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BREAST CANCER CELL CHEMOATTRACTANTS DERIVED FROM OSTEOBLASTS

A Thesis in

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by

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Abstract

Metastatic breast cancer cells have an increased propensity to metastasize to bone. Whether bone has a unique chemotactic potential that attracts breast cancer cells is addressed in this study. Osteonectin has been characterized as a possible bone-derived chemoattractant. Through a series of protein analyses for molecular size, glycosylation, phosphorylation, and amino acid sequence, we determined that there was no detectable unique configuration of bone-derived osteonectin. Although osteonectin did increase cell motility, it did not cause the cancer cells to migrate in a directed manner. Osteonectin did not induce directed cell migration of breast cancer cells in transwell migration assays. Although breast cancer cells were not attracted to osteonectin, they were attracted to and migrated toward the mineralized portion of bone matrix (bone extracts). We first assayed the bone extracts for the presence of other bone matrix proteins and confirmed the presence of fibronectin, vitronectin, osteopontin and bone sialoprotein. Through transwell migration assays, it was also confirmed that breast cancer cells migrated toward fibronectin, vitronectin and osteopontin; this migration was RGD-dependent. We then determined that the breast cancer cell attraction and migration toward bone extracts was also RGD-dependent. The next aim of the dissertation was to analyze the soluble portion of bone. The soluble portion of bone was collected in the form of conditioned media from an osteoblast cell culture. We demonstrated that breast cancer cells were also attracted to and migrated toward osteoblast-conditioned media. This migration was not RGD-dependent and was correlated to the concentration of the chemokine, RANTES. We concluded that osteonectin was not a chemoattractant that directed beast cancer cell migration but increased random cellular motility. In contrast, RGD-containing proteins,

namely fibronectin, vitronectin and osteopontin attracted breast cancer cells and may contribute to their preferential metastasis to bone. In addition to the RGD-containing proteins, osteoblasts also secreted unidentified soluble factors that attracted breast cancer cells.

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Abbreviations

BMP	bone morphogenetic protein
DMEM	Dulbecco's Modified Eagle's Medium
FBS	fetal bovine serum
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
HRP	horseradish peroxidase
IGF	insulin-like growth factor
IL	interleukins
INF	interferon
MCP-1	monocyte chemotactic protein-1
MIP	macrophage inflammatory protein
MMP	matrix metaloproteinase
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
pen	penicillin
PMSF	phenylmethanesulfonyl fluoride
PTH	parathyroid hormone
PTH-rp	parathyroid hormone related peptide
RANK	receptor-activator for NFκB
RANKL	receptor-activator for NFκB ligand
RANTES	regulated on activation normal T-cell expressed and secreted protein
RGD	arginine, glycine, and aspartic acid peptide
SDF-1α	stromal cell derived factor-1 alpha
strep	streptomycin
TGF	transforming growth factor
TNF	tumor necrosis factor

Cell Lines

HBME-1	human bone marrow endothelial
hFOB1.19 (hFOB)	human fetal osteoblast
hTERT-HME1	human mammary epithelial
NIH3T3	mouse fibroblast
MC3T3-E1	mouse osteoblast
MDA-MB-231	human breast cancer
MDA-MB-435	human breast cancer
MDA-MB-468	human breast cancer

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Chapter 1

Introduction

Breast cancer statistics and the biology of metastasis

The American Cancer Society reported that cancer was the primary cause of death for American women in 2002 [1]. The most common location of cancer development women experience is within the breast. In fact, one out of every eight women will develop breast cancer in her lifetime [2]. The impact of breast cancer on world health is substantial due in part to the prevalence of the disease; but even more devastating is the mortality rates that result due to metastasis. Breast cancer claims the lives of more than 40,000 American women each year [3] making it the second deadliest type of cancer after lung cancer [4].

Breast cancer develops from the epithelial tissue of ducts and glands within the breast. Carcinomas that develop from the epithelial cells of either lobules or glands are termed lobular carcinomas. A carcinoma that develops from the epithelial cells of the ducts is designated as ductal carcinoma. Ductal carcinomas are more prone to genetic mutations and therefore have a greater frequency of metastasis [5].

Twenty-five percent of newly diagnosed breast cancer patients will ultimately develop skeletal metastases. At autopsy, 75% of all breast cancer patients are found to have bone metastases [6]. Breast cancer is not the only type of cancer to frequently metastasize to bone. The three most common types of cancers in humans, (lung, breast and prostate) all have increased propensities to metastasize to bone [7]. Upon the diagnosis of skeletal metastases, patients have a mean survival time of five years.

Although this survival time is relatively long compared to other cancer prognoses, breast cancer patients suffer greatly during this time. The morbidity induced by bone metastases includes spinal cord compression, fractures, hypercalcemia, and severe bone pain [8]. Despite the prevalence of breast cancer metastasis to bone and the negative effect it has on quality of life, relatively little is understood about why bone is the preferred site of metastasis.

The most likely cause of death for a breast cancer patient occurs when there is metastasis to a distant organ. The progression of metastasis occurs in a series of specific and interrelated steps (Figure 1.1). First, cells from the primary tumor must detach from neighboring cancer cells and invade the basal lamina surrounding the tumor (Figure 1.1 B). This invasion is possible due to cancer cell secretion of matrix metaloproteinases (MMPs) [7]. The migrant cells then enter the surrounding vascular or lymphatic systems (Figure 1.1 C, D) and drain into nearby lymph nodes (Figure 1.1 D). Once inside a lymph node, cells are then directed into the circulatory system (Figure 1.1 E). As the cells travel through the circulatory system, they must then attach to vascular endothelium within a distant organ. This adhesion usually occurs through attachment molecules such as E-cadherin [7], cellular adhesion molecules, and selectins [9] (Figure 1.1 F) and most often happens in the lung, liver and bone [10] (Figure 1.1 G). The adherent cells next extravasate through the endothelial cell layer and into the organ tissue; the extravasation process is also facilitated by breast cancer cell secretion of MMPs [7] (Figure 1.1 H). Finally, in order for these cells to form tumors at a distant site, cancer cells must survive the host immune system, proliferate and develop a vascular network for nutritional support.

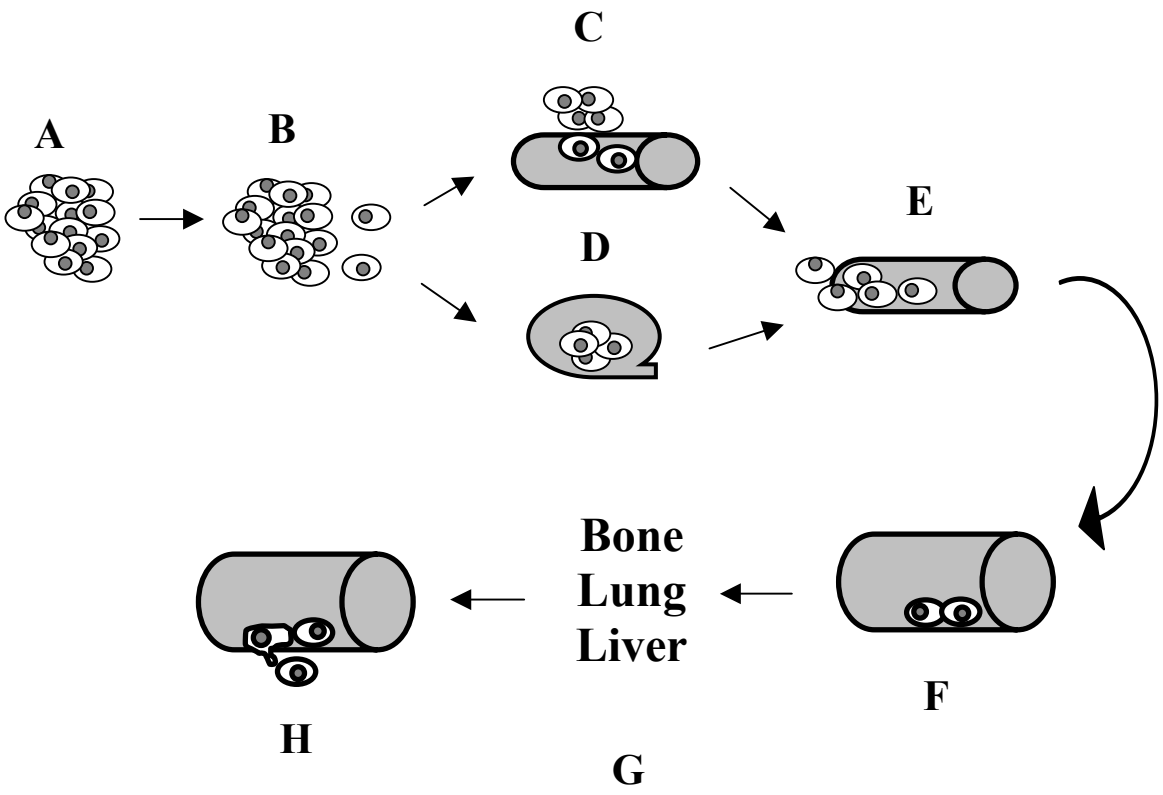


Figure 1.1 Steps involved in breast cancer cell metastasis to distant organs. A,B) Cancer cells detach from nearby cells and invade through the basal lamina surrounding the primary tumor. The detached cells either enter C) the circulatory system through local blood vessels or D) the lymphatic system where they are then drained into a nearby lymph node. E) Cells in a lymph node eventually pass into the blood. F) The migrant cancer cells attach to the endothelial cells of a capillary or sinusoid G) usually within bone, lung or liver. H) Cells then extravasate out of the vasculature and into the organ tissue.

Gross anatomy of bone

The bones of the body can be classified into two groups, long bones (the tibia, femur, and humerus) and flat bones (the skull, ileum, and mandible). In general, these bones are composed of both cortical and trabecular bone. The outermost region of a bone consists of cortical bone, which is characterized by a very dense matrix. Cortical bone is vital to supporting the weight load of the body. The inside of flat bones and ends of long bones are composed of trabecular bone (also called cancellous bone). This bone is less dense and appears spongy to the naked eye. Trabecular bone absorbs most of the impact put on the body and is highly metabolically active. All bone matrix undergoes remodeling but trabecular bone turns over at a greater rate than cortical bone [11].

Bone matrix is composed mainly of type I collagen which accounts for about 90% of total protein content of bone [12]. There are at least 20 other proteins that represent the remaining 10% of matrix proteins [13]. The mineral content of bone is mostly composed of hydroxyapatite [$3Ca(PO_4)_2(OH)_2$] although many other minerals, such as magnesium, sodium and carbonate are also found [12].

Long bones are divided into three areas, the diaphysis (long shaft), metaphysis, and epiphysis (Figure 1.2). The diaphysis is composed mainly of cortical bone whereas the metaphysis is mostly composed of trabecular bone. Furthermore, the trabecular bone of the metaphysis is surrounded by hematopoietic marrow, fatty marrow and blood vessels [14].

Bone development occurs by either intramembranous ossification or endochondral bone formation. Intramembranous ossification occurs at the site of flat formation of a cartilage limb bud, which is later replaced with bone matrix. The length

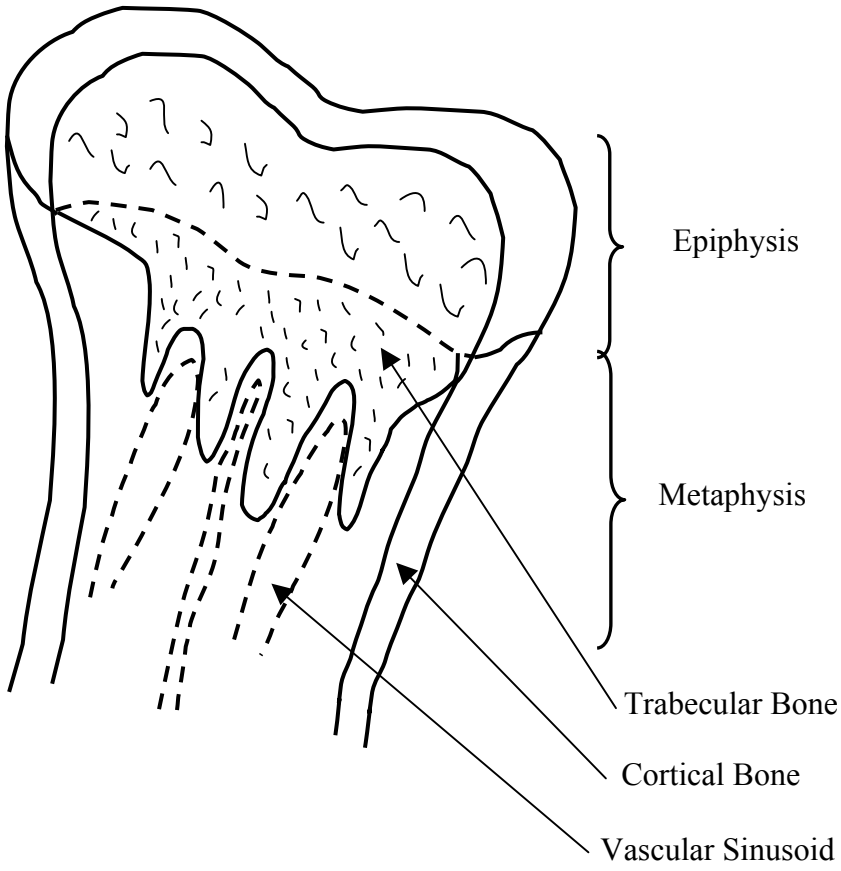


Figure 1.2 Diagram of the end of a typical long bone. Long bones are divided into three regions, the diaphysis (shaft), metaphysis and epiphysis (end of the bone and the site of joint contact). Bone matrix is classified as either cortical bone or trabecular bone. The microvasculature of the trabecular bone is composed of large discontinuous sinusoids.

of the long bones is a result of cell growth at the growth plate; this is the point of contact between the metaphysis and the epiphysis. Cartilage is replaced with bone matrix during endochondral bone formation through a series of cellular events by which chondrocytes proliferate, deposit cartilage, and then hypertrophy. The cellular debris is removed upon vascular invasion and the cartilage framework is degraded while osteoblasts lay down new bone matrix. The vascularization into the cartilage rudiment follows the longitudinal growth of chondrocytes and results in the formation of large vascular sinusoids [15] (Figure 1.2).

Bone is a highly vascularized organ consisting of arterial inputs, venous outputs and microvascularization [16]. The trabecular bone has a unique microvascular system characterized by large sinusoids. Sinusoids are also present within the liver and are usually the sites of breast cancer metastasis in either organ. Vascular sinusoids are characterized by large lumens that span up to 100 microns in diameter and therefore have reduced blood flow. The blood flow measured in a sinusoid is about 30 times slower than the blood flow in a capillary [17]. A unique characteristic of metaphyseal sinusoids are that they are lined with endothelial cells that have 60 Å fenestra [18]. Fenestra are important regulators of vascular permeability and are involved in transendothelial migration of cells [19, 20]. Sinusoids are also characterized by a discontinuous layer of adventitious cells and basal lamina which are involved in the dilation of the vascular barrier to allow the passage of hematopoetic cells [17, 21].

The physiology of bone turnover

Adult bone is an active organ that is constantly being remodeled. Two specialized cell types that either build new or degrade old bone matrix facilitate bone turnover. The bone cells responsible for producing new bone matrix are osteoblasts. Osteoblasts develop from mesenchymal stem cells found within bone marrow. Local growth factors and bone morphogenetic proteins (BMPs) induce mesenchymal stem cells to proliferate and differentiate into preosteoblasts. Preosteoblasts then further differentiate into fully functional osteoblasts [22]. Differentiated osteoblasts secrete many different growth factors, cytokines, matrix proteins and hydroxyapatite. Osteoblasts are also never found alone; they exist in small clusters surrounded by preosteoblasts and mesenchymal stem cells [12]. Some osteoblasts are encased by the developing matrix; these engulfed cells differentiate into osteocytes and play a major role in maintaining bone health. The remaining osteoblasts either become apoptotic or flat bone lining cells in their final phase of differentiation [23].

The cells responsible for bone resorption are osteoclasts. Osteoclasts develop from circulating monocytes in the blood that have been activated by osteoblast secretions. Osteoblasts express receptor-activator for NFκB ligand (RANKL) on their plasma membrane surface, which binds to its receptor (RANK) found on the membrane surface of monocytes. RANKL promotes cellular fusion of several monocytes to form a single, multinucleated osteoclast [24]. Activated osteoclasts (Figure 1.3) bind to bone matrix through $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_2\beta_1$ integrins located on the membrane surface.

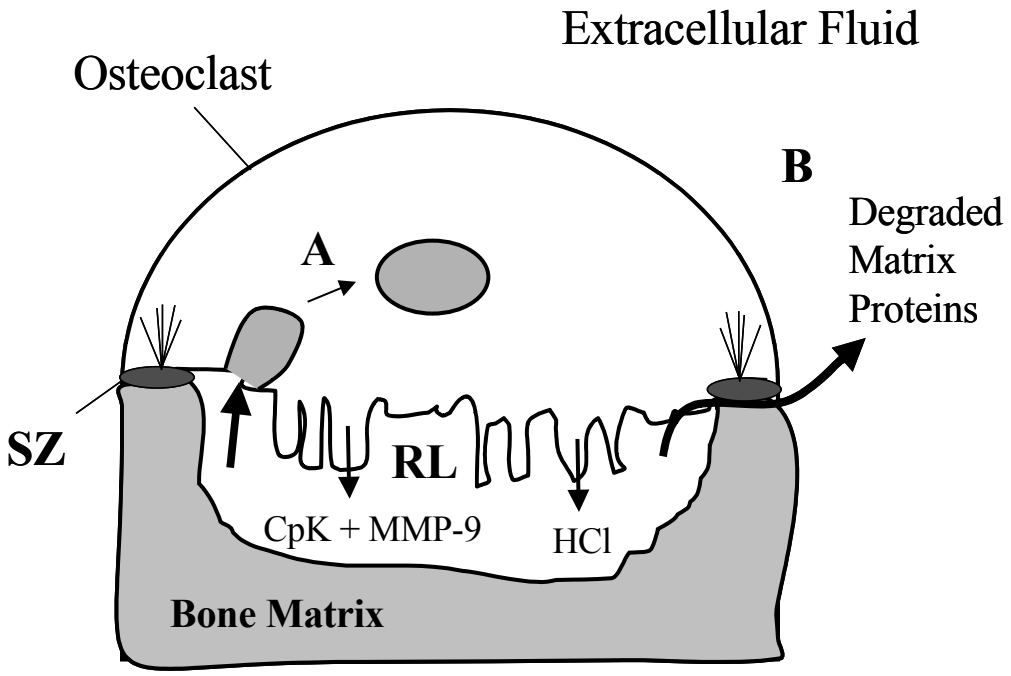


Figure 1.3 Osteoclastic bone resorption. An osteoclast binds to bone matrix through integrins on the cell surface; this point of contact is referred to as the sealing zone (SZ) and is characterized by the presence of an intercellular actin ring. The sealing zone surrounds the resorptive lacuna (RL), which is filled with cathepsin k (CpK), MMP-9 and HCl to degrade the matrix proteins and solubilize mineral. These enzymes and acid are secreted at the ruffled border of the osteoclast plasma membrane. After matrix proteins are degraded, they are either A) endocytosed or B) released into the extracellular fluid from under the sealing zone.

The point of contact between the matrix and osteoclast integrins is referred to as the sealing zone. The sealing zone is characterized by the formation of an intercellular actin ring responsible for stabilizing the cell. In addition to being the primary means of osteoclast attachment and support to the bone matrix, the sealing zone is vital to the creation of a resorptive lacuna. The resorptive lacuna is an isolated microenvironment where an osteoclast plasma membrane is in close proximity to bone matrix. This area becomes highly acidic due to the sizeable quantity of H^+ ions secreted by osteoclasts. The plasma membrane contained in the resorptive lacuna is ruffled in appearance and aptly referred to as the ruffled border. Characterized by deep folds, the ruffled border is highly active during bone resorption containing many V-type ATPases and Cl^- channels used to transport H^+ and Cl^-, respectively, into the resorptive lacuna [25, 26]. Osteoclasts contain many proteolytic enzyme-filled vesicles, which are also released into the resorptive lacuna. Cathepsin K and MMP-9 are the best characterized proteolytic enzymes that are released by osteoclasts and are fundamental to matrix resorption. These enzymes degrade the protein portion of the matrix while the acidic environment solubilizes the mineral content [26, 27].

Bone formation and resorption are kept in balance by local and systemic regulators. Remodeling is initiated when bone is damaged or serum mineral homeostasis is compromised. Locally, growth factors such as interleukins, transforming growth factor-β (TGFβ), fibroblast growth factor (FGF), BMPs and members of the tumor necrosis factor (TNF) ligand family are released from the damaged bone matrix. These growth factors induce mesenchymal stem cells to differentiate into osteoblasts, which will develop new bone matrix. The osteoblasts also stimulate osteoclastogenesis to break

down the old or damaged bone matrix [28]. The systemic hormones, parathyroid hormone (PTH) and 1,25 dihydroxycholecalciferol (vitamin D), are released in response to low serum calcium levels. PTH binds to the PTH receptor on osteoblast plasma membranes to stimulate RANKL expression. As already discussed, RANKL stimulates osteoclastogenesis which in turn restores normal serum calcium levels [29].

