PROSPECTS
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Three-Dimensional in Vitro Model to Study Osteobiology and Osteopathology

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ABSTRACT

The bone is an amazing organ that grows and remodels itself over a lifetime. It is generally accepted that bone sculpting in response to stress and force is carried out by groups of cells contained within bone multicellular units that are coordinated to degrade existing bone and form new bone. Because of the nature of bone and the extensiveness of the skeleton, it is difficult to study bone remodeling in vivo. On the other hand, because the bone contains a complex environment of many cell types, is it possible to study bone remodeling in vitro? We propose that one can at minimum study the interaction between osteoblasts (bone formation) and osteoclasts (bone degradation) in a three dimensional (3D) "bioreactor". Furthermore, one can add bone degrading metastatic cancer cells, and study how they contribute to and take part in the bone degradation process. We have primarily cultured and differentiated MC3T3-E1 osteoblasts for long periods (2–10 months) before addition of bone marrow osteoclasts and/or metastatic (MDA-MB-231), metastasis suppressed (MDA-MB-231BRMS1) or non-metastatic (MCF-7) breast cancer cells. In the co-culture of osteoblasts and osteoclasts there was clear evidence of matrix degradation. Loss of matrix was also evident after co-culture with metastatic breast cancer cells. Tri–culture permitted an evaluation of the interaction of the three cell types. The 3D system holds promise for further studies of cancer dormancy, hormone, and cytokine effects and matrix manipulation. J. Cell. Biochem. 116: 2715–2723, 2015.

KEY WORDS: BONE REMODELING; OSTEOBLASTS; OSTEOCLASTS; METASTATIC CANCER; 3D CULTURE UNIT

Putting aside the general argument of whether it is possible to ever model an in vivo system in vitro, we would like to consider the possibility of recreating at least part of the bone remodeling process in culture. Arguably bone is one of the most difficult organs to study. It is a complex organ, the major component of the skeletal system, with many functions. It is important for mechanical integrity and protection but also for mineral homeostasis. The bone marrow is also the site of hematopoiesis and the generation of cells of the immune system. The skeleton is extensive. In addition, its relative inaccessibility, calcified nature and opacity make it difficult to examine as a whole. An approach used for many organs is to study the composite cells in culture. It was not until the 1970′s that osteoblasts were successfully cultured in vitro [Sims and Martin, 2014]. As reviewed by Gallagher, Russell described a protocol for isolating and growing human cells in the 1980′s [Gallagher et al., 1984, 2003]. Isolation of osteoblasts from mouse bones was carried out much earlier [Peck et al., 1964]. Primary human and mouse osteoblasts as well as cell lines have been used extensively ever since.

One of the most amazing things about bone is its ability to adapt to stress and force, both as the body grows and as it encounters changes. Bone sculpting, as it is called, is carried out by cells in a series of coordinated events of degradation (resorption) and replacement (bone formation). Bone remodeling is carried out by groups of cells contained within bone multicellular units (BMU). A BMU consists minimally of osteoblasts, the bone forming cells, and

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osteoclasts, the bone resorbing cells. In a recent review [Sims and Martin, 2014], the more complete BMU is depicted as containing a canopy of bone lining cells covering the resorption area, and precursors of osteoblasts (mesenchymal stem cells), precursors of osteoclasts (hematopoietic cells), osteocytes, and transient macrophages and lymphocytes. BMU exist as discrete units in many places in the bone and act asynchronously, apparently responding to local forces, growth factors and cytokines. Each BMU is active for many months as bone is resorbed and then replaced. Both cortical and trabecular bone contain these remodeling units. Within each BMU there is a strict coupling of resorption and replacement of bone. Nonetheless, a little excess in bone formation over time allows for bone growth, as happens in the growing young. Too little replacement over time leads to bone loss, as happens in old age. Bone density is also associated with various pathologies. Osteoporosis develops with severe loss of bone. Bone degradation also can be pronounced with cancers that metastasize to bone, for example breast, lung; or cancers that originate from cell in the bone such as multiple myeloma.

Harold Frost is credited with the concept of coupled bone remodeling in a BMU [Hattner et al., 1965]. In a series of measurements taken with human trabecular bone he found that 96.7% of the bone formation processes occurred only after resorption occurred in the same part of the bone surface. He concluded that there was a spatial relationship between resorption and formation, and that the processes occurred in a well-defined sequence. This concept explains the ability of the bone to repair, remodel areas throughout the bone in a coordinated fashion.

BONE REMODELING IN VITRO

Given the importance of bone remodeling to the health of the bone as well as its role in various pathologies, is it possible to establish an in vitro model that would mimic a BMU? A few years ago we began to experiment with a relatively simple growth chamber based on the model of growth and dialysis [Rose, 1966]. We refer to this device as a "bioreactor." We successfully grew primary mouse and human osteoblasts as well as human and mouse pre-osteoblast lines [Dhurjati et al., 2008b; Krishnan et al., 2010, 2011c, 2014; Sosnoski et al., 2015]. We considered the possibility that this model could be used to create a simple BMU in vitro. We were particularly interested in studying the interaction of metastatic cancers with predilection to bone; for example breast cancer. How do cancer cells affect bone remodeling?

