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13. ABSTRACT ***(Maximum 200 Words)***

Malignant breast adenocarcinoma cells mimic osteoblasts, a critical step in their metastatic colonization and destruction of bone. CBFA1, a transcription factor responsible for normal osteoblast differentiation, is expressed inappropriately by the human breast adenocarcinoma cell line MDA-MB-231, and about 2-fold more strongly by a subline M1, recovered from an osteolytic bone metastasis of MDA-MB-231 in a nu/nu mouse. CBFA1 expression was not detected in normal breast epithelium (HMEC cells). Experiments involved semiquantitative RT-PCR and SDS-PAGE immunoblot analyis of nuclear lysates with a specific CBFA1 antibody. Electrophoretic mobility shift with labeled oligonucleotide containing the binding site for CBFA1 demonstrated functional CBFA1 protein in nuclear extracts of MB-MDA-231 cells but not HMEC. CBFA1 expression by adenocarcinoma may explain anomalous expression of "osteoblast-specific" genes (mimicry), because these genes typically have CBFA1 response elements in their 5'-promoters; osteocalcin is a prime example. Ostecalcin expression was analyzed in human breast adenocarcinoma MCF-7 (detectable human osteocalcin), MB-MDA-231 (low levels), and normal breast HMEC (no osteocalcin). Co-culture of MCF-7 with a normal mouse osteoblast line (MC3T3-E1) increased human osteocalcin expression by the tumor cells. This effect could be reproduced by addition of conditioned medium from proliferating MC3T3-E1. Thus, CBFA1 expression appears to provide breast adenocarcinoma cells with an advantage in the bone microenvironment, and may explain their observed mimicry of certain aspects of the normal osteoblast. Microarray survey of gene expression patterns in osteoblasts and adenocarcinoma cells in co-culture revealed that the proximity of tumor and bone cells in a skeletal metastasis may allow paracrine effects to suppress osteogenic repair while enhancing tumor survival and osteolysis.

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Final Report

Bone Factors Regulating the Osteotropism of Metastatic Breast Cancer

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Table of Contents

Abstract (Repeated from Face Page)

Malignant breast adenocarcinoma cells mimic osteoblasts, a critical step in their metastatic colonization and destruction of bone. CBFA1, a transcription factor responsible for normal osteoblast differentiation, is expressed inappropriately by the human breast adenocarcinoma cell line MDA-MB-231, and about 2-fold more strongly by a subline M1, recovered from an osteolytic bone metastasis of MDA-MB-231 in a nu/nu mouse. CBFA1 expression was not detected in normal breast epithelium (HMEC cells). Experiments involved semiquantitative RT-PCR and SDS-PAGE immunoblot analyis of nuclear lysates with a specific CBFA1 antibody. Electrophoretic mobility shift with labeled oligonucleotide containing the binding site for CBFA1 demonstrated functional CBFA1 protein in nuclear extracts of MB-MDA-231 cells but not HMEC. CBFA1 expression by adenocarcinoma may explain anomalous expression of "osteoblast-specific" genes (mimicry), because these genes typically have CBFA1 response elements in their 5'-promoters; osteocalcin is a prime example. Ostecalcin expression was analyzed in human breast adenocarcinoma MCF-7 (detectable human osteocalcin), MB-MDA-231 (low levels), and normal breast HMEC (no osteocalcin). Co-culture of MCF-7 with a normal mouse osteoblast line (MC3T3-E1) increased human osteocalcin expression by the tumor cells. This effect could be reproduced by addition of conditioned medium from proliferating MC3T3-E1. Thus, CBFA1 expression appears to provide breast adenocarcinoma cells with an advantage in the bone microenvironment, and may explain their observed mimicry of certain aspects of the normal osteoblast. Microarray survey of gene expression patterns in osteoblasts and adenocarcinoma cells in co-culture revealed that the proximity of tumor and bone cells in a skeletal metastasis may allow paracrine effects to suppress osteogenic repair while enhancing tumor survival and osteolysis.

Introduction

Osteotropism of metastatic breast cancer (i.e., the predisposition of malignant adenocarcinoma cells to lodge in bone and establish painful osteolytic metastases) is the focus of this Project, and has direct relevance to three of the goals of the USAMRMC/DOD Breast Cancer Research Program:

1. We are defining changes in cell and molecular functions accounting for the development and progression of breast cancer.
2. The findings could be of potential use in guiding diagnosis, prevention, and treatment.
3. The research targets a major economic element (the costs of hospitalization and clinical management of skeletal morbidity) and could lead to more cost-effective health care delivery.

Malignant breast cancer cells exploit the normal bone homeostatic mechanisms in 3 ways for their own benefit: 1) secretion of PTH-rp and other factors stimulates osteolysis, creating space for metastatic tumor growth; 2) growth factor release during pathologic osteolysis drives adenocarcinoma cell proliferation; and 3) angiogenesis caused by osteolysis provides increased blood flow and a plentiful supply of nutrients to growing metastases. Partial mimicry of the osteoblast phenotype by malignant breast adenocarcinoma cells [1-3] is hypothesized to allow the expropriation of normal osteoblast-osteoclast signaling pathways for the exclusive benefit of growing tumor metastases in bone. Release of growth factors and rapid angiogenesis occurs in the immediate vicinity of an active osteoclast.[4,5] Osteoblast-derived bone sialoprotein (BSP), osteopontin (OPN),

and osteocalcin (OC) are required for osteoclast attachment and activation,[5-10] and expression of BSP by primary human breast adenocarcinoma is a recent predictor of metastasis.[3] Little is known about the regulation of such events. We are using *in vitro* and *in vivo* models to characterize this important pathological mimicry by malignant breast cancer cells.

Our ongoing and planned experiments should provide insight into the biology of painful bone metastasis in invasive breast cancer, offering hope for innovative therapy. Given the unchanging survival outlook for patients with metastatic bone disease, any significant improvement in the quality of remaining life would stand as an important therapeutic effect.

Body of Report

Overview of "Statement of Work"

There are three Technical Objectives comprising the original Statement of Work for this project. On the following pages, results for the entire project period have been organized according to these Technical Objectives and the specific Tasks within each section on which progress has been made. Where appropriate, difficulties preventing the completion of individual Tasks are addressed.

Progress on Technical Objective #1

Technical Objective 1: Examine the regulation of malignant human breast adenocarcinoma cell lines by bone extracellular matrix proteins and by normal osteoblasts *in vitro*.

Task 2: Develop baseline data on cell proliferation and gene expression by the MCF-7 human breast adencarcinoma cell line cultured on various bone ECM constructs. Follow this with a parallel study of the more malignant estrogen-independent MDA-231 line.

Task 3: Using Expt.#2, where normal osteoblasts are included in co-cultures with breast adencarcinoma cells, study the process of osteoblastic mimicry. That is, define the expression of bone matrix proteins (BSP, OPN, OC) by adencarcinoma cells. With trans-well chambers, test the role of cell-matrix and cell-cell (adencarcinoma-osteoblast) contact in the regulation of gene expression and proliferation.

Methods: The primary analysis involves comparison of adenocarcinoma cell gene expression and proliferation in the presence and absence of normal osteoblasts. A proven method for this interactive analysis of soluble factors is co-culture, either in the trans-well format, where tumor cells growing on a semi-permeable membrane are placed in proximity to the osteoblast monolayer culture, or it direct cell-cell contact, allowing the analysis of cell-matrix and cell-cell (adenocarcinoma-osteoblast) contact in the regulation of gene expression and proliferation. Our prime focus is to define the regulation of adenocarcinoma gene expression by bone factors. A secondary focus is modulation of the osteoblast phenotype by the tumor cells.

Cells: Human Breast Cancer (BrCa) Cell Lines: **MCF-7** (estrogen-dependent human breast adenocarcinoma); **MDA-MB-231** (estrogen-independent human breast adenocarcinoma, more aggressively malignant and metastatic that MCF-7); **M1**, **M2**, and **Blast-4** sublines of MDA-MB-231 recovered, cultured, and passaged during this project from individual bone metastases in nude

mice; C5 (another MDA-MB-231 subline recovered by M. Tondravi); **HMEC** (normal human breast epithelial cells); **hFOB** (human fetal osteoblasts); and **MC3T3** (clonal mouse osteoblasts).

RNA/RT-PCR RNA extraction was performed using RNeasy Mini Kit (Qiagen, CA). Primers were synthesized to unique regions in the osteocalcin, CBFA1, and GAPDH human cDNA sequences reported in the Genbank (Table 1). PCR reaction cycle conditions consisted of denaturation at 93°C for 60s, primer annealing at 61°C for 60s, extension at 72°C for 60s, and a final extension at 72°C for 7 min in a MJ Research (PTC-100) programmable thermal-cycler. Samples were amplified for 25 cycles. UV photography and densitometry of ethidium bromide stained gels was performed . Semi-quantification was achieved by comparing the amplimer intensities of both CBFA1 and osteocalcin to GAPDH.

Quantitative RT-PCR: This method has only recently become available in our institution. We have now designed and synthesized TaqMan primer sets for human osteocalcin as an initial test of this technology. Using a Perkin-Elmer instrument, we have measured a C_T value of 13.3 for an input of only 40 femtograms of human osteocalcin cDNA target. This means that a single molecule of osteocalcin cDNA (about 2×10^{-17} g cDNA) in an RT sample prepared from cellular RNA should have a C_T of about 28 cycles, well within the limit of 35-40 cycles for a typical run. As warranted we have planned to expand this approach to CBFA1 and other bone genes to complete the analysis of the adenocarcinoma expression profile.

