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

Osteopontin, a secreted phosphoprotein, is a major modulator of the motility of several cell types including macrophages, osteoclasts and tumor cells. Through its interaction with integrins and CD44v, osteopontin can induce metalloprotease, inhibit apoptosis, inhibit NO production and induce cytokine secretion. We have recently isolated a hexa peptide from osteopontin that is chemotactic to tumor cells. Antibodies raised against this peptide neutralize the chemotactic response of tumor cells to osteopontin *in vitro* and *in vivo*. Our hypothesis is that osteopontin or chemotactic peptides released during bone remodeling attract circulating breast tumor cells expressing specific CD44v splice variants. We have demonstrated that expression of human CD44 (v3-v6) in non-metastatic human tumor cells, results in turning these cells into metastatic tumor cells. Further, we have also demonstrated that the novel metastatic tumor line metastasizes predominantly into bone. The expression of CD44 (v3-v6) in breast tumor is necessary but not sufficient for bone specific metastasis. Other genes need to be turned on in tumor cells for these cells to become metastatic. We have isolated a peptide analogue of the chemotactic domain (PepL) that inhibits tumor cell migration, induces Nitric Oxide production and activates apoptosis in tumor cells. We now report that the chemotactic domain of OPN induces the activation of FAK, Protein Kinase Cβ (PKCβ) and PI-3 kinase, while PepL inhibits the activation of FAK, inhibits the activation of PKCβ, but can activate PKCζ. Further mutants of OPN lacking the chemotactic domain cannot stimulate the chemotaxis of tumor cells nor can they activate protein kinase Cβ. However, these mutants can activate PI3 kinase and FAK. We concluded that OPN mediates tumor migration by regulating, FAK, PI3 Kinase and PKCβ. We further conclude that PepL mediates its anti-tumor activity by inhibiting the activation of FAK and the activation of PKCζ. We further demonstrate that activation of PKCζ results in the activation of NKκb and the induction of iNOS. These results provide a novel target for therapeutic intervention of breast tumor metastasis and provide a prototype compound for the development of targeted therapy against tumor metastasis.

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Table of Contents

INTRODUCTION

Several cell surface molecules that modulate cell-cell and cell-matrix interactions have been linked to metastatic spread. These cell surface receptors, which include the laminin receptor, cadherins, Ig superfamily of receptors, integrins, selectins ICAMS and CD44 (Steller and Stevenson *et al.*, 1993),. may participate at specific steps in the metastatic spread of tumor cells and influence cell migration, invasion, spreading and cell growth. Blocking one of these steps may result in inhibition of metastasis. Breast tumors metastasize to bone and brain more frequently than any other organ. The factors responsible for the frequent metastasis of breast tumors to bone are currently unknown. The CD44 gene encodes a transmembrane protein which is expressed as a family of molecular isoforms generated from alternative RNA splicing. Standard form of CD44 is expressed in many normal tissues, whereas the expression of variant isoforms of CD44 (CD44v) is restricted (Tolg *et al.*, 1993). Some CD44v isoforms, which may regulate the homing of lymphocytes and macrophages after antigenic stimulation *in vivo,* have also been implicated in the metastatic spread of tumor cells. Transfection of CD44 isoforms into non-metastatic tumor cells transforms these cells into metastatic ones. Furthermore, antibodies raised against CD44 inhibit the metastatic spread of a variety of tumors (Arch, 1992). CD44v is expressed on the surface of metastatic breast tumors and it is currently being investigated as a possible prognostic tool for patients diagnosed with breast cancer. Recent reports have correlated the expression of CD44v (containing exon v3,v5,and v6) on breast tumors (Kaufman *et al.*, 1995) with metastasis and poor prognosis in patients with breast cancer. It is still unclear which molecule(s) CD44v is interacting with and at what stage in the metastatic cascade is CD44 critical. Osteopontin, a secreted phospho-glycoprotein secreted by bone cells, promotes the chemotaxis of macrophages and tumor cells through CD44v receptor and that the migration of these cells to OPN can be inhibited by antibodies raised against either OPN or CD44. Our hypothesis is that osteopontin or chemotactic peptides released from bone during remodeling attract breast tumor cells expressing specific CD44 splice variants to migrate out of the capillary bed of bone marrow. The extravasated tumor cells can then attach to matrix bound osteopontin resulting in the induction of MMP2 and MMP9 which may further facilitate the invasion and growth of tumor cells within the bone micro-environment by removing connective tissue barriers and releasing matrix bound cytokines.

Invasion though a three-dimensional extracellular matrix is a coupled event requiring cell attachment, detachment and localized degradation of the matrix in the direction of movement. This coordinated process requires the synergistic interaction among several types of receptors with different molecules of the extracellular matrix. This grant has **three Specific Aims**. In the **first Aim** we will examine the invasion of the CD44 transfectants into marrow stroma and MC3T3E1 cell cultures. A chemotactic domain on OPN was defined. In the **second aim** we will examine the role of this chemotactic domain on the migration of breast tumor cells and determine which amino acids within the chemotactic domain are essential for chemotaxis. Once an essential amino acid has been identified, we will mutagenize this amino acid in OPN and determine the biological consequences of the mutagenesis. In the **third Aim** we will assess the *in vivo* role of CD44 splice variants in bone specific metastasis and test whether the anti-osteopontin antibodies will neutralize the metastasis of MDA-MB-231 and MDA-MB-453/CD44 transfectants to bone.

(2) **BODY:**

The approved statement of work is appended.

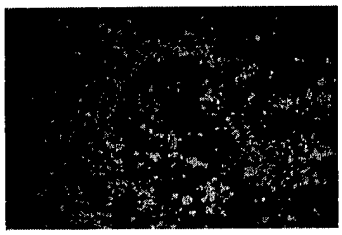
It is anticipated that the control of cellular development *in vitro* will require the interaction of tens if .not hundreds of molecular species varying in concentration and location in time. Mapping the genes necessary for metastasis and developing new anti-tumor drugs depend, then, upon the capacity to

expose tumor cells to permutations of reactive molecule concentrations in high throughput models in real time. For this reason, *in vivo* models alone will be untenable. Furthermore, most existing *in vitro* model systems do not mimic sufficiently the behavior of metastasizing tumor cells in vivo, partially because of the multiplicity of *in vivo* stimuli and the difficulty in reproducing them *in vitro.* A new approach to assay design was developed which better mimics metastasis *in vivo.* These high throughput 3 dimensional bridge assays were developed to mimic discrete steps in the metastatic cascade and allow the rapid screening of novel anti-metastatic drugs and the mapping of essential genes in the metastatic cascade. These *in vitro* assays are based on the ability to recreate in culture three-dimensional ordered structures that mimic the organization and physiology of tissue *in vivo.* for example, pre-osteoblasts in culture can differentiate into matrix producing osteoblasts that synthesize, assemble and mineralize a matrix that has been shown to be nearly identical to bone. Currently we have three bridge assays 1) co-culture of tumor cells with human umbilical cord endothelial cells that mimics the attachment and extravasation of tumor cells in vivo. 2) Attachment and invasion into marrow stromal cells and 3) Invasion into osteoblast cultures that mimic in vivo invasion into osteoid and mineralized bone.

