A TALE OF MICE AND (WO)MEN: DEVELOPMENT OF AND INSIGHTS FROM AN “ALL HUMAN” ANIMAL MODEL OF BREAST CANCER METASTASIS TO BONE

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ABSTRACT

There are 200,000 new cases of breast cancer (BrCa) annually in the United States. Metastasis to bone signals a life-threatening phase of this disease. Little progress has been made in understanding the pathogenesis of metastasis. Few validated drug targets have been identified. So there is a compelling need to understand the molecular mechanisms by which BrCa metastasizes to bone (osteotropism).

There is need for animal models that reflect the complex biology of metastasis in humans. We performed research designed to elucidate the mechanisms of osteotropism from both sides of the tumor–stroma interface. We created an “all human” model in which human bone is transplanted into non-obese diabetic/severe combined immunodeficient immunodeficient (NOD/SCID) mice. Human BrCa cells injected into the mammary fat pad later metastasize to bone.

We also found traffic in the opposite direction: bone marrow stem cells migrate from human bone to human breast tumors in the mouse.

We are identifying osteotropism genes used by BrCa to metastasize to human bone.

INTRODUCTION

Approximately 200,000 new cases of BrCa and 40,000 BrCa deaths occur annually in the United States (1). After the breast itself, the skeleton is the next most common site for BrCa (2, 3, 4, 5). In many respects, the problem of metastasis to bone is more serious than the original tumor; it is one of the most life-threatening complications of BrCa. Other complications include pathologic fractures, disability,
pain, and hypercalcemia (6). Although important advances have been made in understanding and treating the primary malignancy, less progress has been made regarding metastasis. The field of BrCa metastasis lacks sufficient validated targets to propel efforts to discover metastasis-specific therapies. Such treatments, combined with strategies to eradicate the primary malignancy, would have high clinical impact. Hence, there is a compelling need to understand the molecular mechanisms by which BrCa metastasizes to bone, and an urgent unmet medical need for safe and effective agents to prevent or treat BrCa skeletal metastases.

The Metastatic Pathway

An impediment to research on bone metastasis has been the complexity of the process. Spreading from their site of origin to distant organs, tumor cells must first depart the primary colony, migrate through extracellular matrix (ECM), and enter the blood or lymph. Although disseminated widely, cancer cells adhere to the vascular endothelium of only certain organs. They then extravasate into the stroma, where they respond to the new environment. Surviving cells establish themselves first as micrometastases, and then many become life-threatening secondary tumors (7, 8, 9). A feature unique to the bone environment is that, to generate space to expand, cancer cells must stimulate osteoclasts; tumor cells themselves cannot resorb bone (10, 11, 12). Therefore, tumor cells must secrete factors that “hijack” the physiological bone resorption apparatus (e.g., osteoclasts) — a critical feature of the tumor-stroma interaction for bone metastasis (13, 14, 15).

BrCa is one of only a few cancers that metastasize to bone (5). Stromal interactions are important and distinctive for BrCa osteotropism. BrCa cells display cell-surface adhesion molecules, such as the αvβ3 integrin, that bind to bone matrix proteins. BrCa cells may adhere preferentially to the bone marrow vasculature because endothelial cells differ phenotypically across organs (16, 17, 18, 19). Furthermore, BrCa cells secrete parathyroid hormone–related protein (PTHrP), enabling them to recruit a “shell” of osteoclasts around a metastatic colony (10, 12, 20, 21). As the osteoclasts resorb extracellular matrix (ECM), transforming growth factor β (TGFβ) is released, stimulating tumor growth and increased PTHrP secretion in a “vicious” cycle (22, 23). Once within the bone marrow, BrCa cells find an especially hospitable environment for harboring metastases because bone marrow contains mitogens and other factors that normally support hematopoi-
esis and adult stem cells. Also present are numerous chemokines and growth, angiogenic, and anti-apoptotic factors (10, 12, 24, 25, 26, 27, 28, 29). Hence, bone is replete in factors that can select and sustain malignancies.

**Animal Models of Osteotropism**

One barrier to identifying the mechanisms of osteotropism has been the lack of animal models that reflect the complex biology of the metastatic process in humans (30). Murine tumor models exist, but syngeneic models of BrCa rarely generate bone metastasis from the primary site (31). An increasing number of differences between murine and human tumors have been documented; therefore, it is important to use human BrCa cells that metastasize naturally to the skeleton. The most commonly used models of skeletal metastasis, based on intravenous (i.v.) tail vein administration of cancer cells, bypass the early steps of metastasis, i.e., BrCa cells migrating from the primary site and entering the bloodstream. This approach typically generates lung metastases. In other models, human BrCa cells are administered via the intracardiac route or are forced into animal bone through direct inoculation, yielding human cancer “metastases” in mouse bone (32, 33, 34). These models do not select for cells capable of completing the entire metastatic cascade: thus, the phenotype represented may not accurately reflect that of metastatic cells in the bone of patients.