The development of a chemotactic gradient

Because bone is a common site of breast cancer metastasis, researchers have attempted to identify unique properties that may attract circulating breast cancer cells to bone. Bone is composed of many different growth factors and matrix proteins, some of which have fairly limited expression to mineralized tissues such as bone and teeth. It is reasonable to hypothesize that some of these unique factors may be responsible for the migration of breast cancer cells out of the vasculature and into the bone microenvironment.

Chemotactic factors must exist in a gradient to attract distant cells. A gradient is needed to expose a responsive cell to a small amount of chemoattractant. The cell will then bind and migrate toward greater concentrations of the attractive protein. The highest concentration of most bone-derived proteins will likely exist in the matrix. This concept is supported clinically by the observation that bone metastases are often found in close proximity to bone matrix [7].

There are several processes that result in the development of a bone-derived chemotactic gradient. Because osteoblasts produce and secrete bone matrix proteins, it is likely that any bone-derived chemoattractant was originally secreted by osteoblasts

(Figure 1.4). Osteoblasts are polarized cells that secrete most of their proteins in a single direction. Most of the osteoblast-secreted proteins are deposited directly into the developing bone matrix and then mineralized [30]. At this point, these proteins are unlikely to form a chemotactic gradient due to their static placement within the bone matrix. However, some of the smaller proteins, such as osteocalcin (~ 5 KD) can diffuse out of the matrix and into the circulation. Clinically, serum concentrations of osteocalcin are used to measure bone development [31]. Although most of the proteins produced by osteoblasts are secreted into the developing matrix, some, such as osteoprotegrin, are also released directly into the surrounding extracellular fluid [30].

Bone proteins deposited directly into the developing matrix cannot readily form a chemotactic gradient due to their stationary placement. However, during bone resorption, these proteins are released from the matrix and could at this point form a chemotactic gradient (Figure 1.3). Osteoclasts release proteolytic enzymes and acid that break down bone matrix. Degraded matrix proteins are hypothesized to be expelled from the resorptive lacuna by one of two mechanisms. The first mechanism involves osteoclastic endocytosis and subsequent intracellular digestion of the degraded proteins. A second process involves degraded proteins being released from beneath the sealing zone and into the extracellular fluid [26]. Breast cancer cells could detect and migrate toward bone matrix proteins as they are released during the process of osteoclastic bone resorption. Some proteins released during bone resorption include matrix proteins and growth factors such as TGF-β, insulin-like growth factor-1 and 2 (IGF-1, IGF-2), FGF-1, FGF-2, and platelet derived growth factor (PDGF) [8]. Clinicians use serum concentrations of bone sialoprotein to measure bone resorption [31].

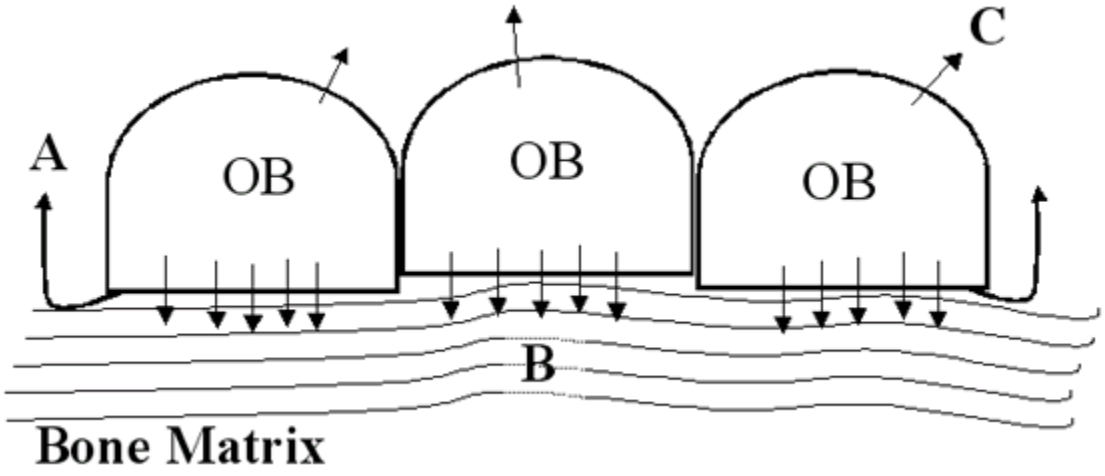


Figure 1.4 Directionality of osteoblast protein secretions. Osteoblast-derived proteins are secreted in three different directions. A) Some small proteins, such as osteocalcin, can diffuse out of the matrix and into the extracellular fluid. B) Most matrix proteins and growth factors are secreted directly into the developing matrix. C) Proteins such as osteoprotegrin are secreted directly into the extracellular fluid.

Once breast cancer cells have migrated into the bone microenvironment, they usually develop osteolytic lesions [7]. Osteolysis occurs when the balance between bone resorption and bone formation favors resorption and ultimately results in significant bone loss. One well characterized mechanism of breast cancer-induced osteolytic bone resorption involves secretion of parathyroid hormone related protein (PTH-rp). PTH-rp binds to the PTH receptor expressed on the plasma membrane surface of osteoblasts and functions similarly to the systemic hormone PTH [8]. PTH and PTH-rp stimulate osteoblast expression of RANKL, which then stimulates osteoclastogenesis. When bone matrix is resorbed, many growth factors such as TGF-β and IGF are released. These growth factors in turn promote breast cancer cell proliferation and hence more localized breast cancer-secreted agents responsible for bone resorption. Other agents that breast cancer cells secrete to increase bone resorption are colony stimulating factor, endothelial growth factor, PDGF and TNF-α [32]. Breast cancer-induced bone resorption is responsible for the morbidity often associated with bone metastases (ie. fracture, hypercalcemia and subsequent bone pain) [33].

Some matrix proteins, produced by osteoblasts and released by osteoclasts, have been described as possible chemoattractants for breast cancer cells. Therefore, these proteins could contribute to the preferential metastasis of breast cancer cells to bone and be exploited for the development of intervention therapy. We assessed the chemotactic quality of the bone matrix proteins, osteonectin and RGD-containing proteins, specifically osteopontin, fibronectin and vitronectin.

Osteonectin as a bone-derived chemotactic factor

The relationship between osteonectin and cancer has gained some attention in recent years. Osteonectin (also called SPARC or BM-40) was originally discovered in bone by its ability to bind to type I collagen [34]. Later studies identified osteonectin in many other normal and neoplastic tissues [35]. This 32-46 kD glycoprotein is characterized by three domains, each with a specific function. The acidic domain (a.a. 1-52) inhibits cell spreading and prevents chemotaxis [36]. A second domain, the follistatin-like domain (a.a. 53-137), inhibits proliferation and disrupts focal adhesions. The extracellular Ca^{2+}-binding domain (a.a. 138-286) also inhibits cell spreading, proliferation, and focal adhesions, but in addition, binds cells and other matrix proteins in a calcium-dependent manner [37].

Although osteonectin binds to many structural proteins, such as type I collagen, type IV collagen [38] and vitronectin [39], it is not responsible for the structural stability of extracellular matrix [40]. Instead, osteonectin has mainly been described as a matricellular protein with de-adhesive qualities [41]. Soluble osteonectin induces the loss of focal adhesion plaques in endothelial cells. The loss of focal adhesions is characterized by the redistribution of vinculin and F-actin within the cytoplasm [42]. Cells remain attached to matrix proteins through integrins despite the loss of focal adhesion plaques. Such cells appear to have a normal morphology, namely, they remain spread over a matrix [43]. When a cell maintains integrin binding to a matrix but loses focal adhesion plaques, it is described as being in a state of "intermediate adhesion" which is considered ideal for cell motility (Figure 1.5) [41]. In contrast, strong attachment, which is characterized by many focal adhesions, prevents a cell from

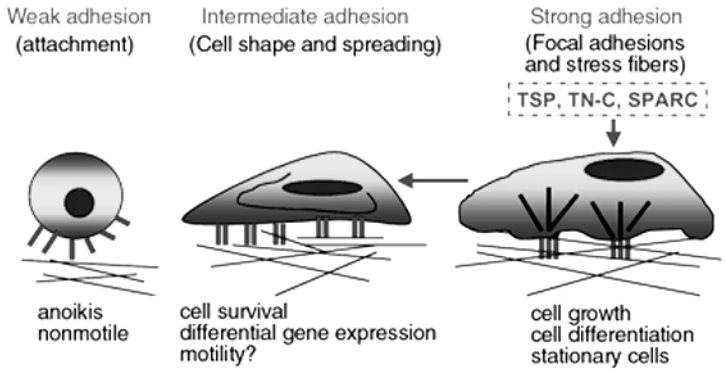


Figure 1.5 Osteonectin-induced intermediate adhesion. Exogenous osteonectin (SPARC), tenascin-C (TN-C), and thrombospondin-1 and 2 (TSP) will induce intermediate adhesion of a strongly adherent cell. This is characterized by the maintenance of integrin binding to the matrix while actin fibers are redistributed and focal adhesions are lost. Intermediate adhesion is distinct from weak adhesion where integrin binding is also lost. Murphy-Ullrich JE (2001) J Clin Invest.

detaching the matrix and consequently renders the cell immobile. When a cell is weakly attached, there is little or no integrin binding and the cell is unable to develop the contractile force needed for movement [41]. Soluble osteonectin is a potent stimulator of intermediate attachment and therefore enhances cell motility. Because of its anti-adhesive properties and effect on cell motility, osteonectin has been considered as a possible chemoattractant.

Clinically, invasive breast carcinomas produce more osteonectin than normal breast tissue [44]. This is also true for prostate cancer cells; osteonectin upregulation is correlated with bone metastases [45]. It has been reported that osteonectin produced by bone cells is a chemoattractant for prostate cancer cells [46] and the main factor responsible for prostate cancer cell migration toward bone extracts [47]. Osteonectin has not been thoroughly examined as a chemoattractant for breast cancer cells; this glycoprotein could be a major contributing factor to their preferential metastasis to bone. However, because many metastatic breast cancer cells also produce osteonectin, it is counterintuitive to postulate that these same cells would be attracted to exogenous osteonectin. In order for a cell to migrate toward a chemotactic factor, a gradient must form so that a responsive cell can move toward greater concentrations.

Because many metastatic breast cancer cells and bone cells produce osteonectin, a chemotactic gradient would not exist unless one of two conditions was present. The first condition is that the bone cells secrete a unique configuration of osteonectin, hence a chemotactic isoform. If tissue-dependent configurations of osteonectin exist, it is reasonable to hypothesize that bone-derived osteonectin could form a gradient and therefore attract breast cancer cells. The second condition is that breast cancer cells that

migrate to bone secrete little or no osteonectin and could therefore respond to the bone-derived osteonectin. This second condition is plausible considering the Woelfle report (2003), which correlated a positive diagnosis of bone micrometastases with primary tumors that have reduced expression of osteonectin [48].

RGD-containing proteins as bone-derived chemotactic factors

Arg-Gly-Asp (RGD)-containing proteins, found in bone matrix, have been described as chemoattractants for breast cancer cells [49-56]. Within bone, the RGD-containing proteins include fibronectin, vitronectin, osteopontin, bone sialoprotein, thrombospondin, and fibrillin. These glycoproteins are distinct from each other but categorized together because of their common RGD domain. RGD peptides bind to integrin receptors on the plasma membrane surface of many cell types and are therefore important for cell attachment [13].

A number of studies have reported that cancer cells are chemoattractant to RGD through $\alpha_v\beta_3$. Breast cancer cells, including the MDA-MB-231 cell line, have been shown to migrate toward fibronectin, vitronectin, and bone sialoprotein in an RGD-dependent manner through the $\alpha_v\beta_3$ integrin [49-52]. The activation of $\alpha_v\beta_3$ integrin is essential for endothelial cell migration toward bone sialoprotein. However, this is not true for some melanoma cells and the MDA-MB-435 cell line which migrate to bone sialoprotein independent of integrin activation [53]. *In situ* hybridization of bone biopsies revealed that the breast cancer cells that colonize bone over express $\alpha_v\beta_3$ [54]. Other studies have reported *in vivo* evidence that circulating cancer cells with reduced expression or blocked function of $\alpha_v\beta_3$ develop fewer bone metastases [55, 56].

Chemokines as bone-derived chemotactic factors

Chemokines are a subset of cytokines known to be chemoattractive to immune cells and are divided into four subgroups: CXC, CC, C and CX_3C. Recently, some chemokines have been described to be chemoattractive for cancer cells and may be responsible for their metastasis to distant organs [57]. Evidence has emerged suggesting that bone-derived chemokines, such as monocyte chemoattractant proteins (MCP), RANTES, and stromal cell-derived factors (SDF) [58-60], may contribute to the preferential metastasis of multiple types of cancer to bone [61-65]. MCP-1 secreted by the endothelial cells of the bone marrow is chemoattractive for multiple myeloma cells [61]. The expression of the CCR2 (MCP-2 receptor) is vital to the migration of multiple myeloma cells toward bone marrow stromal cells [62]. Multiple myeloma cells are also chemoattractive to regulated on activation normal T-cell expressed and secreted protein (RANTES) through expression of the CCR1 receptor [63]. RANTES has also been shown to chemoattract three breast cancer cell lines *in vitro* [64]. The expression of CXCR4 (SDF-1α receptor) by breast cancer cells has been included in a gene expression signature responsible for the development of bone metastases [65].

Aims of Study

The focus of this study is to identify what factors within bone matrix are chemoattractive to breast cancer cells. A clear understanding of the chemotactic factors responsible for the attraction of breast cancer to bone may lead to the development of new therapies to block skeletal metastases.

Hypothesis

Osteoblasts secrete proteins that chemoattract breast cancer cells.

Specific Aim 1: *To determine if a chemotactic isoform of osteonectin exists.*

A chemotactic isoform of osteonectin could attract breast cancer cells to bone. To study this aim, osteonectin was immunopurified from multiple cell lines such as breast cancer, osteoblast, non-neoplastic breast epithelial and bone marrow vascular endothelial cells. The osteonectin from the different sources was analyzed for post-translational differences, specifically, variations in molecular size, glycosylation, phosphorylation and amino acid sequence.

Specific Aim 2: *To determine if bone-derived osteonectin will chemoattract breast cancer cells.*

Bone-derived osteonectin could attract breast cancer cells that secrete reduced levels of osteonectin. This aim was accomplished by measuring cell motility and migration induced by osteonectin. Undirected breast cancer cell motility was induced with soluble osteonectin purified from bone marrow vascular endothelial cells, osteoblasts and cancer cells. Breast cancer cell migration toward osteonectin from multiple sources, including bone, was also measured. The chemoattraction of breast cancer cells to osteonectin and a known chemoattractant, vitronectin, was compared. Finally, the ability of osteonectin to chemoattract breast cancer cells in the presence of other matrix proteins was assayed through the use of bone extracts from wild-type and osteonectin-null mouse bones.

Specific Aim 3: *To determine which factors within bone extracts chemoattract breast cancer cells.*

Bone extracts were found to be chemotactic for breast cancer cells. The bone extracts were analyzed for the presence of fibronectin, vitronectin, osteopontin and bone sialoprotein. Breast cancer cell migration toward fibronectin, vitronectin and osteopontin was measured. The migration toward fibronectin and vitronectin was determined to be RGD-dependent. Finally, the contribution of RGD-dependent migration toward bone extracts was quantified. Through a series of transwell chamber migration assays and RGD blocking peptides, the chemotactic ability of the mineralized portion of bone matrix was measured.

Specific Aim 4: *To reveal possible osteoblast-derived soluble factors that chemoattract breast cancer cells.*

The chemotactic quality of the mineralized portion of bone matrix was quantified but whether osteoblasts secrete soluble chemoattractants was unknown. Conditioned media were collected from mouse osteoblast cultures. These media were utilized to measure breast cancer cell attraction to osteoblast-secreted soluble factors. Conditioned media were also assayed for RGD-dependent migration and the presence of cytokines.

Chapter 2

Materials and Methods

Cell lines

The human breast cancer cell lines used were the MDA-MB-231 [66], MDA-MB-468 and MDA-MB-435 [67]. These breast cancer cell lines and the human bone marrow endothelial cell line, HBME-1 [68] which was derived from iliac crest biopsies, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) and supplemented with 5% fetal bovine serum (FBS), 100 I.U./ml penicillin (pen, Mediatech, Herndon, VA) and 100 μg/ml streptomycin (strep, Mediatech). The human mammary epithelial cell line, hTERT-HME1 (BD Biosciences-Clontech, Palo Alto, CA), was grown using the MEGM® BulletKit® media system (Cambrex, Walkersville, MD) which consists of bovine pituitary extract, hydrocortisone, human epithelial growth factor, and insulin. A human fetal osteoblast cell line, hFOB1.19 (hFOB) [69] was grown in DMEM/F-12 (Mediatech) with 10% FBS, 100 I.U./ml pen and 100 μg/ml strep. NIH3T3 (mouse fibroblasts) were grown in DMEM with 10% FBS, 100 I.U./ml pen and 100 μg/ml strep. The MC3T3-E1 (mouse osteoblasts) cell line was grown in αMEM with 10% FBS, 50 μg/ml vitamin C, 10mM β-glycerophosphate, 100 I.U./ml pen and 100 μg/ml strep.

Protein isolation – osteonectin immunopurified from cell lines

Osteonectin was immunopurified from conditioned media collected from the MDA-MB-435, MDA-MB-468, hTERT-HME1, hFOB, and HBME-1 cell lines. Cells were grown to confluency in their respective media, rinsed three times in phosphate buffered saline (PBS, pH 7.2), and then exposed to DMEM/F-12 with Serum Replacement 3 (Sigma-Aldrich), 100 I.U./ml pen and 100 μg/ml strep for 24 hours; normal growth media was used for the hTERT-HME1 cell line. Serum replacement was required because osteonectin is present in FBS; the hTERT-HME1 growth media does not contain osteonectin and was therefore suitable for use as conditioned media. The Serum Replacement 3 contains human serum albumin, human transferring, and human recombinant insulin; it does not contain growth factors, steroid hormones, glucocorticoids, cell adhesion factors, detectable antibodies and mitogens. Osteonectin produced by the cultured cells was isolated using anti-human osteonectin monoclonal mouse IgG (Haematologic Technologies, Essex Junction, VT) linked to an AminoLink® Plus Immobilization kit (Pierce, Rockford, IL). The monoclonal antibody (1 μg) was coupled to the AminoLink® Plus Gel using the "pH 7.2 coupling buffer" procedure for ligand immobilization as described by the manufacturer. Approximately 250 ml of conditioned media was mixed with the antibody-coupled gel at 4°C overnight while rotating at 160rpm on a rotary platform. Samples were eluted from the gel with 0.1 M glycine, dialyzed against PBS, and stored at -80°C. Concentrations of osteonectin were determined by an ELISA (Haematologic Technologies). Purity of samples was confirmed by a Coomassie blue stained SDS-PAGE gel; only a single band at the molecular weight of osteonectin was detected.

Protein isolation – mouse bone extracts

The femurs and tibias of 7-9 week old 129SV X C57BL/6 osteonectin knockout, heterozygous and wild-type mice were a gift from Dr. Hynda Kleinman (National Institutes of Health, National Institute of Dental and Craniofacial Research) [70]. The mice were euthanized by CO_2 inhalation; their hind limbs were removed and freed of muscle, tendon, and bone marrow. The cleaned bones were then rinsed in PBS, flash frozen in liquid nitrogen, and stored at -80°C.

A modified method of Termine et al. [71] was used to extract protein; the conditions used are known to release native osteonectin that binds to collagen and hydroxyapatite [34]. Frozen bones were crushed into a fine powder under liquid nitrogen using a mortar and pestle. One gram of bone powder was diluted into 50 ml of extraction buffer (4.0 M guanidine HCl, 0.05 M Tris, 0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM phenylmethanesulfonyl fluoride (PMSF) pH 7.2) and mixed on a rotary platform at 160rpm for 24 hours in 4°C. Samples were centrifuged at 1800g for 6 minutes, the supernatant was removed and stored at –80°C until dialyzed. The first bone extraction removed the nonmineralized portion of the bone including remaining cellular debris, vasculature and soluble matrix proteins. The remaining residue was treated with 10 ml volume of extraction buffer, which also contained 0.5 M EDTA. The samples were incubated at 4°C for 72 hours and mixed on a rotary platform at 160rpm. The samples were then centrifuged at 1800g for 6 minutes and the supernatant collected. These bone extracts constitute the noncollagenous mineralized portion of the bone. Bone extracts were dialyzed twice against dH_2O with protease inhibitors (0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM PMSF) at 4°C for a total of 48

hours. The samples were lyophilized and reconstituted in dH_2O with protease inhibitors and stored at -80°C. Protein concentrations were determined using the BioRad Protein Assay system (BioRad, Hercules, CA). The total protein content of the bone extracts was also analyzed by separation on a 12% SDS-PAGE gel, stained with 7.5% SYPRO® orange in acetic acid (Molecular Probes, Eugene, OR) and imaged by a Typhoon™ Variable Mode Imager (Amersham Biosciences, Piscataway, NJ).