Gel Shift Assay (EMSA): Nuclear extracts are prepared by 0.45M KCl extraction. Electrophoretic mobility assays are performed using standard conditions.[11] DNA binding reactions will be performed as described.[12,13] Nuclear extracts are incubated with 10 fmol of the ^{32}P labeled CBFA1 binding site consensus oligonucleotide (5'-CGAGTATTGTGGTTAATACG-3') as the probe and nonspecific competitor DNA. Protein-DNA complexes are resolved on a 4% non-denaturing polyacrylamide gel. Anti-sera supershift experiments will utilize polyclonal anti serum directed to a CBFA1 peptide containing 17 amino acids[13] or preimmune serum (control). (Gift of Dr. Scott Hiebert, Vanderbilt Medical Center Nashville TN/ and Oncogene Research Products, Cambridge, MA).

Osteocalcin Radioimmunoassay (RIA): Human osteocalcin concentrations in conditioned medium samples (1,3,5,7 days) were measured by radioimmunoassay using rabbit anti-monkey osteocalcin (first antibody) and goat anti- rabbit IgG (second antibody).

Osteocalcin Western Blot: This is a totally new method which we developed during the course of this project. It enabled us to solve the difficult question of whether osteocalcin expression, at the protein level, is detectable in breast cancer cells, and whether it changes in response to osteoblast factors. The technical difficulty is that osteoblasts are heavy producers of osteocalcin, so it would be impossible to perform this experiment with human BrCa cells and human osteoblasts. However, we developed two antibodies that recognize osteocalcins of the human (primate) and mouse sequence in a species specific fashion. Therefore, as shown in the western blots below, a coculture of mouse MC3T3-E1 osteoblasts with human BrCa lines can be examined for bidirectional regulation of osteocalcin expression by both osteoblasts and tumor cells.

General Cell Extraction: Cultures were rinsed with PBS, scraped into buffer and collected by centrifugation. Cells were lysed with hypotonic buffer in the presence of protease inhibitors. Nuclei were collected by centrifugation and resuspended in hypertonic buffer and protease inhibitors. Nuclei were extracted on ice, insoluble material was cleared by centrifugation and soluble proteins

were stored at –80°C. Nuclear extracts (30 μg protein per lane) were resolved with 8% SDS-PAGE and electroblotted onto nitrocellulose membrane. Membranes were incubated with a 1:100 dilution of antibody in Tris-buffered saline containing 1% BSA. Affinity purified antibodies specific for CBFA1 were used in these studies. Membranes were incubated with secondary antibody, and protein bands were visualized by streptavidin-HRP chemiluminescence (Pierce, IL).

Results: Human breast adenocarcinoma cell lines have been established in the laboratory and examined by RT-PCR, Northern analysis, immunohistochemistry, and radioimmunoassay to establish the level of expression of osteoblast-specific genes. The focus has been maintained on the MDA-MB-231 adenocarcinoma line, based on our success in obtaining osteolytic metastases with this same line in nude mice (see Technical Objective #3 below).

RT-PCR for CBFA1. (Figure 1) Semi-quantitative PCR was performed comparing the expression of CBFA1 and osteocalcin between HMEC, MDA-MB 231 cell line, and M1. These results were normalized to GAPDH for each sample. There was a two fold increase in both CBFA1 (375bp) and osteocalcin (294bp) in the M1 clone (lanes 5 and 2) compared to the original MDA-MB 231 cell line (lanes 4 and 1). There was no evidence of CBFA1 in the HMEC (lanes 3 and 6) cell line. PCR product identity was confirmed by producing products of expected size.. The CBFA1 amplimer was excised from the gel, purified and underwent automated sequencing to further confirm its identity (see Appendix Figures).

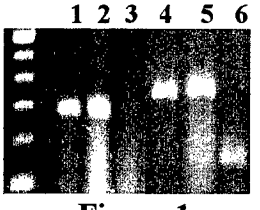


Figure 1

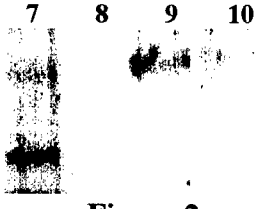


Figure 2

Western Blot for CBFA1: (Figure 2) We examined nuclear extracts from the MDA-MB 231 cell line, HMEC line, and M1 subclone, and MG-63 osteosarcoma cell line (positive control) using antibody to CBFA1. CBFA1 was detected in all of these cell lines except the normal breast line HMEC (Lane 8). A 60-65 kDa species was present in the MDA-MB 231 cells (Lane 10) and M1 subclone (Lane 9). The species was present at a two-fold greater band density in the M1 clone (Lane 9). The MG-63 human osteosarcoma cells (Lane 7) expressed the 60-65 kDa form as well as a prominent 46 kDa form that was not seen in the breast cancer cell lines (see Appendix Figures).

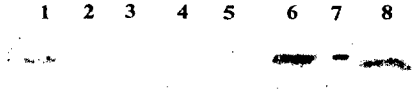
Supershift and Electromobility Shift Assays (EMSA) for CBFA1: The possibility of CBFA1 expression was analyzed by EMSA on nuclear extracts prepared from cultured breast adenocarcinoma and normal cell lines (see Appendix Figures). CBFA1 transcription factor activity is detected by retarding the mobiltiy of a specific radioactive target oligonucleotide containing the CBFA1 binding site that is common to a number of osteoblast-specific gene promoters. The labeled oligonucleotide was shifted to slower mobility by putative CBFA1 activity in MDA-MB-231, M1, M2, and Blast-4 cell extracts. HMEC extracts failed to show CBFA1 activity. MG-63 osteosarcoma served as a positive control for CBFA1, and the EMSA assays showed the expected competition with excess cold target oligonucleotide. Importantly, the specific antibody to CBFA1 caused further retardation of the label (supershifting), allowing us to conclude that active CBFA1 had been

upregulated in the BrCa cells. This finding provides a likely explanation for the osteoblastic mimicry by BrCa cells.

Breast Cancer Cells Express Increased Levels of Osteocalcin Compared to Normal Breast Tissue: The rationale for examining osteocalcin expression is that this is one of the "osteoblast specific genes" that is transcriptionally regulated by CBFA1. Because CBFA1 is upregulated in the tumor lines, we have analyzed the conditioned media from tumor and normal breast cells for osteocalcin by two methods. Human osteocalcin concentrations in conditioned medium samples of the MDA-MB 231 cells line and HMEC cell lines were measured by radioimmunoassay using a specific osteocalcin antibody. Data are reported as ng osteocalcin/ml of medium. Medium (with 10% FCS) served as the baseline control, and this accounts for the background which is 13 ng/ml of cross reacting bovine osteocalcin contributed by the 10% serum (FCS). MDA-MB-231 cells expressed increasing amounts of osteocalcin with increasing confluence. The HMEC cell line did not produce osteocalcin when compared to medium controls. However, other less osteotropic cell lines have not yet been examined to determine if there is an association between osteocalcin production and ability to metastasize to bone. Future experiments will examine less osteotropic breast cancer cell lines, non-osteotropic and non breast malignancies (i.e. colon cancer), as well as the M1 and the LC115 bone metastasis derived cell lines, in order to corroborate the apparent positive correlation between high osteotropism and expression of CBFA1 and osteocalcin.

Western Blot Analysis of Osteocalcin Expression:

Figure 3. Species specificity of osteocalcin detection in condititioned media. The upper blot detects the 6kDa mouse osteocalcin protein in lanes 1 (mouse MC3T3 osteoblasts), 6 (MC3T3 cultured in the mineralizing promoter beta glycerolphosphate), and 8 (mouse osteocalcin standard 5ng). No human osteocalcin in the human BrCa cell line MCF-7 is detected in lanes 3 or 4, nor is the primate osteocalcin standard in lane 7 detected (5 ng monkey osteocalcin). In the lower blot, the human osteocalcin is prominent in the MCF-7 conditioned medium when cocultured with mouse osteoblasts (lane 7), but only weakly detectable in the tumor cells alone (lane 5, 6). Mouse osteoblasts (lane 8) show no background expression of human osteocalcin cross-reactivity.



Western Blot (Goat anti-mouse OC antibody)

1: 3T3 2/19; 2: 3T3 2/19 + MCF 2/24
3: MCF 2/24; 4: MCF 2/24 + GP;
5: 3T3 2/19+MCF2/24+GP; 6: 3T3 2/19+GP
7: Marker; 8: 5ng OC + alpha-MEM

Western Blot
Rabbit anti-monkey OC antibody

1: 3 ng monkey OC 2: Marker
3: 3T3 2/19+GP; 4: 3T3 2/19 + MCF 2/24 +GP
5: MCF 2/24+GP; 6: MCF 2/24;
7: 3T3 2/19+MCF2/24; 8: 3T3 2/19

Other western blots showing the reproducible detection of human osteocalcin in the conditioned media of MCF-7 cells, particularly when cocultured with osteoblasts (MC3T3) or with CM

(concentrated conditioned media) from osteoblasts. The normal breast epithelium (HMEC) shows no osteocalcin expression, while MDA-MB-231 shows extremely low levels (see Appendix Figures).

The western blot data for osteocalcin are in need of corroboration by RIA and quantitative RT-PCR (TaqMan) analysis of gene expression (see Appendix Figures). It should be noted that while the CBFA1 expression levels appear to correlate with the malignancy of the BrCa cell type (M1 > MDA-MB-231 > MCF-7 > HMEC), there is not a similar correlation with osteocalcin protein levels, where MCF-7 > MDA-MB-231 > A2, HMEC. Some of the explanation may lie in the proteinases secreted by each of the cells, as osteocalcin is very susceptible to proteolytic fragmentation which destroys its immunoreactivity.

