cusj COLUMBIA UNDERGRADUATE SCIENCE JOURNAL

The Verification of a Novel Explant System Used to Determine the Role of Osteocytes in the Breast Cancer Vicious Cycle

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Abstract

Breast cancer affects one in eight women per year, and 70% of patients with stage IV breast cancer develop metastases in bone, causing life-threatening side effects. We examine the use of an explant system to mimic breast cancer metastasis to bone without confounding cell types and to determine the role that osteocytes, the bone mechanosensing cells, may have in the development of metastasis. Using the explant system, a custom cell seeder and sterile cell culture techniques, we introduced metastatic MDA-MB-231/GFP cells to a three-dimensional bone matrix with osteocytes only. Confocal imaging confirms that breast cancer cells were, in fact, successfully seeded onto bone cores, mimicking metastasis. Though additional experiments will be necessary to determine the importance of breast cancer-osteocyte interactions, this study shows that the explant system is a viable methodology for studying breast cancer in bone.

Introduction

Cancer is a devastating disease that is responsible for thirteen percent of deaths worldwide and has affected countless families and individuals throughout the world (Cancer, World Health Organization). It affects 1 in 8 women every year (U.S. breast cancer statistics). Breast cancer originates from the inner lining of the lobules that supply the milk ducts in the breast (Wolf et al., 2003). Stage IV breast cancer is metastatic, meaning that it is violent and transcends the host organ (the breast) and spreads to a secondary site. The cancer that metastasizes is still considered breast cancer. It has been reported that up to 70% of stage IV breast cancer patients will experience some form of metastasis of breast cancer to bone (Roth et al., 2009). Patients often experience pathological fractures, intense pain, hypercalcemia, and various nervous compression complications (Zhang et al., 2010). These devastating effects are caused by an imbalance of bone remodeling, which involved the interactions of the three main bone cell types.

Bone is comprised of three types of cells: osteocytes (OCY), osteoblasts (OB) and osteoclasts (OCL). Osteocytes are the primary mechanosensing cells in bones (Burger et al., 1995). They regulate the activity of osteoblasts and osteoclasts. Osteocytes are "trapped" in the mineralized bone matrix, and are thus they are thought to have only signaling functions, both intercellular and intracellular. After osteocytes sense a mechanical load, that load is transduced into a chemical signal is sensed by the cells. This stress is translated into a biochemical signal that is communicated to the osteoblasts and osteoclasts, the bone forming and bone resorbing cells (Burger et al., 1995). Osteoblasts synthesize the bone matrix, which is subsequently deposited and calcified to become bone mineral. When osteoblasts secrete too much matrix, they become stuck in the bone, and as a result they completely differentiate into osteocytes (Saladin, 2007; Buckwalter et al. 1995). Osteoclasts, on the other hand, resorb bone. To accomplish this, they use their "ruffled" membrane (as shown in Figure 1) to create a seal around bone and then pump enzymes and hydrochloric acid to degrade the matrix (Saladin, 2007; Buckwalter et al. 1995).

There are two types of metastasis: osteolytic and osteoblastic. Osteolytic metastases break down bone and are the most common type of metastasis for breast cancer. Osteoblastic lesions, characterized by excess bone formation, affect 15-20% of patients. Mixed types also exist, wherein the patient experiences unnecessary bone excess as well as dearth (Zhang et al., 2010). The large majority of stage IV breast cancer cases end in metastasis to bone because bone has high levels of growth factors that breast cancer uses to survive. We can see that bone is a likely candidate for breast cancer metastasis due to the presence of "transforming growth factor *B* (TGF*B*), insulin-like growth factors I and II (IGF), fibroblast growth factors (FGFs), platelet-derived