Immunoblotting – detection of osteonectin

Purified osteonectin samples (10 ng) derived from cultured media were diluted with Laemelli loading buffer, reduced with 100 mM DTT and boiled for 5 minutes. Following separation on a 15% SDS-PAGE gel, proteins were transferred to nitrocellulose membranes (BioRad). The membranes were blocked with 3% BSA in PBS for 1 hour at room temperature. Membranes were subsequently exposed to primary antibody (anti-human osteonectin mouse IgG, Haematologic Technologies) at a dilution of 1:15,000 in blocking solution and incubated overnight at room temperature. A secondary antibody, sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP, Amersham Biosciences), was used at a dilution of 1:3750 in PBS and incubated for 1 hour at room temperature. Immunolabeled bands were detected using an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

The detection of osteonectin from mouse bone extracts was accomplished by the same immunoblotting method with the following modifications. Blots were blocked with 5% non-fat dry milk and 1% BSA in PBS. The primary antibody, anti-mouse osteonectin rabbit IgG, was diluted to 1:1000 in blocking solution [72]. A horse anti-rabbit IgG conjugated to HRP (Amersham Biosciences) was diluted to 1:5000 in PBS.

Immunoblotting – detection of RGD-containing proteins

The detection of fibronectin and vitronectin in mouse bone extracts was accomplished by utilizing immunoblotting techniques similar to those described for immunopurified osteonectin but with the following modifications. Bone extracts were separated on a 6% (for fibronectin) or 10% (for vitronectin) SDS-PAGE gel, transferred to nitrocellulose and blocked in 5% non-fat dry milk in PBS and 0.05% tween-20. The primary antibody (anti-human fibronectin or anti-human vitronectin rabbit IgG, Santa Cruz Biotechnologies, Santa Cruz, CA) was diluted to 1:1000 in blocking solution and incubated on the membrane overnight at room temperature. A secondary antibody (anti-rabbit IgG raised in donkey and conjugated to HRP, Amersham Biosciences) was diluted 1:10,000 in PBS with 0.05% tween-20 and incubated for 1 hr at room temperature. All washes were done with PBS and 0.05% tween-20.

The detection of bone sialoprotein and osteopontin in the bone extracts was accomplished by immunoblotting with the following modifications. Extracts were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose. Blots were blocked in 5% non-fat dry milk and 1% BSA in PBS for 1 hr at room temperature. Primary antibody (anti-human osteopontin rabbit IgG [73, 74] and anti-human bone sialoprotein

rabbit IgG [75, 76] provided by Dr. Larry Fisher, National Institutes of Health, National Institute of Dental and Craniofacial Research) was diluted 1:5000 in blocking solution and incubated overnight at room temperature. The secondary antibody, anti-rabbit IgG raised in sheep and conjugated to HRP (Amersham Biosciences) was diluted to 1:2500 in PBS and blots were incubated for 1 hour at room temperature. All washes were done with PBS and 0.05% tween-20

Phosphoserine detection

Detection of phosphoserines was accomplished by immunoblotting the immunopurified osteonectin samples collected from MDA-MB-435, MDA-MB-468, hTERT-HME1, hFOB, and HBME-1 cell lines. The samples (10 ng) were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose. The nitrocellulose membranes were blocked in 3% BSA in PBS with 0.2% tween-20 and phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride). The primary antibody was an anti-phosphoserine mouse IgG (Sigma-Aldrich) diluted to 1:5000 in blocking solution. The secondary antibody was diluted to 1:3750 in PBS with 0.2% tween-20 and phosphatase inhibitors; all rinses were done in the presence of phosphatase inhibitors. Rat brain extracts (Biomol, Plymouth Meeting, PA) were used as the positive control.

Deglycosylation

Analysis of osteonectin glycosylation was accomplished with the Sigma-Aldrich Enzymatic Protein Deglycosylation Kit. This kit includes the enzymes, denaturing solution, Triton-X solution, and buffer. Osteonectin (50 ng) immunopurified from conditioned media collected from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines was denatured and mixed with either a.) Reaction buffer alone (control), b.) PNGase F c.) PNGase F with α-2 neuraminidase, d.) O-glycosidase, e.) O-glycosidase with α-2 neuraminidase, f.) O-glycosidase with α-2 neuraminidase and β-galactosidase, or g.) O-glycosidase with α-2 neuraminidase and β-N-acetylglucosaminidase. Samples were enzymatically deglycosylated at 37°C for 3 hours. The deglycosylated samples were then analyzed for shifts in migration by immunoblotting for immunopurified osteonectin as described earlier.

cDNA sequencing

Cell cultures of MDA-MB-435, MDA-MB-468, hFOB, hTERT-HME1, and HBME-1 were rinsed in PBS and lysed using the QIAshredder™ kit (Qiagen, Valencia, CA). RNA was isolated with an RNeasy® kit (Qiagen) and stored at -80°C. Full length cDNA transcripts were generated using the reverse transcriptase Retroscript kit (Ambion, Austin, TX) which included an oligo(dT) primer, dNTP mix, and RNase inhibitors. The MDA-MB-435, MDA-MB-468, hFOB, and hTERT-HME1 osteonectin cDNA coding region (911 bp) was amplified by PCR with the following primers: forward CCT-GCC-TGC-CAC-TGA-GG and reverse TAA-ACA-TTG-GGG-GAA-ACA-CG (MWG Biotech, High Point, NC). Primers were selected from the human osteonectin published

sequence (Genbank accession # NM003118). The HBME-1 cDNA was amplified using the same forward primer with the reverse primer GCA-GAA-CAA-CAA-ACC-ATC-CA (MWG Biotech). PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide; bands were excised from the gel and DNA was extracted with the QIAquick® gel extraction kit (Qiagen). Internal sequencing primers were designed approximately 400 base pairs apart to obtain overlapping sequence verification (see Appendix). Sense and anti-sense strands of the PCR products were then sequenced on an Applied Biosystem Hitachi 3100 Genetic Analyzer (Foster City, CA).

The amino acid sequence was translated from the resulting cDNA sequence using the ExPASy web-based program (us.expasy.org/tools/dna.html).

Cell motility assay

MDA-MB-231 cells were grown to confluency on 4-well permanox chamber slides (Nalge Nunc, International, Naperville, IL). A cross-shaped "wound" was created in each well by scraping the cell layer with a 0.6 mm diameter pipette tip; detached cells were removed. The cross-shape wound provided a point of reference for images collected at two different time points; images were collected at the same distance from the center of the cross-shaped wound at 0 and 6 hours in each chamber. DMEM/F-12 with serum replacement 3 and either vehicle (PBS only) or 500 ng of osteonectin immunopurified from MDA-MB-468, hFOB or HBME-1 cells were added to each well. The chamber slides were incubated at 37°C in a 5% CO_2 humidified incubator. Wounds were imaged using a 10X objective and phase contrast microscopy. Wound closure was calculated as percent change in the distance between the borders of cell growth.

Transwell migration assays – addition of chemoattractants

A modified chemoattraction assay, previously described by Byzova et al. [53] was employed (see diagram, Figure 2.1). The chemoattractant was diluted as described below and air-dried to the lower surface of the membrane of Falcon® FluoroBlock™ transwell chamber inserts (Becton Dickinson, Franklin Lakes, NJ). The transwell inserts were in the 24-well format and were opaque with 8 μm pores. The chemoattractants used were A) osteonectin derived from cell secretions of five cell lines, B) bovine bone osteonectin (Haematologic Technologies), C) protein extracts from mouse femurs and tibias, D) human osteopontin (Chemicon, Temecula, CA), E) human vitronectin (Chemicon), F) human fibronectin (Biomedical Technologies, Stoughton MA), G) conditioned media from mouse fibroblasts (NIH3T3 fibroblasts), H) conditioned media from undifferentiated mouse osteoblasts (MC3T3-E1) or I) conditioned media from differentiated mouse osteoblasts (MC3T3-E1).

In experiment A, osteonectin secreted from MDA-MB-435, MDA-MB-468, hFOB, hTERT-HME1 or HBME-1 was diluted in PBS; 0 ng (vehicle control), 25 ng or 50 ng of protein in a 33 μl volume were air-dried to the lower surface of each well. In experiment B, 0 ng (vehicle control), 50 ng, 100 ng, 200 ng or 1 μg of bovine bone osteonectin in a 10 μl volume was air-dried to the lower surface of each well. Experiment C utilized bone extracts that were diluted in dH_20 with protease inhibitors (0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM PMSF). The lower surface of the wells were coated with either 0 ng (vehicle control), 50 ng, 100 ng, or 200 ng of extract in a total volume of 10 μl of dH_20 with protease inhibitors. In experiments D-F fibronectin, vitronectin, osteopontin diluted to 0, 50, 100, and 200 ng in PBS.

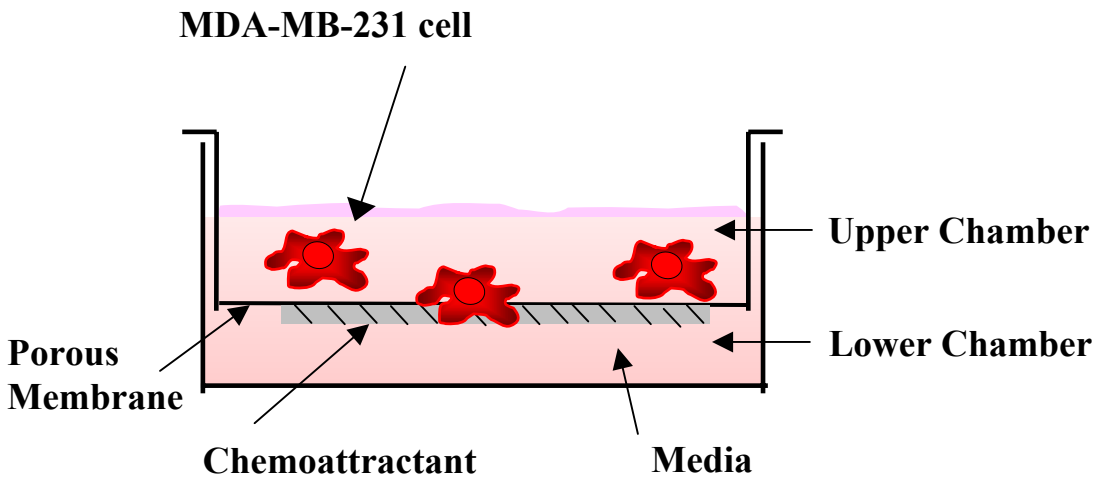


Figure 2.1 Schematic of transwell chamber system for cell migration. The chemoattractant was air-dried to the lower surface of the transwell membrane. Vibrant® DiI-stained MDA-MB-231 cells in serum-free media (either with or without blocking peptides) were added to the upper chamber. Lastly, serum free media (or conditioned media if specified) was added to the lower chamber.

We also used 200 ng of vitronectin, 200 ng bovine bone osteonectin or 100 ng of both the vitronectin and osteonectin (200 ng total protein) diluted in dH_20 with protease inhibitors. When conditioned media were used as the chemoattractant, as in experiments H and I, dH_2O with protease inhibitors was air-dried to the lower surface of the membrane and 800 μl of conditioned media were added to the lower chamber. Conditioned media were collected from a mouse fibroblast cell line (NIH3T3 fibroblasts) and a mouse osteoblast cell line (MC3T3-E1). DMEM with 100 I.U./ml pen, 100 μg/ml strep and Serum Replacement 3 was exposed to confluent NIH3T3 fibroblasts for three days prior to rinsing the cell culture with PBS. αMEM with Serum Replacement 3, 100 I.U./ml pen and 100 μg/ml strep was used on the MC3T3-E1 cells for either undifferentiated (days 3,4,5) or differentiated (days 28,29,30) conditioned media collections. All conditioned media was filtered using a 2mm porous membrane and stored at –20°C.

Transwell migration assays – addition of cells

MDA-MB-231 cells were stained with Vibrant® DiI (Molecular Probes), which is a lipophilic carbocyanine that diffuses into the cell and stains all membranes including the plasma membrane. These pre-stained cells were seeded to the upper surface of the chamber at $5X10^4$ cells per well in the presence of 300 μl DMEM/F-12 with Serum Replacement 3, 100 I.U./ml pen and 100 μg/ml strep. DMEM/F-12 (800 μl) was added to the lower chamber except when conditioned media was utilized as the chemoattractant (experiments G-I). The cells were allowed to migrate in a 37°C, 5% CO_2 humidified incubator for either 6 or 48 hours. The membranes were then rinsed with PBS, fixed with 4% paraformeldahyde, and mounted to a microscope slide with Fluoromount-G

(Southern Biotech, Birmingham, AL). Migrated cells found on the lower surface of the membrane were visualized and counted with the use of 549 nm excitation and 565 nm emission wavelengths at 100X magnification.

In a final set of experiments (experiments C, E-I), GRGESP (RGE) and GRGDSP (RGD) blocking peptides (Gibco, Rockville, MD) were utilized. In 1ml of DMEM/F-12 with Serum Replacement 3, 200 μg of peptides were mixed with $5X10^4$ Vibrant® DiI stained MDA-MB-231 cell. The cell and blocking peptide mixture (300 μl) was then added to the upper chamber of each well.

Cytokine analysis

Wild-type mouse bone extracts and conditioned media collected from mouse fibroblasts, undifferentiated osteoblasts and differentiated osteoblasts were assayed for the presence of 18 cytokines with the X-Plex assay system (BioRad). This bioassay can detect concentrations of cytokines from 10 pg/ml up to 32,000 pg/ml. Undiluted conditioned media sample and 130 ng of wild-type bone extracts were tested. The cytokine assay detects mouse interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 (p40), IL-12 (p70), IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, macrophage inflammatory proteins (MIP)-1α, RANTES and TNF-α.

Statistical analysis

Minitab was used to analyze data for this study. All data are represented with a mean +/- standard error; independent culture replicates were used to generate the mean. The p-values were determined with a student t-test. Each experiment was done in triplicate (total of 9 independent replicates) unless otherwise noted.

Chapter 3

Characterization of Osteonectin Secreted by Breast, Bone and Vascular Cells

Rationale

Because bone cells (osteoblast and bone marrow vascular endothelial cells) and breast cells (neoplastic and normal epithelial cells) both secrete osteonectin, it is unlikely that bone-derived osteonectin could form a chemotactic gradient in the presence of breast-cancer derived osteonectin. We first hypothesized that the bone-derived osteonectin was distinct from the breast cancer-derived osteonectin. By analyzing post-translational and transcriptional differences, we attempted to identify a configuration of osteonectin unique to bone or breast cancer cells. A distinct isoform of osteonectin would support the concept that bone-derived osteonectin is chemotactic to breast cancer cells.

Immunoblotting of osteonectin secreted by various cell types

Osteonectin is secreted by many normal and neoplastic cells in the body; we selected an array of cell lines that represent key cell types involved in breast cancer metastasis to bone. We used a non-neoplastic breast epithelial (hTERT-HME1) cell line and three human breast cancer cell lines (MDA-MB-435, MDA-MB-468 and MDA-MB-231); together these cells provide a useful model of both normal and neoplastic breast tissue. The MDA-MB-435 and MDA-MB-231 cells lines are considered highly metastatic and will metastasize to bone in mice [77, 78]. In contrast, the MDA-MB-468

cell line is reportedly less metastatic compared to the MDA-MB-435 and MDA-MB-231 cell lines [79]. To represent the bone microenvironment, we utilized a human bone marrow vascular endothelial cell line (HBME-1) derived from iliac crest biopsies and a human fetal osteoblast cell line (hFOB).

We first determined which of these cell lines secreted osteonectin. The MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines all secreted detectable levels of osteonectin by immunoblotting (Figure 3.1). The MDA-MB-231 cell line did not secrete measurable levels of osteonectin; however, low levels of osteonectin mRNA were detected by RT-PCR (data not shown). Furthermore, the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines all secreted osteonectin with the same molecular weight (~ 46 kD). Although osteonectin from the various cell types had similar molecular weights, a chemotactic gradient could exist if one of the secreted forms had unique post-translational modifications. Therefore, we further analyzed osteonectin from these cell sources to identify specific post-translational modifications.

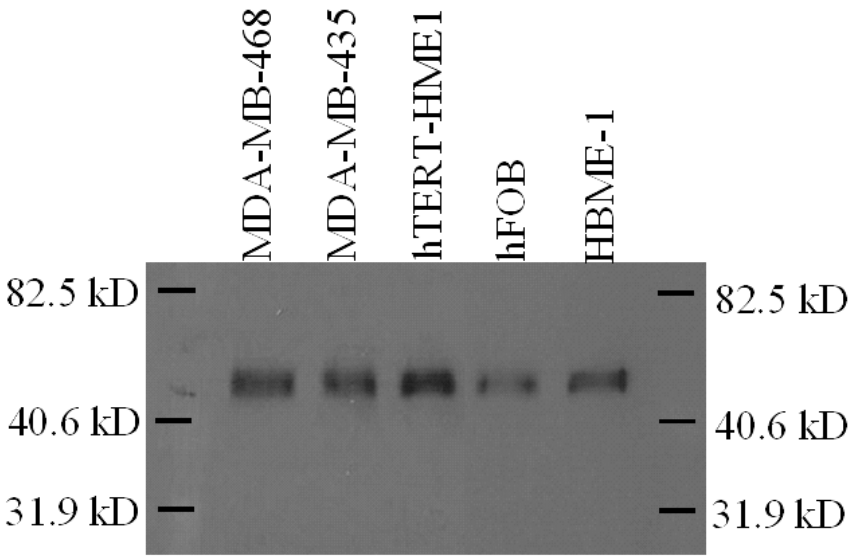


Figure 3.1 Immunoblotting of osteonectin. Osteonectin (10 ng) from conditioned media was reduced and separated on a 15% SDS-PAGE gel. The protein was transferred to nitrocellulose and immunoblotted for the presence of osteonectin. Osteonectin from the MDA-MB-468, MDA-MB-435, hTERT-HME1, hFOB and HBME-1 cell lines was detected at about 46kD.

Identification of post-translational modifications of osteonectin

Two common post-translational modifications that could result in the formation of a unique configuration of osteonectin are glycosylation and phosphorylation. The glycosylation pattern of osteonectin secreted by the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines was generated by using a series of deglycosylation enzymes (Figure 3.2). All of the sources of osteonectin exhibited a marked gel shift when exposed to PNGase F. This pattern of enzymatic deglycosylation demonstrated that the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines secreted osteonectin with N-linked oligosaccharides. There was no detectable gel shift to neuraminidase, O-glycosidase, β-galactosidase, or N-acetylglucosaminidase, which remove sialic acid, O-link oligosaccharides, galactose, and β-linked N-acetylglucosamine residues, respectively. These data demonstrated that osteonectin from all cell lines examined had the same pattern of glycosylation.

Osteonectin has been described as a phosphoglycoprotein [80, 81] with multiple serine residues as the predominant site for phosphorylation [82]. We analyzed osteonectin from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines for the presence of phosphoserines and determined that none of the tested osteonectin samples contained phosphorylated serines (Figure 3.3). However, one limitation of this detection method was that proteins with known phosphoserines may not be recognized by the antibody because of steric hindrance.

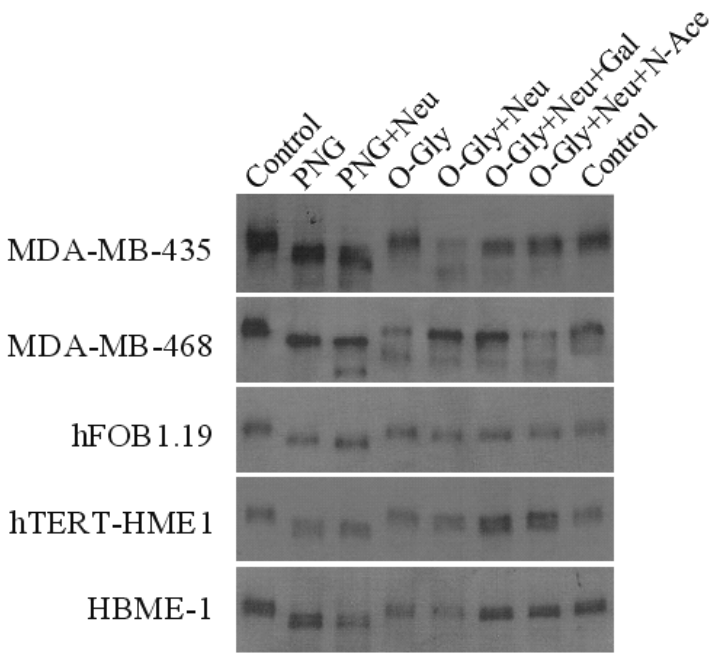


Figure 3.2 Deglycosylation of osteonectin from different cell lines. Osteonectin was deglycosylated with a series of enzymes: PNGase F (PNG), neuraminidase (Neu), O-glycosidase (O-Gly), β-galactosidase (Gal), and N-acetylglucosaminidase (N-Ace). Samples were then separated on a 15% SDS-PAGE gel, transferred to nitrocellulose and immunoblotted. Compared to the control (no enzyme), the MDA-MB-435, MDA-MB-468, hFOB1.19, hTERT-HME1, and HBME-1 osteonectin displayed a molecular weight shift in response to PNGase F only.