Task 4: Study the action of hOP-1 in regulating adenocarcinoma cells.

Tumor cell culture with hOP-1 showed decreasing osteocalcin after day 7 by RIA analysis. Prior to publication, this important observation needs to be repeated, and we will also examine proliferation effects, as hOP-1 also appears to slow the tumor cell growth in vitro. In designing the future experiments, we have obtained two new sources of human osteoblasts [FOB (Mayo Clinic) and NHOst (Clonetics)], along with the normal human breast epithelial line, HMEC (which is negative for osteocalcin).

Progress on Technical Objective #2

Technical Objective 2: Study the bidirectional interaction between breast adenocarcinoma cells and bone cells (osteoblasts and osteoclasts) *in vitro*.

Task 5: Months 6-12. Establish the 3-D co-culture model (Expt. #3) initially with MCF-7 adencarcinoma cells and human MG63 osteoblast-like osteosarcoma cells, testing the variables of cell density and time of contact between cell clusters on proliferation and gene expression of the two cell types. Adapt normal mouse MC3T3-E1 osteoblasts to growth in the 3-D gelatin gel configuration in order to examine the actions of the adencarcinoma cells in perturbing the normal osteoblast phenotype. Follow this with dose-dependence studies of 17-β estradiol and hOP-1 effects in the model.

Task 6: Months 9-15. Establish the resorbing calvaria co-culture model (Expt. #4), initially comparing standard resorption stimuli (PTH, PGE_2) with varying numbers of MCF-7 adencarcinoma cells and MCF-7 conditioned medium. Use blocking antibodies, peptide antagonists of PTH-rp, and indomethacin to define the nature of the resorptive stimulus originating from the adencarcinoma cells.

Task 7: Months 12-18. Investigate the effects of resorbing bone culture medium on MCF-7 and MDA-231 adencarcinoma cells, using appropriate controls for the carry-over of PTH or PGE_2. Focus on differentially regulated cell proliferation and the expression of genes which are known to be correlated with metastasis. Study the comparative effects of 17-β estradiol, tamoxifen, and hOP-1 on the MCF-7 and MDA-231 responses.

Methods: Add tumor cells to established cultures of normal osteoblasts for direct contact co-culture, and search for changes in gene expresion and proliferation of the tumor cells which require cell contact or labile products of living osteoblasts.

Results: The standard method adopted for co-culture studies was to plate MC3T3 mouse osteoblasts, allow them to attach, and then to seed the surface with tumor cells (either MCF-7 or MDA-MB-231) with a delay of between 0 and 45 days. When plated with minimal delay, we observed that both tumor cell types would rapidly attach and outgrow the osteoblasts. In the case of MCF-7, the osteoblasts were adversely affected and a moderate fraction began to undergo detachment and apoptosis within several days of contact. However, MDA-MB-231 were observed to require as much as 5-7 days before causing osteoblast apoptosis, and in this case there was evidence of severe cytotoxicity with virtually 100% of the osteoblasts being killed.

Very different results were obtained for tumor cells plated on established, mineralized osteoblast cell layers. In this situation, both tumor cell types required many days to attach and proliferate to the point where small clonal colonies of 5-10 cells could be distinguished. Because the morphological features of MCF-7 made these colonies very easy to identify, most of the long-term experiments involved MCF-7 rather than MDA-MB-231. Importantly, there was clear evidence of paracrine effects of the tumor cells on the osteoblasts. Those osteoblasts that were in immediate proximity or contact with an MCF-7 colony were found to survive and appeared to produce increased amounts of mineralized matrix. However, a high percentage of osteoblasts that were distant from the tumor cells underwent apoptosis.

Trans-well co-culture experiments with of MDA-MB-231 cells and MC3T3-E1 cells were difficult and therefore abandoned. The principal problem is the rapid growth of the tumor cells and the resulting nutrient depletion and medium acidification which adversely affects the slower-growing osteoblasts. These experiments were discontinued in favor of direct co-culture. It required much trial and error to determine the appropriate plating densities and culture conditions. The optimal conditions for co-culture are established for 24-well plates: 1) plate $1.2x10^5$ MC3T3 cells (day 0); 2) overlay $1.2x10^4$ MCF-7 cells when osteoblasts cover 80% of the culture surface (day 2); 3) fix at day 4 or later for immunohistochemistry.

Table 1. Immunohistochemistry of Breast Adenocarcinoma (MCF-7) and Osteoblastic (MC3T3) Cells

Antigenic Marker	Cells Cultured Alone		Cells Co-Cultured (2d)	
	MCF-7	MC3T3	MCF-7	MC3T3
human cytokeratin-18	+++	—	++++	—
bone sialoprotein (BSP)	++	—	+++	—
osteopontin (OPN)	++	—	+++	—
osteocalcin (OC)	+	±	++	±
alkaline phosphatase	—	+++	±	+++
non-immune serum control	—	—	—	—

Immunohistochemistry: Table 1 shows the results of antibody staining of breast adenocarcinoma (MCF-7) and osteoblastic (MC3T3) cells alone and in co-culture. The human-specific cytokeratin-18 antibody clearly distinguishes the epithelial adenocarcinoma cells from the murine osteoblasts, as expected. The osteoblasts are selectively stained by alkaline phosphatase (BCIP reaction), and weakly for osteocalcin, as expected for this early time point in the culture (day 4). What is important about the data in Table 1 is the relatively strong expression of the bone cell markers by the MCF-7 cells, and the observation that the staining increases when the MCF-7 tumor cells are co-cultured with the MC3T3 osteoblasts. The growth behavior of the MCF-7 cells in co-

culture is in isolated clonal clusters containing 5-50 cells which are surrounded by flattened osteoblasts. The density of the positive immunohistochemical staining for the proteins such as BSP and OPN is greater at the periphery of these clusters, suggesting either a contact-dependent regulation of gene expression, or a possible uptake of osteoblast-produced proteins by the tumor cells. These possibilities will be discriminated by future experiments employing in situ hybridization and species-specific immunostaining.

Bidirectional Interactions of Tumor Cells and Osteoblasts: It is noteworthy that when larger numbers of MCF-7 cells are plated on the osteoblasts (i.e., when the ratio of tumor cells/osteoblasts is greater than 1/2, then the osteoblasts lift off the culture surface and die within 3 days. Even if the medium is buffered by HEPES to prevent overt acidification by the metabolically active tumor cells, rounding and detachment of the osteoblasts is only delayed by 1-2 days.

Conditioned medium experiments showed that both MCF-7 and MC3T3 cells produce attachment factors which increase each other's attachment to plastic in serum-free conditioned alpha-MEM. However neither of the cell types can survive beyond 48-72 hr in these conditioned media, presumably because of the absence of serum growth factors. At this time, we have no evidence of overt cytotoxic factors being produced by either cell type for action on the other.

Gene Expression Array Analysis of BrCa Regulation by Osteoblasts: The major new development of commercially produced Gene Expression Arrays in the past year has provided a comprehensive technique for profiling the total phenotype of normal and cancer cells. These new arrays allow the simultaneous analysis of thousands of genes at a time, rather than the few genes to which most previous studies had been restricted. We have utilized the new advanced arrays of 1176 known cDNAs produced by Clontech (Human 1.2, Human 1.2 II, Human Cancer 1.2, and Mouse 1.2). The small region (about 200 genes) of the array shown below (Fig. 4) demonstrates the density of valuable information that can be obtained. This example involved a survey of the effects of osteoblast conditioned medium on gene expression by MCF7 cells after 24 hr treatment. Each spot contains 10 ng of cDNA from a known human gene, and the black density in the autoradiogram is proportional to the expression level of mRNA for each gene in the MCF7 cell culture. Note that the expression of many genes is not affected by the added osteoblast factors, whereas others are increased or decreased.

Figure 4. Clontech Human Gene 1.2 Array - sector A. Left side is hybridized with ^{32}P-labeled cDNA from control MCF7 cells; right side is MCF7 + osteoblast CM.

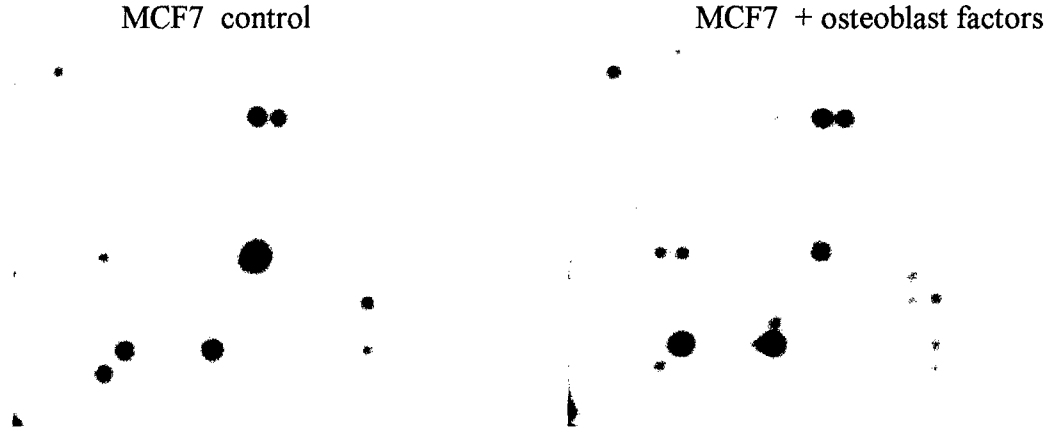


Table 2 indicates a partial list of the important genes for which the expression level is turned up or down at least 2-fold by osteoblast-derived factors in the MCF7 cell line, a well studied breast adenocarcinoma model.

