fashion to activate osteoclasts, leading to bone resorption. Bone resorption is accompanied by release of growth factors and cytokines previously deposited within the matrix during bone formation. These growth factors act, in turn, back on the breast cancer cells to promote their further proliferation, establishing the “vicious cycle” of breast cancer skeletal metastasis (4–7).

The ability of malignant cells to migrate from a primary site, adhere to the bone vasculature, extravasate, and then establish a secondary tumor colony is termed “osteotropism.” This spread of cancer to bone is largely restricted to breast and prostate cancers, and a small number of others, such as lung, thyroid, kidney, and multiple myeloma (4). A major barrier to progress in identifying the mechanisms of breast cancer osteotropism is the lack of animal models that fully reflect the biology of human breast cancer metastasis to bone (7).

Several murine models exist that utilize transgenic mice predisposed to mammary epithelium tumor formation and subsequent metastases, but skeletal metastases are rarely observed (8–12). Another experimental mouse model of metastasis utilizes a cell line derived from a spontaneous mammary tumor (13). This cell line frequently produces lung, but rarely bone, metastases. To overcome this issue, this cell line underwent selection and passing to establish a bone metastatic variant (14).

Currently, human tumor models to study breast cancer metastasis to bone rely on introducing cancer cells directly into the circulation, either by injection into the tail vein or the cardiac left ventricle in immunodeficient mice (15, 16). Whereas these models are very useful for examining and identifying factors involved in proliferation of breast cancer cells that have been directly deposited into the bone environment, they do not replicate the early events in metastasis from the primary tumor site and, therefore, may not encompass the molecular mechanisms by which most breast cancer cells are attracted to bone in patients. In addition, most of these models utilize highly evolved cancer cells (from pleural effusions) that may not necessarily reflect the behavior of tumor cells in vivo.

Recently, important advances have been made in creating animal models of human cancer metastasis to human bone (17–19). Human fetal bone and, recently, adult human rib have been
implanted into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice, a model termed NOD/SCID-hu, and utilized to study prostate and lung cancer osteotropism. In these studies, human prostate cancer cells were administered via tail vein injections or directly introduced into implanted bone (17–19). The human prostate cancer cells formed visible tumors only in the human bone implants and not in the mouse skeleton or in other human or mouse tissues implanted at the same ectopic site. Hence, this experimental model enables the study of human prostate cancer cell metastasis in a tissue-specific and species-specific manner. For these reasons, we sought to extend the NOD/SCID-hu model for the identification and characterization of a breast cancer osteotropic cell line for use in studying the process of breast cancer metastasis to bone.

Materials and Methods

Cells and tissue culture. Cell lines (PC-3, MDA-MB-231, MCF7, MCF10A) were obtained from the American Type Culture Center and cultured under conditions provided by the manufacturer. Cell lines SUM1315, SUM225, SUM229, SUM190, SUM149, SUM159 were provided by Stephen Ethier (University of Michigan) and grown as described (http://www.cancer.med.umich.edu/breast_cell/clines/clines.html).

Cell lines HMLER, HMLER-MMP9, HMLER-TGFβ1 were generated as previously described (20, 21). Briefly, human breast epithelial cells were infected with retroviruses containing the expression vectors for the early region of the SV40 large T antigen, the human catalytic subunit of PKC, and oncocgenic Ras to generate the parental HMLER cells. These cells were subsequently infected with an expression vector that encodes for the human collagenase (MMP9) gene or human transforming growth factor-β1 (TGF-β1) gene (22). HMLER cells were subsequently subjected to retroviral infections of matrix metalloproteinase 9 (MMP9) or transforming growth factor β1 (TGFβ1) with drug selection used to purify polyclonal-infected populations after each infection, as previously described (20, 21). Drug selection of infected cells was done with 700 μg/mL zeocin (zeo). Retroviral constructs for MMP9 and TGFβ1 were generated by cloning the full-length cDNA of human MMP9 (kindly provided by Zena Werb, University of California, San Francisco, CA) or TGFβ1 (provided by R. Derynck, University of California, San Francisco, CA) into the pBabe-zeo vector system (Clonetech, Palo Alto, CA).