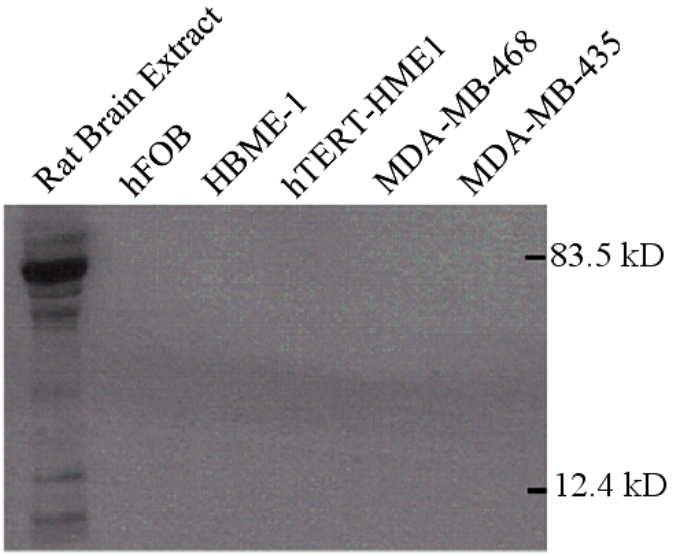


Figure 3.3 Detection of phosphorylated serines of osteonectin from different cell lines. Immunopurified osteonectin (10 ng) from the hFOB, HBME-1, hTERT-HME1, MDA-MB-468, and MDA-MB-435 cell lines was analyzed for the presence of phosphorylated serines. The osteonectin was reduced and separated on a 12% SDS-PAGE gel. Protein was then transferred to nitrocellulose and immunoblotted for phosphorylated serines. Rat brain extract was used as the positive control. None of the examined cell lines secreted osteonectin with phosphorylated serines.

Osteonectin cDNA sequencing and amino acid analysis

Because cells of breast and bone origin secrete osteonectin with the same post-translational modifications, we compared the amino acid sequence for translational differences. Osteonectin cDNA was sequenced from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines. The resulting nucleic acid sequences (see Appendix) were then translated into amino acid sequences (see Figure 3.4 and Appendix). We found the MDA-MB-435, MDA-MB-468, hFOB, and hTERT-HME1 samples had identical cDNA sequences and therefore were assumed to have identical amino acid sequences to each other and to the published human osteonectin sequence (Genbank accession # NM003118). The HBME-1 cDNA sequence had a number of nucleic acid point mutations that resulted in eight amino acids that differed from the published sequence. The published osteonectin amino acid sequence, with the eight substitutions found in the HBME-1 sample, is illustrated in Table 3.1. A Dayhoff scoring matrix was used to generate the scores provided in Table 3.1 (Figure 3.5) [83]. Only substitution #2 resulted in a low score of –4. All the other substitutions were considered to occur with a relatively high frequency through evolution and to not affect the function of the protein. Furthermore, there were no changes in the number and position of cysteines, which indicates that disulfide bonding was unaffected. There was a loss of a single serine (substitution 3) and a single threonine (substitution 1), which could affect phosphorylation. The EF-hands, which sequester Ca^{2+} and bind to other matrix proteins, were not affected by the mutations [84]. In addition, the GHK peptide within the follistatin-like domain that is responsible for stimulating angiogenesis and proliferation was also unaffected by the mutations [37].

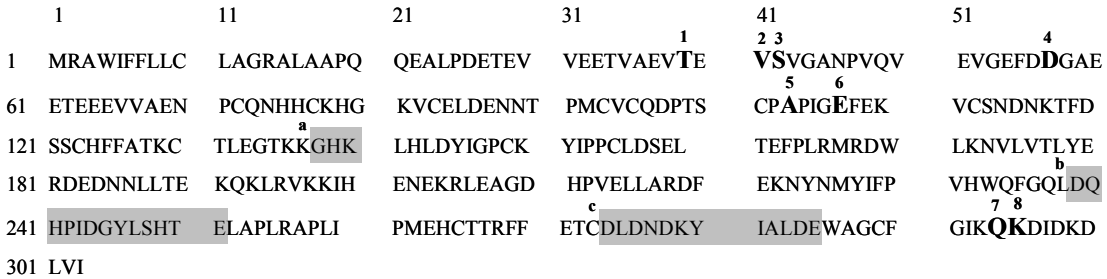


Figure 3.4 The published amino acid sequence of human osteonectin with the location of substitutions found in the HBME-1 osteonectin. MDA-MB-468, MDA-MB-435, hTERT-HME1, and hFOB osteonectin amino acid sequence was identical to the published sequence. There were eight substitutions identified in the amino acid translation of the HBME-1 sample, these substitutions were positioned at numbers 1-8 and in bold type. The "GHK" (a), EF-hand 1 (b), and EF-hand 2 (c) peptides are highlighted.

Substitution Number	Published (Genbank accession # NM003118) Amino Acid	HBME-1 Amino Acid	Dayhoff Score
1	Threonine	Alanine	**-1**
2	Valine	Glycine	**-4**
3	Serine	Proline	**-1**
4	Aspartic acid	Glutamic acid	**2**
5	Alanine	Glycine	**0**
6	Glutamic acid	Aspartic acid	**2**
7	Glutamine	Glutamic acid	**2**
8	Lysine	Glutamine	**1**

Table 3.1 Substitutions found in the HBME-1 osteonectin cDNA compared to the published sequence. The left column contains the substitution numbers assigned to each amino acid in the published sequence where a substitution was identified in the HBME-1 sequence (Figure 3.4). Listed from left to right is the amino acid identified in the published and HBME-1 osteonectin sequence, respectively. In the far right column is the Dayhoff matrix score associated with each substitution pair.

	C	S	T	P	A	G	N	D	E	Q	H	R	K	M	I	L	V	F	Y	W
C	11																			
S	-4	4																		
T	-5	1	5																	
P	-8	-1	-1	7																
A	-2	0	-1	-1	4															
G	-6	-1	-3	-2	0	5														
N	-6	0	0	-2	-1	-1	5													
D	-7	0	-1	-1	-1	-1	2	6												
E	-3	-1	0	-1	0	-2	0	2	5											
Q	-3	-1	0	-2	0	-2	0	0	2	6										
H	-6	-2	-2	-3	-2	-3	2	0	-2	0	8									
R	-2	0	-1	-2	-1	-2	-1	-2	0	1	0	7								
K	-4	-1	0	-1	-1	-3	0	-1	1	1	0	2	5							
M	-5	-4	-2	-6	0	-4	-2	-4	-2	1	-2	-4	-1	8						
I	-4	-3	-2	-4	-2	-5	-3	-3	-3	-5	-5	-3	-3	1	6					
L	-6	-4	-3	-3	-2	-5	-3	-6	-4	-3	-3	-3	-2	3	2	5				
V	-4	-3	-1	-4	0	-4	-4	-4	-2	-2	-2	-3	-3	0	2	1	5			
F	-2	-3	-3	-5	-3	-6	-3	-5	-4	-4	-2	-4	-3	0	1	2	-1	7		
Y	-6	-2	-2	-6	-3	-3	-1	-3	-2	-3	0	-1	-2	-1	-1	-2	-1	3	7	
W	-6	-5	-5	-4	-3	-4	-5	-6	-6	-5	-3	-2	-3	-2	-2	-1	-4	2	2	10

Figure 3.5 Dayhoff scoring matrix used to identify common evolutionary substitutions between amino acids. Listed horizontally and vertically are the twenty amino acids. Two different amino acids were lined up to generate a score. This score represents the frequency with which two amino acids will substitute for each other without affecting the function of the protein. The score is mainly based on similarities in charge and polarity between two different amino acids. This matrix was used to generate a score for each of the amino acid substitutions found in the HBME-1 osteonectin cDNA (see Table 3.1). Dayhoff, M.O. and National Biomedical Research Foundation., *Atlas of protein sequence and structure*. 1979, Silver Spring, Md.: National Biomedical Research Foundation.

We conclude that the amino acid sequence of osteonectin from breast cancer cells (MDA-MB-435 and MDA-MB-468), osteoblasts (hFOB), and non-neoplastic breast epithelial cells (hTERT-HME1) were identical. However, the human bone marrow vascular endothelial cell line (HBME-1) produced osteonectin with eight substituted amino acids. From all indications, the substitutions found in the HBME-1 sample were not significant and would not result in a different configuration of osteonectin.

Chapter 4

The Effects of Osteonectin on Breast Cancer Cell Motility and Migration

Rationale

Even though osteonectin from the cancer cells (MDA-MB-435 and MDA-MB-468) and bone cells (hFOB) appeared to be identical, it was possible that a chemotactic gradient could exist if bone cells secreted higher levels of osteonectin than breast cancer cells. Under this premise, we studied the ability of osteonectin to increase cell motility and chemoattraction on a cell line that secretes low levels of osteonectin. MDA-MB-231 cells did not secrete detectable amounts of osteonectin protein and therefore were utilized to study cellular responses generated by exogenous osteonectin.

Osteonectin–induced MDA-MB-231 cell motility

The ability of osteonectin to induce cell motility was examined. In a "wound-healing" assay, MDA-MB-231 cells were grown to confluency and then treated with either vehicle (PBS) or osteonectin immunopurified from MDA-MB-468, HBME-1, or hFOB cell-conditioned media. The MDA-MB-231 cells displayed greater cell outgrowth in the presence of osteonectin compared to the vehicle control (Figure 4.1). After 6 hours, there was no wound closure in the control treatment. However, the hFOB-derived osteonectin induced a 23% increase in wound closure. There was also a 42% and 46% increase in wound closure by the HBME-1 and MDA-MB-468 derived osteonectin, respectively. These data support the literature that exogenous osteonectin enhanced cell motility.

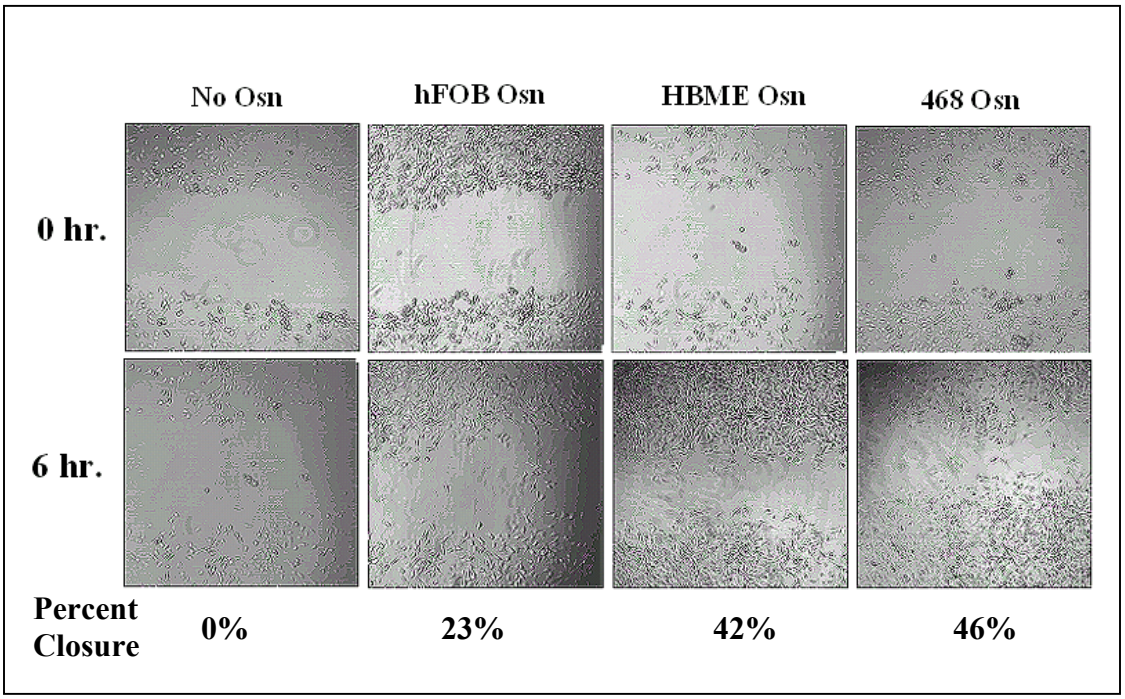


Figure 4.1 Cell motility induced by osteonectin. MDA-MB-231 cells were grown to confluence on permanox chamber slides. A cross-shaped "wound" was then cut into the monolayer and the remaining attached cells were exposed to 500 ng of osteonectin from MDA-MB-468 (468), hFOB, HBME-1 (HBME) cells or PBS only (control) for 6 hours. Compared to the control, the hFOB-derived osteonectin induced a 23% wound closure while the HBME-1 and MDA-MB-468 osteonectin induced 42% and 46% closure, respectively. These images have been 30X magnified and represent typical results from duplicate experiments.

Migration of MDA-MB-231 cells toward osteonectin

The directed migration (or chemoattraction) of MDA-MB-231 cells, which do not secrete detectable levels of osteonectin, toward osteonectin isolated from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines was investigated in transwell migration assays (Figure 4.2). After 6 hours, there was no significant migration toward either 25 ng or 50 ng of osteonectin from the MDA-MB-435, MDA-MB-468, and hFOB cell lines. Cells did migrate toward the highest dose of hTERT-HME1-derived osteonectin (5-fold increase). However, the actual number of cells that migrated to the hTERT-HME1-derived osteonectin was very low, only about 10 cells migrated for every square millimeter. There was also some migration to the HBME-1 derived osteonectin; we observed a 2-fold and 3-fold increase in migration toward the 25 ng and 50 ng doses, respectively. The number of migrated cells toward the HBME-1 derived osteonectin was also low. We observed about 4 cells in every mm^2 in the 25 ng dose while the number of migrated cells toward the 50 ng dose increased to about 6 cells per mm^2.

Chemoattraction of breast cancer cells to purified bovine bone-derived osteonectin was also tested. We extended the migration time to 48 hours and increased the doses of osteonectin to 50 ng, 100 ng, 200 ng, and 1 μg of protein per well. The metastatic breast cancer cells did not display any increase in migration toward the bovine bone osteonectin (Figure 4.3).

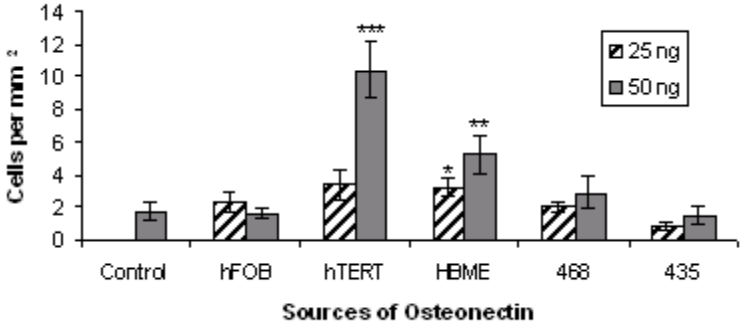


Figure 4.2 MDA-MB-231 cell migration toward osteonectin. Either 25 ng or 50 ng of osteonectin isolated from hFOB, hTERT-HME1 (hTERT), HBME, MDA-MB-468 (468), and MDA-MB-435 (435) cell lines was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells ($5X10^4$ cells) were added to the upper chamber. After 6 hours, membranes were fixed and migrated cells were counted. Data represents three replicate experiments. No significant migration was observed toward the osteonectin isolated from the MDA-MB-435, MDA-MB-468, or hFOB cell lines. There was significant migration to 50 ng of hTERT-HME1 osteonectin (5-fold increase). A 2-fold and 3-fold increase in migration was observed to 25 ng and 50 ng of HBME-1 derived osteonectin, respectively. (N=9 membranes per peptide dose, mean ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to control)

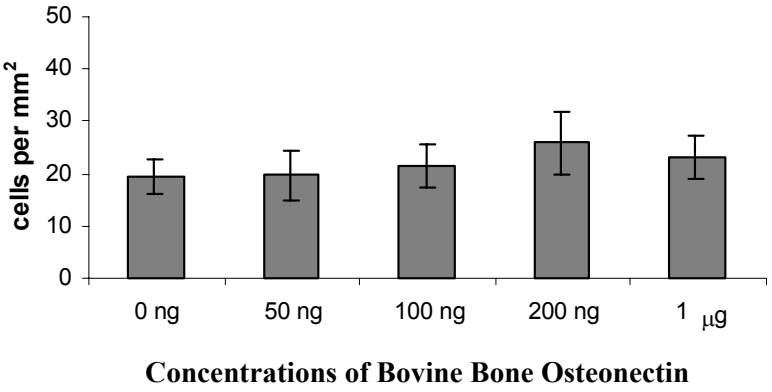


Figure 4.3 MDA-MB-231 cell migration toward bovine bone osteonectin. Either 0, 50, 100, 200 ng or 1 μg of bovine bone osteonectin was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells were seeded at $5X10^4$ cells per membrane. After 48 hours, membranes were fixed and migrated cells were counted. Data represents three replicate experiments. There was no significant migration to any doses of bone osteonectin (N=9 membranes per peptide dose, mean ± SEM*)*

Migration of MDA-MB-231 cells toward vitronectin, osteonectin, and a vitronectin/osteonectin mixture

To confirm that osteonectin is not chemotactic to breast cancer cells, we compared the migration of MDA-MB-231 cells to osteonectin and the known chemoattractant vitronectin [49]. Vitronectin has been shown to chemoattract breast cancer cells through its RGD domain [50]. We conducted migration assays with either 200 ng osteonectin, 200 ng vitronectin or 100 ng of both vitronectin and osteonectin (vitronectin/osteonectin mixture, 200 ng of total protein) as the chemoattractant. The MDA-MB-231 breast cancer cells were pretreated with either GRGESP (RGE) or GRGDSP (RGD) blocking peptides and exposed to the chemoattractants for 6 hours (Figure 4.4). There was no significant migration of the breast cancer cells to osteonectin in the presence of RGE or RGD peptides. In contrast, there was substantial migration to vitronectin in the presence of RGE peptides. When the vitronectin/osteonectin mixture was used as a single chemoattractant in the presence of RGE peptides, breast cancer cell migration was also significantly increased. The migration of breast cancer cells to either vitronectin or the vitronectin/osteonectin mixture was significantly inhibited with RGD peptides. This assay confirms that osteonectin does not chemoattract breast cancer cells but that vitronectin is chemotactic though its RGD domain. Furthermore, the levels of migration to vitronectin (200 ng) compared to the vitronectin/osteonectin mixture were statistically equivalent despite the reduced dose of vitronectin in the mixture (100 ng).

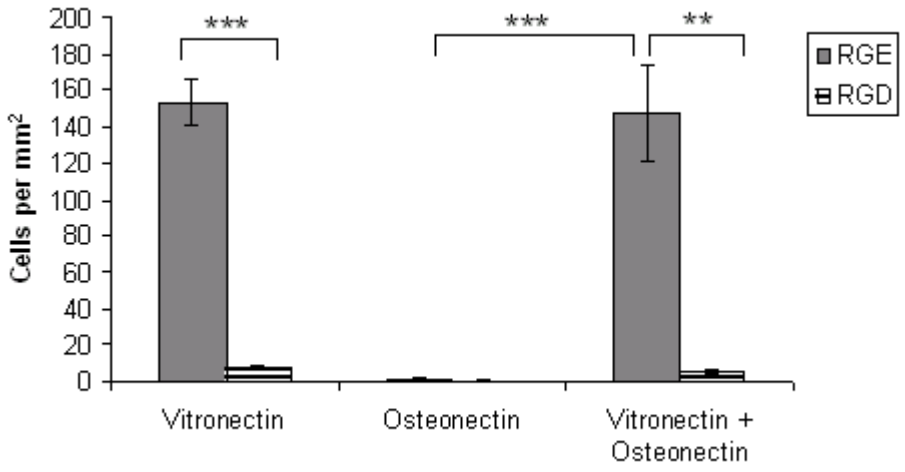


Figure 4.4 Migration of MDA-MB-231 cells toward osteonectin and vitronectin. Either 200 ng osteonectin, 200 ng vitronectin or 100 ng of both osteonectin and vitronectin (vitronectin/osteonectin mixture, 200 ng total protein) was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells ($5X10^4$ cells per membrane) were combined with 200 μg/ml of RGE or RGD peptides. Experiments were done in duplicate. After 6 hours, there was no migration to osteonectin in the presence of RGE or RGD blocking peptides. There was migration to vitronectin and the vitronectin/osteonectin mixture; this migration was significantly inhibited with RGD blocking peptides. (N=6 membranes per peptide dose, mean ± SEM, ** $p \leq 0.01$, *** $p \leq 0.001$)

Migration of MDA-MB-231 cells toward bone extracts

We determined there was no chemoattraction of breast cancer cells toward osteonectin. However, physiologically, osteonectin is in the presence of many other matrix proteins in the bone microenvironment [13]. The attractive quality of osteonectin may be dependent on its proximity to these other matrix proteins. To characterize the chemoattraction of osteonectin in the presence of other matrix proteins, we utilized protein extracts collected from the femurs and tibias of wild-type, heterozygous, and osteonectin-null mice. We first assayed the bone extracts for the presence of osteonectin. In a SYPRO®-stained SDS-PAGE gel, the wild-type sample had a single band that was noticeably absent in the knockout sample and reduced in the heterozygous sample (Figure 4.5 A). There was very little effect on the presence of other proteins as indicated by the number and density of protein bands between the samples. An immunoblot confirmed the absence of osteonectin in the knockout sample (Figure 4.5 B).

We used the wild-type and osteonectin-null bone extracts in a transwell chamber migration assay; De et al. (2003) used a similar method to demonstrate that osteonectin is the main chemotactic factor in bone extracts for prostate cancer cells [47]. In Figure 4.6, the migration of MDA-MB-231 cells toward either vehicle (dH_2O with protease inhibitors) or 50 ng, 100 ng, and 200 ng of wild-type or osteonectin-null extracts is represented. The cancer cells migrated to all doses of wild-type and osteonectin-null bone extracts. There was no difference in the migration toward wild-type or osteonectin-null extracts when compared at equal doses. This experiment indicates that the metastatic breast cancer cells were attracted to bone extracts but that the presence of osteonectin was not required.