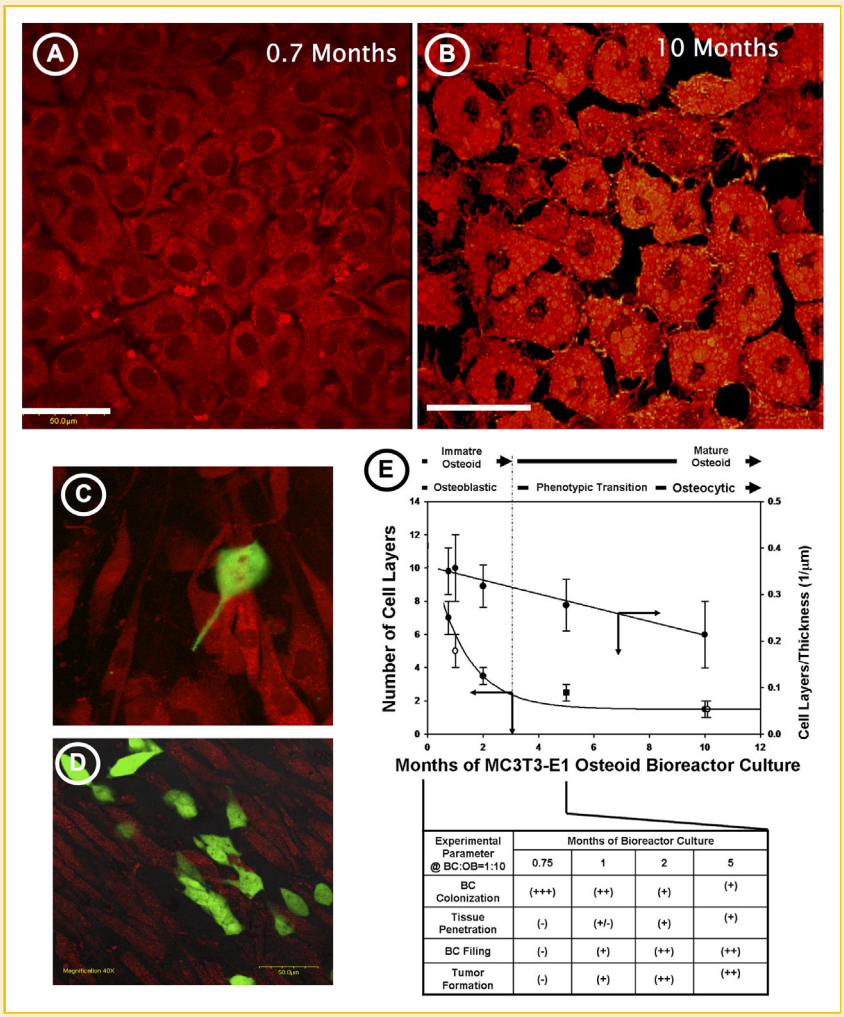
We had evidence from conventional in vitro cell culture studies that, in the presence of breast cancer cells or their medium, osteoblasts no longer make the proteins required for bone repair. Furthermore osteoblasts produce inflammatory cytokines, which in turn can stimulate osteoclasts. Treatment of osteoblasts with breast cancer conditioned medium resulted in a change in osteoblast morphology and adhesion, as noted by reduced focal adhesion plaques and a reorganization of actin stress fibers. These effects were mediated through TGFβ, PDGF, and IGF, all present in the conditioned medium [Mercer et al., 2004].

In principle, a sub-set of this metastatic process can be studied in vitro if the model system under consideration retains sufficient biological complexity to be a reasonable surrogate for host tissue. Effective in vitro models must strike a balance between experimental efficiency and retention of biological complexity. Three-dimensional (3D) tissue models have become a focus of recent investigation for this reason [Weaver et al., 1995; Nelson and Bissell, 2005]. Surrogates for bone tissue are, however, quite challenging to construct [Kuperwasser et al., 2005]. Models based on excised bone are not only technically demanding but also difficult to interface with modern microscopic methods of investigation [Nemeth et al., 1999].

For improved understanding of breast cancer colonization of bone, we implemented the bioreactor to study the critical factors such as the proliferative/differentiated state of osteoblasts and osteoclasts and the interaction of breast cancer cells with these bone remodeling cells. While there are numerous osteoblast cell lines, there are few sources of osteoclasts which presented another challenge.

The overall goal of our study was to determine whether 3D mineralized tissue derived from the co-culture of osteoblasts with osteoclasts ("biosynthetic bone") in a novel bioreactor would be a relevant in vitro bone surrogate for studying the early stages of breast-cancer colonization. This approach might lead to target for effective therapeutic intervention. Thus, we hypothesized that a bone microenvironment model that simulates the in vivo process of bone accretion and resorption (remodeling) could be created in a 3D in vitro cell culture system (bioreactor). Furthermore, addition of osteolytic cancer will upset this in vitro stasis in a manner related to the progression of bone pathology.