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Received 12/22/2012. Accepted 1/31/2012. Published 4/1/2012
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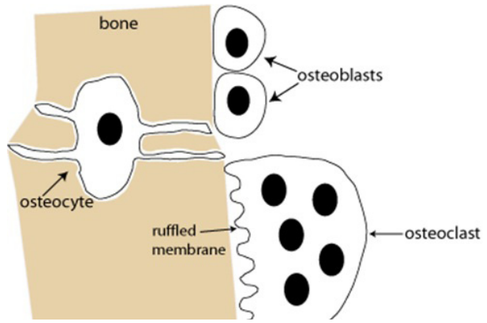

Figure 1 *Schematic depicting the types of cells in bones. The image depicts the osteocytes, trapped in the matrix of the bone, osteoblasts, on the surface, and the multi-nuclear osteoclast, which via the ruffled membrane, resorbs bone. Adapted from http://courses.washington.edu/conj/bess/bone/bone-cells.png.*

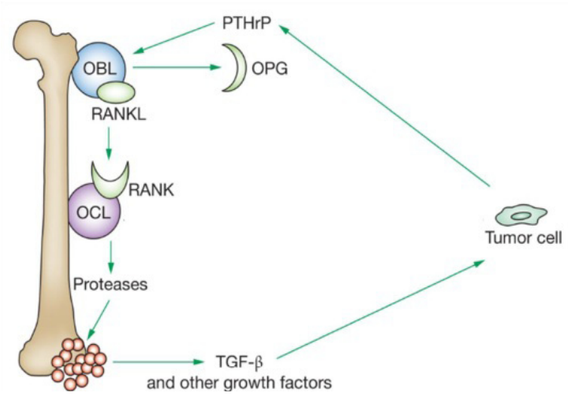

Figure 2 *Schematic of the "vicious cycle" pathway. The Tumor cell produces PTHrP, increasing the amount of RANKL and decreasing the amount of OPG. This activates more osteoclasts, which degrades bone, releasing TGF—β, completing a positive feedback loop. [Adapted from Steeg PS, Theodorescu D, Nat Clin Pract Oncol 2008]*

growth factors (PDGFs), bone morphogenetic proteins (BMPs), and calcium" which make up the microenvironment of bone cells (Zhang et al., 2010). Thus, the bone microenvironment is particularly suitable for cancer cell survival.

When breast cancer cells metastasize to bone, they first proliferate from the primary site, using proteases such as urokinase plasminogen activator (uPA), matrix metalloproteinases (MMPs) and mitogen-activated protein kinases (MAPK) which degrade surrounding matrix proteins, letting the cancer cells migrate more easily. Additionally, cadherin-11 and N-cadherin expression has been shown to have a strong link to bone metastases (Zhang et al., 2010). Next, cancer cells must migrate to the bones. Studies suggest that chemokines may home breast cancer cells to bones. The cells must adhere to the bones via the glycoproteins in the extracellular matrix (Mundy, 2002). The *BvB*3 integrin has been implicated as a key protein in this step. Lastly, the cells must proliferate. This proliferation occurs via a "vicious cycle" (Figure 2) between tumor growth and bone remodeling which utilizes the osteoblasts (bone-forming) and osteoclasts (bone-resorbing) in the bone (Zhang et al., 2010).

The bone remodeling cycle is normally stable, but following the introduction of metastatic breast cancer cells, this cycle creates a positive feedback loop. First, the breast cancer cell produces parathyroid hormone-related protein (PTHrP) that signals osteoblasts to increase the amount of receptor activator of nuclear factor B ligand (RANKL). Additionally, osteoblasts will decrease the amount of osteoprotegrin (OPG), which is a decoy receptor for RANKL (Guise, 2000). As a result of an increase in the amount of RANK ligand and less OPG to competitively inhibit the activity of receptor activator of nuclear factor B (RANK) on osteoclasts, more primary osteoclasts are activated to become osteoclasts. The ratio of RANKL to OPG determines the rate of osteoclast activity. When the bone is degraded, transforming growth factor-*B* (TGF-*B*) is released. TGF-*B* stimulates the production of PTHrP, completing the positive feedback loop. This is generally referred to as the "vicious cycle" between breast cancer and the bone environment (Mundy, 1997).