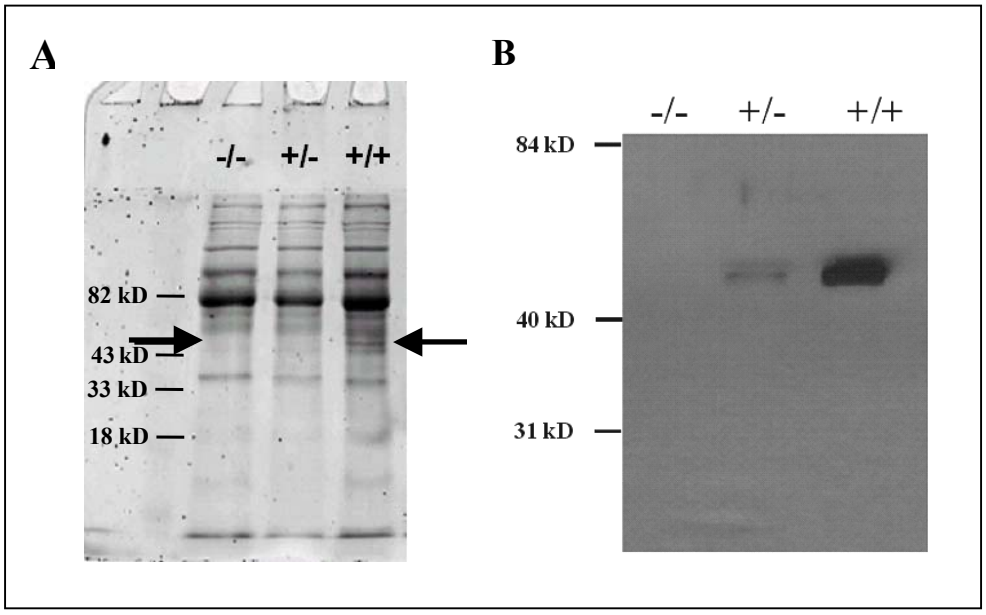


Figure 4.5 Bone extracts from wild-type, heterozygous and osteonectin-null mice. A) The bone extracts were separated by a 12% SDS-PAGE and stained with SYPRO®. There was a single band absent (denoted with an arrows) in the knockout extract (-/-) and reduced in the heterozygous (+/-) sample. Most of the other bands (molecular weight and density) were conserved between the samples. B) Bone extracts were analyzed by immunoblotting. Osteonectin was detected in the wild-type (+/+) and heterozygous extract but absent in the knockout sample.

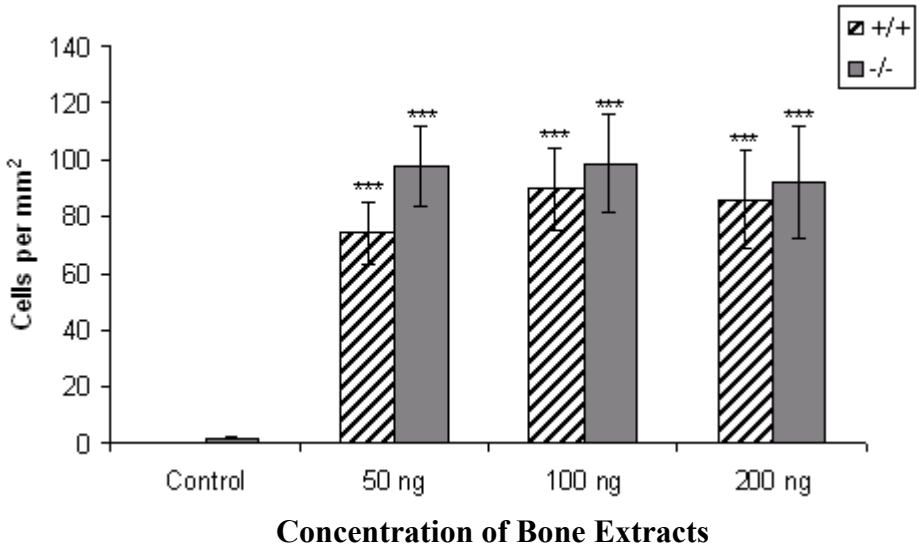


Figure 4.6 Migration of MDA-MB-231 cells toward bone extracts. 0 (Control), 50, 100, or 200 ng bone extracts from the femurs and tibias of wild-type (+/+) or osteonectin-null (-/-) mice were air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells were seeded at $5X10^4$ cells per well and allowed to migrate for 6 hours before being fixed. Data represents three replicate experiments. There was increased migration to all bone extracts (38-50 fold increase). No difference in migration toward the wild-type or osteonectin-null extracts of equal concentrations was detected. (N=9 membranes per peptide dose, mean ± SEM, *** $p \leq 0.001$ compared to control)

Chapter 5

Identification of Chemotactic Factors Within Bone Extracts

Rationale

Osteonectin was determined to not chemoattract breast cancer cells. However, mouse bone extracts (both wild-type and osteonectin-null) were highly chemotactic for the MDA-MB-231 cells. We aimed to determine which proteins within the bone extracts are chemoattractive.

Analysis of mouse bone extracts for fibronectin, bone sialoprotein, vitronectin, and osteopontin

The mineralized portion of the wild-type, heterozygous and osteonectin-null mouse bone extracts was determined to contain many different proteins as demonstrated by the Sypro-stained SDS-PAGE gel in Chapter 4 (Figure 4.5). In addition to osteonectin, these extracts were immunoblotted for the presence of a) fibronectin, b) bone sialoprotein, c) vitronectin, and d) osteopontin (Figure 5.1). These proteins were identified in the mineralized portion of the wild-type, heterozygous and osteonectin-null extracts.

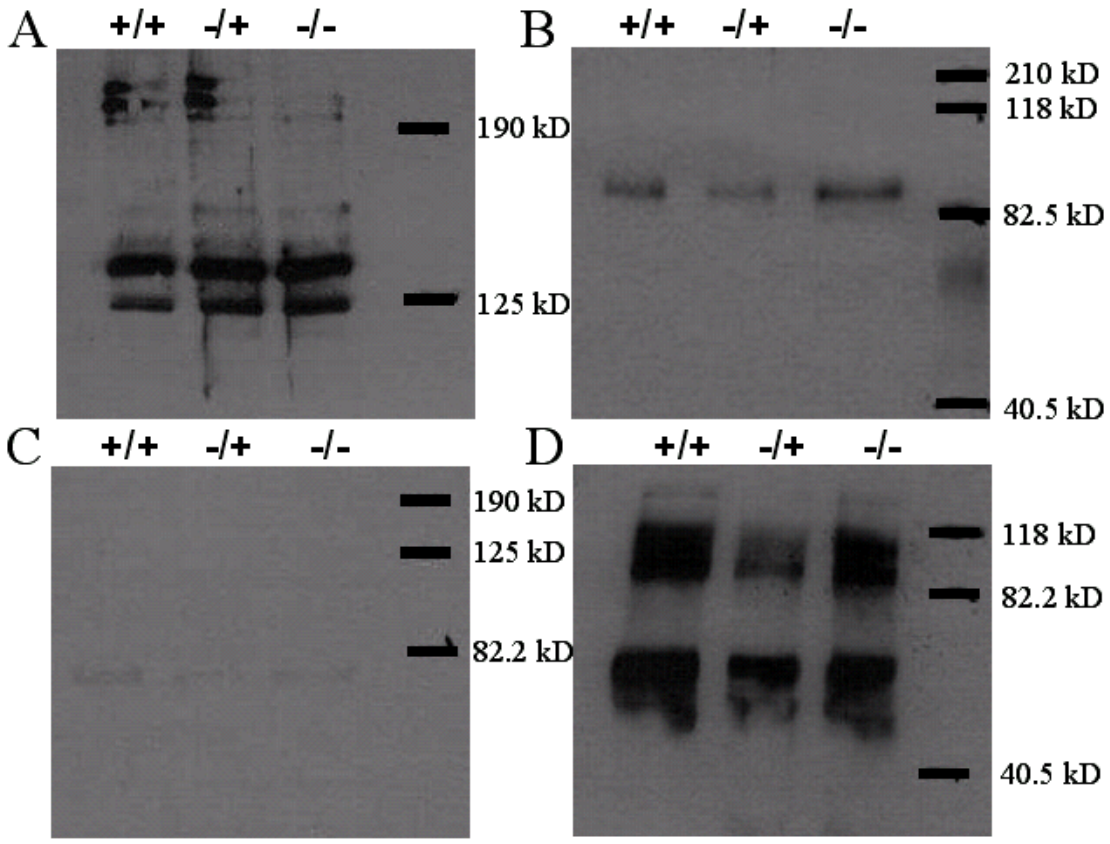


Figure 5.1 Immunoblotting of mouse bone extracts for the presence of A) fibronectin, B) bone sialoprotein, C) vitronectin and D) osteopontin. Bone extracts from wild-type (+/+), heterozygous (-/+), and osteonectin-null (-/-) mice were separated by SDS-PAGE and immunoblotted. Fibronectin, bone sialoprotein, vitronectin, and osteopontin were identified in all tested bone extracts. These are representative images from duplicate experiments.

Migration of MDA-MB-231 cells to fibronectin, vitronectin, and osteopontin

The presence of fibronectin, vitronectin and osteopontin in mouse bone extracts was confirmed (Figure 4.5) and consequently each was assayed for its chemotactic quality. The migration of breast cancer cells to human fibronectin, vitronectin, and osteopontin was tested in the transwell chamber system. After 6 hours, MDA-MB-231 cells migrated to all tested doses of fibronectin, vitronectin, and osteopontin as observed by confocal microscopy (Figure 5.2). There was migration of 45, 163, and 311 cells per mm^2 toward 50, 100 and 200 ng fibronectin, respectively (Figure 5.3 A). These numbers translate to a 273-fold increase in migration of the breast cancer cells compared to the control (Figure 5.3 B). We observed that 50, 100, and 200 ng vitronectin induced the migration of 66, 87, and 91 breast cancer cells per mm^2, respectively (Figure 5.3 A). These cell numbers indicate a 241-fold increase in breast cancer cell migration toward the highest dose of vitronectin (Figure 5.3 B). As levels of osteopontin increased, we counted an average migration of 21, 22, and 30 breast cancer cells per mm^2 (Figure 5.3 A). Osteopontin stimulated up to a 65-fold increase in migration of breast cancer cells compared to the control (Figure 5.3 B). We conclude that fibronectin, vitronectin and osteopontin were chemoattractive to MDA-MB-231 cells.

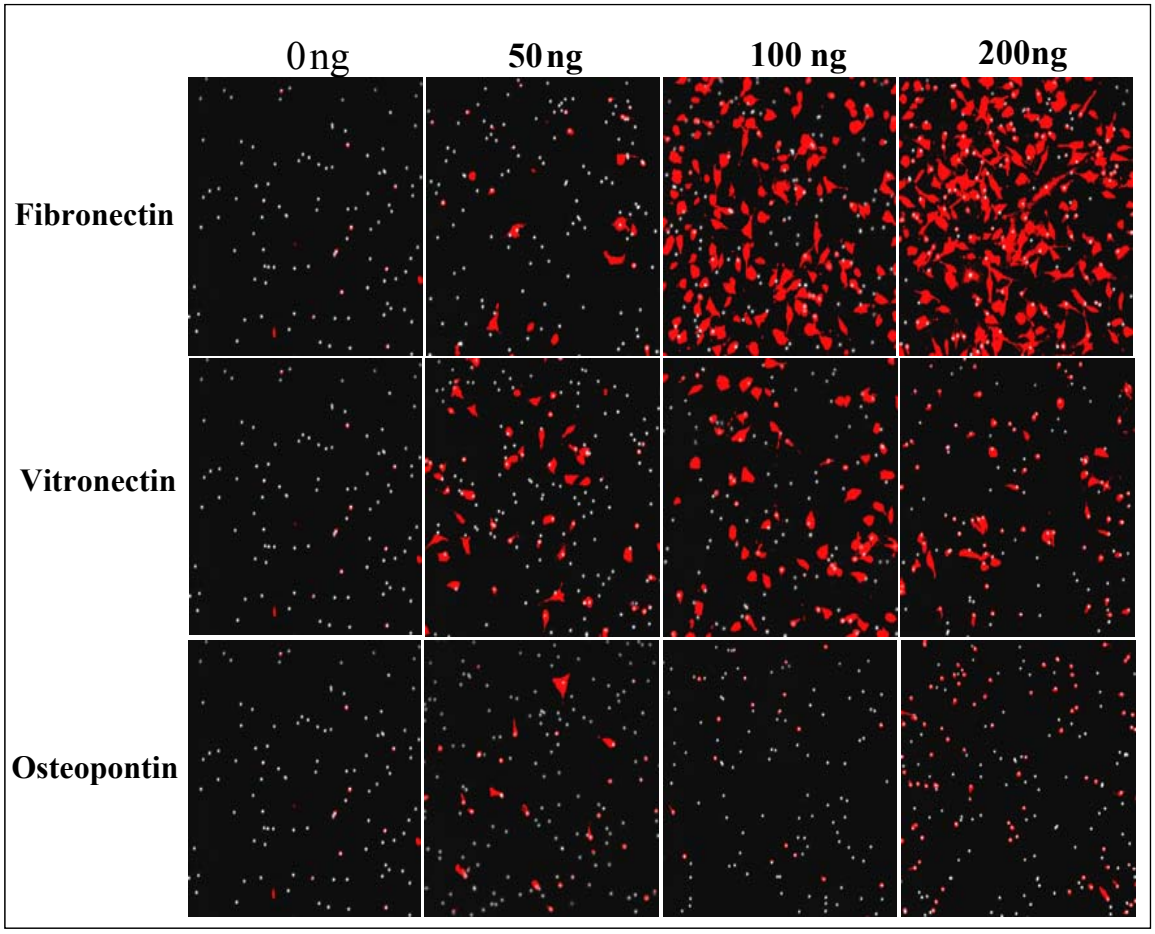


Figure 5.2 Fluorescent images of migrated MDA-MB-231 cells through filters with 8 μm pores. Either 0, 50, 100 or 200 ng of fibronectin, vitronectin and osteopontin were air-dried onto the lower surface of transwell membranes. MDA-MB-231 cells were stained with Vibrant® DiI and seeded at $5X10^4$ cells per well. The membranes were fixed after 6 hours and the center of the lower surface of each membrane was imaged using a 20X objective and 549 nm excitation and 565 nm emission. These images are 60.6X magnification and represent triplicate experiments (n=9 membranes per peptide dose).

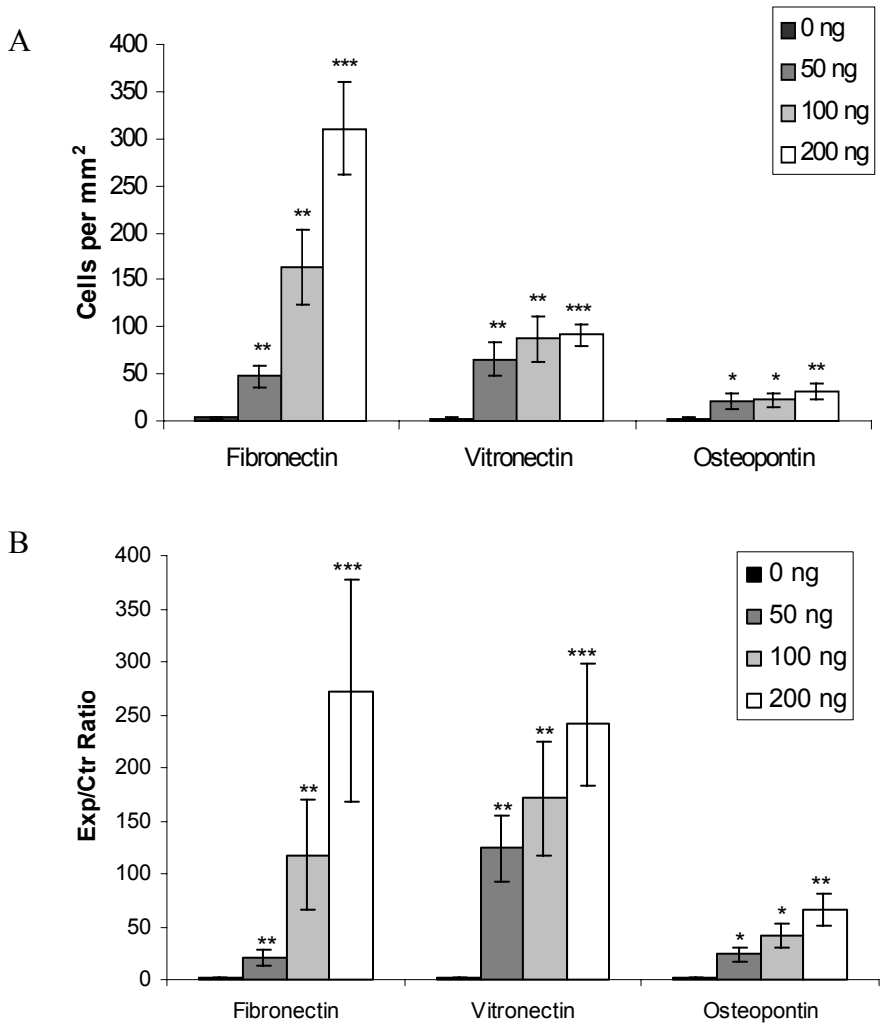


Figure 5.3 Migration of MDA-MB-231 cells toward matrix proteins found in bone extracts. Either 0, 50, 100, or 200 ng of human fibronectin, vitronectin or osteopontin was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells were seeded at $5X10^4$ cells per well. A) migrated cells per mm^2 and B) experimental/control ratios are expressed. Data represents three replicate experiments. After 6 hours, there was significant migration to all concentrations of fibronectin, vitronectin, and osteopontin. (N=9 membranes per peptide dose, mean ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to 0 ng)

RGD-dependence of MDA-MB-231 cell migration to fibronectin and vitronectin

In transwell chamber migration assays, fibronectin and vitronectin significantly increased breast cancer cell migration. The migration of MDA-MB-231 cells toward fibronectin and vitronectin has been described to be RGD-dependent [49, 50]. Through the use of RGE and RGD blocking peptides, we determined if the observed migration in the transwell chamber system was also RGD-dependent. The RGE peptides had no effect on the migration of the MDA-MB-231 cells toward fibronectin (Figure 5.4) or vitronectin (Figure 5.5). However, the blocking RGD peptides significantly inhibited breast cancer cell migration toward fibronectin (Figure 5.4) and vitronectin (Figure 5.5). The results demonstrated that the breast cancer cells were attracted to and migrated toward fibronectin and vitronectin in an RGD-dependent manner.