Toward realization of a 3D model bone remodeling and the effect of the interaction of breast cancer cells with osteoblasts and osteoclasts, we developed [Vogler, 1989; Krishnan et al., 2010] a bioreactor system that permits long-term growth of osteoblasts (MC3T3-E1), at least 10 months, without subculture or perfusion. The result is a mineralizing, multiple-cell-layer tissue that by histological examination exhibits a normal phenotype that strongly resembles normal osteogenic tissue. The pre-osteoblast MC3T3-E1 cells phenotypically mature to form osteocyte-like cells over the 10-months of culture (Fig. 1A, B). Cell morphology changes associated with this maturation process were documented using visual images obtained by confocal microscopy, and by monitoring the gene expression (RT-PCR) of important osteoblast differentiation markers over time [Krishnan et al., 2011]. We showed that, by challenging this osteoblast tissue with metastatic breast cancer cells (MDA–MB-231^{GFP}), important hallmarks of cancer metastasis including cancer-cell penetration of tissue and tumor formation were observed in vitro [Dhurjati et al., 2008] (Fig. 1 C–E). The osteoblasts changed from cuboidal to spindle shaped in the presence of cancer cells in the bioreactor. In these same cultures, the cancer cells exhibited a distinct alignment with the long axis of the osteoblasts. This specific arrangement of cancer cells often referred to as "single cell filing" is characteristic of tumor invasion in pathological tissue [Elizabeth Mallon and Gusterson, 2000]. Also, we found that the osteogenic tissue maturity was a key variable in influencing the manner of breast cancer colonization in the bioreactor. As the culture matured, the osteoblasts formed a multilayered osteoblast tissue that mineralized. Breast cancer interaction was more pronounced with the more mature osteoblast tissue. In addition, the ratio of breast cancer cells to



Experimental Parameter @ BC:OB=1:10	Months of Bioreactor Culture			
	0.75	1	2	5
BC Colonization	(+++)	(++)	(+)	(+)
Tissue Penetration	(-)	(+/-)	(+)	(+)
BC Filing	(-)	(+)	(++)	(++)
Tumor Formation	(-)	(+)	(++)	(++)

Fig.1. Co-culture of MDA-MB-231 metastatic cancer cells in the bioreactor with MC3T3-E1 osteoblasts. Phalloidin-stained MC3T3-E1 osteoblast tissue after 22 days of continuous culture in the bioreactor (A). Upon elongated culture period of ~10 months, a morphological gradient in the tissue was evident from cuboidal cells to stellar exhibited filamentous inter-cell connections reminiscent of osteocyte morphology (B). MC3T3-E1 osteoblast cells (stained with Cell Tracker Orange™) was co-cultured with MDA-MB-231-GFP breast cancer cells. Day 1 post inoculation, breast cancer cells start to invade the osteoblast tissue (C) and day 3 post inoculation, Linear-like organization of breast cancer cells and osteoblasts was evident within the tissue (scale bar = 50 μm). Qualitative aspects of MDA-MB-231 metastatic breast cancer cell (BC) interaction correlate with osteoblast (OB) maturity. An exponential-like decrease in the number of cell layers with time (left-hand axis, graph) translated into a linear-like decrease in cell-layer/tissue-thickness ratio (right-hand axis, graph). This observation was consistent with the process of bone-tissue maturation that resulted in transformation of proliferating pre-osteoblasts into non-dividing osteoblasts that become engulfed in mineralized matrix that mature into osteocytes through a process of phenotypic transformation marked by increased osteoblast apoptosis. The data presented in the table suggested that declining rates of BC colonization and increasing efficiency of tissue penetration, filing, and colony formation were related to OB maturity (E). [Data reproduced from Krishnan et al, 2011 and Dhurjati et al 2008].

osteoblasts was varied (1:10, 1:100, 1:1,000) in an effort to mimic a disease process ranging from micro-metastasis to late stage metastasis. The cultures with higher ratio of cancer cells exhibited a rapid and systematic degradation of the osteoblast tissue accompanied with a change in the osteoblast morphology; whereas, the cultures with lower ratio of cancer cells depicted a much slower and less pronounced destruction of the tissue [Krishnan et al., 2011]. Live confocal imaging was used to monitor the interaction and phenotypic changes induced by cancer cells on osteoblasts. We found up-regulation of the inflammatory cytokine, IL-6, and a reduction in osteocalcin (bone specific differentiation marker) levels, in the cultures challenged with breast cancer cells. Also, a significant reduction in levels of soluble collagen (indicative of collagen formation) was observed [Dhurjati et al., 2008].