Animals and surgery. A colony of immunodeficient (NOD/SCID) mice was maintained in-house under aseptic sterile conditions. Mice were given autoclaved food and water ad libitum. Surgeries were done under sterile conditions on 8- to 10-week-old female mice. Animals received antibiotics (Trimethoprim Sulfam) in the drinking water up to 2 weeks following all surgical procedures.

All human bone tissue for these experiments was procured in compliance with NIH regulations and institutional guidelines, as approved by the Institutional Review Board committee of the New England Baptist Hospital, Beth Israel Deaconess Medical Center, Tufts University School of Medicine, and the Committee on the Use of Humans as Experimental Subjects at Massachusetts Institute of Technology. Human bone was isolated from discarded femoral heads from patients undergoing total hip replacement surgery. Bone was cut into discs 1 to 1.5 cm in width. Two to four hours after removal of the bone from the patient, a bone harvester was used to generate bone cores (1 cm × 5 mm), which were then implanted into the right and left dorsal flanks of 5-week-old NOD/SCID mice that were anesthetized with Avertin.

The bone implants were allowed to engraft in the mice for 4 to 12 weeks. For mice that were subsequently utilized for metastasis assays, cancer cells were introduced into bone-bearing mice 4 weeks after the bone implantation.

In all experiments in which cells were introduced via the tail vein, 2 × 10⁶ cells were resuspended in PBS solution and injected in a volume of 200 μL. For experiments in which cells were introduced i.p. or orthotopically, 1 × 10⁶ cells were resuspended in diluted Matrigel 1:3 (BD Biosciences, Bedford, MA) and injected in volumes of 200 μL i.p., 40 μL in the mammary gland (for breast cancer cells), or 30 μL into the dorsal prostate (for PC-3 cells) using a 31-gauge needle and 50 μL Hamilton syringe.

For primary tumor experiments, four or five animals per cell line (unless otherwise described, with two injection sites per animal) were examined at 8 weeks (in mice given PC3 cells), or when the tumors reached a diameter of 1 cm (for animals injected with breast cancer cell lines). Fluorescence-activated cell sorting analysis and ELISA. Fluorescence-activated cell sorting (FACS) analysis was done on the entire spleen removed from adult female NOD/SCID mice, or female animals harboring human bone fragments at 6 or 10 weeks postimplantation. Spleen cells were isolated by dissociating the tissue with the plunger end of a 10 mL syringe and then passing the cells through a 75-μm cell culture filter. Cells were resuspended in PBS supplemented with 1% fetal bovine serum. One million cells were incubated with antibodies specific for human CD19-FTTC and human IgG-phycocerythrin (PE; BD Biosciences) washed and subjected to FACS analysis.

To assay human immunoglobulins, wells in 96-well Easylight ELISA plates (Corning Incorporated, Corning, NY) were coated with 30 μL of primary rabbit anti-human IgG, specific for heavy and light chain immunoglobulins (Southern Biotechnology Associates, Inc., Birmingham, AL) at a concentration of 2 μg/mL in PBS. The same antibody, conjugated to horseradish peroxidase, was used as a secondary antibody (Southern Biotechnology Associates) at a 1:3,000 dilution in PBS/0.1% bovine serum albumin. Assays were developed using standard techniques with a horseradish peroxidase chromogenic substrate, ABTS (Sigma-Aldrich, St. Louis, MO).

Histology and immunohistochemistry. Immunohistochemistry was done on tissues fixed in 10% neutral buffered formalin and bone tissues were decalcified with 8% fomic acid treatment until soft. Tissues were embedded in paraffin and 5 μm sections were deparaffinized, rehydrated through graded alcohols, and subjected to antigen retrieval for immunohistochemistry (22). Sections were incubated in mouse monoclonal antibodies against smooth muscle actin (Novacstra, Newcastle, United Kingdom), human-specific vimentin (Novacstra), human-specific CD34 (Santa Cruz Biotechnology, Santa Cruz, CA), or pan cytokeratin (DAKO, Carpinteria, CA). Immunocomplexes were visualized by the ABC peroxidase method (Vector Laboratories, Burlingame, CA), and sections were counterstained with hematoxylin or methyl green. Negative controls were carried out on serial sections while omitting the primary antibody. All histologic analyses were examined by a pathologist.