For human BrCa, only a model in which tumor is first established at the orthotopic site obligates BrCa cells to take all the steps in the metastasis pathway.

**Approaches to Identifying Genes and Mechanisms of Osteotropism**

Elucidation of the mechanisms responsible for BrCa osteotropism holds the promise of revealing innovative metastasis-specific therapeutic approaches. It is conceivable that all breast cancers use the same genetic “toolkit” to spread to bone. It is also possible that other malignancies, such as prostate cancer, also use the same toolkit. If this proves true, then it may be feasible to discover drugs that block cancer metastasis to bone, regardless of the cancer type.

To identify osteotropism genes, many practical obstacles must be overcome. A comparison of transcriptional profiles between clinical samples of skeletal metastases and primary malignancies would be revealing. However, bone biopsy specimens are rarely obtained; when they are, samples of the primary are often not available. Autopsy
samples are valuable in theory, but must be acquired quickly post-mortem before mRNA degrades. Human BrCa cells obtained from human bone in a non-obese diabetic/severe combined immunodeficient (NOD/SCID) mouse model are an attractive alternate source of tissue for gene expression profiling. Because the model can be used to compare metastases to primary tumors that are genotypically equivalent, but have distinct phenotypes, the model should facilitate observation of gene differences of genuine interest.

Transcriptome profiling is one important means to identify osteotropism genes and possible targets for discovery of metastasis-specific therapies. Genomic technologies can rapidly generate multiple disease hypotheses by parallel query of thousands of data points and enable correlation of gene profiles with phenotypes defining disease mechanisms at a molecular level. These data have the potential to yield diagnostic disease markers and "gene signatures" (35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46).

Presumably, only a subset of tumor cells express the genetic repertoire necessary to complete the multi-step process of organ-specific metastasis (47, 48). Clinical and experimental evidence indicates that metastasis is an inefficient process: less than 0.1% of cells can generate secondary tumors (13, 49, 50).

MATERIALS AND METHODS

An “All Human” Animal Model of Breast Cancer Metastasis

We created an “all human” model of BrCa metastasis to bone that recapitulates the events observed in patients who have skeletal metastasis: BrCa cells originating at the orthotopic site metastasize to transplanted human adult bone (51). We obtained fragments of human femur from freshly discarded tissue at the time of surgery in patients undergoing total hip replacement. Female NOD/SCID mice were implanted subcutaneously with trabecular bone cores (Figure 1). By 12 weeks, new bone appears to be synthesized, as evidenced by osteoid apposed to mature bone and osteocytes, indicative of osteoblast activity.

Selection of Bone-residing BrCa Cells

We used SUM1315 cells, isolated by Steven Ethier (then at the University of Michigan) from a xenografted metastatic nodule of a patient with invasive ductal carcinoma (52) and bone metastases, for serial passaging via direct injection into human bone in vivo.
SUM1315 cells are negative for estrogen and progesterone receptors, E-cadherin, and high MW cytokeratins. They express P-cadherin and β-catenin, high levels of Her2/Neu and EGF receptor, and are mutant at the TP53 locus. Cells were injected directly into human adult femur fragments implanted in NOD/SCID mice. After 12 weeks, tumors were harvested and transferred to cell culture. After expansion in culture, cells were injected into another set of implanted femur fragments to form bone-residing tumors, and then harvested for transcriptome analysis.

Identification of Candidate Osteotropic Genes by Transcriptome Analysis

To demonstrate the feasibility of identifying candidate osteotropism genes, we used BrCa cells that were injected directly intraosseously. SUM1315 cells, twice passaged through human bone in vivo, were used as the basis for transcriptome analysis by DNA microarray methods.
Creation of an “All Human” Model of BrCa Metastasis to Bone From the Orthotopic Site

We have developed a NOD/SCID-hu model of BrCa metastasis to bone in which the BrCa cells and the bone are both human (51). To test whether SUM1315 form metastases when implanted orthotopically, SUM1315 cells expressing a luciferase gene were injected into the mammary fat pad (MFP) of female NOD/SCID mice bearing implanted human bone fragments. Tumor burden was assessed in anesthetized mice after administration of luciferin using a Xenogen IVIS-200 instrument, a non-invasive optical bioluminescent imaging method best suited for surface anatomy such as bone grafts. The sensitivity of this approach enables us to locate small clusters of BrCa cells and quantify tumor burden.