Most studies assume that proliferation of breast cancer cells is due to osteoclast and osteoblast action and with no other cell types involved. This phenomenon appears to explain the predominance of osteolytic lesions in breast cancer patients (Burger et al., 1995). However, studies have shown that osteocytes also produce OPG and RANKL when regulating bone modeling (Bonewald, 2011). There are some who argue that osteocytes can degrade bone matrix through a rapid and ephemeral mechanism called osteocytic osteolysis (Teti and Zallone, 2009). These links lead us to believe that cancer cells may affect osteocytes, which in turn affect the other cell types. It is possible that the accepted "vicious cycle" has another component: osteocytes. This would suggest that preventing osteocytes from being affected by cancer cells may stop the vicious cycle, because then the osteoclasts and osteoblasts would not have to be blocked from cancer cells.

In order to explore the interactions between breast cancer cells and osteocytes, we utilize a trabecular bone explant system. It is a tractable and novel system that contains only a cleaned bone core and no confounding cell types, ensuring that any variables created by cells' signaling processes are controlled. However, other cell types can be added to the bone cores as this system is seedable, meaning that we can add precise amounts of whichever cells we wish to examine. Although this study does not require it, this system also allows for long-term culture by incorporating a perfusion chamber which prevents the bone cores from dying due to lack of nutrition (Chan et al., 2009). Imaging and 3-D bone reconstruction allow for the determination of how breast cancer cells distribute themselves on the bone and affect bone volume fraction. Figure 3 shows dendritic-like signaling processes that breast cancer cells develop in 3-D culture. These chains are only visible on a 3-D scaffold and are indicative of proliferation (Wolf et al., 2003).

It is clear that the mechanism of the metastasis of breast cancer cells to bone must be evaluated, but this would require a system in which a bone has osteoblasts and osteoclasts, with the variables being the presence of osteocytes and breast cancer cells. This experiment would have to appropriately model metastasis and then indicate a loss or gain in bone. Through this study, we demonstrate a methodology that can be expanded and extrapolated to a larger scale experiment in order to model the breast cancer vicious cycle with the appropriate cell types.

Methods

Preparation of Bone Cores

The tarsal-metatarsal joint was harvested from a calf under sterile conditions. Specimens were sacrificed a day prior to the procedure. This joint is used due to the flat articulating surface found between these two joints, which is conducive to drilling. Cores were obtained by drilling into the articular cartilage and through trabecular bone using a Starlite diamond-tipped brill bit. Drilled cores were 7 mm in diameter (See Figure 4). The cores were lightly cleaned with an Interplak dental water pick running phosphate buffered saline (PBS), a saline solution containing NaCl and NaK. The cores were then cut using an Isomet low-speed saw to a height of 7 mm, avoiding the articular cartilage on top and the less dense bottom half of the cores. The cores were cleaned again thoroughly with the water pick running PBS before undergoing a serial trypsin treatment, which involved the cores being incubated in 3-4 mL of trypsin at 37°C for 8 minutes and then being thoroughly cleaned with PBS via the water pick. This treatment was replicated three times. The trypsin treatment was done to remove the surface cells from the cores. By completely eradicating the surface cells on bones, the cores are free of variable cell types. Twelve cores were harvested and cultured in supplemented media, which consisted of *a*-Minimum Essential Medium (*a*-MEM), 10%