Table 2. Regulation of MCF7 Adenocarcinoma Gene Expression by Osteoblast Co-Culture

Expression Increased (≥2-fold)	Expression Decreased (≥2-fold)
TGF-beta Family Growth Factor Related	
Macrophage Inhibitory Cytokine (MIC-1, pTGFb, PDF)	BMPR-II
	Smad-6
	TIEG
Angiogenesis Related	
Vascular Endothelial Growth Factor (VEGF)	
Angiopoietin 1, 2	
Tyr-61	
Macrophage Stimulating Protein (MSP)	
Macrophage Chemoattractant Protein (MCP-1)	
Interleukin-6 (IL-6)	
Osteoclast Differentiation Related	
Macrophage Colony Stimulating Factor (M-CSF)	Osteoprotegerin (OPG)
Vascular Endothelial Growth Factor (VEGF)	
Interleukin-6 (IL-6)	
Macrophage Chemoattractant Protein (MCP-1)	
Macrophage Stimulating Protein (MSP)	
Tumor Metastasis Related	
	E-cadherin
	GATA-3
Morphology and Ion Transport	
Na/H exchanger (HEN-1)	
Na/K ATPase alpha-1	
Rho E	
Death/Apoptosis	
Defender against cell death (DAD-1)	Annexin V
IEX-1L	Rho B
GRP78	
Tissue Invasion and Extracellular Matrix	
Matrix Metalloproteinase-1 (MMP-1)	
Matrix Metalloproteinase-13 (MMP-13)	
Metalloproteinase inhibitor TIMP-3	
Cathepsin D	
SOX-9	
Aggrecan	
Tyr61	
Lysyl Oxidase	

MCF7 human breast adenocarcinoma cells were co-cultured by plating on monolayers of murine MC3T3 osteoblasts for 48 hr. Brief trypsin treatement released MCF7 cells specifically, and RNA was prepared from these MCF7 cells and verified to be free of murine contamination by RT-PCR with murine and human-specific primers for osteocalcin and Ese-1.

We have now developed a list of important adenocarcinoma genes that are turned up or down by osteoblast contact in an in vitro co-culture paradigm. This provides a window into the vast and interconnected responses of BrCa cells in the bone microenvironment. A complementary set of experiments has analyzed the effects of the BrCa cell-derived factors on osteoblasts.

The gene expression profiles for both MCF-7 and MDA-MB-231 show a number of important markers that have strong implications for their malignant phenotypes, including genes involved in growth factor stimulation, rapid proliferation and protection from apoptosis, activation of angiogenesis and osteolysis, and tissue invasion. The action of osteoblast contact to turn up or down the expression of these tumor-related genes is unequivocal. What is not yet clear is whether a common set of genes is so regulated in every breast adenocarcinoma cell type, or whether each cell model has an intrinsically unique pattern. We are currently searching for these patterns.

Table 3. Regulation of MC3T3 Osteoblast Gene Expression by BrCa-Expressed Proteins

Expression Increased (≥2-fold)	Expression Decreased (≥2-fold)
TGF-beta Family Growth Factor Related	
Activin betaA	
TGF-beta	
PDGF-Receptor alpha	
LIF-Receptor	
Suppressor of Cytokine Signaling (SOCS-3)	
Angiogenesis Related	
Macrophage Chemoattractant Protein (MCP-3)	
Osteoclast Differentiation Related	
Macrophage Colony Stimulating Factor (M-CSF)	
Transcriptional Repressor Protein (FBN1)	
Core Binding Factor (CBFA1)	
RANK Ligand (RANKL) ?	
Macrophage Chemoattractant Protein (MCP-3)	
Tumor Metastasis Related	
VCAM-1	
Osteogenesis Related	
Fas-Associated Factor (FAF-1)	GRP-78
Fas-Ligand Receptor	Kruppel-like Factor (LKLF)
Bak apoptosis regulator	
Stra-13	
Fibrillin 1 (FBN1)	
Transcriptional Repressor NAB1	
Tissue Invasion and Extracellular Matrix	
Osteopontin (OPN)	Laminin Receptor
Osteocalcin (OC)	CD44
Retinoic Acid Receptor (RARgamma)	

Serum-free 48hr conditioned medium from MDA-MB231 human breast adenocarcinoma cells was ultrafiltered to remove <5kDa constituents, concentrated 100-fold, and added to MC3T3 murine osteoblasts at 0.005 ml/ml. Gene expression changes are based on comparison with osteoblasts grown under the same conditions, but without the tumor medium supplementation.

Gene expression analysis (Table 3) of murine osteoblasts reveals a number of changes induced by human BrCa factors that would contribute to the progression of a skeletal metastasis. Admittedly this is an interspecies model whose validity to the human patient requires further study. However, the model is instructive about the osteolytic bone metastases found in nude mice following intracardiac injection of human breast adenocarcinoma MDA-MB-231 cells (see Appendix Figures). Again, from Table 3, the osteoblastic response to BrCa factors is one that promotes osteoclast differentiation, osteolysis, and angiogenesis, while also reducing osteoblastic survival. We were compelled to use conditioned medium from BrCa cells, because was technically impossible to separate out the osteoblasts to study their gene expression in a direct co-culture model as had been done for BrCa cells in Table 2.

Progress on Technical Objective #3

Technical Objective 3: Establish human breast adenocarcinoma tumors at primary and skeletal metastatic sites by inoculation of athymic nude mice. Analyze the gene expression of tumor and bone cells in the recovered tissue. Test the activity of shOP-1 on primary tumor growth and on establishment and progression of skeletal metastases.

Task 8: Months 6-15. Using Expt. #5, inoculate MCF-7 cells and recover primary tumors from two subcutaneous sites (adjacent to fascia *vs.* calvaria). Measure tumor size and perform histology. Test the effect of shOP-1 on tumor growth and osteolysis in the calvarial site.

Task 9: Months 9-24. Establish the skeletal metastasis model (Expt. #6), first with MCF-7, and then with the more aggressive MDA-231 line. Perform a cell inoculation dose study and monitor osteolysis in developing skeletal metastases radiographically. Recover metastases for histology. Compare gene expression patterns by cells at the tumor/bone interface with tumor harvested from soft tissue (eg., lung).

Methods: Animals: 7 week old, female Nu/Nu SCID mice (20-25g) were used following institutional guidelines for the use and care of laboratory animals. MDA-MB 231 cells were injected (1×10^5 cells/100μl) through the left second intercostal space into the left ventricle to produce bone metastases. Animals were radiographed bimonthly to detect the presence of lytic lesion. Bones with lesions were fixed in 4% paraformaldehyde and decalcified. Several lesions were isolated and subcultured to produce a breast cancer cell lines from a bone metastasis. These subclones were further characterized by immunostaining with cytokeratin 18 (mouse anti-human) to confirm both the human and glandular epithelial origin. Reinjection into mice was performed to confirm malignancy. Immunohistochemistry: Monoclonal or polyclonal antibodies to osteocalcin and CBFA1 and appropriate negative controls were employed. The sections were obtained from the distal femur and proximal tibia of mice. Archival human specimens from 10 patients with metastatic breast cancer to appendicular and axial sites were similarly examined. Fixed, paraffin embedded sections from each tumor were immunostained using an indirect avidin-biotin complex method. The appropriate concentrations of the primary antibodies against CBFA1 and osteocalcin were used (1:10 and 1:100, respectively).

Results:

Over two years of experience with the mouse model for osteolytic metastasis of breast cancer has shown that only approximately 20% of mice develop metastases which are osteolytic by

radiographic criteria. In about 2-5% of mice, multiple osteolytic sites are found. Three typical radiographs are shown in the Appendix Figures. The most common sites for osteolytic metastasis in the nude mouse were the femur and tibia. The radiolucent areas are evident, and these result from the osteoclastic osteolysis in the vicinity of the breast tumor metastasis.

Recovery of Malignant Human Breast Adenocarcinoma Cells from Bone Metastases: Osteolytic bone metastases (and one osteosclerotic metastasis) were located radiographically in mice which had been injected by the intracardiac method with MDA-MB-231 cells. At sacrifice, 15 sites were dissected and curetted to obtain cells for tissue culture. Of these candidate cultures, 2 osteolytic ones (M1 and M2) grew vigorously and were verified to be sublines of the human MDA-MB-231 adenocarcinoma by human-specific cytokeratin-18 immunostaining which showed human, glandular epithelial origin. An osteosclerotic metastasis yielded a subline (Blast-4) which is also derived from the parental MDA-MB-231 line.

Characterization of Osteoblast Gene Expression Patterns in Human Breast Adenocarcinoma Cells from Osteolytic and Osteosclerotic Metastases:

Immunohistochemical Investigation of Metastases Recovered from Mice: looked for CBFA1 in osteolytic tumor metastases and found positive evidence of the expression of this transcription factor in tumor foci, with little or no expression in mature bone itself. The CBFA1 expression by osteoblasts is primarily confined to the rapidly growing regions of the skeleton (growth plates, etc.). Osteocalcin expression was observed in the tumor foci more strongly than in the surrounding bone.