Results

Analysis of functional viability of adult human hip bone in mice. Human tissues implanted beneath the skin of immunodeficient mice are often viable and functional for extended periods of time (23, 24). Consequently, we sought to determine the extent to which fragments of adult human femur could be maintained in mice with preservation of functionality.

To do so, we obtained fragments of human femur from freshly discarded tissue at the time of orthopedic surgery in patients undergoing total hip replacement. In each case, the head of the femur was manually dissected and a bone harvester was used to produce cores of trabecular bone. Female NOD/SCID mice were implanted with bone cores (1 cm × 4 mm × 5 mm; see Materials and Methods) s.c. at 5 weeks of age. At different times ranging from 4 to 12 weeks postimplantation, the human bone implants, mouse spleen, and blood were collected from the xenograft-bearing mice. Histologic analyses of the human bone implants were done (4 and 10 weeks postimplantation) to determine if all the cellular and morphologic components of human bone were detectable and viable.

As seen in Fig. 1, bone marrow cells, osteoclasts, mineralized bone, and stromal cells (including fibroblasts, adipocytes, and endothelial cells) were clearly present in the grafts (12 weeks
after implantation). Moreover, newly synthesized bone was evident in grafts at this late time. However, there was a difference in the integrity of the bone grafts at the two time periods. Whereas bone architecture was preserved at 4 weeks, the viability of bone was impaired, as reflected by the absence of osteocytes. By 12 weeks, however, much, if not all, of the mineralized bone contains newly synthesized bone as evidenced by osteoid formation in areas apposed to mature bone and

**Figure 1.** Histologic analysis of human bone implants. Representative H&E-stained section of human hip bone fragment implanted s.c. in NOD/SCID mice 12 weeks postimplantation (×4; A) and ×20 (B), respectively. Normal architecture is preserved in the implants and all the cellular components are maintained and viable. Newly synthesized bone, osteoclasts, and blood vessels are all observed 12 weeks after implantation (B, arrows). Immunohistochemistry was done on serial sections using human-specific CD34 antibodies to delineate human blood vessels in bone xenografts (C, D, brown stain). D, ×20 magnification of the area outlined in (C). Adjacent mouse tissue stained negatively for human-CD34 (E). Arrows indicate blood vessels that are negative. In contrast, as a positive control, blood vessels in the stroma of human breast tissue (F) stained for human-CD34. G, H&E section of human bone implants showing human bone marrow still localized in the marrow spaces, which is rarely observed at 12 weeks postimplantation.
viable osteocytes, indicative of osteoblastic activity (Fig. 1A and B). At this stage following engraftment, the question of whether the osteoclasts are of human origin or derived from circulating mouse progenitors has not been answered. Because little to no necrosis was evident in the grafts throughout the observation period, we examined their vascularization (Fig. 1C-E). Neangiogenesis was detected in and surrounding the implanted bone. To determine the species origin of the endothelial cells of the blood vessels in the bone implants, sections were examined by immunohistochemistry using human-specific CD34 antibodies. Specific blood vessels in the center of the grafts stained positive for human CD34, whereas the blood vessels in the periphery were negative (Fig. 1C-E). As a positive control for specificity of this antibody, human breast tissue was stained. The blood vessels in the stroma and surrounding the ducts stained positive for CD34 (Fig. 1F). This indicates that mouse vasculature was recruited to supply the implanted bone, and that the human vasculature remained viable within the bone for periods of 12 weeks or longer postimplantation.

In 95% of the engrafted bone implants, marrow spaces were preserved. These spaces had a high degree of cellularity (adipocytes and fibroblasts). However, few, if any, mature hematopoietic cells (T cells and B cells) were apparent histologically (Fig. 1G). Therefore, we examined the levels of human IgG in the blood of mice that received bone implants 6 and 10 weeks earlier. Mouse serum was subjected to ELISA assay, which showed that human IgG was present at levels of ~100 μg/mL. This shows that the human B cells or plasma cells in the marrow implanted along with the bone remain viable and continue to produce immunoglobulin for an extended period after implantation. These findings are in agreement with previous studies that reported the detection of human antibodies in the serum of mice implanted with human hematopoietic cells (25).