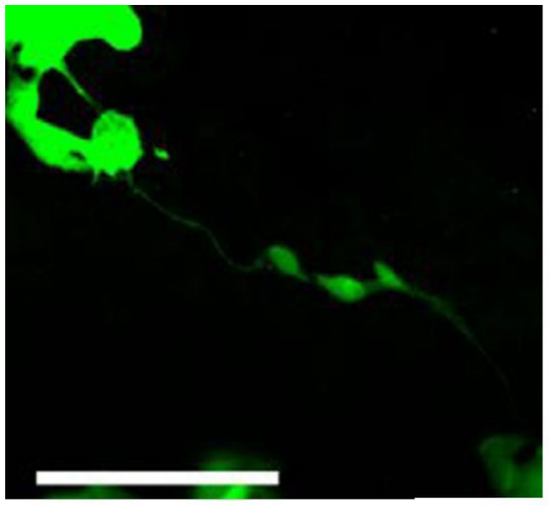

Figure 3 *Confocal imaging of MDA-MB231-GFP cultured in on a bone core. The image highlights the dendrite-like connections made between cells on a 3-D model. Scale bar = 50 μM. [Miller, 2010]*

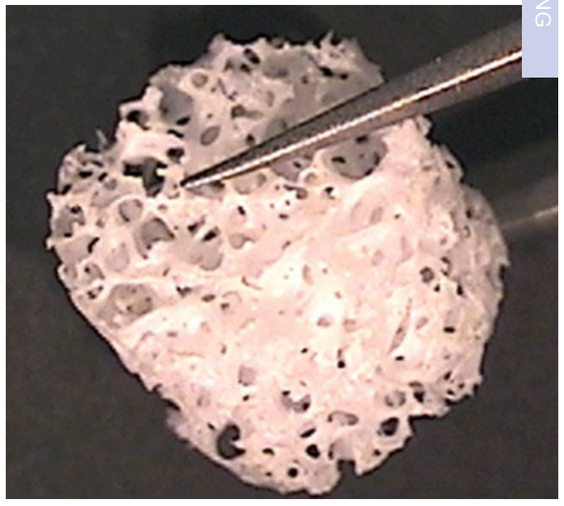

Figure 4 *Cleaned bone core, measuring 7 mm in height and diameter. [Adapted from Chan, ME, Lu, XL, Huo, B, Baik, AD, Chiang, V, et al. (2009). A Trabecular Bone Explant Model of Osteocyte-Osteoblast Co-Culture for Bone]*

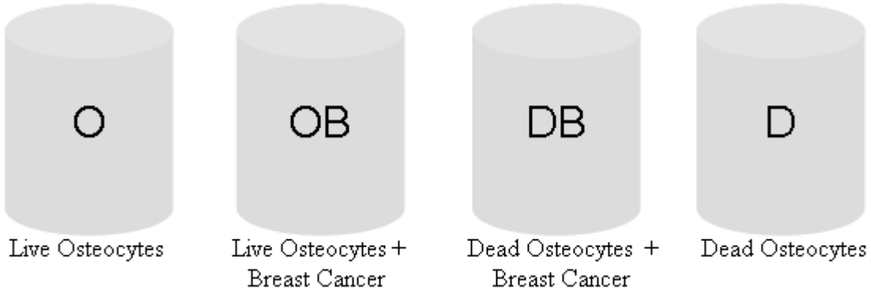

Figure 5 *Visual representation of the experimental design groups. First, we have cores with live osteocytes and no breast cancer cells, as a control. The variable condition is the core with live osteocytes with breast cancer cells. We also included two more groups, both with dead osteocytes. This was because we wanted to see if the mineralized bone, not necessarily the active osteocytes, affected the way breast cancer cells acted.*

Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin (Pen-Strep).

Creation of Experimental Groups

The cores were randomly assigned to four experimental groups, "Osteocytes and Breast Cancer" (OB), "Osteocytes" (O), "Dead Osteocytes and Breast Cancer" (DB) and "Dead Osteocytes" (D) (See Figure 5). Half the cores were put through a repeated freeze-thaw technique in order to kill the osteocytes still in the cores. This was done to see if mineralized bone, but not necessarily active osteocytes, affects breast cancer cell activity. Half of the dead osteocyte and live osteocyte groups were seeded with MDA-MB-231/GFP stage IV breast cancer cells via a custom cell seeder created in the lab on Day 0. The bone cores were stuck onto needles and submerged in a solution containing $5x10^4$ cells for each core. The loader was placed on a stir plate with a magnetic stir fly for an hour to facilitate uniform adheration of the cancer cells. Figure 6 shows a schematic of the cell seeder. This process simulated breast cancer in bone.