SUM1315 cells form bone metastases 8 to 12 weeks after orthotopic inoculation (Figure 2). Because the anatomical location of the human bone in mice is anomalous, our positive result confirms the notion that metastases develop in certain organs in a way that cannot be explained by bloodstream or lymphatic drainage patterns and non-specific entrapment (53, 54, 51). Rather, these findings implicate specific mechanisms and trophic factors as responsible for osteotropism. These experiments indicate that the human BrCa cell line, SUM1315, is able to form a primary tumor in MFP and subsequently metastasize to human bone and, at a later time point, mouse lung.

FIG. 2. Xenogen imaging of mice implanted with human bone grafts. Human BrCa (SUM 1315) luciferase-expressing cells were injected originally in the mammary fat pad. Reprinted with permission from Moreau et al (64).
**Human Tissue-engineered Bone as a Homing-site for BrCa**

We are also interested in elucidating the contribution of bone to BrCa osteotropism. We created human tissue-engineered bone for use as a potential target for BrCa metastasis. Using silk fibroin protein sponges as scaffolds to which are added human bone marrow stromal cells (hBMSCs), we were able to create *in vitro* biocompatible 3-D porous silk-based biomaterials that are bone-like in structure (Figure 3) and biomechanical properties. The experiments we conducted are as follows: specimens of silk-based tissue-engineered bone and native human bone “controls” were implanted subcutaneously in immunodeficient (NOD/SCID) mice, with subsequent injection of the luciferase-expressing human epithelial BrCa cell line, SUM1315, into the orthotopic site. Engineered-bone scaffolds were prepared from silk fibroin, and seeded directly with passage-2 human BMSCs on the day of implantation into

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*Stem cells are contained within the mix of marrow-derived stromal cells. Whether or not these are true stem cells has not been determined definitively. The cells are multipotent, self-renewing, and able to form bone under these circumstances."

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**FIG. 3.** Water-based human tissue-engineered bone scaffold. Inset depicts porosity of scaffold as imaged by scanning electron microscopy. Reprinted with permission from Kim et al (63).
mice. Concurrently, native human bone and engineered-bone were implanted subcutaneously over each shoulder. One month after implantation, labeled SUM1315 cells were injected orthotopically into mouse MFPs and monitored over the course of 12 weeks. Xenogen-imaging of bone implants at final harvest revealed the presence of metastases in both the tissue-engineered scaffolds and native human bone implants in mice (Figure 4). Metastatic spread was exclusive to implanted bone (engineered and native), with no luciferin signal evident in the mouse skeleton (images not shown), despite extensive scanning.

**Engineered Bone for Tumor Tropism**

Silk scaffolds were created and differentiated into tissue-engineered bone (TEB) as previously reported (55). Specifically, scaffolds seeded with $1 \times 10^6$ P2 hBMSCs were differentiated *in vitro* for 2 weeks and reseeded with $1 \times 10^6$ fluorescently labeled P3 hBMSCs 1 day before implantation, which were tracked for tumor tropism.

![Image](image.png)

**FIG. 4.** Xenogen imaging of SUM1315 luciferase-expressing BrCa cells 12 weeks after orthotopic injection. Human bone implant (*right side*) and tissue-engineered bone (*left side*). Reprinted with permission from Moreau et al (64).
hBMSC Differentiation

Osteogenic and adipogenic differentiation media and Oil Red-O and Alizarin Red staining for validation of hBMSC pluripotency have been previously described (51).

Animal Experiments

The Tufts University Division of Laboratory Animal Medicine and Institutional Animal Care and Use Committee approved all animal procedures. Metastasis and hBMSC-tumor homing procedures, including imaging, TEB implantation, tumor formation, and histology were described previously (55). Two weeks after TEB implantation, mice were sacrificed, and tumors and TEB were removed for analysis.