One of the major reasons for the interaction and the penetration of the MDA-MB-231 cells with the more differentiated cultures of the osteoblasts compared with the less mature ones, is likely due to the properties of the extracellular matrix. The results of several studies indicate the importance of cancer cell attachment, stiffness of the matrix [Butcher et al., 2009], matrix degradation, and proteases [Sabeh et al., 2009] in the metastatic process [Marastoni et al., 2008]. Within the bioreactor, extracellular matrix deposited by MC3T3-E1 pre-osteoblasts appears to mimic the physiological lamellar organization of collagen bundles that are arranged in alternating

longitudinal and transverse layers (Fig. 2). In an effort to investigate changes induced in the host osteoblasts extracellular matrix by the MDA-MB-231 cells, we exposed osteoblasts to conditioned medium from the breast cancer cells. RNA was collected for testing with a gene array specific for extracellular matrix related genes (Super-array, SA Biosciences), and an Affymetrix gene array to assess the changes in approximately 239 genes related to adhesion and motility, osteoblast growth and differentiation, proteases, cytokines, and receptors. The presence of the breast cancer cells caused a change in the gene-expression profile of the osteoblasts. Characteristic osteoblast differentiation proteins were down-regulated. There was an increase in inflammatory cytokines and cytokines that attract osteoclasts [Krishnan et al., 2014]. These data along with observed morphology changes in direct co-cultures suggest that the osteoblast matrix was being remodeled as a result of secreted molecules and cell-cell contact indicating that the matrix that forms in the 3D culture is critical to begin to understand the cancer cell colonization of the host tissue.

Normal bone remodeling involves osteoblasts but also osteoclasts as well as several growth factors, cell adhesion molecules, and cytokines. These same cells and factors also make bone conducive for metastatic tumor cells to colonize bone. Osteoclasts prime the bone microenvironment for tumor cell growth by causing bone resorption that releases many of these potential growth-stimulating molecules into the microenvironment [Sasaki et al., 1995; Powles et al., 2002]. To increase the complexity of the bioreactor system to study the three-way interaction of breast cancer cells with osteoblasts and osteoclasts, we first developed a model of osteoblast-osteoclast remodeling in co-culture (Fig. 3). We created an in vitro "bone-remodeling" mimic to which metastatic cancer cells could be added and monitored over time. First, we introduced pre-osteoclasts that we isolated from mouse bone marrow onto mature osteoblasts that had grown and differentiated in the bioreactor for at least two months. The pre-osteoclasts were differentiated by the addition of RANK ligand. After 10 days, the osteoclasts formed multinucleated, giant cells that stained positive for TRAP and displayed characteristic actin rings (Fig. 3B). In the bioreactor containing both the osteoblasts and the osteoclasts, we observed the matrix degradation of the ECM by the osteoclasts. We visualized tracks in the matrix left from the migrating osteoclasts. We stained the cultures for collagen using an antibody, and calculated the matrix thickness based on image reconstruction. The matrix in the presence of the large, TRAP positive osteoclasts was reduced in thickness. In addition, the supernatant contained collagen fragments, C-terminal telopeptides, characteristic of degraded matrix [Krishnan et al., 2014]. The evidence strongly supported the hypothesis that characteristic matrix degradation of an osteoblast culture had occurred in the bioreactor. However, the bone remodeling cycle is not complete until the osteoblasts have replaced the lost bone. In order to recreate this process in vitro, we infused the osteoblast-osteoclast co-culture with fresh MC3T3-E1 pre-osteoblasts. Each cell type expressed a different fluorescent label in order to distinguish the newly infused pre-osteoblasts from those already in the culture. We found that the pre-osteoblasts re-sealed the entire culture with a thin confluent