Confocal Microscopy

The cores were initially stained with Cell Tracker Red and incubated for 45 minutes in order to stain the viable cells in the bone cores before being seeded. The cores were imaged using a Leica confocal microscope on Day 0 after initial seeding, and on Day 4 using confocal microscopy.

The confocal imaging on Day 0 indicates that the cancerous cells do exist on the bone matrix. Figures 7.1 and 7.2 depict cores that were seeded with breast cancer cells, and the green fluorescence indicates that the cells are on the core. In contrast, Figures 7.3 and 7.4 only show the osteocytes in the lacunae of the bone core. The subsequent set of images on Day 4 shows a much higher number of cells on the bone core, as indicated by Figures 8.1 and 8.2. Figures 8.3 and 8.4 again indicate that there are no breast cancer cells in these two experimental groups.

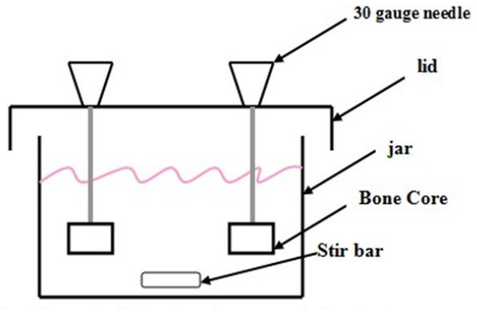

Figure 6 *Schematic of the cell seeding technique. Bone cores were stuck on the needles and suspended in the media. Adheration was facilitated by the stir bar.[Adapted from Chan, ME, Lu, XL, Huo, B, Baik, AD, Chiang, V, et al. (2009). A Trabecular Bone Explant Model of Osteocyte-Osteoblast Co-Culture for Bone]*

Results

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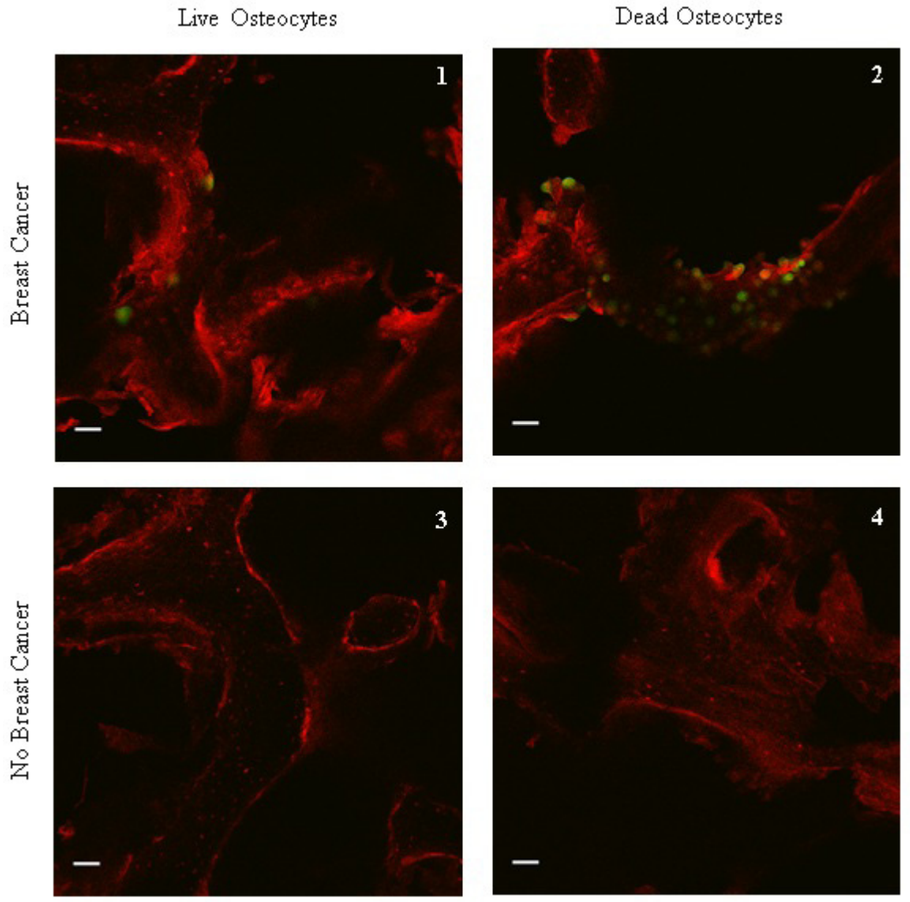