We next wished to determine if human hematopoietic cells from the bone marrow could be observed in this system. During hematopoiesis, hematopoietic stem cells from the bone marrow develop into defined lineages of precursor cells, which ultimately yield fully differentiated cells. During this process, precursor B cells begin to mature in the bone marrow, but then home to the spleen where they complete differentiation. Therefore, the presence in the mouse spleen of human B cells derived from the implanted bone would provide further evidence of the intactness and viability of the engrafted human bone and marrow.

Spleen was isolated from mice that did not receive human bone and from mice previously engrafted with human bone for 6 and 10 weeks. Flow cytometry was done on single-cell preparations of the entire spleens using markers specific for human B cells (human CD19 and human IgG; Fig. 2). Double-labeled positive cells were not detected from preparations of spleen derived from mice devoid of human bone. In contrast, over 4% of the cells from the spleen of a mouse carrying human bone for 6 weeks stained for both human CD19 and human IgG (Fig. 2B). Moreover, 16.5% of the mouse spleen contained a human CD19-, human IgG-positive population of cells from mice carrying bone for 10 weeks. This implies that the systemic machinery necessary for B-cell homing to spleen is intact in this model. The finding that human cells migrate from one site to colonize another shows the feasibility of producing similar results for cancer cells in the model of metastases we set out to create. Furthermore, these experiments confirm the vitality and functionality of human bone fragments...

**Figure 2.** FACS analysis of human B cells in mouse spleen. Cells were isolated from the spleen of NOD/SCID mice not implanted with human bone (control) or mice implanted with human bone 6 or 10 weeks before analysis. Single cell suspensions were labeled with antibodies specific for human IgG-PE and CD19-FTIC. FACS histogram of the PE fluorescence of IgG-positive cells from the total number of gated human CD19-positive cells. The total counts were normalized to the background autofluorescence observed in this assay from mouse splenocytes.
These human cell lines were MDA-MB-231, MCF7, MCF10A cells, various SUM lines derived from primary or metastatic breast cancer samples (SUM 159, SUM 149, SUM 225, SUM 229, SUM 190, SUM 1315), as well as the genetically engineered HMLER lines previously described (8, 20).8

First, we wanted to determine the efficiency with which these various cancer cell lines develop primary tumors at the orthotopic site. Breast cancer or prostate cancer cells were injected into the mature adult mammary gland or prostate, respectively, and monitored for tumor growth until tumors reached a diameter of 1 cm. The tumorigenicity of the various cell lines is summarized in Table 1. As expected from previous studies, MDA-MB-231 and PC-3 cell lines (which could not form lung metastases upon tail vein injection) could form metastases when implanted orthotopically.

Upon i.p. injection, SUM1315 cells also gave rise to microscopic metastases in the lungs of mice 8 weeks after tail vein injection of the breast cancer cell lines. One cell line, SUM1315, did give rise to a large palpable mass within the implanted human bone fragment after tail vein injection. A substantial portion of the mass of this tumor was composed of immune cells (Fig. 3G and H). Upon i.p. injection, SUM1315 cells also gave rise to microscopic metastases in the human bone implants. Again, we observed immune cells surrounding nests of tumor cells (not shown). From these experiments, we conclude that of all lines tested, only cells from the SUM1315 line can colonize both lung and bone when given by either the tail vein or i.p. route.

We next tested whether cells from the SUM1315 or the other cell lines (which could not form lung metastases upon tail vein injection) could form metastases when implanted orthotopically. Breast cancer cells were injected into the mammary glands of NOD/SCID-hu mice or into the prostate gland (for PC-3 cells). The mice were then monitored for development of metastases (Table 2).