To test whether osteopontin has any effect on the invasion process, fluorescently labeled MDA-MB 231 cells were cultured on confluent marrow stromal cells isolated from 4 week old c57 blk mice, upon confluency these marrow stromal cells form discrete environments capable of supporting hematopoeitic and monocyte/macrophage/osteoclasts differentiation. The invasion of the extracellular matrices (ECM) by the labeled MDA-MB 231 cells was monitored by fluorescent microscopy at different times up to 2 weeks. The red fluorescent marker allowed the visual differentiation between the tumor cells and the marrow stromal cells in this co-culture system. Within one hour after seeding, the MDA-MB 231 cells started attaching and spreading on the marrow stromal cultures. By 24 h, MDA-MB 231 cells organized and formed invasion foci (Figure 1A) cells within these foci start proliferating and within three days the tumor cells have invaded into the cultures and have destroyed the marrow stromal layer. This invasion was enhanced by the addition of osteopontin to the cultures (Figure 1b). Addition of osteopontin increased the number of adherent and spread tumor cells. Similarly, MDA-MB-231 can invade mineralizing cultures of MC3T3E1(figure 1C) and can destroy the matrix synthesized by these cells (Figure 1D). Expression of antisense OPN in MC3T3 obliterates the invasion of MDA-MB-231 into these cultures (figure 2). With these two *in vitro* systems, we can mimic the invasion of breast tumor cells into the marrow and into matrix (osteoid) component of bone. We have also determined that the invasiveness of MB-MDA-231 is specific. These cells do not attach to nor do they invade cultures of kidney or fibroblasts cell culture.

Figure 1: Invasion Of MDA-MB 231 Cells into Extracellular Matrix of marrow stromal cultures

A



B

C

Marrow stromal cells were grown on Transwell cell culture inserts of 1.0 μm pore size. At confluency, 10,000 MDA-MB 231, labeled with PKH26-GL red fluorescent cell linker (Sigma BioSciences), were added to the marrow stromal cultures. The attachment of labeled MDA-MB 231 cells and their subsequent invasion into the marrow stromal matrix was monitored visually using an Olympus microscope over a period of 7 days. One, three and 7 days post seeding photomicrographs of representative fields were taken at 200X magnification. Each experiment was performed in triplicates, and repeated 3 times.

A) Fluorescent photomicrograph showing the spreading and arrangement of MDA-MB 231 cells into invasion foci 1 day after seeding.

B) Fluorescent photomicrograph showing the effect of exogenously added osteopontin (10 μg/well) on the increase in number of attached and spread MDA-MB 231 cells, along with the increase in formation of invasion foci

C) Fluorescent photomicrograph showing the effect of exogenously added anti mouse osteopontin antibody (20 μg of purified IgG/well) on the decrease in number of attached MDA-MB 231 cells, and decrease in number of invasion foci.

D) Quantitation of the number of invasion foci/field observed in the above experiments. Each point was the average of five non-overlapping fields and each experiment was done in duplicates. Bold numbers $p < 0.05$

Number of invasion Foci Formed by MDA-MB-231

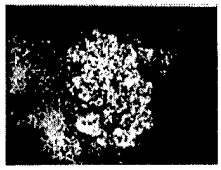
	# of invasion Foci
control	3 ± 1
+ OPN	**16 ± 2**
+ αOPN	**1** ± 0.3

D

A

B

C



D

Figure 2. Destruction of MC3T3E1 extracellular matrix by invading MDA-MB-231. MC3T3-E1 cells were grown on Transwell cell culture inserts of 1.0 μm pore size. At confluency, the cells where treated for 1 week with 10 μg/ml ascorbate and 10^{-8} M Dexamethasone. This treatment results in the synthesis of the extracellular matrix. Subsequent to the formation of extracellular matrix, 10,000 MDA-MB 231 cells were labeled with PKH26-GL red fluorescent cell linker (Sigma BioSciences) and added to the cultures. The attachment of labeled MDA-MB 231 cells and their subsequent invasion of the MC3T3-E1 matrix was monitored visually under an Olympus microscope. 24 h post seeding photomicrographs of representative fields were taken at 200X magnification. Each experiment was performed in triplicates, and repeated 3 times.

A) fluorescent photomicrograph showing the spreading and arrangement of MDA-MB 231 cells into invasion foci.

B) Addition of 10 μg of osteopontin enhances the attachment and invasiveness of MDA-MB-231 and matrix destruction

C)MC3T3 matrix (blue) is destroyed by invading MDA-MB-231. Cultures were stained with toluidine blue to visualize the extracellular matrix. (Destroyed matrix appears white in a blue background)

D) Osteopontin enhances MC3T3E1 matrix destruction by MDA-MB-231 Human breast tumor cells. Cultures were visualized with toluidine blue.

A

B

C

Figure 3. Modulation of the invasion phenotype by overexpression of anti-sense OPN or CD44(v3,v5,v6)

A. **Expression of anti-sense OPN in MC3T3 drastically reduces the invasion potency of MB-MDA-231.** Osteopontin cDNA was expressed in the anti-sense orientation under the control of the CMV promoter. The expression of OPN in the transfected cells was reduced to about one tenth of the normal level of expression of collagen or bone sialoprotein. MC3T3 expression antisense OPN produce a matrix similar to the matrix produced by the parental cell line. However, this cell line does not support the attachment or invasion of MB-MDA-231 human cells.

B. **Invasion of MB-MDA-231 into matrix producing MC3T3.** Assay was performed as described in Figure 1.

C. **Transfection with CD44 (v3,v5,v6) enhances the attachment and invasiveness of the non-invasive human cell line MB-MDA-453.** MB-MDA-453 parental cells are $CD44^-$ that do not recognize OPN nor do they invade matrix producing MC3T3 cell cultures. However, over-expressing CD44 (v3, v5, and v6) in these cells transforms them into invasive cells. Preliminary results also indicate that the transfected cells can now recognize and respond to bone OPN.