Gene Expression: The small size of the human breast adenocarcinoma metastases in mice makes it impossible to perform certain types of molecular analysis, so we have resorted to analyzing the adenocarcinoma cell lines (M1, M2, and Blast-4) recovered from individual metastases. M1 and M2 are positive for CBFA1 expression RT-PCR (Fig.1), EMSA and Westerns (Fig.2). Northern analysis for CBFA1 has been problematic due to comigration of CBFA1 mRNA with the abundant 18s RNA. Blast-4 cells are positive for CBFA1 by EMSA and Western blotting. In summary, we have the parental line (MDA-MB-231), 3 recovered sublines (M1, M2, and Blast-4), and another MDA-MB-231 subline recovered by another laboratory (C5; M. Tondravi). All 5 of these cell lines express CBFA1, while normal breast cells (HMEC) do not.

Analysis of Archival Human Specimens: Archival human specimens have been obtained from 20 patients with metastatic breast cancer. The primary breast lesions and bone metastases have been obtained from the records of the Orthopaedic Oncology Service at the Massachusetts General Hospital. Paraffin block specimens from metastases in the femur have been obtained. Both osteocalcin and CBFA1 were noted to be positive for immunostaining in both archival human specimens and in experimentally induced animal models of bone metastasis. CBFA1 was seen primarily in the breast cancer cells within each metastasis, with minimal staining of the surrounding bone. Osteocalcin was seen in both the breast cancer metastasis and surrounding bone in both the archival human and animal specimens.

Progress Summary for Milestones of the Original Research Plan:

1. Demonstration of bone matrix-dependent and osteoblast-dependent regulation of malignant human breast adenocarcinoma cells. *(Accomplished and extended by gene expression arrays).*

2. Characterization of factors regulating osteoblastic mimicry by breast adenocarcinoma cells. *(Factors demonstrated, eg. CBFA1, but awaiting definitive proof that this is causative)*
3. Demonstration of adenocarcinoma-dependent modulation of the phenotype of normal osteoblasts. *(Work with osteoblasts still ongoing via gene expression arrays. The exciting implication of this approach is the potential discovery of tumor-specific proteins that stimulate the osteolytic reaction to breast cancer metastases. These could be targets for future therapeutic development).*
4. Recovery of bone metastases of human breast adenocarcinoma from athymic nude mice in quantities suitable for histochemical and in situ hybridization studies of gene expression. *(The low frequency of metastases in the animal model did not provide sufficient tissue for statistically valid histochemical and in situ studies. Metastatic sublines were established from many lesions, however, and this has provided a very promising collection of culturable materials for further study)*
5. Proof of anti-tumor efficacy of hOP-1 and shOP-1 in the experimental models. *(As above, the low frequency of metastases in the animal model did not provide sufficient tissue for statistically valid analysis of treatments. Accordingly, because of prohibitive projected costs and uncertain statistical power, this experiment was not performed).*

Key Research Accomplishments:

- Demonstration of gene expression of the bone-specific osteoblast lineage transcription factor CBFA1 in human breast adenocarcinoma but not normal breast epithelium
- Proof that the CBFA1 protein in nuclear extracts of breast adenocarcinoma cells is active
- Provisional correlation of CBFA1 expression with the potential for osteotropic metastasis of breast adenocarcinoma
- Demonstration that the mimicry of the osteoblast phenotype by human breast adenocarcinoma cells includes the anomalous production of osteocalcin, a protein normally viewed as an osteoblastic marker
- Isolation from bone metastases of derivative cell lines of the malignant human breast adenocarcinoma model MDA-MB-231 that produce an osteolytic response in the skeletal site
- Identification of cohorts of human genes expressed by human breast adenocarcinoma cells that are significantly upregulated or downregulated by coculture with osteoblast-derived factors
- Identification of cohorts of osteoblast genes expressed by murine osteoblastic cells that are significantly upregulated or downregulated by coculture with conditioned medium factors produced by human breast adenocarcinoma cells in vitro.

Reportable Outcomes:

Manuscripts, Abstracts, Presentations:

Hecht AC, Adolphson LD, Feinstein MI, Barnes GL, Gerstenfeld LC, George C, Danciu T, Keel S, Skazkina K, Gebhardt MC, and Hauschka PV (1999). Breast Cancer Metastases to Bone Express

Increased CBFA1 and Osteocalcin. Proceedings of the Orthopedic Research Society, Abstract. Selected for oral presentation by Dr. Hecht, February 1-4, 1999, Anaheim, CA

Hecht AC, Barnes GL, Yang QL, Adolphson LD, Skazkina K, and Hauschka PV (2000). Mimicry of the osteoblast phenotype by breast adenocarcinoma cells. Abstract. DoD Breast Cancer Research Program Era of Hope Meeting, June 8-12, 2000, Atlanta, GA

Manuscripts on these topics are in preparation and will be submitted following editorial review.

Development of Cell Lines:

M1, **M2**, and **Blast-4** sublines of the MDA-MB-231 malignant human breast adenocarcinoma were recovered, cultured, and passaged during this project from individual bone metastases in nude mice. Subject to further characterization and analysis, these cells will be made available to the scientific community.

Conclusions:

Our data suggest that 1) the breast adenocarcinoma cell mimics the osteoblast by expressing both osteocalcin and CBFA1, and that 2) regulation of these phenotypic properties may be caused by contact with osteoblast-derived factors in the bone microenvironment. There appears to be increased expression of osteocalcin and CBFA1 in the bone metastasis-derived cell line (M1) when compared to the original MDA-MB-231 tumor line, whereas the normal human breast HMEC cells are negative for both markers. These observations suggest a strong correlation between increasing metastatic potential and the expression of the osteoblast phenotype by the adenocarcinoma cell.

The specificity with which breast cancer metastasizes to the human skeleton is currently unexplained. In this project we have attempted to identify the critical molecular interactions and cellular processes responsible for this osteotropism (homing and metastatic growth in bone) of breast cancer. The central hypothesis is that malignant breast adenocarcinoma cells subvert the cooperative paracrine interactions between normal bone cells, endothelial cells, and the extracellular matrix in order to establish metastatic foci. Our data combined with published reports show that invasive breast adenocarcinoma cells mimic certain hallmarks of the osteoblast phenotype, possibly explaining the facile growth and survival of these tumor cells in bone. Analysis of model human tumor lines, and derivative sublines recovered from bone metastases in nude mice, reveals a correlation between the degree of malignancy and the inappropriate expression of the "bone specific" transcription factor CBFA1. The osteoblast mimicry by the tumor cells may be explained by CBFA1 expression; normal breast tissue does not express CBFA1. Osteoblast factors also have a paracrine effect on altering the phenotype of breast adenocarcinoma cells in the bone microenvironment.

Microcalcification of Breast Lesions: A Possible Consequence of Osteoblastic Mimicry by Adenocarcinoma Cells? An important clinical observation suggesting the existence of a privileged relationship between breast and bone tissue is that deposits of calcium compounds are often seen radiographically and histopathologically among breast carcinoma cells. Ectopic calcifications associated with malignant lesions are formed by hydroxyapatite, $[Ca_{10}(PO_4)_6(OH)_2]$, the basic mineral found in the skeleton. Microcalcifications are an important indication of early breast carcinoma.[14] In many instances, the detection of microcalcifications on mammography is the unique sign indicative of the presence of a breast lesion. However, microcalcification deposits in the breast are not restricted to malignant lesions, but can also be associated with benign conditions such has

fibroadenoma, secretory diseases and fat necrosis.[15] Ultrastructural analysis of breast cancer associated microcalcifications has revealed crystalline deposits in the cytoplasm of malignant cells calcification of cytoplasmic organelles, and membrane bound vessicles with hydroxyapatite crystals.[16] These observations have suggested that hydroxyapatite deposition within breast cancer results from an active mechanism rather than the mineralization of cellular debris and necrotic material. Although mammographically detected microcalcifications are frequently the only sign of malignant breast tissue, the mechanism of their deposition has not yet been elucidated. Apparently, breast cancer cells are able to generate a microenvironment that promotes crystallization of calcium and phosphate into bone-like mineral. This observation led investigators to examine breast cancer cells for the expression bone matrix proteins. Osteonectin, osteopontin and bone sialoprotein have been studied in a series of human breast cancers.[3,15-30] Immunohistochemical evaluation of these three proteins in benign and malignant breast lesions revealed markedly increased expression of BSP/OPN/OSN when compared to benign lesions or normal breast tissue. The detection of OSN and OPN in breast tissues was not surprising due to the ubiquitous presence of these two proteins in many normal and cancerous tissues of the body,[31] however, the expression of BSP was thought to be strictly limited to mineralized connective tissues. BSP was found to be present in numerous breast adenocarcinoma cell lines, both estrogen dependent and independent. In addition, BSP expression was found to be a poor prognostic sign when present in the primary lesion. There was a significantly increased incidence of subsequent bone metastases in patients who expressed higher levels of BSP.[18]

Other matrix proteins may also be important in the metastatic process. Osteocalcin has been implicated, along with BSP, in osteoclast recruitment, activation and attachment. However, there are virtually no reports of non-osteoblastic osteocalcin expression. Our preliminary findings of osteocalcin expression by MDA-MB-231 cells identifies osteocalcin as a potential regulator of osteolytic metastasis.