Whereas we detected visible metastases in lung and other organs after PC-3 administration, no bone metastases were found. Cells from all the breast cancer lines did form primary tumors in the mammary glands of the NOD/SCID-hu mice, but only SUM1315 cells formed lung and bone metastases. SUM159 cancer cells were detected within blood vessels of the mammary gland, but no visible metastases were observed. This suggests that some cell lines that form nodules in the lung after tail vein injection cannot form the same metastases after i.p. or orthotopic injection, supporting the notion that the true metastatic

### Table 1. Primary and metastatic behavior of cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Primary tumor latency (wk)</th>
<th>Primary tumor frequency (%)</th>
<th>Observed metastasis</th>
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<tbody>
<tr>
<td>SUM29</td>
<td>4</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>SUM190</td>
<td>6-8</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>SUM149</td>
<td>6-8</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>SUM159</td>
<td>2-4</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>SUM1315</td>
<td>8-12</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>SUM225</td>
<td>6</td>
<td>75</td>
<td>—</td>
</tr>
<tr>
<td>HMLER</td>
<td>6-8</td>
<td>85</td>
<td>—</td>
</tr>
<tr>
<td>HMLER-MMP9</td>
<td>6-8</td>
<td>80</td>
<td>—</td>
</tr>
<tr>
<td>HMLER-TGFβ</td>
<td>4-6</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>MCF7</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>6</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>MCF10A</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PC-3</td>
<td>4-6</td>
<td>100</td>
<td>—</td>
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Note: Tumor latency is defined by the time in which the tumor reached 1 cm in diameter. Five mice [bilateral injections (10 total sites) for breast cancer cells] were analyzed for each cell line. All cell lines were introduced into mice via tail vein (t.v.), i.p., or orthotopic (ortho) injection and examined for the presence (+) or absence (−) of metastases in the animals and the location of the respective metastases observed (lung or bone).

The engrafted human bone was examined for metastases. Unlike previous reports (18, 19), we were unable to detect bone metastases from the prostate cancer cell line PC-3, either at the macroscopic or microscopic level, perhaps because we used a different kind of bone than used by others. Moreover, with one exception, no bone metastases were detectable following tail vein injection of the breast cancer cell lines. One cell line, SUM1315, did give rise to a large palpable mass within the implanted human bone fragment after tail vein injection. A substantial portion of the mass of this tumor was composed of immune cells (Fig. 3G and H). Upon i.p. injection, SUM1315 cells also gave rise to microscopic metastases in the human bone implants. Again, we observed immune cells surrounding nests of tumor cells (not shown). From these experiments, we conclude that of all lines tested, only cells from the SUM1315 line can colonize both lung and bone when given by either the tail vein or i.p. route.

http://www.cancer.med.umich.edu/breast_cell/Production/sumlines/SUM1315MO2.html

8 http://www.cancer.med.umich.edu/breast_cell/Production/sumlines/SUM1315MO2.html
phenotype cannot be ascertained through the expedient of tail vein injection. Cells that form nodules in the lung are not necessarily the cells that can be trapped mechanically, but rather cells that are intrinsically metastatic from primary tumor sites. These various experiments indicate that one human breast cancer cell line, SUM1315, is able to form a primary tumor when introduced into the mammary gland of mice and subsequently form metastases in human bone and mouse lung. This cell line was unique in displaying these properties and served as the basis for our subsequent experiments.

Characterization of SUM1315 from its orthotopic site. Cells of the SUM1315 line, the only one of the above-described cell lines that formed osteotropic metastases, was originally isolated from a xenografted metastatic nodule of a patient with invasive ductal carcinoma. SUM1315 cells have previously been examined for various breast cancer markers and found to display a profile characteristic of invasiveness. These tumors were highly invasive within the mammary glands and in the surrounding muscle (Fig. 4A and B). These cells are negative for estrogen and progesterone receptors, E-cadherin, and low-molecular weight cytokeratins.
(Fig 4; data not shown). However, SUM1315 cells express P-cadherin and β-catenin, high levels of Her2/Neu and the epidermal growth factor receptor, and are mutant at the TP53 locus (27).

When human bone was implanted into NOD/SCID mice before the introduction of breast cancer cells, a slight delay in latency of primary tumor growth was observed and, accordingly, primary tumors reached 1 cm in diameter at 10 to 12 weeks. Yet, bone and lung micrometastases were observed in the mice (Fig. 4; Table 2). Micrometastases were detected in the implanted human bone in 4 of 20 mice and lung micrometastases and macrometastases were observed in 6 of 20 mice. In all the cases of bone metastases, mice also harbored visible lung metastases, yet there were two cases where lung metastases developed without concomitant bone metastases. SUM1315 represents the first human breast cancer cell line that was not isolated from a pleural effusion reported to possess metastatic capabilities.