Technical Objective 2: Effect **of Chemotactic Peptide on the migration of MDA-MB-231 and transfected clones.**

The formation of metastatic colonies by malignant tumors is an active process that requires local proteolysis of the ECM, cell migration and cell attachment. The report that osteopontin is a ligand for the homing receptor CD44v, and can induce the migration of macrophages and lymphocytes in a CD44 dependent manner (Weber *et al.*, 1996, 1997) both *in vitro* and *in vivo*, prompted our investigation into the role of CD44v and osteopontin in tumor metastasis.

In order to investigate if osteopontin plays any role in the migration of breast tumor cells, we first examined the chemotactic response of the $CD44^+$ human breast adenocarcinoma MDA-MB 231 cells to osteopontin *in vitro*. OPN induces the migration of these cells and this migration was totally inhibited by antibodies to osteopontin or CD44 (Table 1). However, osteopontin was not chemotactic to the $CD44^-$, non-metastatic human breast tumor cell lines, MDA-MB-330 or MDA-MB-453. Nevertheless, if CD44 (v3,v4,v5,v6) was transfected into either cell line, both transfected cell lines now respond to OPN.

To further define the chemotactic domain of OPN, we digested osteopontin (1mg) with 50 μg of trypsin at 37° C over night. The resulting peptides were separated on a c18 reverse phase HPLC column using a linear gradient of 0-80% acetonitrile. Peptides were lyophilized and resuspended in water. 10 ng peptides were then tested for chemotactic properties in a modified Transwell system. The results summarized in Table 1 show that one of the isolated peptides (pep51) was chemotactic to MDA-MB-231.

The chemotactic peptide (pep51) was sequenced using gas-phase sequenator (sequence LVLDPK). To confirm that chemotaxis was due to this peptide a 16 amino acid peptide spanning the chemotactic sequence in OPN was synthesized and tested for its chemotactic properties (Table 1).

Since the 16 amino acid peptide was chemotactic to tumor cells, antibodies against the peptide were raised and tested for their ability to neutralize the chemotactic response to OPN and its chemotactic peptides (Table 1).

TABLE 1: Chemotactic response of CD44 positive MDA-MB 231 cells to bone osteopontin. Chemotactic response of MDA-MB-231 to osteopontin was measured as previously described (Weber *et al.*, 1996). Briefly, uncoated polycarbonated filters with 8 mm pore size were used to separate the upper and lower chamber of chemotactic wells. 2 X 10^4 cells were added to the upper chamber and incubated at 37°C in the presence or absence of 200 ng of bone OPN as the chemotactic factors in the lower chamber. After 4h, the filters were removed, fixed in methanol and stained with hematoxylin and eosin. Cells that have responded to OPN, migrate to various areas of the lower surface and can be counted microscopically. 200 ng of bone OPN significantly ($p < 0.01$) stimulated the chemotaxis of MDA-MB-231. Addition of OPN to the upper chamber inhibited ($p < 0.05$) the chemotactic response of MDA-MB-231, suggesting that the response is not due to increased random migration, but rather to chemotaxis. 1 ug of affinity purified rabbit anti-mouse osteopontin and 1 ug of

		UPPER CHAMBER				
		PBS	*OPN*	*PEP51*	*SP64*	*Ab64*
LOWER CHAMBER	*PBS*	9	6	**243**	23	5
	PEP51	**248**	65	**109**	48	21
	SP64	**217**	**97**	66	66	25
	OPN	**214**	44	**187**	16	36

monoclonal antibody against human CD44 inhibited the chemotactic response of MDA-MB-231 to bone osteopontin ($p < 0.05$). Each data point is reported as the mean number of migrating cells per 6 random high powered fields $\pm$ standard error of the mean. Each experiment was repeated 4 times.

To test the amino acid specificity for chemotactic response, several substitutions were created in the chemotactic sequence and the new peptides tested for their ability to induce chemotaxis. The results are summarized in Table 2.

Table 2: Sequence specificity of chemotactic peptide: Chemotactic response of MDA-MB-231 to osteopontin was measured as previously described (Weber *et al.*, 1996). Briefly, uncoated polycarbonated filters with 8 mm pore size were used to separate the upper and lower chamber of chemotactic wells. 2×10^4 cells were added to the upper chamber and incubated at 37° C in the presence or absence of 200 ng of bone OPN as the chemotactic factors in the lower chamber. After 4h, the filters were removed, fixed in methanol and stained with hematoxylin and eosin. Cells, which have responded to OPN, migrate to various areas of the lower surface and can be counted microscopically. 200 ng of bone OPN significantly ($p < 0.01$) stimulated the chemotaxis of MDA-MB-231. Values are expressed as migratory index (MI) which was calculated by dividing the number of cell migrating in response to chemotactic agent by cells migrating in response to buffer (random migration). Addition of OPN to the upper chamber inhibited ($p < 0.05$) the chemotactic response of MDA-MB-231, suggesting that the response is not due to increased random migration, but rather to chemotaxis. 1 ug of affinity purified rabbit anti-mouse osteopontin and 1 ug of monoclonal antibody against human CD44 inhibited the chemotactic response of MDA-MB-231 to bone osteopontin ($p < 0.05$).

TABLE 2
Sequence Specificity of OPN derived chemotactic Peptide

	chemotactic peptides	MI	MI in presence of inhibitory peptide
peptide 51	LVLDPK	10	10
SP64	KFHSHKDKLVLDPKSK	12	9
pepA	LVVDPK	9	8
pepB	LVPDPK	10	8
pepC	LVPDSK	7	10
pepD	LVIDPK	11	9
pepE	LVLDEK	2	10
pepF	VLDPK	6	7
pepG	LVLDP	0	4
pepH	LELDPK	2	10
pepI	LVLAPK	1	9
pepJ	LVLEPK	6	9
pepK	Acetyl-LVLDP	1	4
pepL	Acetyl-MLDP	1	2
pepM	Ac-HKDKMLDP	0.8	2

To evaluate the potential of peptide analogues to inhibit OPN induced chemotaxis, 100 nmoles of peptide was added to the lower compartment and the response of these cells to OPN was determined as described. Each data point is reported as the mean number of migrating cells per 6 random high-powered fields $\pm$ standard error of the mean. Each experiment was repeated 4 times and scored by at least two independent blinded researchers.