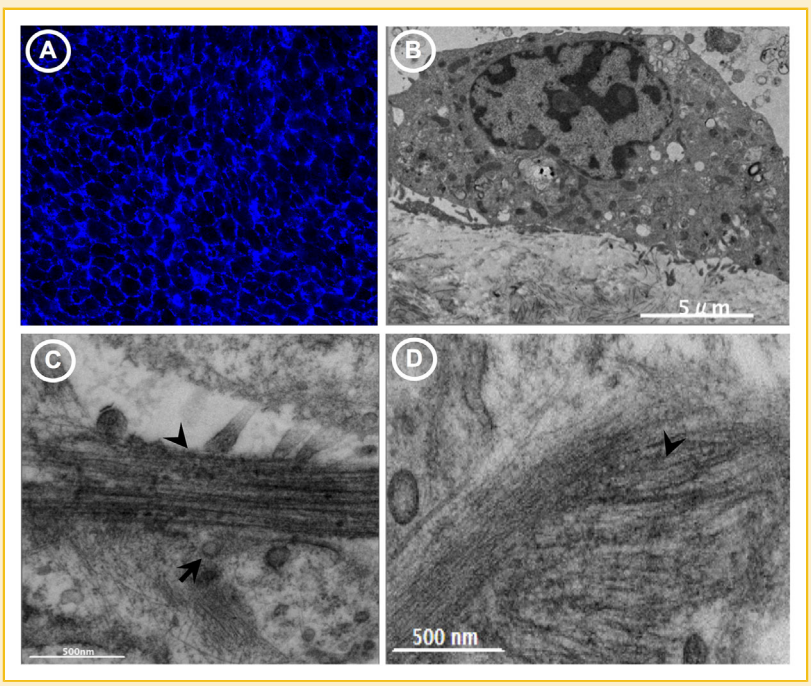


Fig. 2. Micrographs of type I collagen resolving fibrillar formations. A: Confocal micrograph of a 30-day MC3T3-E1 osteoblast tissue cultured in a bioreactor. B–D: TEM of a 22 day MC3T3-E1 cultured in the bioreactor. (B) An osteoblast is seen engulfed in a thick extracellular matrix containing fibrils. (C) The fibrils appear either as round cross-sectioned profiles (arrows) or as elongated, parallel bands (arrowheads). Collagen fibrils intersecting the image plane transversely appear as streaks and fibrils intersecting the plane perpendicularly appear as discs. (D) Collagen fibrils appear to mimic the physiological lamellar organization of collagen bundles that are arranged in alternating longitudinal and transverse layers. [Data reproduced from Krishnan et al, 2014].

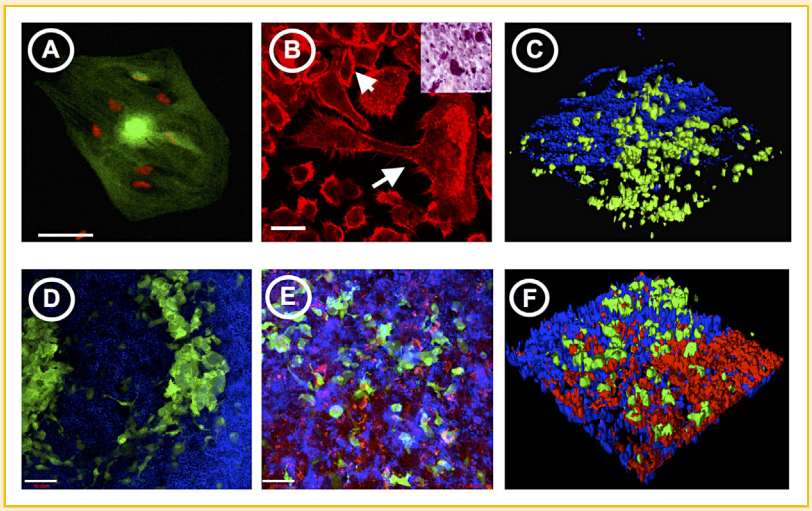


Fig. 3. A multi- nucleated GFP-osteoclast with five prominent red-stained (Draq 5) nuclei derived from osteoclast precursors (A). Osteoclasts were cultured for 3 weeks with 60-day osteoblastic tissue. An actin-stained osteoclast (arrow) migrating on the osteogenic tissue in the presence of osteoblasts (arrowheads). Inset shows a TRAP positive multinucleated osteoclast on osteoblast tissue counter-stained with eosin (B). Confocal reconstruction of GFP-osteoclasts in the presence of collagen (blue); matrix degradation by osteoclasts resulted in a net decrease in tissue thickness from 22 μm to 13.5 μm (C). GFP-osteoclasts assembled into nest-like structures within blue-stained collagen after 3 weeks of co-culture (D). MC3T3-E1 pre-osteoblasts were vital stained (red) and infused into a co-culture of osteoblasts (unstained) and osteoclasts (green). Pre-osteoblasts proliferated within the ECM (blue) after 7 days (E). Confocal reconstruction showed that red osteoblasts filled regions of digested ECM, and restored tissue thickness to 24 μm (F). Scale bars: A and B are 20 μm; C–F are 50 μm. [Data reproduced from Krishnan et al, 2014].

layer of osteoblasts essentially repairing the osteoclast-generated resorption pits (Fig. 3E, F). Thus, the bone remodeling cycle under non-pathological conditions was complete in this 3D system.

What happens when bone metastatic cancer cells are added to this system? We added MDA-MB-231 breast cancer cells and followed the live culture with microscopy for several days. When metastatic breast cancer cells were introduced onto the osteoblast-osteoclast co-culture (bone-remodeling mimic), the breast cancer cells migrated towards sites of active remodeling and clustered as an aggregation of cells that further degraded the osteoblast matrix. Specifically, breast cancer cells appeared to undergo chemotaxis towards active areas of osteoclast activity. They proliferated to form colonies that were a combination of osteoclast, cancer cells, and putative pre-osteoclasts (Fig. 4).

Interestingly, the osteoclasts were seen at the leading edges of the cancer colonies guiding the degradation of osteoblast tissue. Also, there was a significant up-regulation of osteoclast numbers in the presence of breast cancer cells. Although there is anecdotal evidence that cancer cells migrate towards osteoclasts, it has been shown more convincingly that cancer cells migrate to products released by bone resorption. These products presumably are released due to actively resorbing osteoclasts [Orr et al., 1979; Yoneda and Hiraga, 2005]. Mechanisms behind directed migration of cells towards chemo-attractants possibly occur in the following manner: through chemosensing, polarization and locomotion [Roussos et al., 2011]. Asymmetric actin polymerization creates a leading-edge protrusion resulting in extension of cell membrane towards the direction of locomotion, ending with detachment from substrate and contraction of the lagging-edge [Devreotes and Janetopoulos, 2003; Li et al., 2005]. A similar phenomenon is reproduced by cancer cells in their migration towards osteoclasts in the bioreactor (Fig. 4C). Nonetheless, it is not clear whether in vivo, if the breast cancer cells migrate to osteoclasts or are the pre-osteoclasts recruited after the breast cancer cells colonize the bone?