CBFA1, the Transcriptional Regulator of the Osteoblast Phenotype: The molecular mechanisms involved in the selective interaction of breast cancer cells with the bone matrix are not yet identified. The finding that the minor bone proteins; BSP, OPN and OSN, may play a role in this metastatic process may be further illuminated by the discovery a of a novel transcription factor that controls osteoblast differentiation. A transcription factor, CBFA1, was recently shown to control the expression of many osteoblast-specific genes. CBFA1 (core-binding factor), also referred to as PEB2Aα (polyoma enhancer-binding protein) and AML-3 (acute myelogenous leukemia) is one of three mammalian genes (i.e. CBFA1, CBFA2, CBFA3) which encode transcription factors whose DNA-binding domains share homology with the *Drosophila* segmentation gene product runt.[32] The alpha subunit of these heterodimeric protein transcription factors binds to DNA via the runt domain when paired with the ß-subunit which does not directly interact with the DNA. Recent studies have shown that CBFA1 controls the pathway of differentiation into the osteoblast lineage. The promoter regions of the osteoblast phenotype related genes including OPN, osteocalcin, BSP, and type I collagen contain the core binding sequence.[33-37] Deletion of the CBFA1 gene in mice leads to a total absence of osteoblasts owing to an arrest in their differentiation.[38,39] Komori et al. and Otto et al. independently performed a similar study involving the creation of a CBFA1 knock-out mouse.[37-39] The homozygous CBFA1 -/- mice died soon after delivery, cyanotic, due to inability to breathe. The homozygotes were smaller and had shorter limbs, but all remaining organs were proportionate. The most striking finding was the total lack of bone and retention of the partially calcified cartilaginous skeleton. Membranous bones of the skull and endochondral bone in the skeleton were absent. The tibia contained only calcified cartilage where bone is usually formed at this age. Histology revealed an absence of osteoblasts and smaller sized osteoclasts. Investigators performed northern blot

analysis on a number of genes associated with the osteoblast phenotype: osteocalcin, BSP, OPN and alkaline phosphatase. Expression of these genes was greatly diminished. Normal control animals revealed that CBFA1 gene expression was localized during development (via in situ hybridization) to regions destined for bone development. Osteoblast gene expression was examined after BMP-2 stimulation. Both osteocalcin and alkaline phosphatase gene expression was markedly reduced in the mutant calvaria-derived cells. Similarly the genes normally expressed in bone (osteocalcin and osteopontin) were suppressed in CBFA1 -/- mice.

CBFA1 maps to mouse chromosome 17 in mouse and to and to 6p21 in humans[40,41] at the same location as cleidocranial dysplasia (CCD). Otto[39] also noticed that there were abnormalities in heterozygous mice, most prominently: hypoplasia of the clavicle, delayed development of membranous bones, and delayed ossification of cranial bones, causing open anterior and posterior fontanelles, smaller parietal and interparietal cranial bones, and multiple Wormian bones. These features suggested a possible similarity with the human clinical correlate of cleidocranial dysplasia syndrome (CCD). The human CCD syndrome, an autosomal dominant disorder exhibits all the features described above in the CBFA1 +/- mice, plus supernumerary teeth.[42]

CBFA1 was capable of inducing osteoblast related genes in non-osteoblastic tissue. In C3H10t1/2 cells and in skin fibroblasts, transient transfection with a CMV-promoter driven CBFA1 construct up-regulated expression of OPN, osteocalcin, and type I collagen. This essential transcription factor thus controls the lineage specific differentiation of osteoblasts, and may control the breast adenocarcinoma mimicry of the osteoblast phenotype.

We have developed data that point to the role of CBFA1 in controlling the osteoblast phenotype in the metastatic breast adenocarcinoma cell. It is becoming clear that non-osteoblastic cells may be capable of expressing this protein, and it may have different functions in different cell types.[37,43] The unique bone protein osteocalcin was also examined, as CBFA1 directly controls osteocalcin expression. The presence of osteocalcin is indicative of the osteoblast phenotype and this may signify that other key members of the osteoblast program (i.e. PTH/PTHrP receptor, BSP, OPN) are also being expressed by the tumor cells, and that these may also be under the control of CBFA1. The expression of the osteoblast program by the adenocarcinoma cell may allow tumor cells to override the normal osteoblast-osteoclast relationship and to pathologically induce the hormone dependent and cytokine dependent driving of the osteoclast that leads to osteolysis.[46-48]

Importance and Implications:

Bone is the most common site of invasive breast cancer metastasis (over 90% of women dying of breast cancer have bone metastases). The extreme morbidity caused by skeletal metastases (vertebral compression fracture, paraplegia, long bone fracture, severe bone pain) is a major consequence of this disease, causing great personal suffering while consuming an estimated 63% of the total costs of caring for patients with recurrent breast cancer. About 44,300 women died from breast cancer in the U.S. in 1996, and some 184,300 new cases of the disease were diagnosed.[44,45]

We believed that it was essential to focus on defining the limited number mechanisms by which breast adenocarcinoma cells interfere with normal cellular communication in the host skeleton. These mechanisms involve inappropriate ectopic expression of bioactive bone proteins which activate osteoclasts, expression of integrins allowing attachment to the bone ECM, and unregulated growth factor expression. All clinical protocols which target breast cancer with hormone therapy and cytotoxic chemotherapy must contend with the heterogeneity of the malignant adenocarcinoma

phenotype. Diverse mutations and clonal selection of variants evolving in independent sites over the progression of the disease in each patient create an evasive target for chemotherapy. We anticipate that regardless of their detailed phenotypic profile, these malignant cells colonize bone by essentially the same process, and our findings will thus provide a rationale for blocking bone metastasis of breast cancer.

Predictors of future metastasis are of great importance in selecting the high-risk group of *node-negative* breast cancer patients needing careful observation and potentially benefitting from adjuvant therapies. Just as emerging data for microvessel density (angiogenesis) in primary sites correlate well with future recurrence, so may bone protein marker expression eventually predict skeletal metastasis and allow early intervention.

A possible long-term benefit of this Project is the development of molecular approaches to dismantle the pathways by which normal bone cells are coerced into nurturing metastatic cells in the skeletal microenvironment. Importantly, these pathways should be relatively stable targets for therapeutic intervention, in contrast to the highly mutable phenotypes of breast adenocarcinoma cells.

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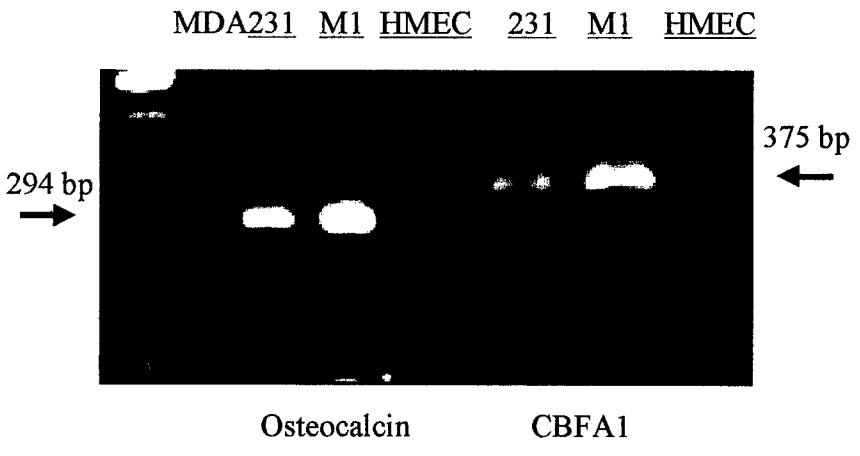
RT-PCR

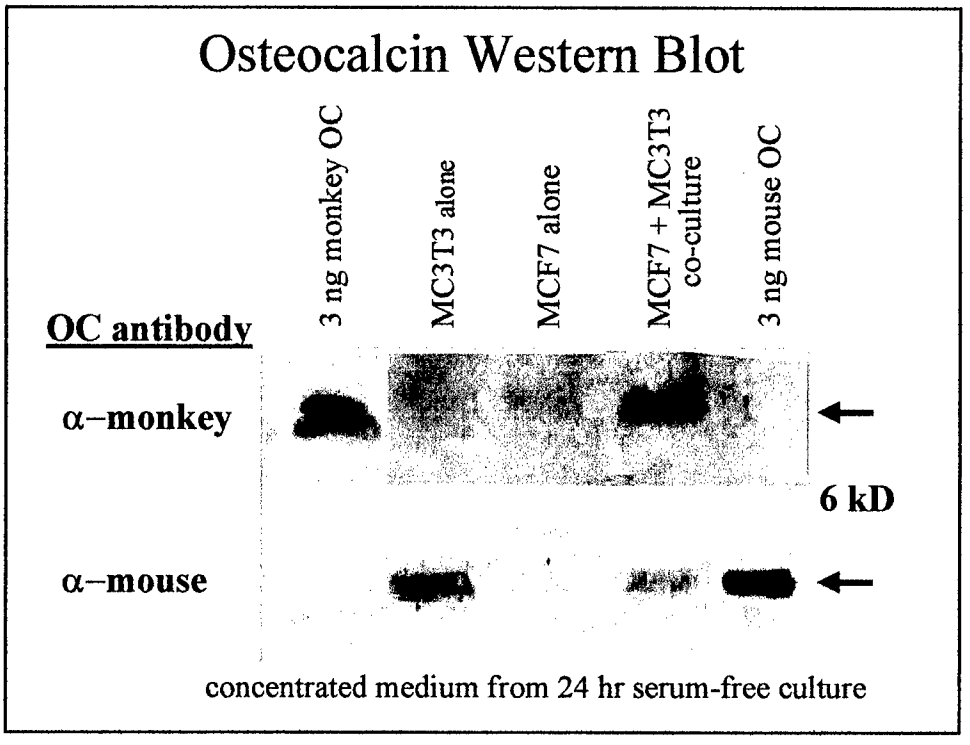


Osteocalcin Expression by QRT-PCR

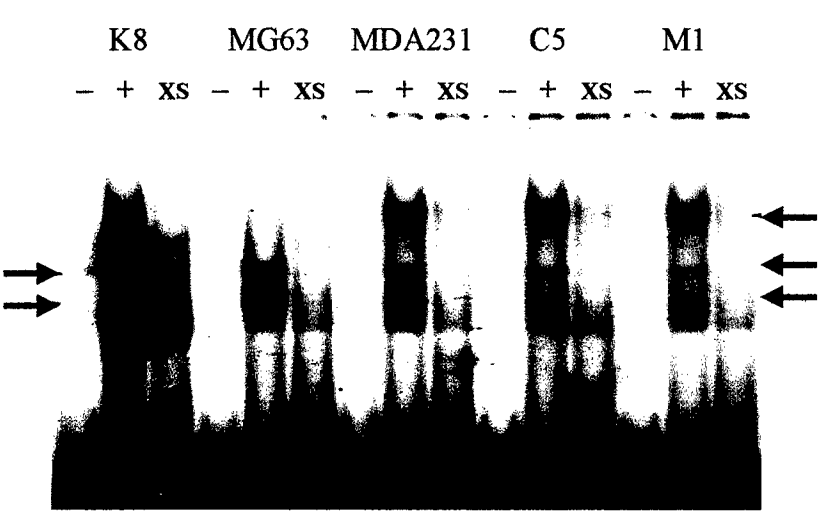
Cell Type	Relative hOC mRNA levels
MDA-MB231 exp 1	87.1
MDA-MB231 exp 2	363.6
MCF-7 exp 1	14.3
MCF-7 exp 2	111.4
HMEC exp 1	31.6
MG-63 hOS exp 1	140.9