Since the peptide LVLDPK was chemotactic to tumor cells and macrophages *in vitro*, we tested whether the peptide was chemotactic *in vivo*. 25 nmols of the peptide were injected into the peritoneal cavity of C57 black mice. After six hours the cells recruited into the peritonium were recovered by lavage and identified by flow cytometry. The results presented in table 3 demonstrate that LVLDPK was chemotactic to macrophages *in vivo* and that replacing the aspartate with alanine in the sequence completely eliminated this activity. A truncated version of LVLDPK, namely VLDPK was also chemotactic but exhibited less activity perhaps due to the faster clearing rate of the peptide. However, the peptide AcMLDP totally abolished the chemotactic response to OPN of LVLDPK if it was co-injected with the chemotactic agent.

Table 3 In vivo recruitment of Immune cells by OPN and its chemotactic peptides

	PBS	OPN	LVLDPK	LVLAPK
Total cells	443,000 ± 150000	1.6 X10^6 ± 321,0000	910,0000 ± 244,0000	512,000 ± 32,0000
macrophages	224,000 ± 43200	667,000 ± 43200	617,000 ± 121,000	98,000 ± 33,500
B-cells	37,300 ± 8,000	24,6300 ± 11,000	11,000 ± 4,000	27,000 ± 9,000
T-cells	18,100 ± 3500	7,800 ± 3,000	22,800 ± 11,000	8,700 ± 1,000

Female C57BL/6 mice, purchased from Jackson Laboratories and housed at the Redstone Animal Facility of the Dana-Farber Cancer Institute, were injected intraperitoneally with 200 µl of PBS containing varying dosages of osteopontin or chemotactic peptides derived from OPN.. Injections of vehicle alone (PBS) served as negative controls. Mice were sacrificed by CO_2-asphyxiation at varying times after injection followed by immediate collection of peritoneal exsudate by intraperitoneal injection and recovery of twice 10 mL PBS. Red blood cells were removed by hypotonic lysis with ACK buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4) for 5 minutes at room temperature. Cells were washed and resuspended in DMEM containing 5% FBS for fluorescent antibody staining at a concentration of 0.2 to 1 million cells in 50 µl. Fluorescence-labeled antibodies (1 µg/1x10^6 cells) were incubated with cells for 30 minutes at 4°C, before washing twice with 200 µl of PBS and fixation in 500 µl of 2% paraformaldehyde in PBS. Analysis for cellular expression of CD44 (Pgp-1, PE) together with CD11b (Mac-1, FITC, macrophage marker), B220 (FITC, B-cell marker), or CD3 (FITC, T-cell marker) was performed by dual-color flow cytometry with antibodies from PharMingen using a Coulter EPICS flow cytometer. Appropriate non-specific antibody controls and single color controls were included.

The results from sequence specificity studies indicate that changing the aspartate (D) to an amino acid other than an acidic amino acid results in loss of biological activity. A hydrophobic tail is necessary for the biological activity of the peptide. The C-terminal K is essential, however, arginine can partially substitute for it. The proline can be replaced by any other amino acid that can adopt a turn conformation. Acetylation of the N-terminal has little effect on the biological activity of the peptide. Deleting the terminal arginine from the sequence results in a peptide that can partially antagonize the activity of OPN. Since one of our objectives was to identify an inhibitory sequence we further characterized this peptide.

Treatment of breast cancer cells with pepL totally abolished the chemotactic response of these cells to OPN. Further, cells treated with PepL or PepM increased NO production within 12 hrs (Figure 4) and resulted in activation of apoptosis. This was confirmed by using TUNNEL and by Caspase 8 assay (Figure 5).

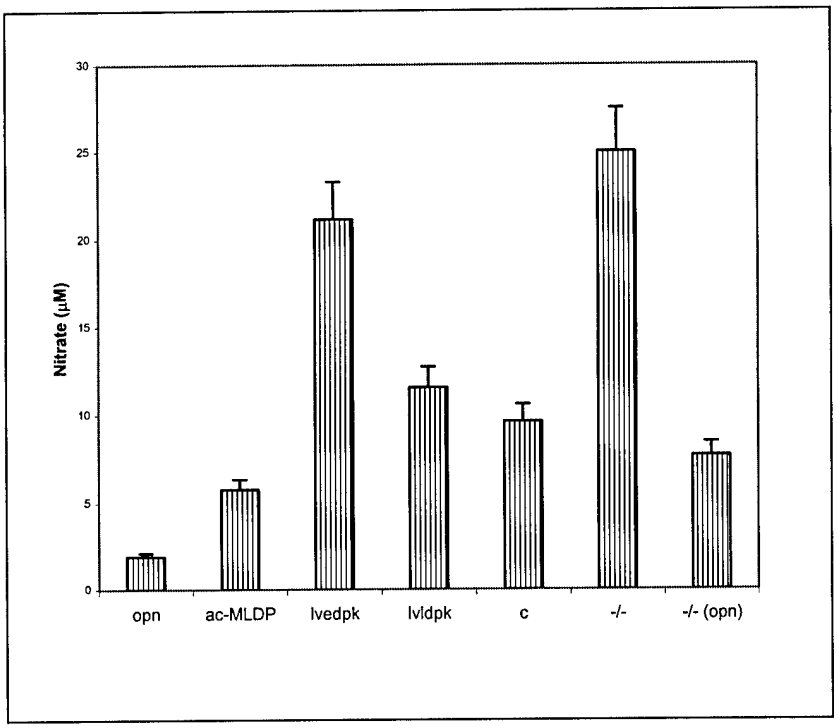


Figure 4: Induction of Nitric Oxide by PepL.
One million MDA-MB-231 cells were incubated with 100 nM of the indicated peptide or protein in DME containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37°C. After 12 hours, the production of nitric oxide (NO) by the breast tumor cells will be assessed by measuring the accumulation of nitrate in culture supernatants. Griess reagents were used as described by Hwang *et al.*, (1993). Results are expressed as μmoles of nitrate/liter, after correction for the amount of nitrate present in cell culture media (0.1 μM). Each assay was done in triplicate and was an average of 2 separate experiments. -/- represents NO production by tumor cells derived from osteopontin knockout mouse. -/- (OPN) represents NO production by tumor cells derived from the OPN knockout mouse and stably tranfected with OPN.