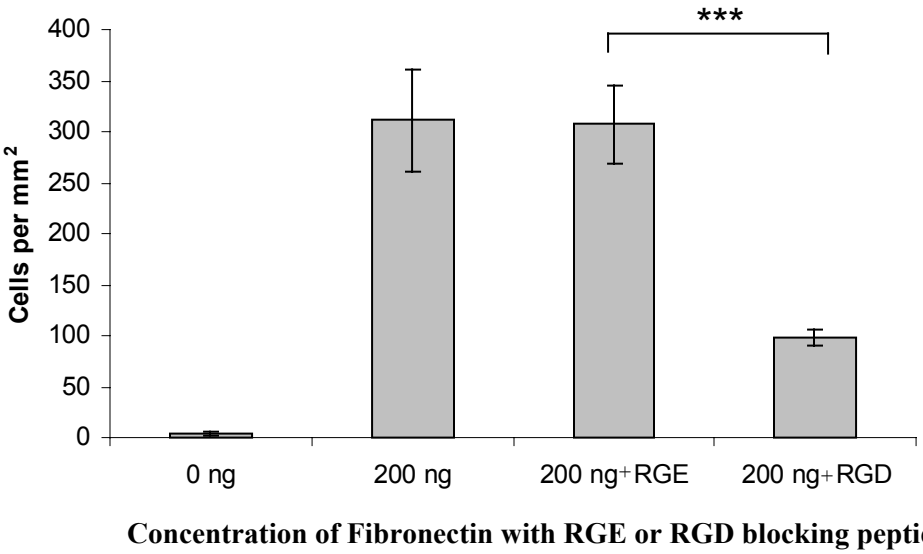


Figure 5.4 Inhibition of MDA-MB-231 cell migration to fibronectin with RGE and RGD blocking peptides. Either 0 or 200 ng of human fibronectin was air-dried onto the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells ($5X10^4$ cells per well) were combined with 0 or 200 μg/ml of RGE or RGD peptides. Experiments were done in duplicate. After 6 hours, the RGE peptide had no effect on the migration of the breast cancer cells whereas the RGD peptide significantly inhibited cell migration. (N=6 membranes per peptide dose, mean ± SEM, *** $p \leq 0.001$)

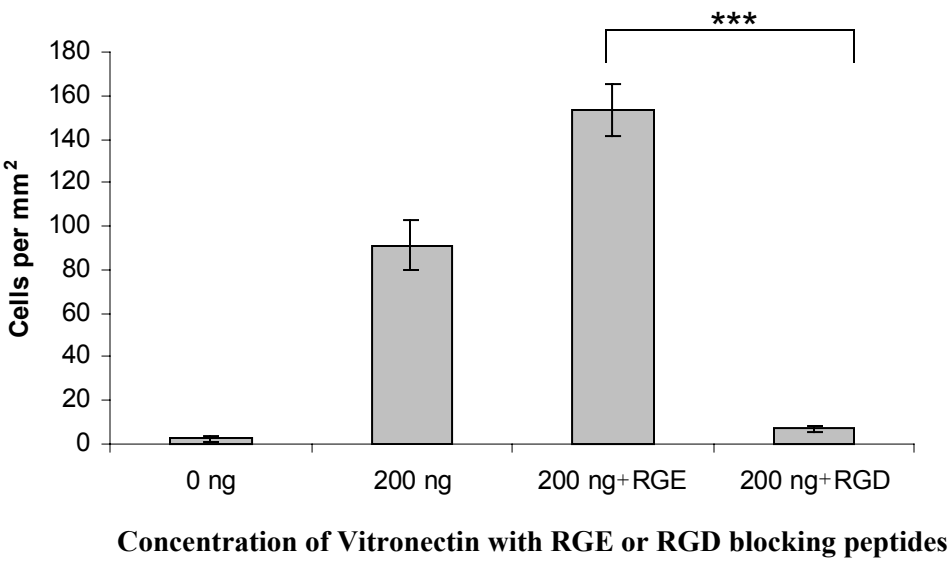


Figure 5.5 Inhibition of MDA-MB-231 cell migration to vitronectin with RGE and RGD blocking peptides. Either 0 or 200 ng of human vitronectin was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells ($5X10^4$ cells per well) were combined with 0 or 200 μg/ml of RGE or RGD peptides. Experiments were done in duplicate. After 6 hours, the RGE peptide had no statistically significant effect on the migration of the breast cancer cells while the RGD peptide significantly inhibited cell migration. (N=6 membranes per peptide dose, mean ± SEM, *** $p \leq 0.001$)

RGD-dependence of MDA-MB-231 cell migration to wild-type extracts

Breast cancer cells were shown to migrate to fibronectin, vitronectin and osteopontin. All of these proteins were identified in the bone extracts and the migration to fibronectin and vitronectin was RGD-dependent. Osteopontin and bone sialoprotein also contain the RGD domain and were identified in the bone extracts by immunoblotting [13]. The contribution of the RGD-dependent migration of breast cancer cells to bone extracts was analyzed. We conducted transwell migration assays in the presence of RGE and RGD blocking peptides (Figure 5.6). The migration of MDA-MB-231 cells toward mouse bone extracts was wholly blocked with RGD blocking peptides; RGD blocking peptides reduced the migration levels to those observed in the vehicle control. The results demonstrated that the migration of breast cancer cells to bone extracts was RGD-dependent.

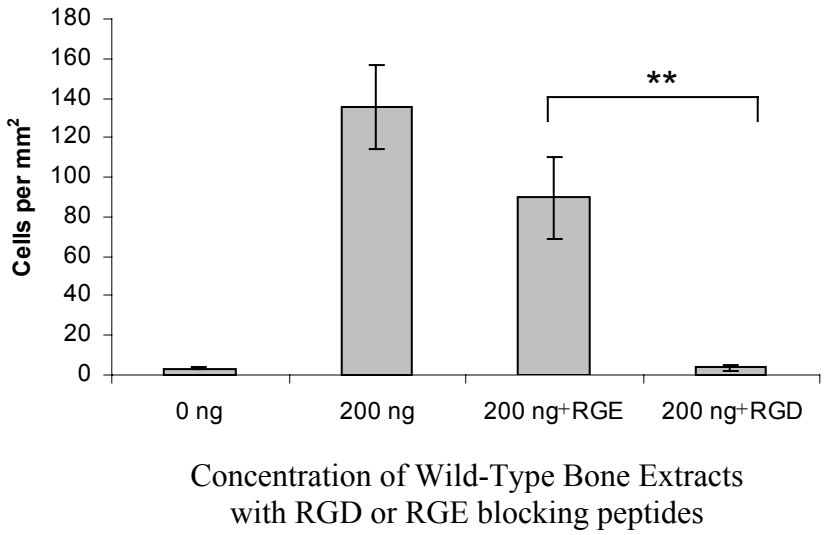


Figure 5.6 Inhibition of MDA-MB-231 cell migration toward wild-type extracts with RGE and RGD blocking peptides. Either 0 or 200 ng of wild-type extracts were air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells ($5X10^4$ cells per well) were combined with 0 or 200 μg/ml of RGE or RGD peptides. Experiments were done in duplicate. After 6 hours, the RGD peptides inhibited cellular migration to the wild-type extracts; the migration was reduced to the levels found in the vehicle control (0 ng). The RGE peptide had no significant effect on cell migration (N=6 membranes per peptide dose, mean ± SEM, ** $p \leq 0.01$)

Chapter 6

Osteoblast-Derived Soluble Factors Influence Breast Cancer Cell Migration to Bone

Rationale

We demonstrated that RGD-containing proteins that exist within the mineralized portion of bone matrix were chemoattractive to breast cancer cells. These RGD-containing proteins are most likely released from the bone matrix during bone resorption and could form a chemotactic gradient. In the next series of experiments, we focus on determining if other soluble factors released from osteoblasts during bone formation could also chemoattract breast cancer cells. We utilized conditioned media from proliferative and differentiating mouse osteoblasts to determine if soluble factors released during bone formation were also attractive to breast cancer cells.

Breast cancer cell migration toward fibroblast and osteoblast-conditioned media

To determine if soluble factors released from proliferative (or undifferentiating) and differentiating osteoblasts chemoattract breast cancer cells, we utilized conditioned media collected from mouse fibroblast (NIH 3T3) and osteoblast (MC3T3-E1) cell cultures. After 6 hours, there was significantly greater migration to both osteoblast-conditioned media samples compared to the fibroblast-conditioned media (Figure 6.1). There was slightly greater migration toward the conditioned media from differentiated MC3T3-E1 cells when compared to the conditioned media from undifferentiated MC3T3-E1 cells.

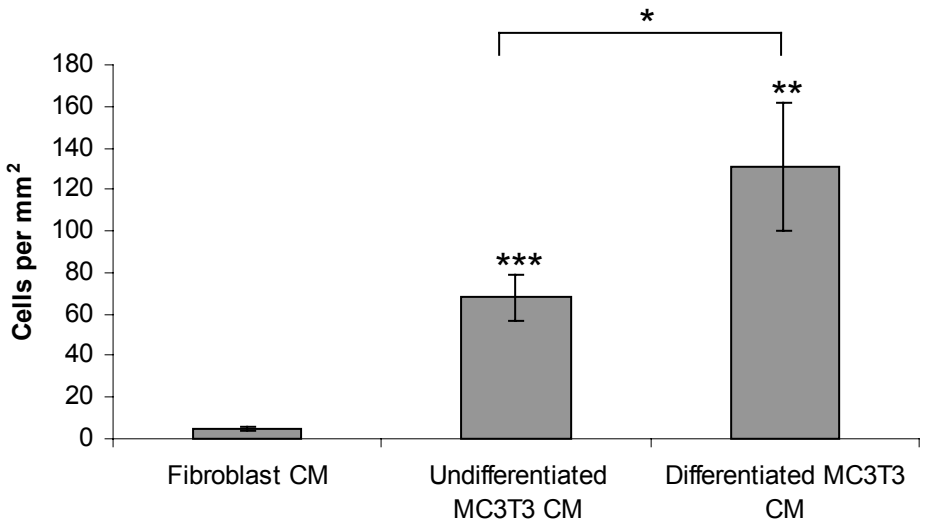


Figure 6.1 Migration of MDA-MB-231 cells toward mouse fibroblast (NIH3T3) and osteoblast (MC3T3-E1) conditioned media. Vibrant ® DiI stained MDA-MB-231 cells were seeded at $5X10^4$ cells per well in transwell chambers. Conditioned media from mouse fibroblast, undifferentiated MC3T3-E1 and differentiated MC3T3-E1 osteoblasts were added to the lower chamber. Experiments were done in triplicate. After 6 hours, there was significantly greater migration toward the osteoblast-conditioned media compared to the fibroblast-conditioned media. Furthermore, there was greater breast cancer cell migration toward the differentiated osteoblast-conditioned media compared to the undifferentiated osteoblast-conditioned media. (N=9 membranes per peptide dose, mean ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$)

Inhibition of RGD-dependent migration toward osteoblast-conditioned media

Because migration of breast cancer cells toward bone extracts was RGD-dependent (Chapter 5), we investigated if the migration toward the mouse osteoblast-conditioned media was also RGD-dependent. After 6 hours, RGE or RGD blocking peptides did not inhibit the migration of MDA-MB-231 cells toward osteoblast-conditioned media (Figure 6.2). However, the migration of the breast cancer cells toward fibroblast-conditioned media was inhibited by RGD blocking peptides. In this study, we demonstrated that the breast cancer cell migration to the osteoblast-conditioned media was not RGD-dependent.

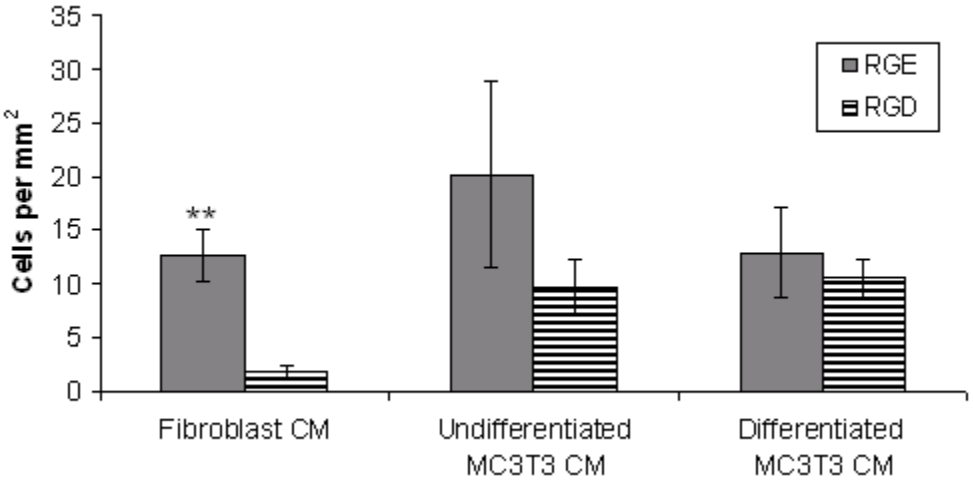


Figure 6.2 Inhibition of MDA-MB-231 cell migration to conditioned media with RGE and RGD blocking peptides. Vibrant ® DiI stained MDA-MB-231 cells ($5X10^4$ cells per well) were mixed with 200 μg/ml RGE or RGD peptides and added to transwell chambers. Conditioned media from mouse fibroblasts, undifferentiated MC3T3-E1 and differentiated MC3T3-E1 osteoblasts were added to the lower chamber. Experiments were done in duplicate. After 6 hours, there was no significant inhibition of breast cancer cell migration toward the osteoblast (both undifferentiated or differentiated) conditioned media. Breast cancer cell migration toward the fibroblast-conditioned media was inhibited with RGD blocking peptides. (N=6 membranes per peptide dose, mean ± SEM, * $p \leq 0.05$ compared to RGD treatment).

Cytokine analysis of fibroblast and osteoblast-conditioned media

To identify some of the soluble factors present in the conditioned media from fibroblast, undifferentiated osteoblast and differentiated osteoblast cultures, we utilized a mouse cytokine bioassay. This bioassay measured the concentrations of 18 cytokines; of these only RANTES, IL-6, KC (homolog to human IL-8), and G-CSF were identified in the conditioned media samples tested (Table 6.1). IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-10, IL-12 (p40), IL-12 (p70), IL-17, GM-CSF, IFN-γ, MIP-1α and TNF-α were not detected in any of the conditioned media samples. None of the 18 cytokines were detected in the mouse bone extracts. RANTES and IL-6 were present in all assayed conditioned media samples. IL-6 was detected at similar levels between samples whereas RANTES was detected at the highest concentration in the osteoblast-conditioned media. Moreover, RANTES concentrations were higher in the differentiated osteoblast-conditioned media compared to the undifferentiated osteoblast-conditioned media. KC and G-CSF were observed only in the fibroblast conditioned media. We concluded that RANTES and IL-6 were possible chemotactic factors present in the osteoblast-conditioned media.

Cytokine	Source of Conditioned Media		
	Fibroblast	Undifferentiated MC3T3	Differentiated MC3T3
RANTES	+	++	+++
IL-6	+	+	+
KC	+++	-	-
G-CSF	+	-	-

Table 6.1 Analysis of cytokines in mouse fibroblast and osteoblast-conditioned media samples. Mouse fibroblast, undifferentiated osteoblast and differentiated osteoblast-conditioned media were assayed for the presence of 18 different cytokines. Of these, only RANTES, IL-6, KC (homolog to human IL-8) and G-CSF were detected. Plus signs (+, ++, and + + +) indicate relative concentrations of these four cytokines in the conditioned media samples.

Correlation of MDA-MB-231 cell migration toward conditioned media samples and concentrations of RANTES

We determined there was increased migration of breast cancer cells toward osteoblast-conditioned media as compared to fibroblast-conditioned media and that this migration was not RGD-dependent. We also detected increased levels of RANTES in the osteoblast-conditioned media. Although this is circumstantial evidence, concentrations of RANTES, which has been previously described as a chemoattractant for immune cells and multiple myeloma [63, 85, 86], correlates with the migration levels induced by the conditioned media samples (Figure 6.3). We concluded that RANTES was a possible chemotactic factor secreted by osteoblasts and should be further investigated to determine the contribution it may have in breast cancer metastasis to bone.

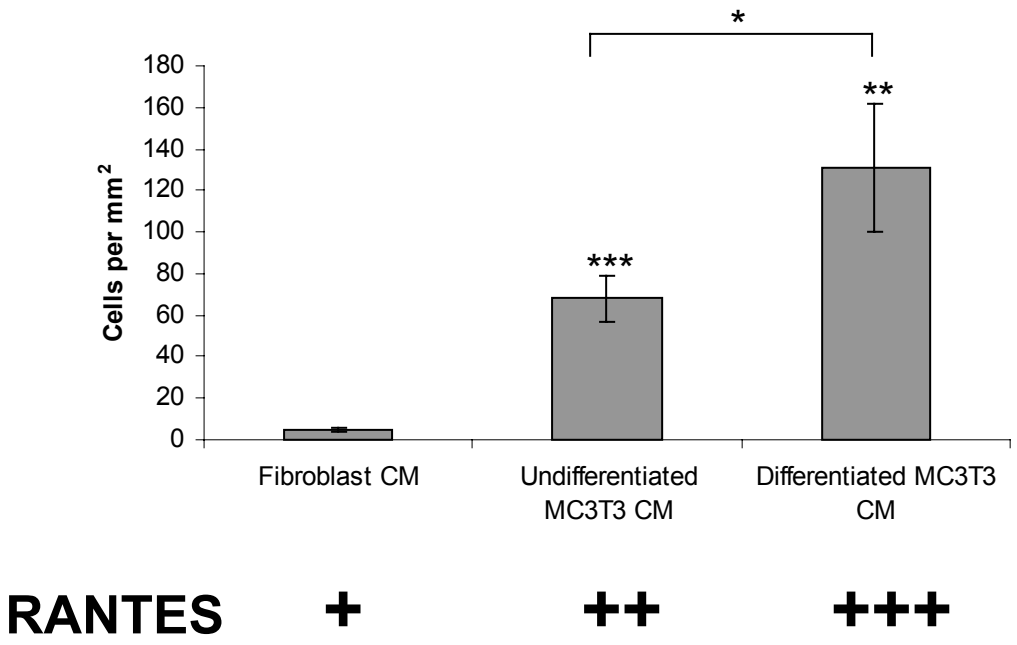


Figure 6.3 Correlation of MDA-MB-231 cell migration to conditioned media and concentrations of RANTES. This figure is a compilation of data represented in Figure 6.1 and Table 6.1. Note the correlation between migration levels and concentrations of RANTES in the conditioned media samples.

Chapter 7

Discussion

Overview

It is well established that breast cancer cells have an increased propensity to metastasize to bone [6]. Whether or not bone has an intrinsic attractive quality that influences breast cancer cell metastasis is not completely understood. Here we hypothesized that there are factors within bone that chemoattract breast cancer cells and we investigated the role of osteoblast-secreted proteins in this phenomenon.

In order for a substance to be chemoattractive, a gradient of the particular substance must form; a cell is then initially exposed to a small amount of that substance. A reactive cell will bind to the chemoattractant inducing actin polymerization near the receptor. The localization of cytoskeletal proteins will reorient the cell toward the chemoattractant and subsequently induce migration to increasing concentrations [87]. Osteoblast-secreted proteins can form a chemotactic gradient by way of three different mechanisms. One way is the diffusion of small proteins, such as osteocalcin, out of the matrix and into the serum [31]. Other proteins, such as osteoprotegrin and some growth factors, are secreted directly into the extracellular fluid. Thirdly, the majority of osteoblast-derived proteins are secreted directly into the developing bone matrix [30]. These proteins can form a chemotactic gradient only after being released from the matrix during osteoclastic bone resorption. Some bone-derived proteins, specifically osteonectin and RGD-containing proteins, have been characterized as chemoattractants for cancer cells.

Osteonectin, secreted by osteoblasts and incorporated into the bone matrix, has been shown to chemoattractant prostate cancer cells [46, 47]. Furthermore, osteonectin has been characterized as the main chemoattractant responsible for the migration of prostate cancer cells toward bone extracts [47]. The possibility that osteonectin has the same effect on breast cancer cells has not been fully examined; such an effect could explain why bone is the preferred site of metastasis. Osteonectin has been shown to increase MDA-MB-231 invasiveness through Matrigel™, a synthetic extracellular matrix [46]. Further evidence supporting a role for osteonectin in the development of skeletal malignancy is the observation that breast cancer cells usually metastasize to trabecular bone where osteonectin is found at its greatest concentration within the bone [88].

RGD-containing proteins, also secreted by osteoblasts and incorporated into the bone matrix, have been shown to chemoattract various cancer cell types including breast and prostate [49-51, 89]**.** The RGD-containing proteins identified in bone matrix are fibronectin, vitronectin, osteopontin, bone sialoprotein, thrombospondin, and fibrillin [13]. An RGD domain binds to integrin receptors on the plasma membrane surface of many cell types and regulates cell attachment and migration [13]. The migration of MDA-MB-231 cells toward RGD-containing proteins is dependent on the expression of $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ integrins by the cancer cells [9]. Migration to fibronectin, vitronectin, bone sialoprotein and osteopontin can be blocked with the use of competitive RGD peptides [90]. These extraneous peptides bind to the integrin and subsequently prevent cellular adhesion and migration toward the RGD domain of a chemoattractant. Blocking antibodies, designed against the integrin receptors, have also been used to prevent ligand binding and therefore chemoattraction [50, 51]

Osteonectin and RGD-containing proteins, such as fibronectin, vitronectin, osteopontin, bone sialoprotein, thrombospondins, and fibrillins, are secreted by osteoblasts and integrated into the developing matrix during bone formation [13]. These proteins are too large to freely diffuse out of the matrix; they range from 35 kD (osteonectin) up to 450 kD (thrombospondin) [13]. Consequently, the most likely mechanism by which these proteins form a chemotactic gradient occurs during osteoclastic bone resorption. A chemotactic gradient of osteonectin or RGD-containing proteins will develop when osteoclasts degrade the matrix and release these proteins into the extracellular fluid. This concept is supported by the clinical use of bone sialoprotein as a serum marker for measuring bone resorption [31].

A circulating breast cancer cell that has entered the microvasculature of the metaphysis could easily detect and respond to a bone-derived chemotactic gradient. The endothelial layer of the vascular sinusoids are highly porous; they are characterized by a discontinuous basal lamina and endothelial cells with large fenestra [17, 19, 20]. Degraded matrix proteins can easily pass through the porous vasculature and into the serum. Conversely, cancer cells can readily extravasate through the dilated endothelial layer. Circulating breast cancer cells bind to the endothelial layer through cellular adhesion molecules and selectins [9]. Once stationary, the cell detects low levels of a chemotactic protein, extravasates through the vasculature and migrates toward increasing concentrations of the chemoattractant. This study aimed to identify bone-derived chemoattractants that could influence breast cancer cell migration into the bone microenvironment. Osteoblast-derived proteins, either secreted into the developing matrix or into the extracellular fluid, were assayed for their chemotactic potential for

breast cancer cells. Specifically, we determined that RGD-containing proteins, but not osteonectin, chemoattracted breast cancer cells.

Evaluation of osteonectin for a chemotactic isoform

In order for a substance to act as a chemoattractant, a diffusible gradient must form, thus attracting a responsive cell toward higher concentrations. A paradox exists in that osteoblasts and metastatic breast cancer cells both secrete osteonectin. This dual production of osteonectin could negate the formation of a gradient to which breast cancer cells respond, assuming that both forms of osteonectin are identical and secreted at similar concentrations. We analyzed osteonectin from an array of cell types to determine if a tissue-dependent configuration and hence a chemotactic isoform exists.