Figure 7 *Day 0 confocal imaging of bone cores. Images taken on Leica Confocal microscope. Red fluorescence is Cell Tracker Red and shows osteocytes (dead and alive), while green fluorescence indicates GFP tagged MDA-MB-231. These images were taken to ensure that the Cell Tracker Red stain did stain osteocytes, which is clear through the masses of red signals near the lacunae, and to ensure that the breast cancer cells were properly seeded onto the bone cores. Scale bar = 50 µm.*

Discussion

Confocal Imaging of the Bone Cores

Confocal imaging of the bone cores on Days 0 and 4 indicate a simulated metastatic process. The first step to metastasis is the adheration of foreign, cancerous, metastatic cells into a new environment. The images, taken on Day 0, indicate that the cells were added in an effective amount that attaches to the bone core appropriately. Figures 7.1 and 7.2 show this adherence and verify that we were able to force adheration of cancerous cells onto the matrix. Additionally, the negative controls are verified in Figures 7.3 and 7.4, which do not have green fluorescence. After this, Day 4 imaging indicates that the breast cancer cells were sustained on the bone cores as they maintained viability and also expanded in quantity on the bone cores. Figures 8.1 and 8.2 show the proliferation of the cells, as there are many more cells that effectively take over the lacunae. When looked at in conjunction with Figures 7.1 and 7.2, it is clear that the cancerous cells are thriving on Day 4. These images suggest the proliferation of the cancerous cells, indicating that metastasis was effectively mimicked in this system.