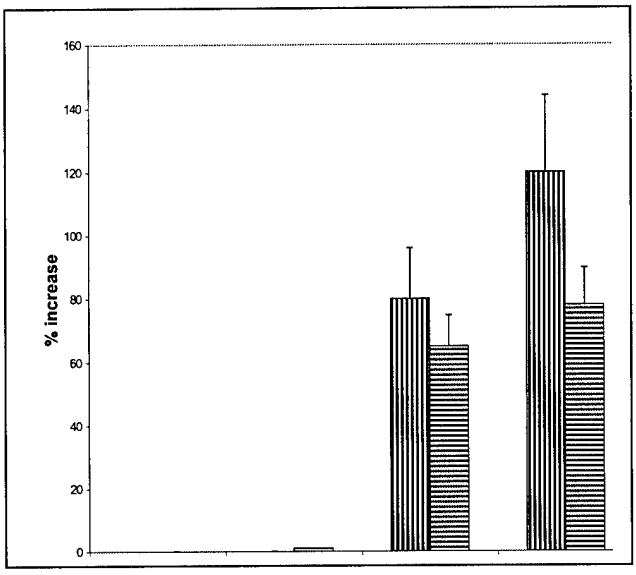


Figure 5: Induction of Caspase 8 and apoptosis in cells treated with peptide analogues. One million MDA-MB-231 cells were incubated with 100 nM of the indicated peptide or protein in DME containing 10% FBS in a humidified atmosphere of 5% CO2 at 37oC. After 12 hours, Caspase 8 and the % of apoptotic cells were determined. Caspase was determined by a fluorescent assay using Chemicon's FLICE/Caspase-8 Fluorometric Protease Assay Kit as described by the manufacturers. Apoptosis was determined using TUNEL using the "In situ cell death detection Kit" (Boehringer-Mannheim, Germany). Results are expressed as % increases. Each assay was done in triplicate and was an average of 2 separate experiments.

These results suggest that the chemotactic antagonists can activate program cell death in susceptible tumors. Peritoneal macrophages, fibroblasts or osteoblasts treated with PepM did not undergo apoptosis, suggesting that the this induction is specific and possibly receptor mediated.
It is not clear if there is any relationship between NO production and apoptosis in tumor cells, however, preliminary results indicate that PepL and PepM did inhibit the activation of PI3 kinase.
During inflammation, activated macrophages kill invading microorganisms, tumor cells and damaged tissues by two separate pathways that involve the production of nitric oxide and reactive oxygen species.

Under specific redox conditions, nitric oxide and superoxide undergo a diffusion-limited reaction generating peroxynitrite (Huie and Padmaja, 1993), a powerful cytotoxic compound that can cause lipid peroxidation (Radi *et al.*, 1991), oxidation of protein sulfhydryls (Smith and Beckman, 1992), and nitration of protein tyrosine residues (Beckman et al., 1994). Tyrosine nitration been found in a variety of pathological tissues such as atherosclerotic lesions (Abe *et al.*, 1992), Amyotrophic Lateral Sclerosis (Shigenaga *et al.*, 1997) and Alzheimer's disease (Kong *et al.*, 1996). Tyrosine kinase activation plays a critical role in the signal transduction pathways induced by e.g. growth factors and integrins. Since it has been demonstrated that nitration of a tyrosine residue impairs the subsequent phosphorylation of the same residue, it is likely that phosphotyrosine-regulated signaling proteins will prove to be important targets for tyrosine nitration. Increasing amounts of evidence implicates that an activation of the PI 3-kinase and its downstream target Akt-I kinase are involved in anti-apoptotic signaling. Tyrosine nitration alters these signaling pathways effectively, shutting them down. Hellberg *et al.*, (1998) demonstrated that tyrosine nitration of PI3 kinase occurs in activated macrophages shortening their life span. Several investigators have correlated NO production (by a variety of mechanisms) with tumor death (Boggio *et al.*, 1998; Janssen and Van den Berge., 1998). However, very little is known about tyrosine nitration during tumor progression. Since it has been our hypothesis that tumor metastasis is cellular mimicry of immune cells, especially macrophages, and since NO production and tyrosine nitration result in inhibition of macrophage proliferation and activation of cell death, we propose that activation of this pathway in metastatic tumors may result in the inhibition of tumor growth and metastasis.

Technical Objective 3: **Testing the anti-metastatic potential of anti chemotactic domain antibody *in vivo* and testing of novel CD44 cell lines for their metastatic potential *in vivo*.**

We have constructed the following cells line to

- MB-231b: human adenocarcinoma cell line cloned from a bone metastasis in nude mice of the parental cell line MB-MDA-23; CD44s, Cd44E, CD44 (v3-v6), integrin alpha v, 4, 6, b1, b5, b3, Sialyl lewis, ICAM, Muc-1, HER2, p53, ER^-
- MDA-MB-453. human adenocarcinoma cell line $CD44^-$, Sialyl lewis, ICAM, Muc-1, HER2, p53 integrin a2, av, a6(low), b1, b5, b3(low), cell lines that do not metastasize *in vivo* :
- MB-453 (CD44s): Tumor cells expressing the standard form of CD44. Detected in all breast tumors but not normal breast tissue (Fichtner et al., 1997).
- MB-453 (CD44E): Tumor cell line expressing the epithelial form of CD44. Detected in 85% of breast tumors (Fichtner et al., 1997)
- MB-453 (CD44v (3,5,6): Tumor cell line expressing CD44 splice variant containing exons 3,5 and 6 that has been linked to metastasis of breast tumors and poor prognosis (Fichtner et al., 1997).
- MB-453i: A clone of MB-453 (CD44v (3,5,6) that expresses integrin b3. This cell line is highly metastatic.
- MB-453 (CD44v (6,7) Tumor cell line expressing CD44 splice variant containing exons 5,6. A splice variant more prevalent in estrogen receptor negative tumors (Fichtner et al., 1997).
- MB-453 (CD44v (9, 10) Tumor cell line expressing CD44 splice variant containing exon 9, 10. Expressed in some tumors and correlates with histological grading (Fichtner et al., 1997).

metastasis iv procedure from experiments run from 1-4 98

cell line	metastatic colonies				
	Lung	long bones	spine	kidney	liver
MDA_MB-231	1	4	6	1	1
MB-231b	0	8	11	0	0
MDA-MB-453	0	1	0	0	0
MB-453i	0	16	22	0	0
MB-MDA-CD44(356)	0	6	8	0	1
MB-MDA-CD44(9,10)	0	1	0	0	0