To examine whether the SUM1315 cells could also metastasize to the mouse skeleton, whole body X-ray analysis was done on mice injected with SUM1315 cells at the orthotopic site that formed tumors (12 weeks following injection). Ten animals were examined for the presence of metastases in the mouse skeleton. No lesions were detected in any of these animals. Specific attention was paid to regions known to display osteolytic lesions after intracardiac injection of human breast cancer cells. In these regions, no evidence of metastasis was detected (Fig. 4G). This suggests that although SUM1315 breast cancer cells are metastatic to the mouse lung in this model, osteotropism is species specific.

Irradiation of mice with human bone decreased immune response against SUM1315 metastases. The finding of immune cells in the vicinity of SUM1315 bone metastases suggested to us that the relatively weak observed metastatic propensity of these breast cancer cells might be caused by an immunologic response against them. Therefore, we attempted to enhance the metastatic frequency or burden through partial ablation of the human bone marrow, hoping thereby to retain bone marrow functions needed for cancer osteotropism while weakening the antitumor immune response. NOD/SCID mice were implanted with human bone as previously described. Four weeks postimplantation, mice were irradiated with a sublethal dose of γ radiation (3 Gy). This dose was chosen because of its immunosuppressive consequences without adverse side effects. One million SUM1315 cells were implanted orthotopically 24 hours after irradiation. Mice were sacrificed 12 weeks postinjection and analyzed for metastasis.

The frequency of lung metastases was not enhanced as a result of irradiation, but an increase in the number of bone metastases was observed from 20% (4 of 20) to 38% (3 of 8; Table 2). Moreover, the bone metastases were larger in irradiated mice compared with unirradiated mice. More importantly, the apparent immunologic response against the SUM1315 cells was absent (Fig. 4H and I). Unlike the situation in which animals were implanted with bone but not irradiated, there were no immune cells surrounding the nests of cancer cells in irradiated mice. Instead, the bone metastases were now osteolytic and in direct contact with the bone tissue without a rim of immune cells around the cancer nodules. Irradiation enabled larger outgrowths of metastatic nodules and significantly dampened the immune response. This suggests that the general low frequency of metastasis in the model is due, at least in part, to the immune reaction of human marrow cells against the arriving cancer cells.

**Discussion**

A major impetus to the study of breast cancer osteotropic metastasis has been the difficulty in obtaining clinical skeletal metastases. Ideally, one would like to compare skeletal metastases to primary malignancies from patients, but bone biopsies are rarely obtained and autopsy samples are generally unsatisfactory for studies such as gene transcriptional analysis. We report here the creation and characterization of a mouse model of human breast cancer skeletal metastasis that replicates all of the steps in the process of breast cancer migration from its orthotopic site to bone. Critical to creating such a model is the engraftment of functional and viable human bone in immunodeficient mice and the identification of a human breast cancer cell line that displays the osteotropic phenotype.

Based on morphologic and immunohistochemical evidence, we showed that adult human bone fragments are both viable and functional for at least 12 weeks post-transplantation. All bone cell types (osteoblasts, osteoclasts, osteocytes, endothelial cells, adipocytes, fibroblasts, marrow, and stromal cells) are present and apparently viable. The marrow is functional, as evidenced by high levels of human immunoglobulin (IgG) and the presence of circulating human B cells in the mouse circulation. Human blood vessels are also present in the bone xenografts, as indicated by the display of human CD34 on vascular endothelial cells. In addition, an angiogenic response is elicited in the mouse, as evidenced by extensive neovascularization that arises to supply the xenograft.

We also wished to determine if any organ tropism could be shown in this system before evaluation of breast cancer cell lines for the osteotropic phenotype. Therefore, we examined the ability of human B cells from the implanted human bone marrow to migrate into the mouse spleen. Flow cytometry was done on cells isolated from spleen using CD19 and human IgG as human-specific markers. By 10 weeks post-transplantation, 4% to 16.5% of the cells in the spleens of mice were shown to be human B cells. This indicates that the engrafted human marrow is functional and associated bone marrow cells can home to their appropriate target organ, all of which made it plausible that osteotropic metastases could be observed in this model.