We put forward a novel in vitro model that can accommodate all the key cell types of the vicious cycle of bone metastases. By monitoring the colonization process using biochemical and microscopy techniques, a rich amount of information on the interaction between the cancer cells and the simulated bone can be gathered. Also, we propose that this could be a very useful model for future mechanistic research and for testing potential therapeutic agents.

In summary, in the bioreactor as well as in standard cell culture, we have been able to differentiate osteoclasts from hematopoietic progenitor cells isolated from murine bone marrow. The osteoclasts formed by exposure to RANKL and M-CSF were multi-nucleated, TRAP positive cells. Pre-osteoclast cells when introduced onto the osteoblast tissue grown in the bioreactor, differentiated to form multinucleated bone-resorbing osteoclasts. The osteoclasts were positive for TRAP and actin ring staining. They degraded the osteoblast matrix. Re-infusion of this co-culture system with pre-osteoblasts resulted in re-filling of osteoclast resorption pits with proliferating osteoblasts which presumably would produce new matrix given more time. When metastatic breast cancer cells were introduced into the osteoblast-osteoclast co-culture, the breast cancer cells migrated towards sites of active remodeling and clustered as an aggregation of cells that further degraded the osteoblast matrix, an observation that is typically seen in vivo.

Cancer-related bone loss appears to occur through multiple pathways, including osteoclast-mediated resorption [Sanchez-Sweatman et al., 1997; Mundy, 2002; Kozlow and Guise, 2005]. In

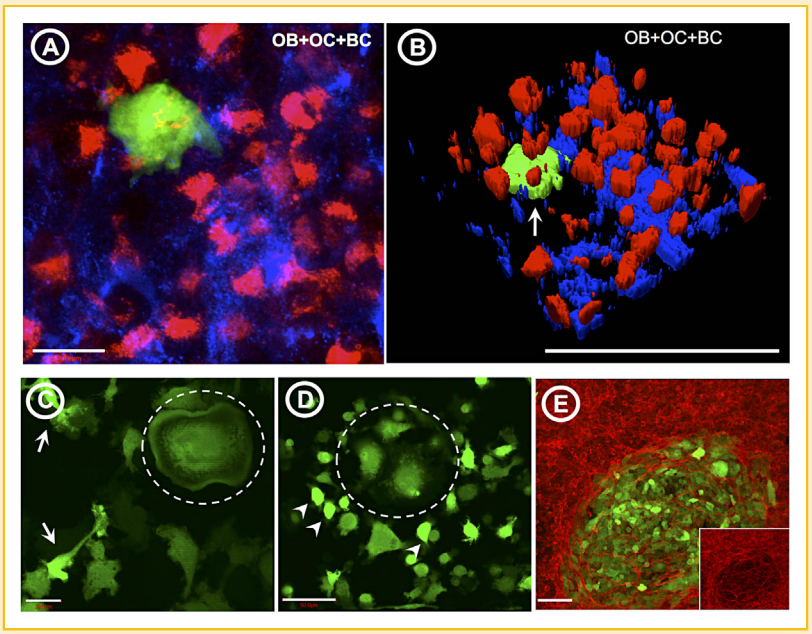


Fig. 4. MC3T3-E1 cultured for 2 months were co- cultured with non-adherent bone marrow cells from dsRED mice enriched for pre- osteoclasts and supplemented with RANKL (50 ng/mL) and MCSF (100 ng/mL). Metastatic breast cancer cells, MDA-MB-231-GFP, were added to the osteoblast-osteoclast co-culture to create a tri-culture system at the 10 day interval and cultured for an additional 10 days (A, B). Cultures were stained for type I collagen (blue). 3D reconstruction of A shows the depth of the tissue. Addition of breast cancer cells (green) to osteoblast-osteoclast (red) co-cultures resulted in the aggregation of cancer cells and osteoclasts to collectively further degrade the osteoblast matrix. Confocal reconstruction revealed that cancer cell colonies (green, arrow) in combination with osteoclasts (red) migrated to the bottom of the osteoblastic tissue. Shown are representative images from three bioreactors. (C) and (D) are live confocal images showing MDA-MB-231-GFP breast cancer cells' (arrows) migration towards GFP osteoclasts (dotted circles) in tri-cultures created by adding breast cancer cells to osteoblast-osteoclast. Breast cancer cells and osteoclasts congregated in nest-like structures formed in phalloidin-stained osteoblastic tissue after 10 days of addition of cancer cells to the osteoblast-osteoclast co-culture (inset shows osteoblasts only) (E). Scale bars for A, C, D, and E indicate 50 μm. B indicates 150 μm. OB- Osteoblasts, OC- Osteoclasts, and BC- Breast Cancer cells. [Data reproduced from Krishnan et al, 2014].