RESULTS AND DISCUSSION

Potential Osteotropism Genes

A comparative genomic hybridization of a bone-residing BrCa cell population versus the MFP-residing xenografts was performed and found 160 genes down-regulated and 337 genes up-regulated by > 1.4-fold. We are currently focusing on three for validation: the interleukin-17B receptor (IL-17BR), matrix metalloproteinase-13 (MMP-13 or collagenase 3), and hormonally up-regulated Neu-associated kinase (HUNK). IL-17BR is a cytokine secreted by bone marrow mesenchymal stem cells and hence of interest in tumor-stroma interactions. The ligand-receptor interaction is a pro-inflammatory response activating NF-κB, while promoting osteoclastogenesis, a key tumor-stroma interaction in bone. MMP-13 may play a role in BrCa invasion by helping to resorb ECM, creating expansion space for metastases. MMP-13 is expressed by BrCa cells, is elevated in bone with metastases, and is associated with osteolysis. HUNK expression accompanies changes in breast tissue during pregnancy. The gene set we identified in preliminary fashion (IL-17BR, MMP-13, and HUNK) has no overlap with the osteotropism set (IL-11, MMP-1, CXCR4, and CTGF) identified by Kang et al (34). However, the functional categories represented in each set are similar: homing, invasion, and osteoclast-mediated osteolysis. Although functions are similar, differences in specific genes between the two sets may arise because the tissue we analyzed is a human BrCa cell population selected by human bone, not mouse skeleton. Importantly, both laboratories interrogated genes identified by the other, so the differences detected appear authentic.
“Homing” of BrCa Cells to Tissue-engineered Bone

We have now demonstrated that human BrCa cells will metastasize from the orthotopic site to human-engineered bone implanted in NOD/SCID mice (Figure 4). This finding indicates that engineered bone is sufficiently replete in bone elements to function as a target site for human BrCa osteotropism. Our observations also demonstrate that it will be possible to manipulate the “bone-side” of the tumor-stroma interaction.

Implanted TEB retained a bone phenotype and fluorescent hBMSCs for the duration of the experiment (2.5 weeks; Figure 5). Clinically,
TGFβ1 is elevated in the plasma of breast cancer patients and is linked to increased cancer progression and metastasis (56). In addition, TGFβ1 is produced by aggressive BrCa cells and can attract hBMSCs in in vitro assays and physiologic bone development (57, 58) (Figure 6). We hypothesized that hBMSCs migrate toward BrCa cells in response to elevated TGFβ1 levels and confirmed this in vitro (Figure 6). In addition, blockade of TGFβ1 in BrCa cell conditioned media by using a neutralizing antibody significantly reduced hBMSC migration (Figure 6). TGFβ1 blockade did not inhibit hBMSC migration toward MDA-MB-231-CM, perhaps due to the abundance of other cytokines in the supernatant. These results further support the theory that inflammation attracts hBMSCs to tumors and suggest that TGFβ1 may play a large role in attracting hBMSCs.

“Homing” of Bone Cells to BrCa

By passaging SUM1315 BrCa cells through human bone, we created the SUM1315-BP2 cell line with a unique gene expression signature that represents a “bone-educated” cell line. To analyze the ability of hBMSCs to home from the bone environment to MFP tumors in vivo, we incorporated previously developed TEB into a novel hBMSC-tumor homing model (51). In the model, TEB delivers fluorescently labeled hBMSCs that migrate to breast cancer tumors as assessed by confocal imaging and fluorescent antibody cell sorting (FACS).

Consistent with published reports, hBMSCs showed tumor-type dependent effects on BrCa proliferation in vitro and in vivo (59).

Because tumor growth does not necessarily correlate with metastatic outcome, we next investigated whether hBMSCs cocultured with BrCa cells could increase BrCa migration metastasis in vivo. To assess metastasis after 10 weeks in vivo, specifically to bone, we used a humanized model of breast cancer metastasis to bone developed in our laboratory (51). Coinjection of MDA-MB-231 BrCa cells and hBMSCs into the MFP increased the frequency of metastasis to human bone, lung, and liver. SUM1315-BP2 BrCa’s coinjected with hBMSCs exhibited an increased skeletal metastasis frequency.

The gene expression signature of SUM1315-BP2 BrCa cells is enriched for genes that may promote metastasis. One of these genes, IL-17b receptor (IL-17BR), is a prognostic indicator of BrCa progression and metastasis and, along with its ligand, IL-17b (IL-17B), is linked to bone turnover and tumor progression (60, 61, 62).