Table 4. **Invasion potential of CD44 expressing cells.**

1. Nude mice were anesthetized (protocol # A97-07-053) by injection with Ketamine/Xylazine, 90mg/10mg mixed together with sterile H_20 or saline, 20g, IP, then scrubbed for aseptic surgery. A 30 gauge needle mounted onto a tuberculin syringe will be inserted in the second intercostal space 2 mm to the left of the sternum and aimed towards the heart. The entrance of bright red blood into the syringe will indicate proper positioning of the needle in the left ventricle of the heart. 2 X 10^5 cells were then injected into the left ventricle of nude mice. The mice will be allowed to recover from anesthesia. Six mice were sacrificed at each time point of 7 and 14, and 28 days after tumor cell injection. Mice will be radiographed in the prone position at 35 KVP for 6s using standard radiographic techniques (this protocol is pending per IACUC approval, Animal Radiography is a service provided by trained veterinary technicians at our ARCH animal facility). Radiographs will be digitized using either OsteoMetrics digitizing table (OsteoMetrics Inc., Atlanta GA) or captured using a high resolution Sony video camera equipped with zoom lens. The area of osteolytic lesions will then be measured using either Osteomeasure Image Analysis System (OsteoMetrics Inc., Atlanta, GA) or Optima 5.2 image analysis software. Statistical significance will be determined by standard analysis of variance. P values of less than 0.05 is considered significant.

The results, presented in table 4 clearly indicate that expression of CD44 (v3-v6) is necessary for tumor metastasis to bone. Further, co-expression of integrin beta 3 with CD44 v3-v6 results in a highly (additive) aggressive tumors.

Technical Objective 2**: Effect of Chemotactic Peptide on the migration of MDA-MB-231 and transfected clones.**

Construction of OPN mutants: To evaluate the contribution of the chemotactic domain to the biology of osteopontin, we adopted a two-tier strategy. In the first tier, we constructed several forms of OPN, lacking the chemotactic domain. These constructs are presented in figure 1. OPN 286 was constructed by cloning amino acids (1-285) into PCDNA3.1 (Invitrogen Corporation, Carlsbad, California).. OPNdcc was constructed by cloning amino acid 291-314 in frame into the 5' end of OPN286 cDNA. The resulting protein has the LVVD replaced by LRP. The resulting OPN286 and OPNdcc clones were separately transfected into LL64, a small cell carcinoma cell line established from the OPN knockout mouse. Stable transfectants over-expressing either OPN286 or OPNdcc were used to purify OPN286 or OPNdcc protein by a modification of our OPN purification method (Ashkar et al., 2000). In the second tier we mutagenized the aspartate in the sequence LVVDPK into an alanine or a glycine. These clones were sequenced and characterized.

OPN

MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDH
MDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYD**GRGDS**VVYGLR
SKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKAND
ESNEHSDVIDSQELSKVSREFHSHEFHSHEDM**LVVDPKS**KEEDKHLKFRISHELDSASSEVN

OPN286

MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDH
MDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLR
SKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKAND
ESNEHSDVIDSQELSKVSREFHSHEFHSHEDMRA

OPNDCC
MRIAVICFCLLGITCAIPVKQADSGSSEEKQLYNKYPDAVATWLNPDPSQKQNLLAPQNAVSSEETNDFKQETLPSKSNESHDH
MDDMDDEDDDDHVDSQDSIDSNDSDDVDDTDDSHQSDESHHSDESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLR
SKSKKFRRPDIQYPDATDEDITSHMESEELNGAYKAIPVAQDLNAPSDWDSRGKDSYETSQLDDQSAETHSHKQSRLYKRKAND
ESNEHSDVIDSQELSKVSREFHSHEFHSHEDMARPKSKEEDKHLKFRISHELDSASSEVN

Figure 6: Sequence of Human OPN, OPN 285 and OPNdcc: RGD integrin binding sequence and LVVDPK chemotactic sequence are in Bold.

Regulation of Tumor cell migration by OPN285 and OPNdcc. OPN285 and OPNdcc were tested for their ability to induce tumor cell chemotaxis and cell spreading. The results summarized in table 5 clearly show that both OPN286 and OPNdcc can support the spreading of MDA-MB-231 cells. However, neither protein can induce the chemotaxis of these cells.

Protein	Chemotactic Index	% Spread
Control	1 ± 0.2	10% ±3
OPN	**10.5 ± 2.2**	**87% ± 18**
OPN286	0.9 ± 0.2	**96% ± 21**
OPNdcc	1.1 ± 0.3	**91% ± 11**

TABLE 5: Chemotaxis of tumor cells to OPN286 and OPNdcc.

A) Directed migration of cells was determined in multi-well chemotaxis chambers. Two-well culture plates (Transwell) with polycarbonate filters (pore size 8-12 μm) separating top and bottom wells were coated with 5 μg fibronectin. 2 X 10^5 MDA-MB231 cells were added to the upper chamber and incubated at 37° C in the presence or absence of osteopontin in the lower chamber. After 4 h, the filters were removed, fixed in methanol, stained with hematoxylin and eosin and cells that had migrated to various areas of the lower surface were counted microscopically. Controls for chemokinesis included 200 ng of the appropriate form of osteopontin in the top well. Data are expressed as migratory index (cells migrating in response to osteopontin/cells migrating in response to buffer). Values are expressed as mean ± standard error, numbers in bold are significantly different from control values with $p < 0.05$ or better. All assays were done in triplicates and are reported as mean ± standard error.

B) 24-well plates were coated overnight at 4°C with 10 μg/ml of the indicated ligand then blocked for 1 h at room temperature with 10 mg/ml BSA in PBS. To preserve the integrity of adhesion receptors MH-S monocytic cells were harvested from subconfluent cultures by non-enzymatic cell dissociation solution (Sigma, St Louis MO). Cells were washed twice with PBS and resuspended at a concentration of 1 X 10^5 cell/ml of sterile Ca^{2+}- and Mg^{2+}-free PBS supplemented with 0.1% BSA and 1 mM sodium pyruvate. 5 x 10^4 cells were incubated in each well and, after 1 h at 37°C, the wells were washed 3 times with 0.5 ml PBS to remove non-adherent cells, fixed in 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 1 hour then stained with toluidine blue and hematoxylin. The total number of attached or spread cells in each well were counted microscopically using a Nikon Eclipse microscope equipped with a Sony digital Camera. Total number of attached or spread cells were quantitated using Optima 5.2 image analysis system. Each experiment was done in triplicates and is reported as mean ± standard error. To minimize variability inherent to cell attachment studies we scored cells as attached only when a defined nucleus was observed accompanied by a transition from round to cuboidal cell morphology. Round cells that are loosely attached with no defined nucleus were scored as non-attached. These cells can be removed with repeated washes. The viability of the cells was measured before and after the termination of the experiments and only data from experiments with greater than 95% cell viability were used. Further, under the conditions used in these experiments, cell attachment was temperature dependent, inhabitable by trypsin treatment and was not affected by inhibitors of protein synthesis or secretion. Cell spreading was determined by membrane contour analysis and was scored according to increase in cell volume/surface area. In some experiments, cell spreading was also assessed by the formation of stress fibers. Each experiment was performed in quadruplicate wells and repeated 3 times.