The results indicated that metastatic breast cancer cells (MDA-MB-435 and MDA-MB-468), osteoblasts (hFOB), non-neoplastic breast epithelial cells (hTERT-HME1) and vascular endothelial cells (HBME-1) secreted osteonectin that had a similar or identical molecular weight (~ 46 kD). We also found that osteonectin from these cells had similar post-translational modifications. All forms of osteonectin tested in this study had similar glycosylation patterns, specifically, all had N-linked oligosaccharides but lacked sialic acids and O-linked oligosaccharides. N-linked glycosylation is important because it has been correlated to the binding affinity of osteonectin to collagen [91, 92]. Osteonectin from these cell types also lacked detectable phosphorylated serines.

The breast cancer, osteoblast and normal breast epithelial cell lines generated identical osteonectin cDNA sequences; only the vascular endothelial cells produced a distinctly different osteonectin cDNA. However, at the amino acid level, these

differences were minor. Substitutions # 1,3,4,5,6,7, and 8 are common evolutionary changes; these substitutions resulted in amino acids of similar polarity and mass and probably did not affect the function of the protein [83]. There were also no changes in the number and position of cysteines, which indicated that disulfide bonding was unaffected. The eight substitutions did not occur in the EF-hands or the GHK domain (Figure 7.1) [93]. EF-hands, found in the EC domain, are vital to the function of osteonectin by sequestering Ca^{2+} and binding to other matrix proteins [84]. The GHK peptide, located in the follistatin-like domain, is responsible for stimulating angiogenesis and proliferation [37, 93]. We concluded that the substitutions in the HBME-1 osteonectin cDNA were insignificant. Furthermore, our investigations did not reveal notable differences between the osteonectin secreted by breast cancer cells or osteoblasts; therefore, a chemoattractant gradient based on a unique configuration of osteonectin was unlikely.

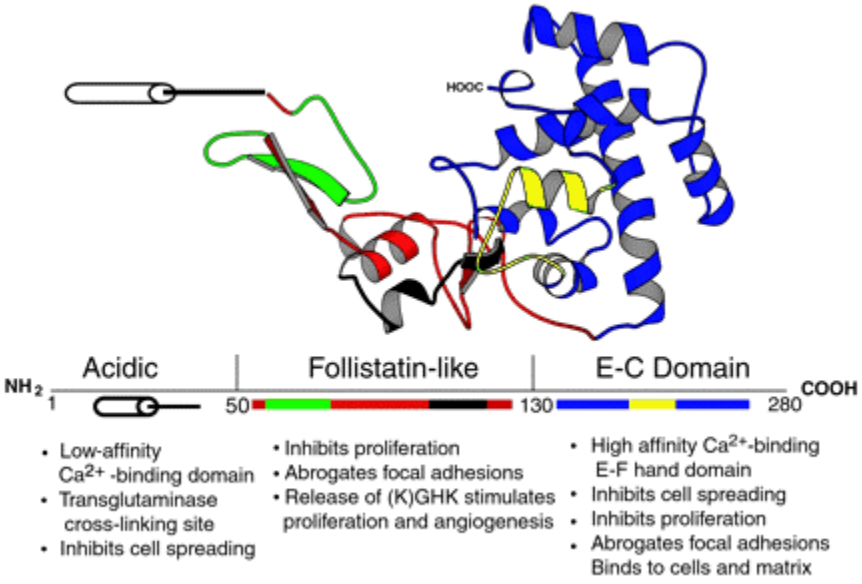


Figure 7.1 Structure of osteonectin. This diagram is derived from crystallographic data to demonstrate the three domains of osteonectin with their corresponding cellular activities. The GHK peptide within the follistatin-like domain is denoted in black. Bradshaw AD and Sage EH (2001) J Clin Invest

The effects of osteonectin on breast cancer cell motility

We were unable to detect a unique configuration of osteonectin and therefore concluded there was no chemotactic isoform. However, one can argue that a gradient of osteonectin can form due to variable secretion levels as opposed to the presence of a tissue-dependent configuration. If cancer cells secrete reduced levels of osteonectin, while osteoblasts secrete high concentrations, a chemotactic gradient could still exist. This concept is supported by clinical observations; researchers have correlated bone micrometastases with low osteonectin gene expression at the primary tumor site [94]. To test this possibility *in vitro*, we analyzed the ability of osteonectin to increase cell motility and migration on a breast cancer cell line (MDA-MB-231), which did not secrete detectable levels of osteonectin.

Osteonectin has been described as a matricellular protein with de-adhesive properties [41] that influence cell motility [43, 93]. Murphy-Ullrich [41] describes the ability of osteonectin to promote an "intermediate state of adhesion" which favors cell motility. Intermediate adhesion is commonly characterized by the disassembly of intracellular stress fibers and focal adhesion complexes while maintaining integrin binding to a matrix [42]. A cell will appear to have a normal spreading morphology over matrix but be in a heightened state for cell movement [41].

We tested the ability of osteonectin to induce motility in a "wound-healing" assay. Osteonectin from osteoblasts, breast cancer cells and vascular endothelial cells increased MDA-MB-231 cell motility, as determined by the ability of osteonectin to enhance cell outgrowth into the wound. Notably, osteonectin from cancer and vascular endothelial cells induced high levels of motility; nearly half of the wound area was

covered with cells by six hours. Osteonectin from the osteoblasts had a strong impact on breast cancer cell motility but induced less cell outgrowth when compared to osteonectin from the other cell sources. These results supported the current literature that exogenous osteonectin enhances cell motility. However, the ability of a single concentration of soluble osteonectin to increase cell motility is distinctly different from chemoattraction, a process that is dependent on a gradient. To test for chemoattraction, or "directed" cell migration, we needed to measure cell movement toward a distant source of osteonectin.

Osteonectin from several cell lines was tested for its ability to chemoattract MDA-MB-231 cells using a transwell chamber assay. This system is ideal for qualifying a cell's response, namely cell migration, to a distant source of a chemoattractant. Osteonectin from several cell types was analyzed for its chemotactic effect on MDA-MB-231 cells. The osteonectin derived from hTERT-HME1 cells induced a 5-fold increase in migration. Although the migration toward the normal breast epithelial osteonectin was statistically significant, the actual number of migrated cells was low (~ 10 cells per mm^2). One can postulate that the increase in migration can actually be attributed to an increase in random cell motility rather than true chemoattraction as a result of the anti-adhesive properties of osteonectin. Nevertheless, if we assume there was actual migration and not merely increased motility, the questions concerning breast cancer metastasis to bone remain unanswered. However, these results do address the impact osteonectin may have had on cancer cells at the primary tumor site. Epithelial-derived osteonectin could assist in the de-adhesion of cancer cells from the original tumor. Osteonectin also upregulates matrix metalloproteinase-2 activation which is important for proteolysis of the extracellular matrix and hence contributes to tumor invasion [46, 95]. Although

osteonectin may play an important role in the progression of cancer metastasis at the primary site, in context of this study, we concluded that epithelial-derived osteonectin does not direct cancer migration into the bone microenvironment.

Osteonectin from the endothelial (HBME-1) cells also increased migration as much as 3-fold; this could again be related to the anti-adhesive properties. In wound healing assays, HBME-1 derived osteonectin was shown to increase random cell motility. This increase in cell motility could account for the low numbers of migrated cells observed in the transwell migration assays. The migration to HBME-1 derived osteonectin in the transwell assays could therefore be a result of increased cell motility and not chemoattraction.

Although some migration to osteonectin from normal breast epithelial cells and vascular endothelial cells was observed, there was no migration to breast cancer or osteoblast-derived osteonectin in transwell chamber assays. Even when the dose of bone-derived osteonectin was increase to 1 μg and the cells were allowed to migrate for 48 hours, we were still unable to detect an increase in breast cancer cell migration. We concluded that bone-derived osteonectin did not chemoattract breast cancer cells.

Role of osteonectin in breast cancer cell migration toward other matrix proteins

The lack of breast cancer cell chemoattraction toward osteonectin was further demonstrated by comparison of the osteonectin-induced migration levels to those stimulated by vitronectin. Vitronectin is known to chemoattract MDA-MB-231 cells through the binding of $\alpha v\beta_3$ on the cancer cell surface to the RGD domain of vitronectin [49, 50] in contrast to osteonectin which does not contain the RGD domain. RGD and RGE blocking peptides were used to demonstrate the RGD-dependence of the cancer cell migration to vitronectin; RGE peptides provided a negative control. There was no migration of the breast cancer cells to bone-derived osteonectin; this was consistent with our previous findings that MDA-MB-231 cells did not migrate to high doses of bone osteonectin after 48 hours. There was marked breast cancer cell migration toward vitronectin in the presence of RGE peptides. RGD peptides significantly inhibited MDA-MB-231 migration toward vitronectin as expected [49, 50]. We also observed increased cancer cell migration toward the vitronectin/osteonectin mixture. In fact, the number of migrated cancer cells toward the vitronectin/osteonectin mixture was equal to the migration levels induced by vitronectin alone. These findings were surprising considering there was half the amount of vitronectin (100 ng) in the vitronectin/osteonectin mixture compared to when vitronectin was used alone (200 ng). In previous experiments, cancer cell migration was shown to exhibit a dose response to increasing amounts of vitronectin. Migration toward the vitronectin/osteonectin mixture was also inhibited with RGD blocking peptides and was therefore deemed RGD-dependent.

The migration of the MDA-MB-231 cells toward the vitronectin/osteonectin mixture was mainly induced by the presence of vitronectin as demonstrated by blocking RGD-dependent migration. However, there appeared to have been a synergistic effect induced by the presence of osteonectin; the number of migrated cells toward the vitronectin/osteonectin mixture (100 ng vitronectin) was equal to the migration levels produced by vitronectin (200 ng). This observation supports the concept that osteonectin enhanced cell motility through its anti-adhesive properties. Murphy-Ulrich describes that intermediate adhesion can only exist when a cell is bound to a matrix through integrins. In this experiment, the MDA-MB-231 cells bound to the RGD-domain of vitronectin through integrins. Osteonectin was then able to generate an intermediate state of adhesion which fostered a greater cellular response to the chemoattractant. Although this is speculation based upon an observation and not a tested hypothesis, it is important to consider the indirect effects osteonectin may have on cancer cell metastasis to bone.

Despite the lack of migration to purified osteonectin, physiologically, osteonectin is in the presence of many other matrix proteins in the bone microenvironment. The chemoattractive properties of osteonectin may be dependent upon its proximity to these other matrix proteins. This concept was supported by our observation that osteonectin increased breast cancer migration toward vitronectin. As stated earlier, osteonectin increases cell motility when the cells are bound to a matrix through integrins. The ability of osteonectin to enhance chemoattraction toward other matrix proteins, in addition to vitronectin, may be based on its de-adhesive properties.

We further assayed the chemoattractive ability of osteonectin in the presence of other bone matrix proteins by utilizing bone extracts from wild-type and osteonectin-null

mice. We detected high migration toward all bone extracts regardless of the presence of osteonectin. At equal protein doses, the metastatic breast cancer cells migrated to the wild-type or osteonectin-null bone extracts at the same rate. This experiment provided strong preliminary evidence that metastatic breast cancer cells were attracted to bone extracts and therefore were attracted to factors within the mineralized portion of the bone matrix. However, it also demonstrated that osteonectin was not a relevant contributor to the chemotaxis of breast cancer cells to bone. These results were in contrast to the observation that osteonectin enhanced RGD-dependent migration toward vitronectin. Enhanced migration to the vitronectin/osteonectin mixture occurred when osteonectin and vitronectin were present at equal doses. Osteonectin is not present in equal concentrations with the other matrix proteins in the bone extracts. Osteonectin only accounts for 23% of the matrix proteins isolated in bone extracts [34]. Any synergistic effect osteonectin had in enhancing MDA-MB-231 cell motility to the bone extracts was most likely lost due to the imbalance in protein concentrations.

Other studies have reported osteonectin to be the primary chemoattractive force in bone extracts for prostate cancer cells [47]. We utilized a chemoattraction assay similar to De et al. (2003) who studied PC3 (a prostate cancer cell line) migration toward wild-type and osteonectin-null bone extracts. They concluded that prostate cancer cells are attracted to bone extracts only when osteonectin was present. In this study, the migration of breast cancer cells toward bone extracts occurred independent of the presence of osteonectin. Because breast and prostate cancer have a high propensity to metastasize to bone, it is assumed that their mechanism of bone metastasis is the same. This assumption is misleading; these two different cell types exert distinctly different bone phenotypes

upon development of bone metastases. Breast cancer cells tend to develop osteolytic bone lesions while prostate cancer cells are usually osteoblastic in nature [7]. Because breast and prostate cancer cells form different types of bone metastases, it is reasonable to hypothesize that their mode of bone metastasis may also be different from each other. Our research indicates that osteonectin is not a significant chemoattractant for breast cancer cells while other researchers have found it is a chemoattractant for prostate cancer cells. This is evidence that these two different cancer cell types may metastasize by different mechanisms.

Although we concluded that osteonectin was not a relevant contributor of the chemoattraction of breast cancer cells to bone extracts; we also found evidence that there were other unknown factors within the mineralized portion of bone matrix that were responsible for the attraction of breast cancer cells.

RGD-dependence of MDA-MB-231 cell migration toward bone extracts

The aim of this study was to identify chemoattractive factors within bone that may be responsible for breast cancer metastasis. We determined that, although osteonectin was secreted by osteoblasts and found in bone matrix, it was not chemoattractive to metastatic breast cancer cells. Osteonectin may have enhanced breast cancer cell migration toward the RGD-containing protein vitronectin. However, any effect osteonectin had on breast cancer chemoattraction toward other matrix proteins was not observed in the migration assays utilizing bone extracts. From these migration assays, we determined there were chemotactic factors present in the mineralized portion

of bone matrix directing metastatic breast cancer cell migration. We then aimed to identify those chemoattractive factors.

Previous studies indicated that RGD-containing proteins chemoattract breast cancer cells directly through the RGD complex [49-52]. We had confirmed that MDA-MB-231 cells were chemoattracted to vitronectin through its RGD domain. The next aim of this study was to qualify the role, if any, RGD-containing proteins had on the migration of breast cancer cells to bone extracts. The presence of RGD-containing proteins in the bone extracts was confirmed. Through immunoblotting, we detected fibronectin, bone sialoprotein, vitronectin and osteopontin in the wild-type, heterozygous and osteonectin-null bone extracts. Fibrillin and thrombospondin, two other RGD-containing proteins associated with bone matrix were not tested for their presence in these bone extracts.

The MDA-MB-231 cell line is reported to express the $\alpha_v\beta_3$ integrin which binds to RGD and directs their migration [49-51]. However, it was necessary to demonstrate that the breast cancer cells in our laboratory would also migrate to RGD-containing proteins; the migration to vitronectin had been established but migration to fibronectin and osteopontin needed to be confirmed as well. We observed increased migration of the MDA-MB-231 cells to all tested doses of fibronectin, vitronectin and osteopontin. These data support the current literature that metastatic breast cancer cells were attracted to and will migrate toward the RGD-containing proteins fibronectin, vitronectin and osteopontin.

The migration of MDA-MB-231 cells toward fibronectin and vitronectin was determined to be RGD-dependent. RGD blocking peptides were used to bind the $\alpha_v\beta_3$

integrin on the breast cancer cell surface and demonstrate the importance of the RGD domain in this migration. A single concentration of soluble RGD peptides combined with the cancer cells negated the chemotactic effect created by the RGD-containing proteins in the lower chamber. An RGE peptide, which did not bind to integrins, had no inhibitory effect on cell migration. These data supported the current literature that the breast cancer cells were attracted to and migrated toward vitronectin and fibronectin in an RGD-dependent manner.

We had established that our bone extracts contained four RGD-containing proteins: vitronectin, fibronectin, osteopontin and bone sialoprotein. We also demonstrated that the MDA-MB-231 cell line was attracted to osteopontin, vitronectin and fibronectin and that this migration was RGD-dependent. We next aimed to measure the contribution of RGD-containing proteins in the migration of breast cancer cells toward bone extracts. When RGE blocking peptides were present, there was no inhibition of breast cancer cell migration to bone extracts. However, in the presence of RGD blocking peptides, we observed a complete inhibition of breast cancer cell migration toward the bone extracts. In fact, the number of migrated breast cancer cells was reduced to levels found in the vehicle control treatment. These data demonstrates that the chemoattraction and subsequent migration of breast cancer cells toward bone extracts was RGD-dependent.

It is important to consider one key distinction between proteins isolated in the bone extracts and proteins released during bone resorption. The proteins isolated in the bone extracts are whole proteins whereas those released from the bone matrix during bone resorption are peptide fragments. Osteoclasts secrete proteolytic enzymes (MMPs

and lysosomal cysteine proteinases) that degrade the organic matrix and result in the release of peptide fragments [26]. An important future experiment designed to measure breast cancer cell chemoattraction toward degraded matrix proteins and whether this migration is also RGD-dependent, should include transwell chambers with active osteoclasts degrading bone chips in the lower chamber. This assay would provide a more accurate representation of breast cancer cell chemoattraction into bone.

We concluded that *in vitro*, the ensemble of RGD-containing proteins in the bone extracts chemoattracted breast cancer cells. It is possible that *in vivo*, the presence of RGD-containing proteins in bone matrix may direct breast cancer cell metastasis into bone (Figure 7.2). RGD-containing peptides are most likely released into the circulation during bone resorption. Breast cancer cells that have attached to the vascular endothelium through selectins and cellular adhesion molecules, will bind the RGD complex with available surface integrins [50-52]. Upon integrin binding, the cell will migrate toward increasing concentrations of RGD-containing peptides. This series of events is supported by the observation that bone metastases are often found at sites of high bone turnover where matrix proteins are degraded and released into the serum continuously [11]. Bone-specific metastasis could result from the steady release of RGD-containing peptides from the metaphysis into the circulation. This constant source of chemoattractants for circulating breast cancer cells could result in their preferential migration and metastasis to bone.

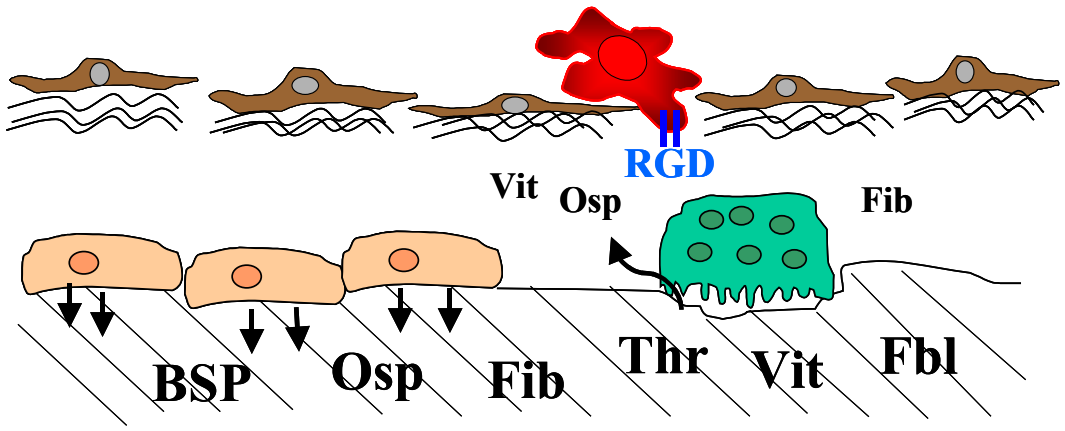


Figure 7.2 RGD-dependence of breast cancer cell migration into bone. RGD-containing proteins, bone sialoprotein (BSP), osteopontin (Osp), fibronectin (Fib), thrombospondin (Thr), vitronectin (Vit) and fibrillin (Fbl), are secreted by osteoblasts into the developing bone matrix. These proteins are most likely degraded and released during osteoclastic bone resorption. A metastatic breast cancer cell, attached to the vascular endothelium of a metaphyseal sinusoid, binds the RGD-domain through surface integrins such as $\alpha_v\beta_3$. Because the vascular endothelial layer is porous and has a discontinuous basal lamina, the breast cancer cell can easily migrate toward the bone matrix.

Osteopontin, fibronectin, thrombospondin and vitronectin are not bone specific; these proteins are found in most tissues throughout the body [13]. It can be argued that because they are not bone specific, they probably are not responsible for bone-specific metastasis. Conversely, bone sialoprotein is more limited to bones and teeth [13]. Recently, it has been identified in the salivary gland [96]. The more limited expression of bone sialoprotein could be a significant factor in bone-specific metastasis.

MDA-MB-231 cell migration to osteoblast-derived soluble proteins

We determined that RGD-containing proteins were responsible for the *in vitro* migration of breast cancer cells toward the mineralized portion of bone matrix. *In vivo*, breast cancer cells most likely respond to these proteins when they are released into the serum during bone resorption. However, we were also interested in identifying other chemotactic factors released during bone formation. In other words, do osteoblasts secrete soluble proteins directly into the circulation that attract breast cancer cells? If so, metastatic breast cancer cells would be attracted to areas of not only high bone resorption, but also high bone formation.

Proteins secreted into the extracellular fluid by osteoblasts were collected in conditioned media from proliferating and differentiating mouse osteoblast (MC3T3-E1) cultures. The chemoattractive capacity of the osteoblast-conditioned media was verified in transwell chamber migration assays. We demonstrated that osteoblast cultures, both proliferating and differentiating, contained factors that attracted breast cancer cells when compared to conditioned media from cultured mouse fibroblasts. We concluded that

proliferating and differentiating osteoblasts secreted soluble factors into their extracellular fluid that chemoattracted breast cancer cells.