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**Table 2. Metastatic frequency of cancer cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Frequency</th>
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<tbody>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>SUM159*</td>
<td>2/3</td>
</tr>
<tr>
<td>SUM149*</td>
<td>2/3</td>
</tr>
<tr>
<td>SUM1315</td>
<td>6/20</td>
</tr>
<tr>
<td>PC-3</td>
<td>6/10</td>
</tr>
<tr>
<td>MDA-MB-231*</td>
<td>3/3</td>
</tr>
<tr>
<td>SUM1315 + IR†</td>
<td>2/8</td>
</tr>
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*Metastasis only detected from tail vein injections.
†Metastasis only examined from orthotopic injections.
To identify a cell line that can metastasize to human bone, cells from a panel of 12 human breast cancer lines were injected via the tail vein. These cell lines were selected because they display various degrees of local invasiveness and propensity to metastasize to different organs. Of all the tumor cell lines tested, osteotropic metastases were only observed following injection of SUM1315 cells, which are derived from a metastasis in a patient with infiltrating ductal carcinoma.

Eight weeks after tail vein injection of SUM1315 cells into NOD/SCID mice, micrometastases were observed in the human bone graft. Surprisingly, no lung metastases developed. Subsequently, we conducted experiments in which breast cancer cells were introduced into the orthotopic (mammary pad) site instead of the tail vein. Of the eight cell lines injected, the SUM1315 cell line again was the only one that metastasized to human bone at the 10-week time point. Lung metastases were also detected at a later time. We examined the mouse by X-ray analysis and did not observe metastasis in the mouse skeleton, suggesting a species-specific mechanism for osteotropism, as has been reported in earlier models based on prostate cancer (17–19).

The fact that bone metastases to the human implants were not observed more frequently may be because only a certain

Figure 4. Histologic analysis of SUM1315 tumors. One million SUM1315 cells in matrigel were injected into the fourth inguinal mammary gland of NOD/SCID females harboring human bone implants. Eight weeks following injection, tumors were removed and examined by H&E. Tumors were highly invasive into the muscle (A) and the adjacent normal mammary tissue (B). Arrows illustrate the invasive front. SUM 1315 tumors are negative for pan-cytokeratin (C), suggestive of an epithelial-to-mesenchymal transition, as the endogenous mammary epithelium is positive for keratin (arrows). Lung metastases were detected grossly and by histology 8 to 12 weeks after injection (D−F). H&E-stained sections of lung metastases (D and E) as well as keratin immunohistochemistry (F) demonstrating the metastases are also not positive for keratin, like the primary tumors. G, a representative X-ray micrograph of the lower vertebrae, hind limbs (typical sites of breast cancer metastases) of an animal bearing SUM1315 tumor, showing no evidence of osteolytic metastases. H, H&E-stained section of an osteolytic bone lesion that was observed in a human bone implant, which was irradiated before cancer cell injection. I, >20 magnification of (H), demonstrating osteolytic resorption of bone.
proportion of cells within the SUM1315 cell population are metastatic, and an even smaller percentage are metastatic to bone. Therefore, we are currently attempting to isolate more highly metastatic variants of the SUM1315 cells, selective for bone or lung.

When examined microscopically, colonies of metastatic cancer cells in the bone were observed in interface with or were infiltrated by immune cells in what seems to be transplantation rejection or “graft versus graft” reaction (because the two human organs—bone and breast cancer—are histoincompatible because they originate from different patients (Fig. 3). It was not surprising that the marrow of the bone implant mounts an immune response to the metastatic cells. This finding was suggested in earlier reports using other human cancers (17). Our observation provides further evidence that the engrafted human marrow is functional. The immune reaction cannot result from circulating mouse cells populating the marrow of the human bone implant because NOB/SCID mice lack such cells.

This immune reaction may create some challenges for the refinement of this experimental model and development of quantitative assays. It is possible that >1 of the 12 cell lines we tested is intrinsically capable of seeding osteotropic metastases. It is possible that >1 of the 12 cell lines we tested is intrinsically capable of seeding osteotropic metastases. It is possible that >1 of the 12 cell lines we tested is intrinsically capable of seeding osteotropic metastases.

References
A Mouse Model of Human Breast Cancer Metastasis to Human Bone

Charlotte Kuperwasser, Scott Dessain, Benjamin E. Bierbaum, et al.


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