Modulation of PI3 kinase and FAK PKC activity by the chemotactic peptide (PepA) and its antagonist PepM

A proximal mediator of osteopontin dependent signal transduction through integrin receptors is the 125 kD focal adhesion kinase (Hruska et al. 1995) which associates with integrin $\alpha_\varsigma\beta_3$ and, in synergy with the cytoplasmic tail of integrin α_ς, activates pp60c-Src (Chellaiah et al. 1996). Two downstream processes ensue which both modulate the cytoskeleton. Tensin and paxillin may be activated by p125 FAK and pp60c-Src (Lopez et al.,1993). Src also activates phosphoinositide 3-hydroxyl kinase by tyrosine phosphorylation of the Src homology 2 domain in the p85 subunit (Hruska et al. 1993). PI 3-kinase may regulate the arrangement of actin filaments through gelsolin in a process that is inhabitable by wortmannin (Chellaiah/Hruska 1996). While both pathways have been associated with cell motility and spreading, their individual contributions to these processes are not known.

Many protein kinases require phosphorylation within their activation loops in order to express full catalytic potential. Such activation loop phosphorylations are also important for protein kinases regulated acutely by allosteric effectors. This is exemplified by PKC, where the Ca2+/diacylglycerol (DAG)-dependent isotypes PKC α and PKC β display an absolute requirement for phosphorylation in their respective activation loops. Suppression of phosphorylation at these PKC sites *in vivo* correlates with the induction of apoptosis in certain cell types (Garcia-Paramio et al., 1998), demonstrating the essential role of this phosphorylation *in vivo*. One potential kinase that has been implicated in the phosphorylation of PKC is the PtdIns(3,4,5)P3 dependent kinase PDK1. PDK-1 has been shown to phosphorylate PKC within their activation loop (Good et al., 1998). The effect of PDK1 is PI 3-kinase-dependent, and is inhibited *in vivo* by LY294002. PKC, therefore, is controlled through a PI 3-kinase

pathway, operating through PDK1-dependent phosphorylation of activation loop sites in the PKC isotypes.

Earlier studies have identified a role for PKC in cell crawling. Treatment of the colon carcinoma cell line HT29-D4 with PMA increased the rate of cell spreading and induced the migration of these cells towards purified matrix proteins in Boyden chamber-based haptotaxis assays. HT29-D4 cell haptotaxis was a direct consequence of PKC activation and not secondary to quantitative or qualitative changes in the cell surface integrins (Rigot et al. 1998). In crawling T cells, triggered via cross-linking of integrin LFA-1, two PKC isoenzymes, β(I) and δ, are targeted to the cytoskeleton with specific localization corresponding to the microtubule-organizing center and microtubules. Cells of a PKC-β–deficient clone derived from the parental PKCβ-expressing T cell line can neither crawl nor develop a polarized microtubule array upon integrin cross-linking. However, their adhesion and formation of actin-based pseudopodia remain unaffected (Volkov et al. 1998).

Since OPN regulates both FAK and PI3 kinase, we examined the effect of the chemotactic domain and its antagonist on the activation of FAK, PI3 kinase and PKC. The results (Fig 7) indicate that OPN, OPN286 and OPNdcc can activate FAK and PI3 Kinase, while PepA (Chemotactic peptide) and PepL can not. Further, OPN and PepA can activate PKCβ but not the zeta or gamma isoforms, while PepL only activates the zeta isoform. OPN286 and OPNdcc failed to activate any of the PKC isoforms tested. (Figure 2).

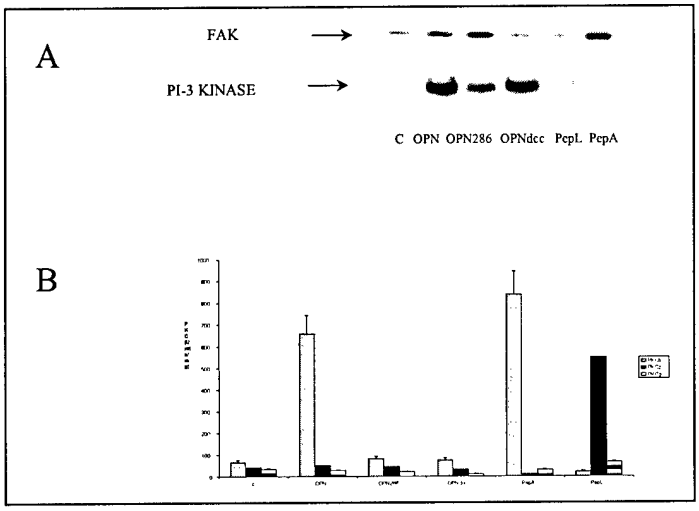


Figure 7 Regulation of Signal transduction by OPN and its analogues.
A) 1 million MDA-MB-231 cells were incubated with either OPN (5 mM), OPN286 (5 mM), OPNdcc (5 mM), PepA (5 mM) or Pep L(5 mM). After 12 hours the cells were harvested and lysed in 1 ml buffer A (10 mM Tris-HCl buffer, pH 7.2, containing 300 mM sucrose, 100 mM KCl, 1% Triton X 100 5 mM $MgCl_2$, 10 mM EGTA, 0.1 mM sodium orthovanadate, 0.1 M ε-amino-n-caproic acid, 5 mM benzamidine, 1 mM p-hydroxymercuribenzoate, 5 mg/l pepstatin, 1 mg/l leupeptin) at 4°C. After 10 min, fractions containing 2 mg protein were pre-cleared by incubation with 100 µl of insoluble protein A at 4°C for 1 h. After centrifugation at 5000 x g for 10 min., the

resultant supernatant was incubated with 0.1 mg of rabbit polyclonal antibody raised against either the p85a subunit of PI-3-Kinase (Upstate Biotechnology, Lake Placid, N.Y.) or FAK monoclonal antibody (BD Transaction Laboratory, Franklin Lakes, NJ) . The immune complexes were collected by incubation with 10 µl insoluble protein A for 1 h at 4°C followed by centrifugation at 5000 x g for 10 min. The protein A-immunocomplexes were washed 5x with lysis buffer, then once with 20 mM Tris-HCl. The immune complexes were released from the protein A beads by boiling the beads in 20 ml of SDS sample buffer containing 0.1% fresh 2-mercaptoethanol. The samples were resolved by on an 8% SDS-polyacrylamide gel and then transferred onto ECL-membrane by semi-dry blotting as described by the manufacturer. Phosphorylation of PI-3-kinase and FAK were assessed by probing the membranes with anti-phosphotyrosine (BD transduction Laboratory, Franklin Lakes, NJ).