particular, destruction of devitalized bone directly by cancer cells has been reported; and it has been found that, late in metastasis when bone degradation rate is highest, there is a rapid decline of osteoclast cell numbers [Sanchez-Sweatman et al., 1997; Mundy, 2002; Kozlow and Guise, 2005; Phadke et al., 2006]. These lines of evidence support the idea that osteoclasts are not solely responsible for excessive bone degradation and that cancer cells directly contribute to bone loss. Degradation of the osteoblast-derived tissue by co-culture with breast cancer cells observed in the model system presented here strongly suggests that yet another mechanism of bone loss is related to disruption of the bone accretion process by destruction of osteoblastic tissue. There are clinical and experimental literatures to support this concept. For example, quantitative histomorphometric analyses of bone biopsies from patients with hypercalcemia due to bone metastasis indicated a dramatic decrease in osteoblast activity [Stewart et al., 1982]. A similar finding was seen with mice inoculated with bone metastatic MDA-MB-231 cancer cells. As the bone lesions progressed, there was a loss of osteoblasts [Phadke et al., 2006].

In another study, histomorphometric analysis of rodents inoculated with lytic human breast cancer cells (MDA-MB-231) indicated that, even though administration of risedronate (a bisphosphonate) reduced the number of osteoclasts, slowed bone lysis, and significantly reduced tumor burden, there was no evidence of new bone deposition or repair. Similarly, administration of bisphosphonates to humans with osteolytic metastasis slows lesion progression but does not bring about healing [Lipton et al., 2000]. Taken together, these observations strongly suggest that normal osteoblast function (i.e., deposition of matrix) is not only impaired in the presence of breast cancer cells but, in fact, osteoblastic tissue is degraded by cancer cells, possibly by enlisting a cooperative response by osteoblasts themselves. As osteoclast activity increase and osteoblast activity decreased, there is a net loss of bone.

APPLICATIONS OF THE BONE MIMIC MODEL

High throughput drug screening. 3D in vitro and/or in vivo tissue-engineering models that are designed to resemble the physiology of tissues could be used to study disease pathogenesis of tumors [Feder-Mengus et al., 2008; Hutmacher et al., 2009] We emphasize the requirement to study within 3D culture systems before preforming pre-clinical animal studies for therapeutics development.

Cells react differently to therapeutics depending on their spatial (2D vs 3D) arrangement. Additional differences in biological parameters may account for how cells are exposed and their consequent reaction to various drugs. Much research has been conducted to analyze the influence of microenvironment on cell

behavior. Weaver et al performed an experiment using non-malignant HMT-3522 breast cells and malignant HMT-3522 type cells. When these cells were grown in 3D using Matrigel®, normal and cancerous cells proliferated. Similar to those seen in healthy breast tissue, the normal cells formed organized, polarized acini. In contrast, the cancer cells, formed disorganized, loose aggregates. Following this observation, both cell types were treated with antibodies against the surface receptor β1-integrin, which is overexpressed in the cancer cells. The antibody treatment resulted in apoptosis of normal cells. The cancer cells, in the other hand, underwent a reversal and adopted a phenotype reminiscent of normal cells, with normal shape and growth patterns. While this was encouraging, a similar result could not be recapitulated when normal and cancer cells were grown in 2D. The difference in the results in the 2D vs 3D growth suggest that extracellular cues affect cellular phenotype; and, as observed in this particular case, cellular phenotype was proposed to be dominant over genotype [Weaver et al., 1997; Bissell et al., 2003; Breslin and O'Driscoll, 2013].

In a different study, a bioengineered tissue model of Ewing's sarcoma, human bone was first engineered from human mesenchymal stem cells cultured in a native bone extracellular matrix for 4 weeks. Cancer cells of the bone were prepared to mimic micro-tumors in form of small aggregates which were then introduced into this in vitro engineered human bone and further cultured. In an additional 4 weeks, the bone tissue context resulted in strong up-regulation of cancer related genes, the expression of a hypoxic and glycolytic tumor phenotype, and angiogenic and vasculogenic mimicry, suggesting that even in culture, cancer cells of the bone are strongly influenced by their bone environment [Villasante et al., 2014; Villasante and Vunjak-Novakovic, 2015].