We detected slightly greater migration toward the differentiating osteoblast-conditioned media compared to the proliferating osteoblast-conditioned media. One could argue that the differentiating osteoblasts secrete unique factors not secreted by proliferating osteoblasts. Although this is a compelling argument, the increase in migration was most likely not a result of unique factors. Instead, we expected the increase in migration to be a result of greater protein concentrations in the differentiated osteoblast culture. Both sets of conditioned media were collected over the course of a 72-hour time period. In the proliferating culture, the cells grew and divided during the entire 72 hour duration and further divided following the media collection. Therefore, we expected a greater number of cells in the differentiated osteoblast culture than in the proliferating osteoblast culture and hence a greater concentration of secreted proteins in the differentiated osteoblast-conditioned media. An increase in breast cancer cell migration toward the differentiated osteoblast-conditioned media was probably a result of increased concentrations of chemotactic factors rather than the presence of unique chemotactic factors synthesized only by late-stage osteoblasts.

We have established that the breast cancer cells were attracted to soluble factors released from osteoblasts. We postulated that since RGD-containing proteins are directionally secreted into the developing matrix and should not be present in the conditioned media, the migration to osteoblast-derived conditioned media was due to a substance other than RGD-containing bone matrix proteins. Through the use of RGE and RGD blocking peptides, we confirmed that breast cancer cell migration to osteoblast-

conditioned media was not RGD-dependent. We detected no change in MDA-MB-231 cell migration in the presence of RGE or RGD blocking peptides. In contrast, migration toward the fibroblast-conditioned media was reduced with RGD peptides suggesting that perhaps fibroblasts secreted RGD-containing proteins. However, we concluded that the metastatic breast cancer cells are attracted to and migrated toward osteoblast-derived soluble factors independent of RGD-containing proteins.

Having demonstrated that metastatic breast cancer cells were attracted to osteoblast-secreted factors that did not contain the RGD domain, we aimed to identify what secreted factors were responsible for the increased breast cancer cell migration toward the osteoblast-conditioned media. Many chemokines, including stromal cell derived factor 1α (SDF-1α), monocyte chemotactic protein-1 (MCP-1) [59-62], and RANTES [64, 97] have been shown to increase cancer cell migration. These chemokines are secreted by osteoblasts and may contribute to breast cancer cell metastasis to bone [58, 98-100]. Chemokines are a subset of cytokines known to chemoattract a number of different cell types including cancer cells [57]. We aimed to identify which, if any chemokines were present in the osteoblast-conditioned media. We utilized a cytokine assay that had the capability to simultaneously detect up to 18 different cytokines in the conditioned media samples. From the 18 cytokines analyzed, only four were detected in any of the conditioned media samples. RANTES, IL-6, KC (homolog to IL-8) and G-CSF were identified. Of these four cytokines, only RANTES levels correlated with the degree of breast cancer cell migration to the conditioned media samples. RANTES was detected in its highest concentration in the differentiated osteoblast-conditioned media; the breast cancer cells also migrated at a greater rate to this conditioned media. RANTES

is a member of the CC chemokine family which has been suggested to play a role in the migration of tumor cells to secondary sites [57]. Although the correlation of RANTES and breast cancer metastasis was an observation and not a tested hypothesis, this preliminary evidence warrants further investigations.

The effect of RANTES or other osteoblast-derived soluble factors could direct cancer metastasis to areas of bone formation (Figure 7.3). Soluble factors, secreted by osteoblasts, are released directly into the surrounding extracellular fluid. Breast cancer cells attached to the vascular endothelium, could bind to chemoattractants through receptors on the plasma membrane surface. Reactive cells will then migrate toward increasing concentrations of the chemoattractant, thus leading them through the porous vascular layer and toward active osteoblasts.

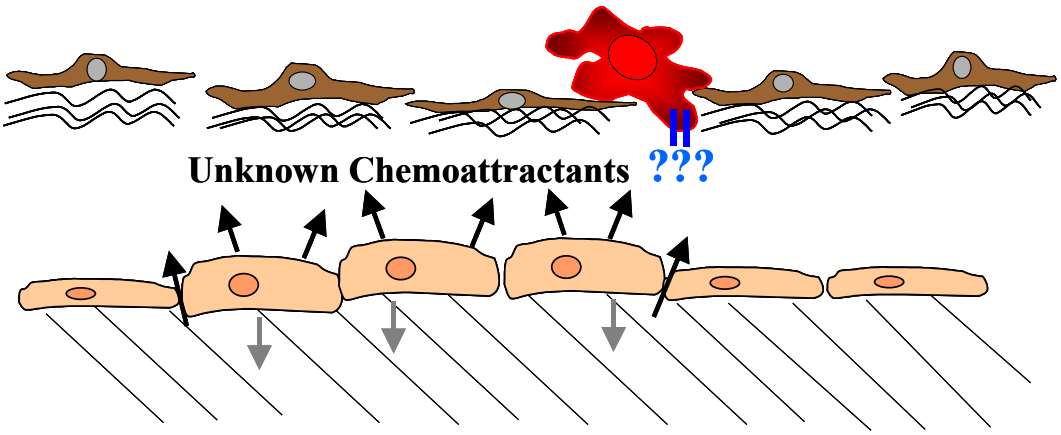


Figure 7.3 Osteoblast-derived soluble factors chemoattract breast cancer cells. Osteoblast secrete chemotactic factors directly into the extracellular fluid. Breast cancer cells, bound to the vascular endothelium, detect the soluble chemoattractants. A reactive cancer cell will then migrate through the vascular barrier toward greater concentrations of the chemoattractant. Note the discontinuous basal lamina of the vascular endothelium, this fosters the easy passage of migrating breast cancer cells.

Summary and conclusions

The focus of this study was to identify bone-derived factors that chemoattract breast cancer cells. Here we provided strong evidence that osteonectin does not chemoattract breast cancer cells. When osteonectin was present in equal doses with vitronectin, it appeared to enhance cancer cell migration. However, this effect was not observed when bone extracts were analyzed with or without osteonectin. The assays utilizing bone extracts provided preliminary evidence that there were chemotactic factors within the mineralized portion of bone matrix. These chemotactic factors within the bone extracts were determined to be the RGD-containing proteins, fibronectin, vitronectin, osteopontin and bone sialoprotein. We concluded that RGD-containing proteins, known to be released from the matrix during bone resorption, may contribute to the preferential metastasis of breast cancer cells. In addition to the chemoattraction that would occur during bone resorption, we determined that during bone formation osteoblasts secrete factors directly into the extracellular fluid that may also contribute to metastasis. It is possible that the chemokine RANTES may be involved in the migration induced by osteoblast-derived soluble factors however further investigations are needed. We concluded that there are factors in bone, both soluble and in the mineralized matrix, that chemoattract breast cancer cells. Therefore, we accept our hypothesis that osteoblasts secrete proteins that chemoattract breast cancer cells.

Future directions

It is clear from this study that osteoblasts secret many factors that chemoattract breast cancer cells. Some of the chemotactic factors are secreted into the matrix to be released during bone resorption while others are secreted directly into the extracellular fluid. Any of these chemotactic factors could contribute to the preferential metastasis of breast cancer cells to bone.

The concepts studied in this thesis are not only applicable to breast cancer metastasis; prostate cancer and multiple myeloma have a high propensity to metastasize to bone. Prostate cancer is generally osteoblastic in nature while breast and lung cancer are usually osteolytic [32]. However, these different cancer types have increased rates of bone metastases formation and could be attracted to the bone microenvironment by the same mechanisms.

We demonstrated that RGD-containing proteins were chemoattractive to breast cancer cells. These proteins are most likely released from the mineralized matrix during bone resorption, placing breast cancer patients at a greater risk for developing bone metastases during the resorptive process. Bisphosphonates, which inhibit the activity of mature osteoclasts and the development of new osteoclasts [101], are currently used in cancer treatment although their exact mode of action against cancer metastases remains unclear. Some researchers have shown that bisphosphonates inhibit the development of bone metastases through the reduction in bone resorption and subsequent release of growth factors [33, 102]. Others have even demonstrated the anticancer effect bisphosphonates have through promoting cancer cell apoptosis [33]. In this study, we offer an additional mode of action of bisphosphonates that may contribute to their

efficacy in the prevention of bone metastases. An inhibition of osteoclastic bone resorption by bisphosphonates will result in a reduction of free RGD-containing proteins and subsequently a reduction in the chemoattractive quality of bone.

This dissertation has also addressed the role of soluble factors released by osteoblasts into the extracellular fluid and their possible contribution to breast cancer cell metastasis. Although we only generated preliminary evidence concerning this concept, further studies should be done to identify those substances secreted directly into the circulation by osteoblasts that may attract cancer cells. Once these chemotactic factors are confirmed and identified, future therapies could involve reducing bone formation or osteoblast differentiation during cancer treatment to prevent bone metastases.

It is important for the medical community and pharmaceutical industry to understand the entire complex metastatic process. Once a clear understanding of metastatic pathways is established, patients could be prescribed individualized treatments based on the profile of their specific cancer. Optimized therapy reduces the stress and risks involved in over treating an already critically ill cancer patient. A physician's hope is to administer the strongest and most appropriate therapy to a cancer patient based on the progression and prognosis of their specific disease. Understanding all the steps involved in cancer metastasis is essential for the development of individualized medicine. Therefore, by identifying chemoattractive factors that influence breast cancer metastasis to bone and better characterizing the metastatic process, we are one step closer toward the development of novel treatments and preventative therapies for optimal cancer medicine.

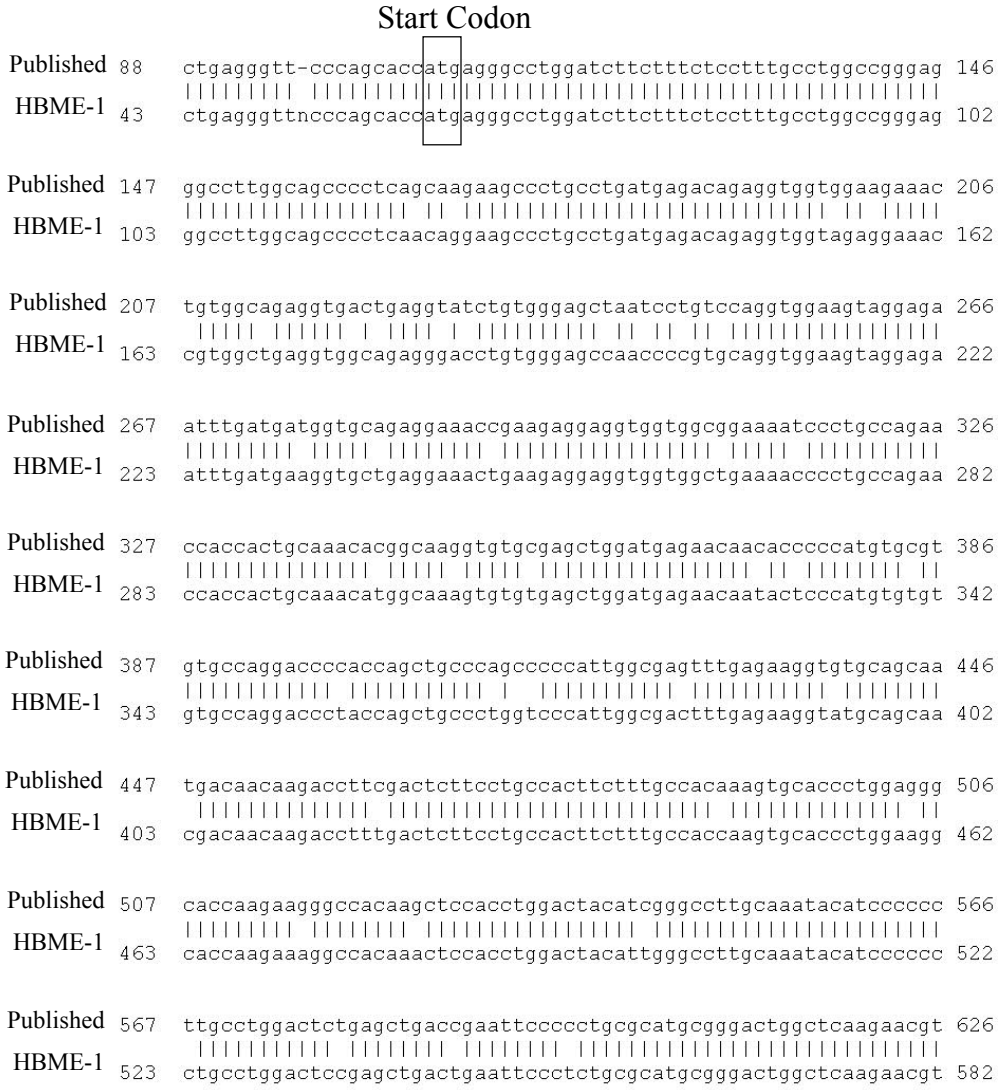
Appendix

Internal Primers Used to Generate cDNA Sequences

Cell Type	Sense Primers	Antisense Primers
hFOB	CCT-GCC-TGC-CAC-TGA-GG CCC-CAT-TGG-CGA-GTT-TGA AGT-TCG-GCC-AGC-TGG-ACC	TAA-ACA-TTG-GGG-GAA-ACA-CG TGT-ACA-GGG-AAG-ATG-TAC GCA-CAT-GGG-GGT-GTT-GTT
HTERT	CCT-GCC-TGC-CAC-TGA-GG CCC-CAT-TGG-CGA-GTT-TGA AGT-TCG-GCC-AGC-TGG-ACC	TAA-ACA-TTG-GGG-GAA-ACA-CG TGT-ACA-GGG-AAG-ATG-TAC GCA-CAT-GGG-GGT-GTT-GTT
MDA-MB-435	CCT-GCC-TGC-CAC-TGA-GG CCC-CAT-TGG-CGA-GTT-TGA AGT-TCG-GCC-AGC-TGG-ACC	TAA-ACA-TTG-GGG-GAA-ACA-CG TGT-ACA-GGG-AAG-ATG-TAC GCA-CAT-GGG-GGT-GTT-GTT
MDA-MB-468	CCT-GCC-TGC-CAC-TGA-GG CCC-CAT-TGG-CGA-GTT-TGA AGT-TCG-GCC-AGC-TGG-ACC	TAA-ACA-TTG-GGG-GAA-ACA-CG TGT-ACA-GGG-AAG-ATG-TAC GCA-CAT-GGG-GGT-GTT-GTT
HBME-1	CCT-GCC-TGC-CAC-TGA-GG CAT-GAG-GGC-CTG-GAT-CTT-CT AGT-TCG-GCC-AGC-TGG-ACC	GCA-GAA-CAA-CAA-ACC-ATC-CA TGT-ACA-GGG-AAG-ATG-TAC

Comparison of Osteonectin cDNA Sequences

The cDNA sequence of published human osteonectin was identical to the sequence generated from the hFOB, MDA-MB-435, MDA-MB-468 and hTERT cell lines. The HBME-1 osteonectin cDNA sequence is displayed and aligned with the published osteonectin sequence. These two cDNA sequences were compared by "Blast-2 Sequence software" provided by the National Center of Biotechnology Information (NCBI) of the National Institutes of Health. Sequence mismatches between the published and HBME-1 code are denoted with the absence of a connecting line. The published and HBME-1 osteonectin cDNA sequences were 91% identical. The start and stop codons are marked.


Start Codon
Published 88 ctgagggtt-cccagcaccatgagggcctggatcttctttctcctttgcctggccgggag 146
HBME-1 43 ctgagggttncccagcaccatgagggcctggatcttctttctcctttgcctggccgggag 102
Published 147 ggccttggcagcccctcagcaagaagccctgcctgatgagacagaggtggtggaagaaac 206
HBME-1 103 ggccttggcagcccctcaacaggaagccctgcctgatgagacagaggtggtagaggaaac 162
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HBME-1 163 cgtggctgaggtggcagagggacctgtgggagccaaccccgtgcaggtggaagtaggaga 222
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HBME-1 223 atttgatgaaggtgctgaggaaactgaagaggaggtggtggctgaaaacccctgccagaa 282
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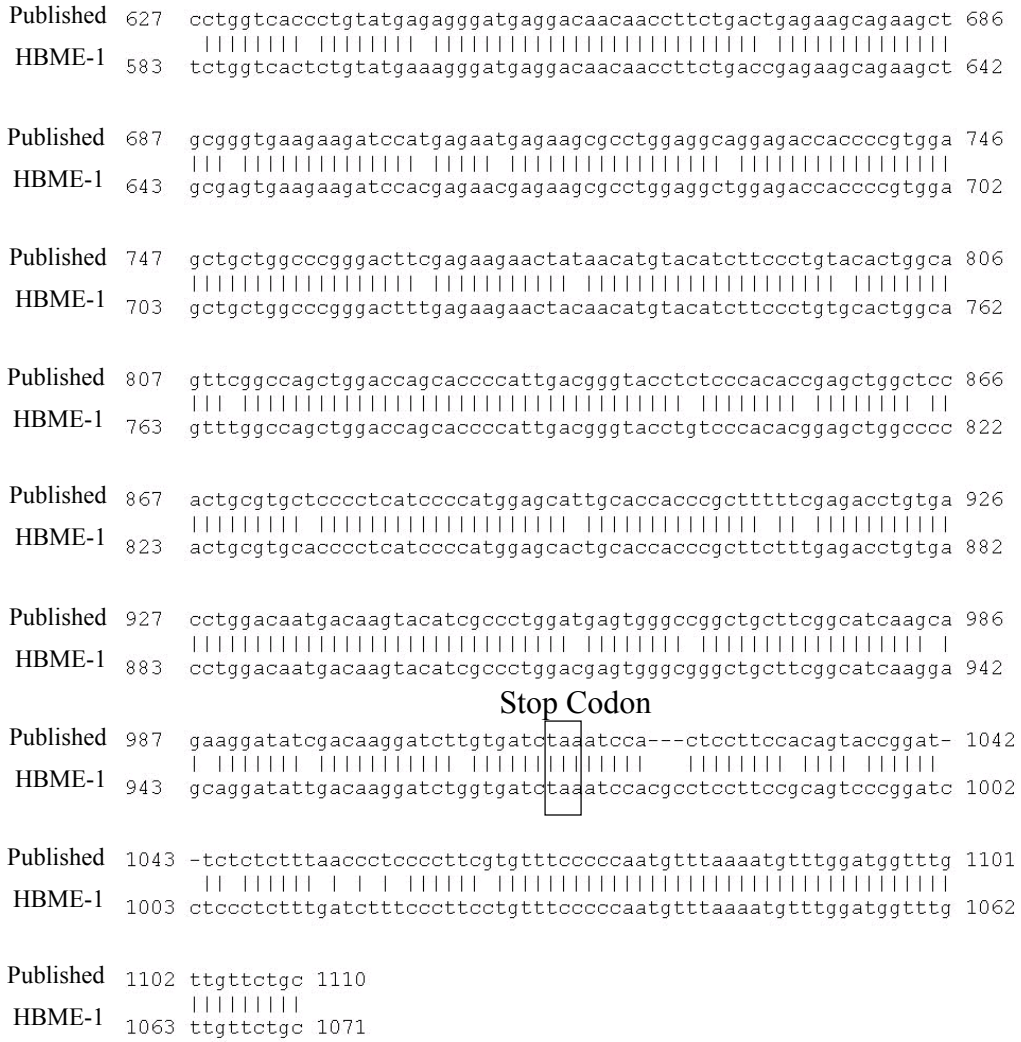
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Published 1102 ttgttctgc 1110
HBME-1 1063 ttgttctgc 1071

The Deduced Amino Acid Sequences from the cDNA of Osteonectin from Human Sources

The amino acid sequence from hFOB, MDA-MB-435, MDA-MB-468, and hTERT:

MRAWIFFLLC LAGRALAAPQ QEALPDETEV VEETVAEVTE VSVGANPVQV EVGEFDDGAE
ETEEEVVAEN PCQNHHCKHG KVCELDENNT PMCVCQDPTS CPAPIGEFEK VCSNDNKTFD
SSCHFFATKC TLEGTKKGHK LHLDYIGPCK YIPPCLDSEL TEFPLRMRDW LKNVLVTLYE
RDEDNNLLTE KQKLRVKKIH ENEKRLEAGD HPVELLARDF EKNYNMYIFP VHWQFGQLDQ
HPIDGYLSHT ELAPLRAPLI PMEHCTTRFF ETCDLDNDKY IALDEWAGCF GIKQKDIDKD
LVI

Molecular weight: 34632.16 daltons

The amino acid sequence from HBME-1:

MRAWIFFLLC LAGRALAAPQ QEALPDETEV VEETVAEVAE GPVGANPVQV EVGEFDEGAE
ETEEEVVAEN PCQNHHCKHG KVCELDENNT PMCVCQDPTS CPGPIGDFEK VCSNDNKTFD
SSCHFFATKC TLEGTKKGHK LHLDYIGPCK YIPPCLDSEL TEFPLRMRDW LKNVLVTLYE
RDEDNNLLTE KQKLRVKKIH ENEKRLEAGD HPVELLARDF EKNYNMYIFP VHWQFGQLDQ
HPIDGYLSHT ELAPLRAPLI PMEHCTTRFF ETCDLDNDKY IALDEWAGCF GIKEQDIDKD
LVI

Molecular weight: 34557.01 daltons

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