B) One million tumor cells were treated as above. The cells were harvested and lysed and the activity of protein kinase C was measured with a kit obtained from Panvera. This assay distinguishes isoforms of protein kinase. We performed two separate experiments with duplicate samples.

Since PepL cannot induce PKCβ, We examined the effect of PepL on OPN induced PKCβ. The results show that in 1umols of PepL completely abolishes the induction of PKCβ by OPN (581± 23 units/mg to 21 ± 11 units/mg in the presence of 1 umol of pepL). These results suggest that PepL is a potent inhibitor of pkcb activation and explains in part it anti migratory properties. Based on the results collected so far, we propose that pepL could be a potent anti-tumor agent. We are currently testing these peptides for their antimetastatic properties in vivo.

(3) **KEY RESEARCH ACCOMPLISHMENTS:** Bulleted list of **key** research accomplishments emanating from this research.

- Transfection of CD44 (v3-v6) turns non-metastatic tumors into metastatic tumors
- CD44 v3-v6 cells metastasize predominantly to bone
- In vitro, regulation of OPN expression modulates invasion of tumor cells into bone
- A peptide, pepL, that induces apoptosis in tumors have been isolated
- Initial analysis indicates that pepL induces tyrosine nitration in susceptible tumor cell.
- Mutants of OPN lacking chemotactic domain cannot induce migration of CD44(v3-v6) tumor cells.
- Chemotactic domain of OPN induces the activation of PKCβ.
- PepL, which antagonizes tumor migration, induces Nitric oxide, and inhibits PKCβ.activation.
- Treatment of tumors with PepL results in the Induction of IL-12 by resident macrophages.
- PepL may by a good candidate for anti-tumor therapy.
- CD44, integrins and MMP form a complex, an invasion complex, with other molecules that drive metastasis of tumor cells. The disruption of this complex inhibits metastasis and activates programmed cell death in tumor cells.

(4) **REPORTABLE OUTCOMES:** Provide a list of reportable outcomes to include:

1- Weber, G.F., and **Ashkar, S.** (2000). Stress Response Genes - The Genes That Make Cancer Metastasize. Journal of Molecular Medicine. 78: 404-408.

2- Weber, G.F and **Ashkar, S.,**. Molecular Mechanisms of Tumor Dissemination in Primary and Metastatic Brain Cancers (2000). Brain Research Bulletin. 53:421-424.

(5) **CONCLUSIONS:**

Through this grant, we gained unique insight into the molecular biology of tumor migration and metastasis. We are beginning to decipher the signal transduction pathways that are operating in mediating tumor metastasis. Based on our results and those of others, we propose that tumor metastasis is mediated by a complex of molecules that form a functional unit that transmits information from the environment and regulates tumor behavior. This invasion complex appears to mimic, in part, the migration complex on immune cells and function in a manner similar to it. We have also gained insight into the complex biology directing organ specific tumor metastasis. CD44 does play a role in homing, however, other gene products are also necessary for organ specific Metastasis. Further the tumor invasion complex or invasome makes a novel target for treatment of advanced cancer

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(7) APPENDICES:

Statement of Work

Technical Objective 1: **CD44 expression and invasion into Bone marrow and MC3T3E1 Cultures (COMPLETED)**

Task 1a: (Month 1-4) Analyze the invasiveness of CD44 cell lines (described above) into MC3T3E1 and Marrow stromal cultures. Correlate expression of specific CD44 with attachment and invasion

Task 1b: (Month 4-6) Analyze the effect of CD44 antibodies (both general and exon specific) on the invasiveness of the CD44 cell lines.

Task 1c: (Month 7-9) Analyze the effect of osteopontin antibodies (polyclonal and monoclonal) on the invasiveness of the CD44 transfected cell lines.

Task 1d: (Month 10) From the above data Examine the physical and temporal relationship between the expression of CD44 splice variants and invasiveness into marrow and bone cultures.

Task 1e: (Month 10-14) Examine the role of CD44 splice variants in the attachment and migration into endothelial cell culture.

Technical Objective 2: **Effect of Chemotactic Peptide on the migration of MDA-MB-231 and transfected clones. (Completed)**

Task 2a: (Month 1) Synthesize chemotactic peptide and peptides altered in one or more amino acid.
Task 2b (Month 2-4) Test chemotactic peptide and its derivatives on chemotaxis of MDA-MB-231 cells and protease secretion
Task 2c: (Months 9-15) Refine peptide sequences based on results from task 2b and repeat analysis.
Task 2d: (Month 17) In vitro mutagenesis of OPN chemotactic domain.
Task 2e: (Months 17-24) Express Mutant in osteopontin negative C3H10T1/2 and purify protein
Task 2f: (Months 24-29) Testing the recombinant mutant protein from C3H10T1/2 for biological properties.
Task 2g: (Month 15-24) Test chemotactic response of new CD44 cell lines to chemotactic peptide and its derivatives and test for protease secretion by the CD44 cell lines in response to the peptides

Technical Objective 3 : **Testing the anti-metastatic potential of anti chemotactic domain antibody *in vivo* and testing of novel CD44 cell lines for their metastatic potential *in vivo*. (COMPLETED)**

Task 3a: (Month 2) Inject mice with cells.
Task 3b: (Month 3) Radiological examination of mice. Finish sacrificing the mice
Task 3c. (Month 4) Histological examination of carcasses.
Task 3d: (Months 12) Inject novel CD44 cell lines in nude mice
Task 3e (Month 13) Radiological examination of mice. Finish sacrificing the mice
Task 3f: (Month 14 -20) histological examination of carcasses

Task 4. (Month 32-36) Finish analysis, write reports and manuscripts