We have created a model that reflects the complete process of human breast cancer metastasizing to adult human bone from a primary
FIG. 6. TGFβ1 secretion from BrCa cells may attract hBMSCs to the primary site. (A) In vitro conditioned medium samples from more aggressive BrCa cells, i.e., SUM1315 and MDA-MB-231, contain increased levels of TGFβ1 when compared with weakly metastatic BrCa cells (MCF7). Conditioned medium was collected in serum-free medium for 48 hours from cells grown to 80% confluence (N = 3 independent plates). Medium was concentrated 10X and TGFβ1 expression was quantified using ELISA (eBioscience). TGFβ1 levels are shown relative to MCF7 levels. (B) Exogenous TGFβ1 can attract hBMSCs in vitro and a neutralizing antibody to TGFβ1 (α-TGFβ1) can block migration of hBMSCs towards BrCa cell–conditioned medium. Conditioned medium was collected in serum-free medium from cells grown to 80% confluence. Medium was used as a chemoattractant with or without α-TGFβ1 (60 μg/mL). 30,000 serum-starved hBMSCs were plated above conditioned medium and allowed to migrate for 6 hours. Migration filters were fixed and stained and the number of cells migrated were counted from three fields of view/well and averaged. Addition of exogenous TGFβ1 (32 ng/mL) to serum-free medium increases migration of hBMSCs; this increased migration can be blocked by addition of α-TGFβ1. N = 5 or 6 for each group. Reprinted with permission from Goldstein et al (59).
orthotopic site in the breast. Such a model should have advantages in enabling the identification of osteotropism-associated genes in human breast cancer and in evaluating the role of such genes and efficacy of potential therapies targeted at skeletal metastases. Using this system to identify lines that can metastasize to the lung or the bone with higher frequencies and selectivity will serve as a valuable resource for studying tissue-specific metastasis.

Combining a mouse model of breast cancer metastasis with tissue-engineered bone has enabled us to demonstrate, for the first time, that engineered scaffolds are capable of functioning as homing sites for metastatic spread. This novel platform will enable future study of bone stromal factors essential for or contributing to metastasis. Moreover, our findings suggest that engineered tissues can be integrated as advantageous components in emerging models of disease.

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DISCUSSION

Schiffman, Providence: Mike, great presentation. Two questions: (1) What were your control tissues besides bone? Did you put stomach, tendon, anything else, connective tissue instead of the engineered bone?

Rosenblatt, Whitehouse Station: Yes. I didn’t show you that. We took pieces of lung, since that’s another site, and we did experiments with pieces of lung and breast cancer didn’t go there.

Schiffman, Providence: (2) My second question is on the bisphosphonate story. There were data in the past that bisphosphonates may interrupt this metastatic cascade, more recent data saying maybe that’s not the case. Can you explain or maybe elaborate on this?

Rosenblatt, Whitehouse Station: We have tried bisphosphonates in this model and they actually work, so maybe that means that there is data on both sides. I don’t know if that means that our model is clinically predictive or not, and the question that Fred is getting at is that these cancer cells can’t actually make their own expansion space inside bone. They have to hijack the normal apparatus, which are called osteoclasts, to tear down bone and make expansion space. The bisphosphonates are particularly effective at stopping osteoclasts in their tracks.

Lippman, Miami: I really enjoyed that greatly. I’d like to make two brief comments that are cautionary. We’ve developed models of human breast cancer put into the mammary gland which does metastasize and a different immunosuppressed model that gives mets everywhere. Bone, brain. . .

Rosenblatt, Whitehouse Station: Is that human breast cancer?

Lippman, Miami You bet, and widely metastatic, and we’ve developed gene array patterns for looking at those and some of the genes that you get for bone we see in bone metastases but many of the ones on your list are actually critical for mets everywhere. For example, IL-11, so it’s not bone-specific, and the specificity you see by using this explant really isn’t specific when we look at. . .there is a bone signature and there is a core group of genes that we find about 18 of which are required for metastasis to all sites. That’s the first, sort of, cautionary note. The second thing, which I think is critical, is looking at stromal genes that change. We have developed a robust platform for inter-
mingled human and mouse cells, which is what a met is, we can tell which are the human and which are the mouse cells. Several of the genes on your signature are actually stromal genes that develop an MDSC, myeloderived stem suppressor cell, signature that’s been described by others. The summary point that I am making is that, while looking at bone tropism is essential and very interesting, unless you look at the metastasis and compare with other sites, you may be slightly misled. You may be looking at genes that are critical for the metastatic process but not necessarily bone-specific.

**Rosenblatt, Whitehouse Station:** Thank you for your comments and I find them very interesting. I must say we have a very different experience. First of all, most mouse tumors, as you know, won’t spread to bone in the mouse and we had a very, very hard time finding any human tumor that would spread to bone; then we had the other interesting problem when these cancers did arrive at bone, you have a human bone and you have breast cancer cells from a different human being arriving in that bone marrow. We actually get a “graft-versus-graft” reaction. We see an immune rejection around the breast cancer cells. So to tell you the truth, I thought we were very lucky to get any metastases at all, which is quite the opposite from what you have gotten.

**Lippman, Miami:** It’s a different immunosuppressed model. We should talk offline about this.

**Rosenblatt, Whitehouse Station:** Yeah that would be fun. Thank you.