Specific human osteocalcin fluorescent TaqMan probe (135R23: 5'-6FAM, 3'-TAMRA) with forward (91F20) and reverse (154R18) primers

Normalized to 3 pt. standard curves by 2-color fluorescence using 18S RNA signal in same sample


Osteocalcin Western Blot
3 ng monkey OC
MC3T3 alone
MCF7 alone
MCF7 + MC3T3 co-culture
3 ng mouse OC
OC antibody
α–monkey
6 kD
α–mouse
concentrated medium from 24 hr serum-free culture

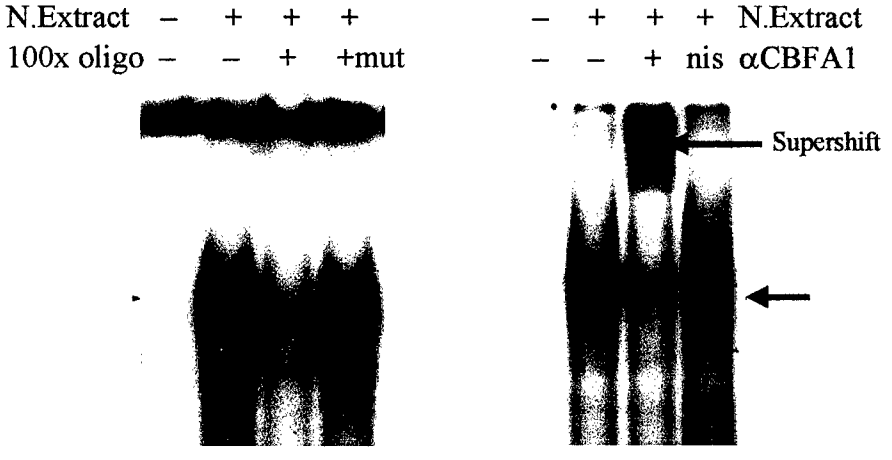
EMSA



5'-AGCTGCAATCACC**AACCACA**GCA-3' dsDNA probe

Murine Osteocalcin OSE-2 consensus CBFA1 binding site

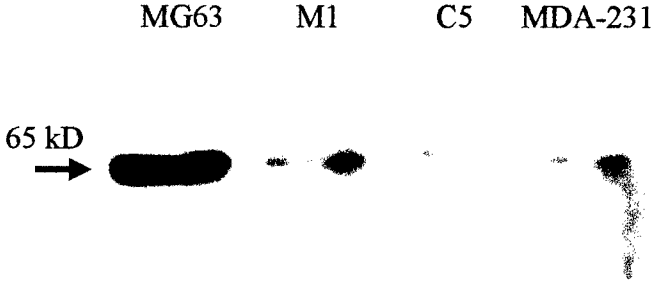
EMSA - MDA231



5'-AGCTGCAATCACC**AACCACA**GCA-3' dsDNA probe

5'-AGCTGCAATCACC**AGACACA**GCA-3' mutated oligo

Western Blot: CBFA1

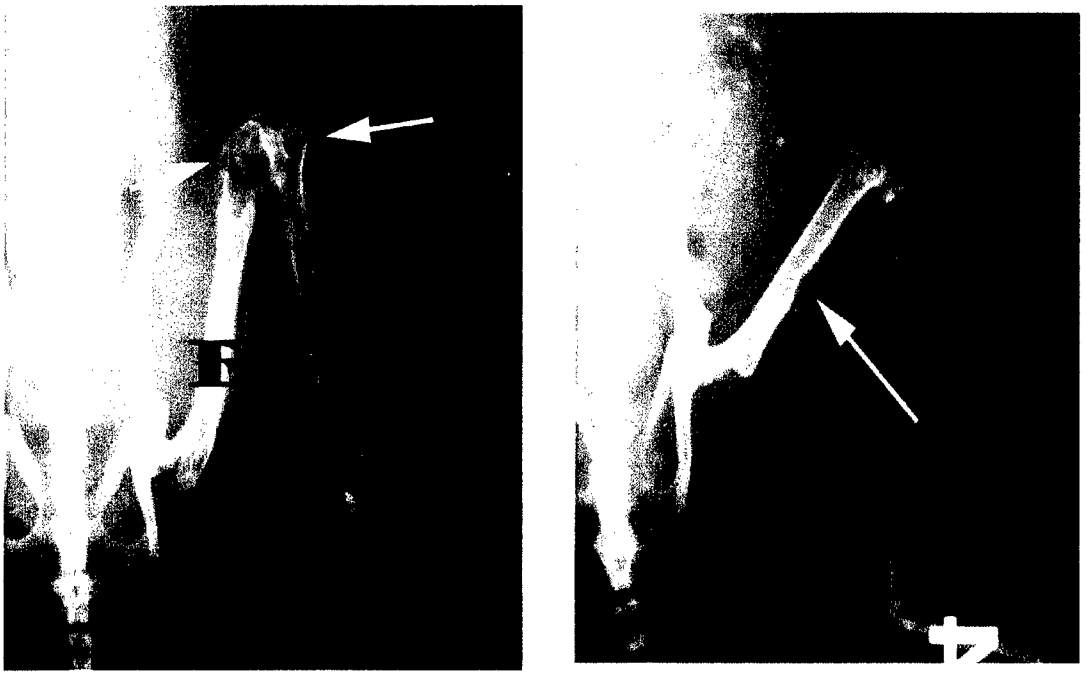


30 µg nuclear protein per lane; 8% SDS-PAGE;
affinity purified antibody to CBFA1 (S. Hiebert)

Upper Radiograph. Osteolytic metastases in mouse femur and tibia of 2 separate animals following injection of MDA-MB-231 human breast adenocarcinoma cells. After longitudinal monitoring of the lesions, animals were euthanized and the metastatic sites dissected, dispersed in sterile medium, and subcultured in vitro.

Lower Radiograph. Strong osteoblastic reaction in response to an osteolytic metastasis of MDA-MB-231 cells. The white arrows show the dense, radioopaque new bone formed in the tumor site. The scalloped area (black arrows) results from tumor-mediated osteolysis. This lesion was the source of the Blast-4 cell line.

Osteolytic Metastases (MDA-MB231)



Mixed Osteogenic and Osteolytic

Abstract to 45th Orthopedic Research Society, February 1-4, 1999, Anaheim, CA
Accepted for oral presentation.

Breast Cancer Metastases to Bone Express Increased CBFA1 and Osteocalcin.

Hecht AC, Adolphson LD, Feinstein MI, Barnes GL, Gerstenfeld LC, George C, Danciu T, Keel S, Skazkina K, Gebhardt MC, and Hauschka PV.

Introduction: Bone Metastases are common in breast cancer. Large autopsy studies estimate their frequency at 85%. Complications of bone metastases include pain, pathologic fracture, loss of mobility and vertebral compression syndromes. The osteotropism of breast cancer metastases remains poorly understood. Several investigators have demonstrated that breast cancer cell lines as well as primary and metastatic tumors synthesize bone proteins such as osteopontin (OPN) and bone sialoprotein (BSP). The transcription factor, CBFA1, regulates the expression of the osteoblast related genes (OC, OPN, BSP). We examined the expression of the osteoblast transcription factor CBFA1 and its target gene osteocalcin (OC) in the MDA-MB 231 cell line, a subclone of this line isolated from a bone metastasis (M1), and archival human specimens of patients with bone metastases from breast cancer primaries. Our central hypothesis was to determine if the expression of the osteoblast phenotype correlated with increased metastatic potential.

METHODS: Cell Lines: MDA-MB 231 (ATCC, MD) and Human Mammary Epithelial Cell (HMEC) (Clonetics, CA)were cultured according to recommended guidelines. The M1 subclone was cultured under conditions identical to the MDA-MB 231 cell line. Animals: 7 week old, female Nu/Nu SCID mice (20-25g) were used following institutional guidelines for the use and care of laboratory animals. MDA-MB 231 cells were injected (1×10^5 cells/100ul) through the left second intercostal space into the left ventricle to produce bone metastases. Animals were radiographed bimonthly to detect the presence of lytic lesion. Bones with lesions were fixed in 4% paraformaldehyde and decalcified. Several lesions were isolated and subcultured to produce a breast cancer cell line from a bone metastasis (M1). This subclone was further characterized by immunostaining with cytokeratin 18 (mouse anti-human) to confirm both the human and glandular epithelial origin. Reinjection into mice was performed to confirm its malignancy.