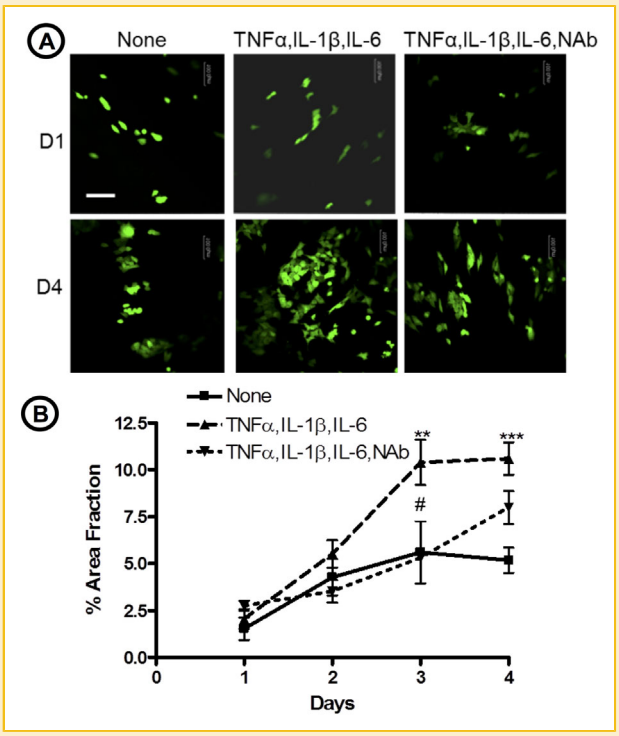


Fig. 5. Images and area fraction graph of BRMS1 osteoblast co-cultures incubated with TNFa, IL-1b, and IL-6 with and without addition of neutralizing antibodies (NAb) to the three cytokines. Shown are representative images from days 2 and 4 of co-culture (n = 3). [Data reproduced from Sosnoski et al 2015].

CANCER DORMANCY IN VITRO

There are at least two examples of the use of 3D cultures to study dormancy, an important aspect of metastasis and recurrence. Why do some cancer cells remain dormant in the bone, often for long periods, even decades after the primary tumor? There are cases studies and anecdotal evidence to suggest that bone trauma or fractures leading to bone repair/remodeling may be a catalyst in awakening the dormant cells [Sosnoski et al., 2015]. We used the 3D bioreactor model to examine a human metastatic cell line, MDA-MB-231 and the isologous, metastasis-suppressed line, MDA-MB-231BRMS1. The BRMS1 cells persist in the bioreactor culture in the presence of osteoblasts but generally do not proliferate. However, the addition of bone remodeling cytokines, TNFα, IL-1β, stimulated them to grow. The key downstream molecule appeared to be prostaglandin E2 (Fig. 5).

Recently, researchers constructed organotypic models of lung- and bone marrow- microvascular niches with the aim of studying disseminated breast tumor cell dormancy. HUVECs (human umbilical vein endothelial cells) were used for creating microvascular niches. These were seeded on top of either bone marrow mesenchymal stem cells or lung fibroblasts. Additionally, breast cancer cells were seeded on top of the newly formed microvasculature. Conclusions based on studying these organotypic 3D models further verified previous in vivo observations made in mice and zebrafish. The latter study had shown that while the stable trunk of the microvasculature made breast cancer cells dormant, in areas where the vessels were sprouting, tumor cells were able to exit their state of dormancy and resume growth. These data strongly suggest that a stable microvasculature induces sustained dormancy in breast cancer cells [Ghajar et al., 2013; Weigelt et al., 2014].

LIMITATIONS

For most 3D models, a lack of vascularization is a severe impairment, since the size of engineered tumors is limited by diffusional restrictions of nutrient and oxygen supply which consequently affects cancer cell viability and function. Vascularization is also required for most tumor metastasis. Notable differences between normal vasculature, and vasculature in solid tumors which are immature, tortuous, and hyper-permeable vessels, offers a novel target for anti-cancer therapy [Villasante and Vunjak-Novakovic, 2015].

Various approaches for creating vascularized tumor models have been employed by co-culturing cancer cells, endothelial cells and supporting cells. As an example, a tumor model was developed as a multi-culture of breast and colon tumor cells, human fibroblasts and endothelial cells in a fibrin matrix,

resulting in a network of sprouting vessels, and cancer cell invasion of the surrounding matrix [Ehsan et al., 2014; Villasante and Vunjak-Novakovic, 2015].

Effective drug testing will require tumor models incorporating a capillary bed, stromal cells, the components of immune system, and mechanotransduction signaling. The bone marrow also contains hematopoietic stem cells (which provide white and red blood cells to the vasculature), adipocytes and nerve cells. However, data from 3D models have shown that, even in the absence of these other cell types, bone remodeling can be studied.

OTHER CANCERS

Although we have focused on breast cancer cells that metastasize to bone, we have examined the appearance of other cancers that grow in the bone. We have successfully grown B16 mouse melanoma, human prostate LNCaP, and PC3 and 5TGM-1, mouse myeloma in co-culture in the bioreactor with two month old MC3T3-E1 osteoblasts. Each of these different lines displayed characteristic growth patterns. In an effort to make a more complex culture model we have also experimented with the addition of endothelial cells and stromal cells in the bioreactor in place of osteoblasts. These lines grew readily in the bioreactor. Added MDA-MB-231 cells grew in co-culture with stromal and endothelial cells; however, we did not detect the same patterns of growth as co-culture with the osteoblasts (data not shown).

SUMMARY

Summary In summary, bone remodeling can fundamentally be studied in vitro. A 3D model allows the production of matrix by osteoblasts, the degradation of the matrix by osteoclasts, and the replacement of the matrix by new osteoblasts. In addition this model can be used to study bone remodeling that occurs in a pathological state.

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