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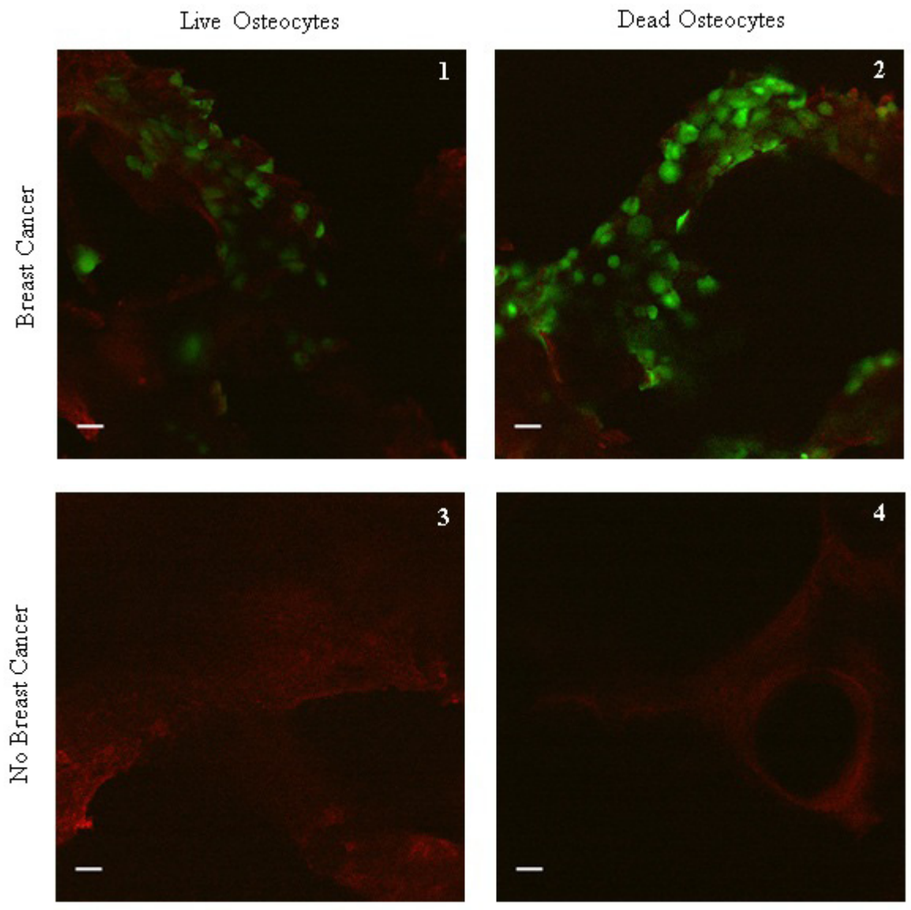

Figure 8 *Day 4 confocal imaging of bone cores. Images taken on Leica Confocal microscope. Red fluorescence is Cell Tracker Red and shows osteocytes (dead and alive), while green fluorescence indicates GFP tagged MDA-MB-231. The Cell Tracker Red stain begins to fade at this point. These images were taken to ensure that the breast cancer cells were properly proliferating across the bone core, which is clear given the number of cells present on the cores. Scale bar = 50 μm.*

Modifications to be made

As the results indicate that the methodology is sound, we must look to future studies and the next step in the larger scale study. The harvest technique, while sterile (given that there were no infections), was time consuming and would benefit by becoming more efficient, so instead of 8 cores being harvested at a time, we could harvest 32 at a time. Day 4 confocal imaging shows a decreased Cell Tracker Red signal, and a longer culture would require a more stable imaging technique. A longer culture would provide for more complicated culture conditions involving all three bone cell types. Using more bone cell types would require more than two different fluorescent stains, relying on the use of the lab's four-color microscopy technology.

Conclusions

Explant system as a viable in vitro model for study

The qualitative (confocal images) data suggests that the explant system is a good method to simulate metastasis of breast cancer, proving that it can be used to further study this topic. The study presents a starting point for future experiments, as it demonstrates our methodology is viable and efficient. Future studies would rely on the use of μCT tomography to determine the Bone Volume/Total Volume, also known as the Bone Volume Fraction (BVF). BVF is a good measure of the amount of bone mineral present in a sample as it determines the space of bone mineral versus total space of the core. Differences in BVF between seeded and nonseeded cores would indicate cancer-induced lesions. The incidence of these lesions would determine the extent of a variable cell's role on the vicious cycle.

In the future, the study would start to include other cell types, creating different experimental groups such as a core with osteoclasts, osteoblasts and breast cancer cells, but no osteocytes. This condition would simulate a bone without osteocytes, and the resulting BVF, as compared to a bone core with all three cell types and breast cancer, would indicate how osteocytes affect bone cell activity in the presence of breast cancer cells. The future implications of this experiment are promising, as it is the first step in determining the individual role of each cell type; determining finally how responsible osteocytes are for metastatic breast cancer spreading to bones.

Acknowledgments

SB thanks XEG for allowing her to work in Bone Bioengineering lab and GNB for guidance and teaching her the cell techniques. SB was funded by the Columbia Department of Biological Sciences from the Summer Undergraduate Research Fellowship.

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