RNA/RT PCR RNA extraction was performed using RNeasy Mini Kit (Quiagen, CA) Primers were synthesized to unique regions in the OC, CBFA1, and GAPDH human cDNA sequences reported in the Genbank. PCR reaction cycle conditions consisted of denaturation at 93°C for 60s, primer annealing at 61°C for 60s, extension at 72°C for 60s, and a final extension at 72°C for 7 min in a Perkin-Elmer thermocycler (Model 9600). Samples were amplified for 25 cycles. UV photography and densitometry of ethidium bromide stained gels was performed . Semi-quantification was achieved by comparing the intensities of both CBFA1 and osteocalcin to GAPDH.

OC radioimmunoassay. Human OC concentrations in conditioned medium samples (1,3,5,7 days) were measured by radioimmunoassay using monkey anti-human osteocalcin (first antibody) and goat anti-monkey osteocalcin (second antibody). Data are reported as nanograms of OC/ml of medium. Alpha MEM with 10% FCS was used as a control.

Western Blot: Cultures were rinsed with PBS, scraped into buffer and collected by centrifugation. Cells were lysed with hypotonic buffer in the presence of protease inhibitors. Nuclei were collected by centrifugation and resuspended in hypertonic buffer and protease inhibitors. Nuclei were extracted on ice, insoluble material was cleared by centrifugation and soluble proteins were stored at –80°C. Nuclear extracts (30ug per lane) were resolved with 8% SDS-PAGE and electroblotted onto nitrocellulose membrane. Membranes were incubated with a 1:100 dilution of antibody in Tris-buffered saline containing 1% BSA. Affinity purified antibodies specific for CBFA1 (gift from Dr. Scott Hiebert, Vanderbilt Cancer Center, Nashville, TN) were used in these studies. Membranes were incubated with secondary antibody followed by streptavidin-HRP chemiluminescence (Pierce, IL).

Immunohistochemistry: Monoclonal or polyclonal antibodies to OCN and CBFA1 and appropriate negative controls were employed. The sections were obtained from the distal femur and proximal tibia of mice. Archival human specimens from 10 patients with metastatic breast cancer to appendicular and axial sites were similarly examined. Fixed, paraffin embedded sections from each tumor were immunostained using an indirect avidin-biotin complex method. The appropriate concentration of the primary antibody against CBFA1 and OC were used (1:10 and 1:100).

Results: RT-PCR. (Figure 1) Semi-quantitative PCR was performed comparing the expression of CBFA1 and OC between HMEC, MDA-MB 231 cell line, and M1. These results were normalized to GAPDH for each sample. There was a two fold increase in both CBFA1 (375bp) and OCN (294bp) in the M1 clone (lanes 5 and 2) compared to the original MDA-MB 231 cell line (lanes 4 and 1). There was no evidence of CBFA1 in the HMEC (lanes 3 and 6) cell line. PCR product identity was confirmed by producing products of expected size.. The CBFA1 amplimer was excised from the gel, purified and underwent automated sequencing to further confirm its identity.

Figure 1

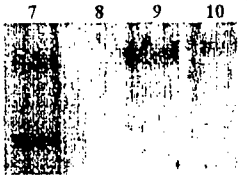


Figure 2

Western Blot: (Figure 2) We examined nuclear extracts from the MDA-MB 231 cell line, HMEC line, and M1 subclone, and MG-63 osteosarcoma cell line (positive control) using antibody to CBFA1. CBFA1 was detected in all of these cell lines except the normal breast line HMEC (Lane 8). A 60-65kDa species was present in the MDA-MB 231 line (Lane 10) and M1 subclone (Lane 9). The species was present at a two-fold greater band density in the M1 clone (Lane 9). The MG-63 line (Lane 7) expressed the 60-65 kDA form as well as a 46 kDA form that was not seen in the breast cancer cell lines.

Radioimmunoassay: MDA-MB 231 cells produced increasing amounts of osteocalcin. OC concentrations from medium + serum control was measured at 12.8 ng/ml. OC from tumor conditioned medium revealed increasing osteocalcin concentrations. Day 1 produced 14.6 ng/ml; Day 3, 18.4ng/ml, Day 5, 19.7 ng/ml; Day 7, 22.4 ng/ml.

Immunohistochemistry. Both osteocalcin and CBFA1 were noted to be positive for immunostaining in both archival human specimens and in experimentally induced animal models of bone metastasis. CBFA1 was seem primarily in the breast cancer metastasis, with minimal staining of the surrounding bone. Both intra and extracellular staining was noted. CBFA1 was noted to be only in the breast cancer metastasis in the archival human specimens with no staining in the surrounding bone. OC was seen in both the breast cancer metastasis and surrounding bone in both the archival human and animal specimens.

DISCUSSION: Several studies have found correlations between an increase in osteonectin, OPN and BSP expression with the degree of breast cancer invasiveness. In previous studies, the osteoblast transcription factor CBFA1, has not been found in non-skeletal tissues. CBFA1 plays a pivotal role in osteoblast differentiation and bone formation. This study reveals for the first time that invasive breast cancer is able to synthesize CBFA1 and OC. In addition adenocarcinoma cells isolated from bone expressed a two fold increase in both CBFA1 and OC. The expression of bone related genes may have implications in the pathologic destruction of bone as well as in its osteotropism. OC has been shown to increase the recruitment of osteoclasts and promote bone resorption. Paget in 1889 proposed that implantation of a given population of circulating cancer cells at a selected site of the organism is dependent on a suitable environment ("the soil") in which compatible tumor cells ("the seed") could proliferate. The molecular mechanisms underlying this well accepted theory remain poorly understood. The expression of the osteoblast phenotype by the breast cancer cell may give it a survival advantage in the bone microenvironment. In addition, CBFA1 mediates both BSP and OPN expression. These proteins may facilitate the targeting and attachment of circulating tumor cells to areas of ossification. It is tempting to speculate that the expression of CBFA1 may play a key role in explaining the increased osteotropic phenomenon of breast cancer, as well as breast cancer microcalcification.

MIMICRY OF THE OSTEOBLAST PHENOTYPE BY BREAST ADENOCARCINOMA CELLS

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Establishment of osteolytic metastases of breast adenocarcinoma in skeletal sites is a complex process wherein tumor cells interfere with normal interactions between osteoblasts and osteoclasts. Mimicry of some osteoblastic functions by malignant adenocarcinoma cells is hypothesized to be central to the biology of the metastatic process in bone.

CBFA1, a transcription factor responsible for normal osteoblast differentiation, is expressed inappropriately by the human breast adenocarcinoma cell line MDA-MB-231, and about 2-fold more strongly by a subline M1, recovered from an osteolytic bone metastasis of MDA-MB-231 in a nu/nu mouse. CBFA1 expression was not detected in normal breast epithelium (HMEC cells). Data were obtained by semiquantitative RT-PCR with specific primers, and by SDS-PAGE immunoblot analyis of nuclear lysates with an antibody that recognizes CBFA1. Electrophoretic mobility shift assay (EMSA) with labeled target oligonucleotide containing the putative binding site for the CBFA1 transcription factor demonstrated band shifting by active CBFA1 protein in nuclear extracts of MB-MDA-231 cells, but not in HMEC. Preincubation with the specific antibody to CBFA1 caused the expected supershifting of the labeled complex to a higher molecular weight.

Expression of CBFA1 by malignant breast adenocarcinoma cells may explain observations of their anomalous expression of "osteoblast-specific" genes (mimicry), because many of these genes have CBFA1 response elements in the 5'-promoter sequences; the osteocalcin gene is a prime example. We analyzed the expression of osteocalcin by SDS-PAGE immunoblotting samples of concentrated 48 hr serum-free conditioned media with specific antibodies that can distinguish between the 6kDa osteocalcin protein sequences of mouse and human. The human breast adenocarcinoma line MCF-7 produced detectable human osteocalcin, while MB-MDA-231 produced marginally detectable levels, and normal HMEC did not appear to express osteocalcin. Co-culture of MCF-7 with a normal mouse osteoblast line (MC3T3-E1) increased the human osteocalcin in the co-culture medium. This effect could be reproduced by addition of conditioned medium from proliferating MC3T3-E1.

We conclude that CBFA1 expression may provide malignant breast adenocarcinoma cells with an advantage in the bone microenvironment, and may explain their observed mimicry of certain aspects of the normal osteoblast. We also infer that the proximity of adenocarcinoma cells to osteoblasts in a skeletal metastasis may allow paracrine effects to enhance mimicry.

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Final Report Data

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Hecht AC, Adolphson LD, Feinstein MI, Barnes GL, Gerstenfeld LC, George C, Danciu T, Keel S, Skazkina K, Gebhardt MC, and Hauschka PV (1999). Breast Cancer Metastases to Bone Express Increased CBFA1 and Osteocalcin. Proceedings of the Orthopedic Research Society, Abstract. Selected for oral presentation by Dr. Hecht, February 1-4, 1999, Anaheim, CA

Hecht AC, Barnes GL, Yang QL, Adolphson LD, Skazkina K, and Hauschka PV (2000). Mimicry of the osteoblast phenotype by breast adenocarcinoma cells. Abstract. DoD Breast Cancer Research Program Era of Hope Meeting, June 8-12, 2000, Atlanta, GA

Manuscripts on these topics are in preparation and will be submitted following editorial review.

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Recd 7/24/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

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FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl